9TH EDITION

Manual of CLINICAL MICROBIOLOGY

PATRICK R. MURRAY ■ ELLEN JO BARON
JAMES H. JORGENSEN ■ MARIE LOUISE LANDRY
MICHAEL A. PFALLER

VOLUME 1

Manual of
CLINICAL MICROBIOLOGY
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Manual of
CLINICAL MICROBIOLOGY

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Acknowledgment of Previous Contributors

The *Manual of Clinical Microbiology* is by its nature a continuously revised work which refines and extends the contributions of previous editions. Since its first edition in 1970, many eminent scientists have contributed to this important reference work. The American Society for Microbiology and its Publications Board gratefully acknowledge the contributions of all of these generous authors over the life of this Manual.

Preface

It seems that much of my professional career has revolved around the *Manual of Clinical Microbiology*, first as an author, then as a section editor, and finally for the last four editions as the editor in chief. I have learned a great deal from this experience, although I must confess that I have forgotten far more science than I have retained. My most valuable lesson has been to rely on my fellow editors. I have been privileged to have worked on all four editions with Ellen Jo Baron and Mike Pfaller, with Jim Jorgensen for the last two editions, and with Marie Landry, who ably filled Bob Yolken's shoes, for this edition. I have also relied on the help of 29 section editors, including 7 new editors for this edition, and hundreds of authors. Almost 40% of the authors of the ninth edition of the *Manual of Clinical Microbiology* (MCM9) are new. For each edition I have purposely selected new editors and authors with the conviction that they will bring a new perspective to their assignment. I have systematically added non-U.S. authors, representing almost 30% of the authors of this edition, so that this Manual will be truly international in scope. These changes have not come without difficulties. My fellow editors and I have frequently had to curtail our authors' "creativity" for the sake of consistency, taught them that eloquence can equate to brevity, and reminded them that deadlines are not the fantasies of publishers. Many unpopular decisions are made in the creation of any work as comprehensive as MCM9, so I must thank the editors and authors for their understanding. If I have offended anyone in the process, it was unintentional.

I am very proud of what we have accomplished with MCM9, and I hope that this feeling is shared by the entire editorial board and all the authors. Although the field of clinical microbiology is undergoing dramatic changes with the confluence of traditional techniques and exciting advances in genomics and proteomics, I believe that the *Manual of Clinical Microbiology* should form the road map for our understanding of this evolving scientific discipline. I hope that the readers share the opinion of the authors and editors that MCM9 successfully meets this goal. An additional feature for the ninth edition is a CD-ROM with close to 500 illustrations from the book. It is available for purchase through ASM Press.

I would be remiss if I did not acknowledge the wisdom and guidance provided by Susan Birch, the ASM Press Production Manager whose name should more appropriately be listed on the cover of MCM9, and Jeff Holtmeier, the director of ASM Press who makes everyone's job a little easier and more pleasant. The entire staff at ASM Press was very supportive and helpful during the process of preparing this edition of the Manual.

PATRICK R. MURRAY

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GENERAL ISSUES IN CLINICAL MICROBIOLOGY

I

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Microscope used during medical school by Joseph W. Mountin, founder of the Communicable Disease Center (from CDC image library).

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Introduction to the Ninth Edition of the *Manual of Clinical Microbiology*

PATRICK R. MURRAY

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The eighth edition of the *Manual of Clinical Microbiology* (MCM8) was a dramatic change from previous editions, growing from one to two volumes and increasing in length by more than 20%. Even though the changes in the ninth edition of the *Manual of Clinical Microbiology* (MCM9) are not as obvious, they are no less substantial. One new volume editor, Marie Louise Landry, and seven new section editors joined the editorial board. Of the 269 authors, almost 40% are new and 28% represent 22 non-U.S. countries. Although we retained the same 11 sections that were introduced in MCM8 and the algorithm chapters that have proved to be practical identification tools, we have expanded the chapters from 141 to 152. The MCM8 chapter "Pathogenic and Indigenous Microorganisms of Humans" was removed in MCM9 because we believed that this topic could not be satisfactorily covered in a limited chapter. To compensate for this deletion, the discussion of the topic was expanded in the individual organism chapters. A new chapter, "Microscopy," was added to section III, Diagnostic Technologies in Clinical Microbiology. This topic was covered in the sixth edition of the Manual but not in subsequent editions, a decision that the editorial board felt should be rectified. Section IV, Bacteriology, was increased by three chapters—coverage of mycobacteria was expanded from two chapters to three; coverage of anaerobes was expanded from three chapters to four; and *Tropheryma* was assigned to a new chapter, chapter 69. The most significant changes in MCM9 occurred in section VI, Virology, reflecting the influence of a new volume editor and three new section editors. The discussion of hepatitis A and hepatitis E viruses was consolidated into one chapter, and three new chapters were added—chapter 91, "Coronaviruses"; chapter 96, "Hendra and Nipah Viruses"; and chapter 98, "Hantaviruses." Coverage in other chapters was expanded to include metapneumoviruses and parechoviruses. Two new chapters were added to section VIII, Mycology—chapter 127, "Mycotoxins"; and chapter 128, "*Lacazia, Pythium*, and *Rhinosporidium*." Finally, section X, Parasitology, was reorganized with the addition of four new chapters. *Cryptosporidium* was transferred to a new chapter, chapter 142, and the coverage of helminths was expanded from two to five chapters.

All chapters in MCM9 were updated with the most current taxonomic and diagnostic information, which is not an insignificant feat in this era of molecular classification and diagnostic tools. Nearly 4,000 of the literature citations in MCM9 were published after the last edition went to press. Despite these efforts, we recognize that by the time that MCM9 will be published, some data will be dated and some statements will be inaccurate. This occurs despite the best efforts of the authors, editors, and ASM staff and is the unfortunate reality of publishing a major reference text. We ask for your understanding, as well as for your help in remedying the inaccuracies. If you discover an error, please contact ASM Press. A system will be established to post both the error and the corrections for the readership. The editorial board hopes that through the efforts of our authors and your careful review of the text, MCM9 will be an accurate, valuable reference source.

Laboratory Management*

W. MICHAEL DUNNE, JR., AND MARK T. LAROCCO

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The diagnostic microbiology laboratory, regardless of demographics, is a business and needs to be managed with the same basic principles and tenets used by businesses. True, the products provided by microbiology laboratories belong to the service industry, but the day-to-day management of resources, productivity, finances, quality, and clients is no different. Whether the laboratory is for profit, not for profit, academic, or community based, it needs to follow the same financial rules and standards that businesses do. To develop a primer for medical microbiologists on the basics of business theory, economics, financial accounting, organizational behavior, and marketing, etc., within the confines of this chapter is impractical given the wealth of excellent contemporary reference materials currently available (9, 25, 31). Rather, this chapter will highlight four major management issues: operations, finance, personnel, and regulations.

OPERATIONS

The efficient operation of a clinical microbiology laboratory and effective delivery of diagnostic services to clinicians and their patients are vitally dependent on the management and communication skills of laboratory directors, managers, supervisors, and technologists. Quality laboratory services result directly from the performance of competent laboratory scientists, but the key element lies in the delivery of these services, a highly complex management activity. The clinical microbiology manager's task is to integrate and coordinate laboratory resources so that quality laboratory services can be provided as effectively and efficiently as possible. Laboratory management may be described in terms of four basic functions: planning, organization, direction, and control. In practice, the boundaries between these functions are often obscure because of the interdependence of the functions for effective management.

Strategic Planning

Strategic planning is the process of deciding on objectives for the organization that fulfill its mission, selecting and allocating the resources needed to obtain those objectives, and assigning responsibilities and accountabilities to those involved in the process. The microbiology laboratory will likely be included in the strategic plan of the overall department that in turn embraces the plan of the hospital or other parent organization. Nonetheless, the microbiology director or manager should have an appreciation of key features of the hospital's strategic plan.

A mission statement is a concise description of the overall goals and values of the organization. A mission statement may be thought of as the hospital's credo on which all of its actions are based. In a sense, the mission statement serves as the cornerstone of the organization's strategic plan as it represents the highest level of success that the organization hopes to achieve.

Planning at the department level is an essential requirement of laboratory management. Each section of the laboratory should establish a set of goals and objectives, formulate policies to carry out those objectives, develop intermediate and short-range plans to implement policies, and develop detailed procedures for implementing each plan. During the planning process, managers must be aware of any changes that occur in the operational environment because such awareness keeps decisions realistic and expectations achievable (37). An analysis of the operational environment can be accomplished by conducting an objective assessment of the laboratory's strengths, weaknesses, opportunities, and threats (SWOT analysis). Let's say, for example, that the microbiology laboratory is asked to develop a strategic plan to incorporate molecular diagnostic testing into its service offerings. A preliminary SWOT analysis may reveal the following.

Strengths

1. Strong technical leadership in the laboratory facilitates the implementation of new technologies.
2. The hospital supports a solid-organ and stem cell transplant program with patients that will likely produce a high demand for the service.
3. A teaching affiliation with a regional school of medical technology provides recruitment opportunities for trained technologists.

Weaknesses

1. The skill sets of most of the current staff do not include molecular diagnostics.

* This chapter contains information presented in chapter 2 by David L. Sewell and James D. MacLowry in the eighth edition of this Manual.

2. Having no record of performance in molecular diagnostics may place the laboratory at a marketing disadvantage.
3. The lack of experience with managing a molecular diagnostic service in the context of the overall laboratory budget.

Opportunities

1. A new academic affiliation will immediately provide resources (space, personnel, and equipment) for supporting a molecular diagnostic service.
2. There is a paucity of facilities in your geographic area that offer molecular diagnostic testing, and physicians are not happy with the service provided by a regional commercial laboratory. The potential for developing outreach volume is high.

Threats

1. Inappropriate utilization of the technology may create a financial hardship for the laboratory.
2. Competition from other commercial laboratories that call on physicians' offices directly.

Specific goals and objectives provide laboratory management with a clear direction before the planning process begins. When policies are set with these goals and objectives in mind, constraints are in place to guide the planning strategies prior to the major plan development stage. A few examples of goals and objectives for the clinical microbiology laboratory may be as follows.

1. Ensure the quality of diagnostic microbiology services by monitoring key functions and processes to determine improvements for quality and cost.
2. Support research and development by reviewing and testing new methods and equipment.
3. Expand volume by researching new markets for outpatient business.
4. Act as consultants to medical staff and outcomes managers to ensure that the most appropriate diagnostic tests are ordered.
5. Communicate, through results, a philosophy of customer service to patients, physicians, family members, and coworkers.
6. Support the strategic plans of the hospital through the laboratory services provided.
7. Employ personnel who are responsive to the needs of patients, physicians, clients, guests, and coworkers.
8. Provide for the future of laboratory service by participating in educational and training programs for medical technology.

Once a goal is identified, a plan for completion of the project and short-term interim plans are needed. Finally, the details necessary for implementation and the expectations of performance are defined. High performance cannot be achieved, however, without accountability. When there is a lack of accountability, needed information may be missed, decisions are not made when action is required, and people do not receive guidance or support when faced with new challenges. Accountability means that people can count on one another to keep performance commitments and communication agreements. It is the basis for an environment of trust, support, and dedication to excellence (27).

During implementation of any department plan, it is important to periodically measure success. Such an exercise affords management the opportunity to step back and revisit elements of the planning strategy while at the same time providing staff with appropriate feedback and motivation to see a difficult project through to its conclusion.

Organization

Organization in the clinical laboratory refers to both structure and process. Structure exemplifies stated relationships or framework, and process deals with interaction. There are three key elements of organization: the tasks to be performed, the individuals who are to perform the tasks, and the clinical laboratory as a workplace.

A scope of services document provides a detailed list of services provided by the microbiology laboratory. The document should contain general information on the hours of laboratory operation and on staffing. It may also include test-specific information such as specimen collection and transport requirements, reference ranges, locations of testing, (in-house versus reference laboratories), and cutoff times for receipt of specimens. The scope of services provided by the laboratory is often a reflection of the type of hospital or facility it serves.

Organizational charts are common to most business structures as they provide a visualization of who is doing what and the chain of command. Although nobody likes to think of oneself as being below other people, these charts can serve a useful purpose in the clinical laboratory by defining the relationships among tasks, individuals, and the workplace. The organizational chart attempts to show relationships between line and staff. A line position is one in which a superior exercises direct supervision over a subordinate. A staff position is advisory, supportive, or auxiliary. These terms were defined in industry, and health care institutions seldom use the same connotation. When laboratory managers speak of staff, they are usually referring to technologists who are, in fact, serving in line positions.

Written policies and procedures, displayed in printed manuals or made available electronically, are an essential component of the clinical microbiology laboratory. Every procedure should be clear, easy to follow, and consistent in its content and organization. These documents provide direction for the many day-to-day tasks performed by the staff, serve as a teaching tool for students and new employees, and provide information to inspectors from accrediting agencies. The Clinical and Laboratory Standards Institute (formerly NCCLS) has a published guideline, GP2-A4, for writing laboratory procedures (23). The guideline provides instructions and recommendations for writing procedures for the full scope of the laboratory's workflow. The document also contains information about organizing procedure manuals, archiving and managing documents, and using manufacturers' procedures for automated purposes. As a testament to the document's universal acceptance, the College of American Pathologists requires that all written laboratory procedures closely follow the GP2-A4 format.

As defined by Brown (4), process design is a broad plan that is developed to provide a blueprint for completing work or a task at hand. Process design in the clinical microbiology laboratory involves an analysis of size and setting, laboratory design, equipment, test methodology, regulations, and staffing. How the work transitions through the laboratory is defined as workflow, and although workflow analysis is part of process design, it is more detailed. Policies and procedures are developed during workflow analysis (4). Workflow can be divided into three phases of the test cycle: preanalytical, analytical, and postanalytical. The preanalytical phase consists of events that occur prior to actual testing, such as selection, collection, transport, and accessioning of specimens. The analytical phase

involves the actual testing. The postanalytical phase consists of events that occur after testing is completed, such as results reporting and interpretation. The preanalytical phase is often the most critical aspect of the test cycle and can be the hardest part of workflow to control.

Strategies to effectively manage and minimize variances associated with the three phases of the test cycle should be included in the clinical laboratory's quality management plan. Quality management is a system for continuously analyzing, improving, and reexamining resources, processes, and services within an organization to produce the best possible outcome (28). Microbiologists have, for many years, concerned themselves with the quality of events that take place within the clinical microbiology laboratory. These events include such things as ensuring the performance of equipment, reagents, stains, and media and monitoring the accuracy of tests performed in the laboratory. These activities fall under the category of quality control (QC). Quality assurance (QA) broadens the scope of traditional QC to encompass processes and events in the preanalytical and postanalytical phases. QA includes such things as determining specimen quality and evaluating the competency and training of personnel. Both QC and QA can be considered aspects of quality management that also includes quality improvement (QI) or quality enhancement. Although applied successfully in industry for many years, QI and quality enhancement are relatively recent concepts in the clinical laboratory setting. Instead of focusing on inspection, identifying poor performance, and taking corrective action, as in traditional QC and QA programs, QI programs emphasize thorough training and prevention and view the system for improvement opportunities rather than the people (8). Continuous QI (CQI), sometimes referred to as total quality management, is an organized, systematic approach to productivity improvement that uses objective methods and a team approach toward improving the quality of all processes, products, and services. The origin of CQI-total quality management is industry based. The approach was promulgated by W. Edwards Deming and grew out of his experience with Japan's industrial reconstruction after World War II. Central to CQI philosophy is the premise that improvement in quality leads to increased productivity, increased customer and employee satisfaction, and decreased cost. The CQI approach is to examine process performance, not people performance, and utilize specific methods of analysis (tools) to better understand and improve those processes. It is a top-down style of QI that requires management commitment and support and involves the participation of all employees. Finally, CQI is viewed as an ongoing initiative.

While these broad definitions may suggest that CQI would be an ideal QI technique for the clinical laboratory, its implementation in clinical laboratories has not been easy. This is due, in part, to the additional time and resource requirements associated with CQI, at least initially, but also relates to an insufficient understanding of how to properly apply CQI to laboratory testing. CQI requires innovative thinking; the traditional internal quality monitors familiar to most laboratories cannot simply be shoehorned into a CQI format. Above all, do not lose sight of the fundamental objective: to improve patient care.

One way for laboratories to avoid this pitfall is to design a CQI program that strives to connect process improvement to clinically relevant patient outcome measures. Outcomes measurement has emerged from the health services research community in response to the changing economic environment in medicine, beginning with the advent of diagnosis-related group (DRG) classifications, and more recently as an important aspect of managed care delivery. Outcomes measurement identifies variations that may adversely affect patient care and can sometimes determine corrective actions for minimizing those variations. Although patient outcomes come in many forms, measurements relevant to laboratory testing generally involve the length of stay, cost, and customer satisfaction, with the customer being both the patient and the physician. Changes in laboratory services and policies that decrease lengths of patient stays, reduce cost, or increase customer satisfaction should be the focus of a laboratory CQI program. Those that have the most impact on outcome measures should be selected for process improvement.

A process is a set of activities that transforms inputs into outputs. Inputs include the needs of patients and physicians, the skill of laboratory personnel, equipment, supplies, and financial resources. Outputs involve laboratory data, diagnoses, and management decisions that are associated with outcomes realized by patients and physicians. Process analysis is often accomplished with techniques such as flowcharting and the use of cause-and-effect diagrams (fishbones), i.e., the tools of CQI, but it is important to remember that the goal of process analysis is to design strategies whereby modifications of the process lead to a measurable improvement in outcome. In clinical microbiology, elements of processes that may be selected for analysis and improvement include specimen rejection criteria, appropriateness of testing, and turnaround times for smear results that acutely affect patient management. Process analysis should include events related to the patient from the time of presentation to the end of an episode of care. For the laboratory, this requires an understanding of how multiple processes related to patient care, including those external to the laboratory, are connected (the output of one process may be the input of another). Failure to understand the interactive dynamic between processes will likely blunt the impact of laboratory improvement initiatives on patient outcomes.

A critical feature of the CQI paradigm is the need to carefully construct and assess any improvement modality before implementing costly changes in policies or procedures on a large scale. Referred to as the Plan-Do-Study-Act cycle for improvement (5, 17), the steps include first stating a specific aim and identifying criteria that will be used to determine if the change represents an improvement. After selection of a change that is practical and most predictive of a positive effect, a plan for evaluating the change in the form of a small pilot study is put forth. The results of the pilot study are assessed for their effect on performance, and then action is taken in the form of process redesign, or if needed, additional pilot studies are conducted. These never-ending cycles of improvement, gradually built into the daily workflow, represent the "meat and potatoes" of a proactive CQI program.

Selection and Implementation of New Equipment and Procedures

As laboratory managers are directly responsible for the quality and productivity of their departments, they must constantly evaluate new technology and equipment for applicability and practicality. Managers must also assess the impact of any new technology on patient care by focusing on attributes such as turnaround time, productivity, and cost. There are several steps involved in this process, including the performance of a needs assessment, research of available technology, and evaluation of performance and cost data gathered before making a decision on implementing a new

procedure or making a new instrument purchase (3). This process is discussed in greater detail elsewhere in the Manual (see chapter 15).

FINANCE

Medicare, Medicaid, prospective payment, DRGs, preferred-provider organizations (PPOs), health care maintenance organizations (HMOs), managed care, fixed versus variable costs, return on investment—the financial side of the health care industry possesses a vocabulary that in many cases is as mind-boggling to the clinical microbiologist as real-time PCR is to the hospital financial officer. In order to be successful, however, clinical microbiology laboratory managers must be able to speak both languages and be as concerned about positive cash flow, cost control, and budget variance as they are about QA and procedural controls.

Laboratory Reimbursement

Medicare Part A provides reimbursement for hospital services related to an inpatient stay. Payment is made based on DRG classifications, a prospective payment system (PPS) of reimbursement. The Centers for Medicare and Medicaid Services (CMS) assign a weight to each DRG based on the severity of the diagnosis, the type of procedure, the number of laboratory and other diagnostic tests, the number and types of drugs prescribed, and the presence of complications or comorbid conditions (6).

Based on this method of payment, hospitals are able to determine how much reimbursement they will receive for Medicare patients and will make a profit if they can manage these patients at a cost below the reimbursed amount. This fixed reimbursement system and incentive to reduce the cost of care has put pressure on hospital support services such as the laboratory to lower expenses.

Medicare Part B covers physician services and outpatient ancillary health care services including clinical laboratory testing. In July 2000, Medicare introduced a new PPS for the outpatient setting and developed ambulatory payment classifications as a basis for reimbursement for outpatient services. The ambulatory payment classification system provides payment predictability and promotes efficiency; however, at this time clinical laboratory tests are excluded from the outpatient PPS. Instead, laboratory tests performed on outpatients are paid for from the Medicare Part B laboratory fee schedule. Fees are assigned based on current procedural terminology (CPT-4) or health care common procedure coding system (HCPCS) codes established for each procedure. The actual Medicare payment is the lowest of either the actual charge, the fee schedule amount set by the contractor, or the national fee cap termed the national limitation amount (la).

Private insurers, also affected by increasing health care costs, have also undergone revision in their reimbursement methodologies. Managed care is defined as a means of providing health care services within a network of service providers (6). Although there are a variety of managed care plans, they usually all require that insured individuals see a physician who is part of the plan or else pay a higher copayment. The most common type of managed care plan is the HMO. Reimbursement under managed care plans can occur in several ways. Capitation, usually applied to physician reimbursement, pays a certain amount per patient per month. Payment can cover the cost of all services related to the member's outpatient care, including any necessary diagnostic or therapeutic tests. The physician can make a profit or lose money depending on the numbers of visits with and tests performed on each patient. Inpatient care costs may be reimbursed on a per diem, or per-day, basis. Under this arrangement, the hospital negotiates with insurers a set amount of reimbursement per day during the patient's hospitalization. The hospital staff will use the cost of care per day to help set the per diem rates and can increase the profit margins if they can decrease costs without compromising patient care. Reimbursement per case, similar to the Medicare PPS, is based on a set reimbursement rate for specific diagnoses or procedures. In some instances where hospitals offer specialized procedures or unique types of care, there may be "carve-outs" whereby reimbursement occurs separately from the per diem or per-case rate, usually on a fee-for-service basis.

In all of this discussion, the key message for the laboratory manager is the focus on cost control. In the hospital's financial landscape, the laboratory is considered a cost center. Although it may produce revenue from its outpatient and outreach workload, the reality is that it represents a necessary but significant expense on the hospital's overall balance sheet. Thus, laboratories that focus on providing high-quality service in a cost-effective and efficient manner will be valued by the organization and those that do not will likely be targeted for redesign.

Laboratory Compliance Programs

In 1998 the Office of the Inspector General, in response to costly and at times fraudulent billing errors on the part of some laboratories, issued compliance program guidance for clinical laboratories. Its purpose was to instruct laboratories on how to conduct their business in a lawful and ethical manner. The guidance document requires that every laboratory have a formal compliance program that addresses several essential elements (2). At a minimum, the laboratory compliance program should (i) develop and promote standards of conduct for employees, (ii) promote a commitment to zero tolerance of fraud and abuse, (iii) designate members of a laboratory compliance committee, (iv) identify education and training programs available to employees, (v) promote communication with employees to ensure that the program is effective, (vi) outline monitoring activities to ensure that the program is effective, and (vii) describe the potential disciplinary measures for those violating standards of conduct and compliance procedures.

The laboratory compliance committee has the responsibility to oversee compliance with standards of conduct and other procedures as they relate to lawful and ethical business practices. The membership of the committee should consist of the medical director, the administrative director, section managers or supervisors, a laboratory information systems (LIS) manager, and the manager of outpatient services if one exists. The duties of the compliance committee are to effectively communicate the standards of conduct to all employees and to implement monitoring systems to detect any possible illegal or unethical conduct as it may relate to medical necessity or billing issues. The committee should also be available to employees who believe that they are aware of unethical or illegal activities that violate the standards of conduct and ensure that the enforcement of these standards is consistently done through appropriate disciplinary mechanisms. On a routine basis, the compliance committee should review any appropriate fraud alerts as periodically issued by the Office of the Inspector General and inform and educate employees appropriately as well as keep current with legal and regulatory compliance issues and revise compliance policy as needed.

The laboratory should communicate regularly with the institution's financial services, governmental reporting, and medical records departments regarding coding and billing

issues. Laboratory managers are expected to take every reasonable precaution to ensure that the CPT-4 codes for laboratory tests accurately describe the tests that were ordered and performed and to choose the code that most accurately describes the ordered and performed test. Intentional up coding, the selection of a code to maximize reimbursement when such a code is not the most appropriate descriptor of the service, is not allowed. A complete review of CPT-4 codes should be performed at least annually to update codes and descriptions, delete obsolete codes, and validate reference lab coding using the American Medical Association's *Current Procedural Terminology* manual (1) and updates from carrier and fiscal intermediary bulletins.

The laboratory should encourage that physicians order only tests that are medically necessary. Diagnostic information for each test should be submitted by the ordering physician or authorized representative in narrative form or codes provided through the International Classification of Diseases, 9th Revision, Clinical Modification (ICD-9-CM). Laboratory employees should never suggest or supply ICD-9-CM codes. As a means to ensure physician knowledge of compliance issues, the laboratory should disseminate a "notice to physicians" to physician clients on an annual basis. The notice should outline the individual component of every laboratory profile that includes a multichannel test or other automated multiple test result and the CPT-4 codes that the laboratory uses to bill Medicare for each such profile. Physicians should also be told that when ordering tests for which Medicare, Medicaid, or other federally funded reimbursement is sought, they should order only those tests which they believe are medically necessary.

Costs

Operating expenditures in the laboratory may be divided into broad categories of fixed and variable costs. These terms reflect the sensitivity of costs to increases or decreases in test volume. A cost that changes more or less in proportion to the test volume is variable. A cost that remains unchanged for some period of time despite volume fluctuations is fixed. Examples of variable costs include technologist salaries, costs of reagents and supplies, and costs on printed requisition forms. Salaries for nonexempt employees, benefits, and the cost of depreciation of equipment are examples of fixed costs. Costs are also classified as either direct or indirect. Costs that can be specifically linked to a test are considered direct costs. These include technologist salaries, costs of clerical support and overtime, courier fees, and costs of reagents and bacteriological media. Costs that cannot be directly traced to a test but are still part of the laboratory's expenses are indirect costs. Examples of indirect costs include those of depreciation, building and equipment maintenance, and utilities. Laboratory managers are more often concerned about unit costs when making financial decisions because understanding how costs fluctuate in producing a specific unit of service (usually defined as a test) permits measurement of productivity. Unit costs are also divided into fixed and variable categories that behave differently as volume changes. Variable costs remain the same per unit regardless of volume, and fixed costs are reduced per unit when volume increases because the total fixed cost is spread over a larger number of tests. A busy clinical laboratory benefits from economies of scale. In general, variable and direct costs are subject to some degree of management control and fixed and indirect costs are much less so. Regardless of cost classifications, laboratory managers must be continually looking for opportunities to control any and all costs. Accurate cost accounting is an imperative for financial decision making, and in today's managed care environment, correct cost information ensures participation in profitable contracts. The laboratory manager must be efficient in defining and itemizing costs associated with the testing services provided by the laboratory. Moreover, accurate test cost analysis provides the manager with a rational mechanism for selecting the most cost-effective testing method among several new procedures whose suppliers each claim that their test is the least expensive to use. Finally, laboratory budgeting becomes more effective with a reliable cost analysis system in place. A simple test cost analysis worksheet is shown in Appendix 1.

Operating Budgets

The development of an operating budget for the hospital is highly dependent upon clearly stated institutional goals and objectives within which the laboratory has a known role and upon which it can base its own specific objectives. The manager should expect to receive, either directly or indirectly from the hospital administration, budget guidelines that include expected inpatient and outpatient census information for the budget year and any anticipated changes in the total service program of the organization. Clinical service line managers, in particular, should communicate new program plans to support service managers, including laboratory managers, because support services may be impacted by these new programs. If, for example, the hospital has plans to begin performing human stem cell transplants, then the microbiology laboratory needs to budget for the additional volume and specialized testing that patients receiving these transplants will require. In return, managers may be required to provide the laboratory and/or hospital administration with information related to program changes anticipated in their areas that arise because of changes in technology or clinical practice. Examples of such changes include procedures that may diminish in volume because of obsolescence or physician education and increasing volume estimates for newly implemented procedures such as those of molecular diagnostics.

There are several types of operating budgets (18). A fixed budget assumes a single level of output or activity and builds the budget around that level. A fixed budget, however, cannot be used to monitor and control resources during changes in test volumes that the laboratory may experience during the year. A flexible budget reflects expected laboratory revenue and expenses and anticipates the impact of volume changes on both. In this type of budget, some of the expenses are fixed and some are variable. The flexible budget recognizes the difficulty of establishing a flat level of expenditure and provides a tool for controlling costs. Quite simply, a flexible budget allocates dollars for resources based on volumes of tests performed. The accuracy of the flexible budget as a predictive tool, however, is only as good as the test component cost analysis performed during the budget's construction. A program budget is based on a specific program matrix. The matrix includes all proposed services and the resources required to deliver them. The program can include a set of activities, services, staffing, and equipment. It is often used for short-term planning purposes when new programs are launched. A zero-based budget calls for management to reevaluate all activities to decide if they should be re-funded for the next budget cycle. Each manager is required to justify the entire budget for their area of responsibility as if all of its activities were entirely new.

Although there is no single formula for the budgetary process, there are some basic elements that will help ensure

success (18). First, establish clear goals and objectives to guide resource allocation. Second, obtain detailed data on existing and potential clients. Third, establish a defined budget period and procedures for development of the budget. Fourth, compile reports with financial and statistical information for comparison with budget information and for variance analysis. Furthermore, when planning a budget, managers should remember the following principles: (i) expenses are charged to the department or cost center that incurs them, (ii) every item of expense must be under the control of someone in the organization, (iii) managers responsible for complying with a budget should participate in the budget's preparation, (iv) managers should not be held accountable for expenditures over which they have no control, and (v) ordinarily, unused funds do not carry over from one budget year to the next.

Variance Analysis

Once the operating budget is established and approved, the extent to which the actual revenues and expenses differ from the budget represents a variance. Controllable variances can be resolved by appropriate management action (32). Salary variances can be addressed by reviewing time cards for excessive overtime. Supply cost variances may prompt an examination of inventory par levels. Suppliers may be approached for deeper discounts, or alternative vendors may be sought. Some variances are not controllable. These often result from unanticipated volume increases due to higher-than-expected numbers of patient admissions, unanticipated (and unbudgeted) price increases, unplanned repair expenses, or an unusual increase in the number of tests requested. A good example of the latter would be an unanticipated epidemic of influenza's producing a large increase in the number of requests for rapid flu testing and the need for the laboratory to purchase an excessive number of kits within a short span of time. As discussed above, a flexible budget should account for month-to-month fluctuations in test volumes and test complexity if properly constructed. Managers may be periodically called upon to communicate their budget performance to the laboratory administration and should be able to accurately record and explain budget variances on a line-by-line basis in their monthly financial statements.

Capital Budgeting

In addition to the operations budget, there must be a process for allocating monies for major investments in facilities and equipment. In accounting terms, a capital item is generally any fixed asset expected to provide service for more than 1 year and has a minimal expense, usually between $500 and $1,000. Items that qualify as capital include new space or renovation of existing space, replacement of equipment, purchase or lease of new equipment, and information technology (hardware, licensing fees, and interfaces, etc.). Capital acquisitions represent a significant investment for the organization and, as such, should be evaluated and justified by a budgetary process. Generally, a fixed amount of funds available for capital is established each fiscal year by the institution. Invariably, the total dollar amount requested for competing capital projects will far exceed the total of funds available. The capital budget process must therefore winnow, select, and prioritize; it is an enormously complex and politically charged task if not handled correctly. But by using a team approach whereby the management maintains focus on the goals and objectives of the organization, the capital budget process can be a successful and rewarding experience.

Prior to the budget process, the pool of total capital dollars available may be divided into smaller pools designed to support different aspects of the institution. For example, a $50 million capital budget may be allocated to allow $5 million for strategic projects, $10 million for information technology, $15 million for patient care, $10 million for facility improvements, and $10 million for ancillary services. If the laboratory is considered part of ancillary services, then the numbers of available dollars is much lower than the overall budget and the laboratory will likely be competing with several other departments for this lesser amount. The evaluation process will seek to classify capital projects in different ways, and managers should be aware of how to appropriately categorize and describe any project submitted for consideration. For example, some projects may represent new or incremental business, some may enhance quality, and some may reduce cost. Finally, capital requests may be prioritized according to need. A nondiscretionary project is a project that must be funded to keep the facility operating. An example would be a renovation project to make the laboratory compliant with regulatory and accreditation mandates. Levels of priority then follow, with level I projects being most critical.

Generally, capital projects of $10,000 or less need minimal analysis. All that is normally required is a brief description of the project and its intended use (new item or replacement, etc.) and the requested dollar amount. Projects with costs exceeding the minimum dollar amount require a higher level of analysis and more detailed documentation supporting the request. In addition to a description of the project and its intended use, proposals for these projects should include an estimate of the life span of the item, an estimate of all costs associated with acquisition, an estimate of yearly incremental cash outflows, and an estimate of yearly incremental cash inflows or savings. Depreciation expense must also be considered. Straight-line depreciation analysis is the most commonly used method of determining the expense of a fixed asset. In this method, the estimated useful life span of the item is divided by the cost of acquisition. A piece of equipment costing $100,000 with a useful life expectancy of 5 years would have a depreciation expense of $20,000 per year.

The financial analysis of large capital projects may have several elements. An illustrative case study follows. Suppose a clinical microbiology laboratory that has historically performed manual bacterial identifications and disk susceptibility tests wishes to purchase an automated system because a new outreach program will significantly increase test volumes. First, a pay-back analysis reveals that with a purchase price of $125,000 and a net cash flow of $137,000 after 3 years, the pay-back period is calculated at 2.68 years. The pay-back method does not take into account the time value of money but serves as a crude measure of risk because it favors projects with a short pay-back horizon. The average rate of return is calculated by dividing the average annual return by the amount of the initial investment. Projects with a higher average rate of return may be rated more favorably, but the method also fails to recognize the impact of time. The concept of present value is very important for capital investment analyses because it takes into account the increase or decrease in the value of a capital investment over time.

The most common method used in the financial analysis of a capital proposal that accounts for the time value of money is the calculation of net present value (NPV). NPV is the present value of all cash flows minus the initial investment. If the NPV is positive or zero, the project is usually considered financially acceptable. This is because all future cash flow has been converted into current dollars, with the use of an expected rate of return (usually set by the institution's

financial department), and has been compared with the initial investment. However, projects with a small negative NPV may still be worthwhile since other factors may be considered, such as the size of the initial investment and the benefit to patients. A favorable NPV for any capital proposal may result in a ranking higher than those for projects with similar costs. Laboratory managers would be well advised to support their capital project proposals with sound financial as well as clinical justification. In the end, it may make the difference between getting the equipment the laboratory needs and limping along for another year without it.

Financial Bench Marking

Increasing economic pressures on today's laboratory operations have created the need for managers to be aware of their financial performance in comparison to some internal or external bench mark. Financial bench marking is but one of many tools that the laboratory employs to improve performance, focus on customers, survive, and thrive in an environment with limited resources (39). In selecting indicators for bench marking, the use of ratios is much preferred over the use of raw numbers because the data are viewed in terms of a common denominator, usually some unit of service. The typical unit of service for the laboratory is the billed test because most financial systems are capturing volumes according to CPT codes that have standardized characteristics. A denominator that is often used outside the laboratory is patient-days or patient discharges. Ratios can be used to assess performance in a number of ways. For example, laboratory workload may be measured by calculating the total number of billed tests per calendar day, per patient-day, or per patient discharge, and examining the number of billed tests per full time equivalent reveals service intensity. Labor productivity can be determined from the number of hours worked per 100 billed tests or per patient discharge. Lastly, important cost data may be ascertained from ratios such as labor and supply expenses per 100 billed tests.

The simplest method of bench marking is to use your own laboratory as the standard and measure performance over time. This type of internal bench marking provides consistency of measurement over time and gives managers control over assumptions. In effect, it affords managers a means to continuously monitor performance and make improvements in productivity and cost on an ongoing basis. External bench marking involves a comparison of the laboratory's performance with that of a peer group. The composition of the peer group is critical. Members of the peer group should be alike in as many critical characteristics as possible. Otherwise, whatever performance expectations are placed on an individual laboratory being compared to the peer group may be unreasonable. Sometimes, voluntary agreements are established with other institutions to share productivity and cost information. This arrangement allows control of the size and composition of the peer group and ensures a fair comparison of whatever characteristics are being measured. There are a number of commercial companies that provide bench-marking products, some specific for the laboratory (see http://www.chisolutionsinc.com) and others more broadly applicable to the institution but with drill-down capability for the laboratory (see http://www.solucient.com).

Now that cost efficiency is a watchword in most health care environments, a comprehensive financial management system is an imperative, rather than an option, for laboratory management. Managers who set goals, measure progress, and identify trouble spots will utilize their limited resources more intelligently than those who do not.

PERSONNEL ISSUES

The study of organizational behavior encompasses all of the human factors encountered in the workplace, including motivation, leadership, organizational models, and responsibilities (31). Organizational behavior is defined as the actions and attitudes of people in organizations. When it comes to personnel issues, most diagnostic microbiology laboratories will fall under the auspices of the human resources department (HR) of the parent institution. In that regard, directors, managers, supervisors, technologists, and laboratory assistants alike are obligated to become familiar with and follow institutional policies relative to hiring and firing practices, compensation and benefits, job descriptions, organizational structure, and grievance procedures, etc., and of course to be compliant with federal, state, and local labor laws.

Hiring

Needs Assessment

Before the microbiology manager goes through the expense and time required to hire a full-time employee, he or she should determine whether that position can be eliminated without compromising service and quality expectations. Could the work be redistributed among existing staff? The solution may involve creative management in terms of subdividing assignments among other stations or individuals (or even among different laboratories), but this approach may not be practical in areas such as specimen processing where the workload is constant and could not be easily transferred elsewhere. It may be possible, however, to rearrange schedules such that individuals are transferred into high-volume work areas during times of peak utilization.

A second option may include the use of temporary or part-time employees to fill the shortage. This alternative can be quite attractive from a convenience standpoint but may be more expensive depending on training and agency costs. An excellent source of part-time help may include previous employees who have left the full-time ranks for alternative pursuits and would like to supplement their income. Finally, the hiring process may present an opportune time to examine laboratory productivity. A number of consultant firms are available to perform bench-marking analysis with laboratories having similar workloads and demographics to determine efficiency.

Analysis, Design, and Description

The job description is a summary of two administrative processes: job analysis and job design. Whereas the analytical and design phases involve proactive evaluation and/or reconfiguration of the nature of the work to be performed, the written job description is a document that defines the required skills, functions, conditions, and hierarchy associated with a specific position (35). Poor job analysis and design will lead to a flawed job description.

A job analysis should be comprehensive and detailed such that the nature of the work to be performed is clearly understood. The information required to complete the analysis can be gained through a structured process that measures and documents the number of tasks performed and the time required to complete those tasks, or informally through observation (10). One can also hire consultants to perform job analysis, request input from current staff, or contact laboratory administrators from demographically similar facilities. Job analysis and design should focus on the essential elements of the position, including the array of duties, the workflow and volume, the technological skills required, the

degree of employee interaction, the reporting relationships, and the working conditions. This, in turn, will make it easier to define minimum education, experience, and personal attributes appropriate for the job. If done properly, job analysis and design will drive the completion of a formal job description. As an example, consider the following scenario. The microbiology manager is allowed to fill a vacancy for a laboratory assistant on the evening shift. The primary responsibilities of that position are specimen processing and reagent preparation. The manager could use the existing job description to recruit for the position. After careful analysis, however, the manager finds that most laboratory assistants on that shift complete their assigned duties nearly 2 h before the shift ends. At the same time, the volume of *Clostridium difficile* toxin testing by enzyme immunoassay on the day shift has exceeded capacity for months, necessitating overtime for qualified technologists. If the new position was expanded to a technologist level, it would allow for testing of excess *C. difficile* specimens on the evening shift without the loss of sample processing capacity at a fraction of the overtime costs alone.

The job description serves to define the specifics of the position and the requirements for the prospective employee (10). It also provides a tool for communication between the employer and the prospective employee and serves as a recruiting tool (35). It can be used to schedule and set staffing requirements, to assess employee performance and competency, to define wage and salary structure, and to act as a template for future corrective actions (35). It should be flexible to allow for future growth of the position and focus on minimum requirements to prevent the loss of high-end applicants (16). There are no rules to writing a good job description unless institutional guidelines are in place. At the very least, the job description should include the items listed in Table 1 (10, 35). Optional information that can be added includes exempt status (time clock or salaried position), personnel and payroll codes (but not actual dollar ranges), and U.S. Department of Labor (DOL) occupational codes and/or U.S. Employment Service functional job analysis codes (34). Bear in mind that the Clinical Laboratory Improvement Amendments of 1988 (CLIA '88; see below) established only six categories for laboratory personnel; director, technical consultant, clinical consultant, technical or general supervisor, testing personnel, and cytotechnologist. The laboratory can expand on the qualifications required for each of these positions but not reduce them below the minimum requirements as outlined in CLIA '88. All job descriptions on file should be reviewed at least annually in conjunction with the employee's performance review or when the position is vacated and a replacement is sought.

Recruiting, Interviews, and Hiring

Once a job description has been drafted and approved, the recruitment and hiring process can begin. As before, it is best to coordinate activities with the organization's HR personnel, who are more familiar with local, state, and federal regulations pertaining to recruitment and hiring. HR can also provide valuable services such as background checks and validation of a candidate's credentials (14).

The job description should be abstracted to provide the essentials for a classified advertisement. The advertisement should contain the job title, minimum education and licensing requirements, a brief summary of the duties, and if desired, the salary ranges (15, 16, 35, 38). The laboratory has basically two candidate pools to choose from: internal and external. The former is usually an attractive source because current employees have already received system orientation and are familiar with general corporate policies and culture. Mobilization of internal candidates usually takes less time because job listings can be posted in organizational newsletters or on institutional websites. Overall, internal hires are generally less costly, but external candidates can infuse the laboratory with new ideas, experiences, and skills. Generally, external searches take longer and may require the use of recruitment bonuses, employment agencies, the Internet, or advertising in trade magazines such as *ASM News*/*Microbe* or *Medical Laboratory Observer*. Other possible external recruitment sources include professional schools and organizations such as the American Society for Clinical Pathology, the Canadian College of Microbiologists, and the American College of Microbiology (the certification and training

TABLE 1 Components of a job description

Items	Example[a]
Organization	Central States Medical Center
Department	Microbiology
Position	Microbiologist, day shift
Education	B.S., medical technology or microbiology
Certification	M.T. (ASCP); SM, NRM, or equivalent
Responsibilities	Responsible for processing of specimens, interpretation of culture and susceptibility results, participation in quality management program, and teaching of medical technology students, laboratory medicine residents, and fellows; nonsupervisory position
Competency	Requires semiannual (first year) and annual (thereafter) competency assessment via departmental program
Working conditions/exposure hazards	Modern clinical microbiology laboratory; no heavy lifting; potential exposure to human specimens, including blood and body fluids, and work with BSL 1–3 agents
Pay code	5, Nonexempt

[a] M.T., Medical Technologist; ASCP, American Society for Clinical Pathologists; SM, Specialist Microbiology; NRM, National Registry of Microbiologists; BSL 1–3, biological safety levels 1–3.

branch of the American Society for Microbiology [ASM]). Parent institutions can also use online automated recruitment web pages for both internal and external candidate searches. By logging on to a website, potential candidates can review job openings within the system and create a personal profile that can be used by HR for reviewing qualifications or for matching individuals with future positions as they become available. The ultimate goal is to provide the microbiology administrator with a qualified pool of candidates while trying to maintain workforce diversity according to a U.S. DOL directive (http://www.dol.gov/dol/compliance/comp-eeo.htm). For individuals applying for supervisory or higher positions, a search committee should be convened to expand the sources of potential candidates. Once a candidate pool of qualified individuals has been identified, it is time to begin the interview process.

The interview is a means of allowing the employer and candidate to determine whether they are suited for each other. It is a very important process because a badly conducted interview may scare off a highly qualified candidate. This is also the part of the hiring process in which the unskilled interviewer may pose a liability and one that requires close attention to regulations. There are several questions shown in Table 2 that the Equal Employment Opportunity Commission prevents an employer from asking a potential employee (14, 16, 35, 38). The interview should be organized and standardized to permit a fair evaluation of each candidate. There are three basic formats for an interview: structured, in which the format is predetermined with a list of questions and a review form; unstructured, in which a set panel of questions is not used but the interview consists primarily of a running conversation with the candidate; and pressurized, in which the candidate is asked to provide analysis of various job-related scenarios or to solve a series of hypothetical problems (34). The interview can be a combination of all three and can be conducted individually or by a panel of interviewers. In some organizations, HR conducts a preliminary interview to screen candidates before the microbiology administration conducts final interviews. The ultimate goals are to meet the candidate, check credentials (usually an HR responsibility), collect additional information not provided on the application form, and gather a sense of personality fit. In management, we tend to think of the interview process from one side only. Holland (14) provides an overview of how to prepare for an interview from both the employer's and the employee's perspectives. If there is an evaluation form to be completed, it should be done immediately after the interview so that impressions are not lost with time. Documentation of the search, selection, interview, and orientation processes should be kept on file for at least 4 years should any improprieties be called into question.

Employee-Related Activities

Orientation

Once a candidate is hired, the next phase involves the assimilation of the new employee into the microbiology workforce. The orientation process must be organized and synchronized to keep the new hire active and engaged.

According to Vernadoe (34), there are three basic aspects of employee orientation. The first is at the organizational level to familiarize the new employee with institutional policies, procedures, and benefits. The second focuses on department-specific policies and codes of conduct. The final aspect concentrates on the microbiology laboratory and addresses job responsibilities, reporting structure, and working relationships that exist therein. There are at least four basic approaches to conducting orientation (16, 34). These include (i) formal meetings and training sessions, usually conducted at the institutional level by HR (these cover such topics as risk management, fire and chemical safety, benefits, and infection control); (ii) microbiology supervisor-led sessions to discuss laboratory-specific issues such as competency assessment, biological safety protocols, dress code, telephone conduct, breaks, and scheduling; (iii) a checklist approach; and (iv) a mentor-driven process.

In addition to a systemwide, general orientation program, many microbiology managers choose to develop a stepwise laboratory-specific orientation process that is checklist driven. First is the preorientation checklist, which is preparatory in nature. It allows the microbiology supervisory staff to organize all the materials that the new employee will receive during orientation. The training orientation checklist guides the assigned mentor and trainee through the basic components of orientation. The specific elements of the microbiology laboratory are covered by a series of modules that may include topics such as scheduling orientation, trainer and trainee expectations, supervisor's review procedures, a special shift rotation checklist, and a LIS orientation, to name a few. The entire process is completed with a postorientation checklist to ensure that each component has been covered and entered into the employee's personnel file.

Above all, the orientation period is the process of welcoming. For this reason, the choice of mentors selected to provide training should be carefully considered and limited to individuals with a great deal of experience and respect.

TABLE 2 Topics to be avoided during a hiring interview

Topic	Examples
Age	Date of birth, year of high school graduation
Weight	Waist size, dietary habits
Financial history	Outstanding loans or debts, bankruptcy
Citizenship	Country of origin, immigration status, parental ancestry
Marital status	Maiden name, number of children
Criminal and military records	Military discharge status, court convictions
Organization memberships	Religious or political groups
Mental/medical/physical disabilities	Current medications, therapy, workman's compensation benefits, missed workdays secondary to disabilities

Staffing and Scheduling

The process of adequately staffing a microbiology laboratory and scheduling individuals with widely ranging abilities to provide maximum coverage on each shift is one part science and one part art. It requires advanced planning, and no one strategy works for every facility because peak activity levels, workloads, and the technical complexity of testing differ significantly. Poor planning can lead to low productivity and high labor costs when staffing is excessive. Inadequate staffing may generate high productivity but also lead to increased overtime and employee burnout (21). To avoid these extremes, a number of good guidelines on staffing and scheduling are available for review (4, 36).

Scheduling is simply the process of assigning work responsibilities to a group of individuals and requires the right number of employees and skill levels to complete the work. Most medical laboratories work under the 8/80 rule for full-time employees, i.e., employees work a total of 8 h per day and 10 days over a 2-week pay period. This plan limits the number of hours to be worked in a day but permits the employee to work more consecutive days and is unique to medical facilities. A 40-h work week permits an employee to work any number of hours per day but not to exceed 40 h per week. Other scheduling systems include the use of part- or variable-time help, job sharing, self-directed work teams, and exempted employees (21, 36). In designing a schedule, it is important to choose a plan or combination of plans to provide the greatest degree of flexibility. A mixture of employees on the 8/80 plan and the 40 plan can be used, but a single employee must choose only one option.

The scheduler must know workload patterns and future trends. This is most often done retrospectively by reviewing LIS-generated billing for an estimate of test volume and plotting these data over time for trend analysis for each shift. Recording workload units periodically over time can also provide a helpful bench mark of test volume. However, keeping pace with method advancement is also necessary for projecting future labor needs. The development of molecular and amplification technology has greatly streamlined a number of processes in the microbiology laboratory—most significantly in clinical virology. Armed with this information, the laboratory manager must calculate how many people for each shift and skill set will accommodate the mean test volume, not peak or trough volumes of work (4). From here, the scheduler starts with a rough draft based on ideal staffing levels by using a matrix for each shift where rows correspond with the services to be provided and columns represent the days of the pay period. With the 8/80 plan, a shift is 8 h times 14 days or 112 h per pay period. Full-time employees can cover 80 of these hours (8 h times 10 days), while part-time staff can be used to fill in the balance (generally 24 h per week or less). Once a rough schedule has been outlined, it can be fine tuned by naming the individuals who will fill each slot and the work assigned. From this working draft, the schedule can be modified to accommodate vacations, family leave, illness, earned time off, and shift switching. Schedulers and managers must also remember that work schedules affect employee families and morale (30). Part-time and variable-time employees can be used to fill in gaps, or cross shift coverage can be applied to relieve peak workload stress. A detailed description of this process can be found elsewhere (4, 30, 36). During new employee orientation, a review of the scheduling process should be included so that there are no surprises when the individual is eventually assigned duties.

Winn and Westenfeld (41) provide an excellent summary of scheduling do's and don'ts as follows: (i) define the absolute minimum number of employees required for each situation (weekends, routine, and holidays); (ii) strictly define the duties for each of the positions required for every type of shift, and if a position is open, try to fill it with existing personnel based on the duties assigned to that slot; (iii) allow scheduling to be orchestrated by a coordinator with input from the staff; (iv) solicit vacation requests well in advance (>6 months) and be clear that not all preferences can be accommodated; (v) publish work schedules in advance to allow for personal adjustments; (vi) if allowed, use earned time off instead of overtime; and (vii) fill empty slots with volunteers or part-time help, with a draft as a last resort. In this context, certain laboratories have developed a rotational "hit list" among staff to cover for open slots on the work schedule. The next person up on the list is informed of their standing and allowed one deferral or may switch their obligation with another employee.

In a situation in which the microbiology workload is high or increasing and staffing is static or decreasing, there are a few options left to the administration to relieve the pressure. The first of these is outsourcing, such as finding reference laboratories to perform a variety of low-volume, esoteric, or technically complex testing, e.g., forwarding all mycobacterial identifications with the exception of probe-confirmed identification of *Mycobacterium tuberculosis* and *Mycobacterium avium* complex organisms to a public health mycobacteriology laboratory. Elimination of low-volume testing may not necessarily prove to be cost efficient but maintaining proficiency when the frequency of testing is low should also factor into outsourcing decisions.

Microbiology laboratories can even outsource testing to other laboratories within their own system. For example, automated blood culture systems can be physically located near a rapid-response laboratory. Signal-positive blood cultures can then be removed during all shifts to be subcultured and stained. Gram stains can be read by trained rapid-response technologists, and cultures can be evaluated during the next microbiology shift.

The second option is to examine the extent of evaluation. In many instances, microbiology laboratories can get into the bad habit of providing too much information, such as identifying isolated organisms that are unlikely pathogens in a disease process. This process not only generates an unnecessary workload but may also give the clinician a false sense of importance.

Unfortunately, staffing and scheduling often go hand in hand with absenteeism. As a result, each facility should have a well-defined absentee policy and management plan that are neither onerous to the employee nor subjective and that can be fairly applied. A laboratory policy on absenteeism requires a working definition, equitable and realistic standards (e.g., less than one unscheduled absence per month), and a management plan that uses the same organizational framework for corrective action for issues such as behavioral or performance problems (36). Absenteeism can be chronic, high frequency, and/or patterned (e.g., Mondays, Fridays, or the day after payday). If not addressed and corrected, chronic absenteeism can generate poor morale among other microbiology staff.

Performance Appraisal

A job description defines the duties of an employee and how the employee's success will be measured. The performance appraisal, on the other hand, asks how the employee is succeeding. A performance appraisal has at least five ingredients: (i) an employee with a job description and appropriate qualifications, (ii) a series of clearly defined goals and

performance standards, (iii) an objective way of measuring achievements, (iv) someone trained to evaluate the employee's performance, and (v) a feedback loop to gain input from the employee (38). The standards used to judge performance must be outlined in the job description and should be objective and consistent with those used to evaluate other employees. Basically, four markers can be measured either alone or in combination to generate a performance appraisal: results, behavior, skills, and peer comparison (33). However, objectivity in performance appraisal is often difficult to achieve. We all have a tendency to rate individuals we like highly and those we do not like less highly. The actual data recorded in the performance appraisal should include employee information, a list of goals and objectives from the previous and for the next review, a scoring system, a place for the employee to provide his or her own feedback, and a summary section that allows for the appraiser to recommend promotions and salary adjustments and that includes a date for the next appraisal and signatures (11, 33). When complete, the form should become an official part of the employee's personal file (22).

If the parent organization permits, there are a number of alternatives to performance appraisals, including feedback sessions, team or section appraisals, self-appraisal, and process improvement sessions, all of which would require some form of documentation.

Competency Assessment

The directive for competency assessment of laboratory personnel was written into CLIA '88. This legislation was signed into law in 1992 and has been revised five times to provide the final ruling in 2003. It mandates that all laboratories falling under the jurisdiction of the CLIA '88 guidelines must develop a program to document the competency of each laboratory employee in every aspect of the employee's assigned duties. As is the case with many pieces of federal legislation, CLIA '88 did not define how this process is to be accomplished nor did they suggest which core competencies should be monitored. They did, however, place the responsibility for establishing a program and documenting competency assessment squarely on the shoulders of the laboratory director and technical consultant or technical supervisor (11). Fortunately, the Clinical and Laboratory Standards Institute (formerly NCCLS) has published a clear and logical approach to the design of a laboratory-based training and competency assessment program that provides a wealth of sample documents used in the process (22). CLIA '88 clearly define the frequency of personnel assessment: semiannually for new employees and annually thereafter unless changes in procedures and protocols occur. At that point, competency for and familiarity with the new protocol must be documented. All agencies approved by the CMS for the purpose of laboratory accreditation have a competency assessment module included in the review process. These agencies include the College of American Pathologists, the Joint Commission on Accreditation of Healthcare Organizations, and the Commission on Office Laboratory Accreditation. For more details on the history and evolution of competency assessment, please refer to the fine review by Sharp and Elder (29).

The final CLIA rule (2003) consolidates commercially marketed in vitro diagnostic assays into two functional categories, waived and nonwaived. When it comes to competency assessment, however, nonwaived testing is subdivided into moderate- and high-complexity tests for the purpose of defining the educational background and training requirements of those individuals performing the testing (40).

CLIA '88 go so far as to define a choice of methods to be used in the evaluation process (13, 29). These include but are not limited to (i) directly observing an individual performing laboratory functions including testing, plating, culture evaluation, and instrument operation and maintenance; (ii) verifying the accuracy of transcribed test results; (iii) checking the integrity of data entered by the employee for QC records, proficiency tests, preventive maintenance charts, and preliminary patient test results; (iv) challenging the employee with internal and external proficiency series; and (v) evaluating an individual's ability to solve laboratory-related dilemmas. Hundreds of individual skills need to be mastered before a technologist-level employee can be considered a competent microbiologist, and there is no way that each of these can be pinpointed let alone evaluated in a year's time. That being said, there are at least three excellent publications outlining a competency assessment program specifically for the microbiology laboratory, and any of these could be used as a template for design (19, 20, 29). General laboratory-wide plans are also available for review (16, 22). In one model, a database in a spreadsheet format (e.g., an Excel file) is established for each active employee upon hire. All of the potential competencies that the job description encompasses are listed in the first column of the spreadsheet (*y* axis), and the first row (*x* axis) indicates the year of review. Each new employee begins with a series of X's in each competency cell for which training has not yet been conducted or in the cells corresponding to competencies not included in the job description. As training for a particular skill is accomplished, the X is removed and the method of measurement, the date, and the initials of the trainer or observer are recorded in the cell. Methods of measurement can be coded as DO for direct observation, Q for quiz, DR for document review, PT for proficiency test (internal or external), PM for preventive maintenance review, and QC for quality control review, etc. By using the spreadsheet approach, the competency assessment report for each employee is readily available for review and update and can also be used as an orientation tool. A copy of each report can be printed on a yearly basis and added to the employee's personal file.

Continuing Education

The Clinical and Laboratory Standards Institute guidelines on training and competence assessment distinguish between training and education in the following way (22). Education is "schooling for the purposes of gaining knowledge," and training is defined as the acquisition of "a set of skills for the purpose of being able to put the knowledge to a practical use." For the purpose of this chapter, however, continuing education will be defined as the extended process of gaining knowledge and improving skill sets. Continuing education constitutes one of the mandatory requirements for competency assessment and professional development dictated by CLIA '88 and necessary for the maintenance of professional licensure by most accreditation agencies. How can microbiology personnel maintain yearly continuing education requirements under the pressure of reduced operating budgets? A number of opportunities are available to satisfy these requirements, including attendance at local meetings such as branch meetings of the ASM. ASM branches usually sponsor at least one annual educational meeting, and the location and time of each are posted on each branch's website (http://www.asm.org/MemberShip/index.asp?bid=1914). In some cities, local microbiologists have formed clubs that meet during the year and invite guest speakers (usually sponsored) to present topics of interest for continuing education

credits. Many medical centers provide weekly opportunities for continuing education purposes such as grand rounds, clinical pathology conferences, infectious diseases case conferences, and microbiology rounds. At one author's facility (W.M.D.), the microbiology laboratory sponsors a weekly case conference open to technologists, infectious diseases staff, and pharmacy personnel. At the organization level, many health centers provide tuition reimbursement for college courses that pertain to the profession of the health care worker.

Other organized microbiological programs for continuing education include teleconferences, workshops, self-assessment materials such as Tech Sample and Check Sample (American Society for Clinical Pathology; http://www.ascp.org/index.asp), and ASM-organized wet laboratory demonstrations, online lectures, and audio lecture series that are available for purchase or subscription (http://www.asm.org).

Termination

Termination refers to the end of a formal business relationship between an employer and employee and can be either voluntary or involuntary (16). The departure of an employee from the workplace can be either amicable or contentious. In any case, procedures are usually developed by HR for employee separation that reflect the situation. For voluntary and amicable termination, the process may include documentation of the date of departure and a forwarding address; discontinuation of payroll, benefits, and parking privileges; and the return of security materials. HR may also wish to conduct an exit interview with the employee to gain insight on laboratory operations and administration (34).

There are good reasons to support the involuntary termination of an employee, including incompetence, chronic absences, violent behavior or verbal abuse, falsification of resume, theft, and insubordination (16). A fair employer will make every reasonable attempt to correct the problem prior to taking disciplinary actions. Detriments to job performance including substance abuse of any kind, stress-related illness, mental illness, or physical disabilities secondary to job activities fall under the auspices of the Americans with Disabilities Act (http://www.usdoj.gov/crt/ada/adahom1.htm) and are not acceptable causes for dismissal (16). A number of variations in the process of progressive disciplinary action have been described for laboratory managers, and each is designed to allow sufficient time for the employee in question to mend his or her ways and for the employer to adequately assess the true nature of the situation (16, 34, 38). The steps generally include (i) verbal counseling, (ii) written counseling, (iii) a penalty phase, and (iv) dismissal. A number of variations on the theme exist. Walters (38) outlines a more creative approach to the resolution or termination process that includes five phases: forewarning, investigation, proof, reflection, and penalty. Importantly, though, employees have the right to representation during any disciplinary process as determined by the National Labor Relations Act (http://www.nlrb.gov/nlrb/legal/manuals/rules/act.asp). If the conclusions of the progressive disciplinary action lead to employee dismissal, the process is usually handled by HR to ensure that all legal steps are followed and documented (16). It is also wise for the employer to retain the personnel file of the departing employee for future reference. Likewise, the departing employee should be informed of the Consolidated Omnibus Budget Reconciliation Act. This act gives workers and their families who lose health benefits the right to continue group health care provided by their current plan for limited periods of time under certain circumstances (http://www.dol.gov/dol/topic/health-plans/cobra.htm).

REGULATIONS

This section will serve as a brief review of those regulations and regulatory agencies that have the most profound effects on the operation of the clinical microbiology laboratory. A more extensive review of laboratory regulations is available in the works of Roseff et al. (26), Walters (38), and Ehrmeyer and Laessig (7).

CLIA '88

CLIA '88 (or simply CLIA) establish baseline performance standards for all U.S. laboratories involved in the testing of human materials for the diagnosis, prevention, or treatment of disease (7). The intention is to ensure the accuracy, reliability, and timeliness of test results no matter which laboratory is performing the testing. They were signed into law in 1988 (12), but five successive final revisions of the initial legislation have been published since, the last of which appeared in the *Federal Register* in 2003 (13, 34). In the first final version of CLIA, laboratory testing was divided into three complexity levels that established the expertise level of personnel performing those tests. Those levels were waived, moderate complexity (including provider-performed microscopy), and high complexity. In the most recent final version of CLIA, testing is now lumped into waived and nonwaived tests (most of the latter were high complexity). However, relative to the qualifications of testing personnel, CLIA still recognize moderate- and high-complexity levels (7, 40).

The administration and implementation of CLIA are under the purview of the CMS (http://www.cms.hhs.gov) in collaboration with the Centers for Disease Control and Prevention (http://www.cdc.gov). The U.S. Food and Drug Administration (http://www.fda.gov) is responsible for classifying the complexity of laboratory testing protocols and the CMS set fee schedules for laboratories providing services to Medicare.

U.S. laboratories wishing to perform and receive compensation for testing of human specimens must apply for a CLIA certificate in one of three complexity categories. If the laboratory meets compliance criteria as judged by the CMS or by one of the recognized professional accreditation agencies, it receives a permanent certificate of compliance or accreditation and becomes subject to the rules, regulations, and inspections of the agency issuing the certification. Organizations that the CMS have deemed suitable for CLIA accreditation of laboratories include the Joint Commission on Accreditation of Healthcare Organizations (http://www.jcaho.org), the Laboratory Accreditation Program of the College of American Pathologists (http://www.cap.org/apps/cap.portal), and the Commission on Office Laboratory Accreditation (http://www.cola.org/).

CLIA lay the foundation for laboratory participation in a proficiency testing program, qualifications of and competency assessment of laboratory personnel, and, establishment of a QC and QA program and ensure the integrity of laboratory testing through all phases of testing. They also call for routine inspection of certified laboratories and the enforcement of penalties for laboratories found to be noncompliant. CLIA dictate that outside or reference laboratories to which specimens are referred from a U.S. health care institution also be CLIA certified or the equivalent. Those wishing to review CLIA '88 in their entirety or to query individual components of the amendments can do so at http://www.phppo.cdc.gov/clia/regs/toc.aspx or http://www.cms.hhs.gov/clia/default.asp. A general description of the program is available at http://www.cms.hhs.gov/clia/progdesc.asp.

HIPAA

Public law 104-191, or the Health Insurance Portability and Accountability Act (HIPAA, Title I) of 1996, was originally written "to improve portability and continuity of health insurance coverage in the group and individual markets, to combat waste, fraud, and abuse in health insurance and health care delivery, to promote the use of medical savings accounts, improve access to long-term care services and coverage, to simplify the administration of health insurance, and for other purposes." However, in 2002, the Department of Health and Human Services issued the HIPAA Privacy Rule (Title II) that set highly restrictive standards on the use and protection of individual demographics and health information (protected health information [PHI]) contained in patient medical records (24). Details are available at http://www.hhs.gov/ocr/hipaa/finalreg.html. The Privacy Rule dictates that all "covered entities" including health insurance plans and health care institutions and providers must obtain written permission from a patient to use or disclose PHI and that the patients must be notified of their right to restrict the use of or disclose PHI (24). Covered entities must also make every attempt to limit PHI disclosure to only that required for any given purpose. From the standpoint of the microbiology laboratory, this means that the release of any PHI attached to bacterial or fungal isolates sent for research or surveillance purposes that can be traced in any way to the patient requires disclosure and consent unless waived by the human studies committee of the institution. The Privacy Rule does not supersede the federal "Common Rule" (see also http://www.hhs.gov/ohrp/humansubjects/guidance/45cfr46.htm) that mandates Institutional Review Board approval of research studies conducted under its auspices (24). As with CLIA '88, the CMS are charged with the responsibility of administering the provisions of HIPAA. For a summary of the HIPAA health insurance reform (Title I) or the HIPAA administrative simplification (Title II), one can access the CMS website at http://www.cms.hhs.gov/hipaa.

Laws Covering Equal Pay and Compensation Discrimination

Several laws have been passed over the past half century to guarantee employees freedom from discrimination in the workplace with regard to wages, compensation, and working conditions. Collectively, these laws are enforced by the U.S. Equal Employment Opportunity Commission and include the Equal Pay Act of 1963, Title VII of the Civil Rights Act of 1964, the Age Discrimination Act of 1967, and Title I of the Americans with Disabilities Act of 1960.

Equal Employment Opportunity Commission

The Equal Employment Opportunity Commission is a federal agency created in 1965 to enforce the Civil Rights Act of 1964 (26). As of 1999, the agency had published new guidelines to further define unlawful harassment in the workplace (including sexual harassment) against the same groups protected by the Civil Rights Act (26, 38; http://www.eeoc.gov). To avoid risk, laboratories and/or HR of the parent institution should have a training module on unlawful harassment in place for employees and supervisory personnel, avenues for rapid reporting and investigation of complaints, and a documentation process for each of these.

Equal Pay Act of 1963

The Equal Pay Act is part of the Fair Labor Standards Act and makes unlawful the practice of wage discrimination between men and women performing the same duties at the same institution in a similar work environment. This act is directed and enforced by the Equal Employment Opportunity Commission and is gender neutral such that it protects males and females equally (38).

Title VII of the Civil Rights Act of 1964

Title VII of the Civil Rights Act of 1964 prohibits the use of race, color, creed, religion, nationality, and sex as a basis for the discharge of or failure to hire an employee, for segregation or classification that would deprive an employee of opportunities or advancement, for the failure or refusal of an employment agency to refer an individual for employment, or for the limitation of training or retraining of an employee.

Age Discrimination in Employment Act of 1967

The Age Discrimination in Employment Act of 1967 applies to individuals 40 years of age and older and, like the Civil Rights Act, prohibits the use of age as a reason for discrimination in the workplace. Interestingly, this law also applies when two candidates over the age of 40 apply for the same job and the younger of the two is hired if the employer has used older age as a negative factor in the selection process (38).

Americans with Disabilities Act

The Americans with Disabilities Act was passed in 1990 and forbids discrimination against an individual who has or has had a disability, is thought to have a disability, or associates with another disabled individual. According to the Americans with Disabilities Act, a disability is a physical or mental deficiency that affects a life function such as walking, talking, employability, and hygiene (38). The Americans with Disabilities Act mandates that an employer reasonably accommodate the needs of any qualified candidate so that he or she can perform the job. For example, the current dimensions of bench tops, shelving, and foot wells in the microbiology laboratory may not be appropriate for wheelchair access by a wheelchair-bound microbiologist and, therefore, require modification. Acceptable dimensions for work-related desk surfaces and shelving are provided in the Americans with Disabilities Act standards for design (http://www.usdoj.gov/crt/ada/stdspdf.htm).

Other Regulations Related to the Workplace

Family and Medical Leave Act

Passed in 1993, the Family and Medical Leave Act provides allowances to eligible employees of an eligible employer to take up to 12 months of unpaid leave during any 12-month period for one or more of the following situations: (i) birth and care of the employee's newborn child, (ii) placement with the employee of a child for adoption or foster care, (iii) care of an immediate family member (spouse, parent, or child) with a serious health problem, and (iv) medical leave secondary to the employee's own serious health problem. The Family and Medical Leave Act falls under the auspices of the U.S. DOL, and definitions of which employers are covered by this act and which employees are eligible can be found at http://www.dol.gov/esa/whd/fmla/.

Fair Labor Standards Act

The DOL also administers the Fair Labor Standards Act, which is legislation that sets basic minimum wage and overtime pay schedules. These regulations are enforced by the

Wage and Hour Division of the DOL, which is a program of the Employment Standards Administration. Pay for overtime (defined as work in excess of 40 h in a work week) is set at a rate of not less than one and one-half times the regular rate of pay. Certain exemptions apply to specific types of businesses or specific types of work. This act does not set requirements for severance pay, sick leave, vacations, or holidays. For more details, one can consult the DOL website at http://www.dol.gov/dol/topic/wages/index.htm.

Affirmative Action

An executive order was issued in 1965 that prohibits contractors engaged in federal contract work exceeding $10,000 from discriminating in hiring decisions against the same protected groups included in the Civil Rights Act. This order has since become known as affirmative action and was updated in 2000 by the Office of Federal Contract Compliance Program of the DOL. In addition, contractors securing more than $50,000 in government business and having more than 50 employees must write and regularly update an affirmative action plan to reflect current guidelines (38). Affirmative action guidelines would apply to many health care institutions and laboratories meeting the criteria defined above because of their participation in federal contracts for care provided to Medicare and Medicaid patients. However, many institutions that do not meet the qualifications outlined in this executive order have chosen to develop a voluntary affirmative action plan. The Office of Federal Contract Compliance Program maintains a website at http://www.dol.gov/esa/regs/compliance/ofccp/faqs/faapfaqs.htm.

APPENDIX 1

Sample test cost analysis form

Test cost analysis worksheet

Test: _____

Patient tests per year _____

Other tests per year (QC, repeats, etc.) _____

Total tests per year _____

Estimated time to perform test _____

Cost analysis

Labor

Direct: _____

Indirect: _____

Supplies

Direct: _____

Indirect: _____

Equipment: _____

Overhead: _____

Cost/Test: _____

Cost/Patient Test: _____ = (cost/test × total test volume)/patient test volume

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Laboratory Design

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A well-designed laboratory is a safe, pleasant, and efficient place in which to work, as well as an enjoyable place to visit. When a new laboratory is being designed or renovation of an existing one is being planned, the design must meet the needs of laboratory staff who spend all or most of their working time there, the needs of workers who spend part of their time there, and the needs of visitors who have to interact with the staff. Above all other considerations, a clinical laboratory must be a safe place. No other aspect of laboratory design carries a higher priority, both for the staff and for visitors. A pleasant environment improves staff morale and productivity, minimizes unnecessary distractions (thereby reducing laboratory errors), and enhances employee recruitment and retention. An efficient clinical laboratory helps to improve productivity, to reduce errors, and to improve patient care by shortening laboratory test turnaround time (TAT). An efficient laboratory also helps to improve staff morale and contributes significantly to a pleasant work environment.

Good laboratory design is based on the concept that form follows function, so the unique functions of a clinical microbiology laboratory (CML) should be reflected in the laboratory design. When designing a CML, it should be remembered that there are three key differences between a CML and other types of clinical laboratories. First, clinical microbiology involves the isolation, propagation, and handling of pathogenic microorganisms that pose a risk to laboratory personnel. To minimize this risk, the entire CML must have the facilities and processes necessary to meet biosafety level 2 criteria and, depending upon the extent and scope of services provided, all or part of the laboratory must also meet the requirements necessary to meet biosafety level 3 criteria (4, 15). Second, the interpretation of cultures and other microbiologic test results is based on the ability of the laboratory to isolate pathogenic microorganisms while minimizing microbial contaminants. Again, the laboratory design must be such that the necessary precautions can be taken to minimize contaminants. Third, laboratory design must accommodate specialized equipment used only in microbiology laboratories.

This chapter is intended to be an introduction to the processes of laboratory design, construction, and renovation. Reading this chapter should enable the microbiologist who is beginning a construction or renovation project to communicate with all of the parties who are involved. The informed microbiologist not only will understand the terminology of design and construction, but also will be able to make better decisions as the processes unfold. Being well informed also reduces the likelihood of costly mistakes. Not everything can or should be learned by experience.

GENERAL DESIGN PRINCIPLES

The needs of each laboratory will change over time, often many times between the initial construction of the laboratory and any subsequent renovations. Thus, the best approach to designing is to use the most generic design possible, minimizing features that are unique to current circumstances. Not only does a more generic design facilitate the changing needs of the laboratory, but also it reduces the costs of construction, renovations, and ongoing maintenance. Moreover, a generic design is the most flexible one. As stated by Crane and Richmond (5), "the biggest challenge to the laboratory design team is to keep the design simple, not to overdesign the laboratory. . . ." Meeting this challenge requires strong leadership by the laboratory administration and the architect. In particular, leading a design project for either new construction or renovation requires the ability to say "no" to requests for special design features.

Laboratory Location

CMLs providing services for a hospital must be fully integrated with the main hospital laboratory (7, 11, 13). This is a critical factor in providing adequate staffing, ensuring proper specimen processing and handling, reducing errors, and increasing efficiency and productivity. While it is true that off-site reference laboratories serve a useful role in providing esoteric testing and/or routine microbiology services for outpatient clinics and physician offices, we believe that there are important reasons why off-site microbiology laboratories do not serve hospitals well. First, use of off-site laboratories may result in delayed specimen processing, which can adversely affect microbial recovery and timely reporting of results. Second, use of off-site laboratories decreases interaction between microbiologists and clinicians. This is of particular concern when there is a lack of clinician input into specimen processing, which directly affects the clinical interpretation of microbiological tests. Third, as previously mentioned, loss of integration with the rest of the clinical

laboratory, particularly specimen receipt and processing areas, increases the risk of incorrect specimen processing and medical errors. Fourth, laboratory staff members in different laboratory sections are unable to interact on a frequent basis, provide coverage for one another, and act as a cohesive unit. Fifth, added complexity and cost are associated with off-site laboratories (e.g., reliable, timely transport of specimens). Sixth, location of the CML close to the main laboratory facilitates access during off-hours and simplifies security. A laboratory that is remote to the main laboratory is unlikely to be visited by clinicians during off-hours, if ever. Last, location of a CML distant to the site of patient care precludes meaningful training of clinical microbiologists, infectious disease physicians, and other health care professionals (7, 11).

Laboratory Size

Only broad generalizations can be made regarding laboratory size. As a general rule, each bench technologist needs a minimum of 50 ft^2 in which to work, excluding space for large pieces of equipment, walls, corridors, storage, lockers, and offices. Areas such as that used for specimen processing require ample space to accommodate the necessary equipment, the specimen receiving bench itself, and foot traffic. Laboratory sections such as mycobacteriology, mycology, and virology require a larger amount of space relative to the number of technologists who work there. Areas such as a dedicated autoclave room or medium preparation room require additional space. If the number of technologists and supervisors is proportional to the test volume and the laboratory is well designed with little wasted space, the space allocated for the laboratory should be between 150 and 200 ft^2 per staff member.

Laboratories should be designed with more space than is needed for current workloads and types of services provided. This serves three purposes. First, an efficient laboratory always has the space needed to accommodate short-term expansion of the types and volumes of services provided by that laboratory. Second, extra space (often referred to as swing space) can be used when part of the laboratory is being renovated or repaired. Third, extra space can be used for future expansion of services. It is unwise to design and build a laboratory to meet current needs only.

Transportation

As with other laboratory sections, an effective transportation system is crucial for providing clinical microbiology services. A variety of transportation systems are in use in hospitals today, including manual transportation, robotic systems, and pneumatic tube systems. Manual systems are notoriously unreliable, particularly in large medical centers, and the associated personnel costs make this approach one of the most expensive. While the capital costs of robotic and pneumatic tube systems are high, over time these costs may be less than those associated with a manual transportation system. Robotic systems are not as widely used as pneumatic tube systems for transportation throughout hospitals. Pneumatic tube systems are widely used in hospitals and have been proven to be an effective method for transporting some types of specimens to clinical laboratories. If they are well designed and maintained, they can play an important role in reducing the costs of transportation, decreasing the number of lost or delayed specimens, and reducing test TAT. In general, however, dedicated pneumatic tube systems are not as useful for CMLs as they are for chemistry and hematology laboratories. This is because most microbiologic tests do not have the same expectation for test TAT, many pneumatic tube systems cannot transport specimens as heavy as full blood culture bottles, and containers used to collect specimens such as urine are prone to leakage when shipped through pneumatic tube systems. Other automated transportation systems are available, but, as with pneumatic tube systems, any benefit to a CML is not as great as it is for other clinical laboratory sections.

Building Codes and Architectural Standards

Laboratorians who have never been involved in the construction or renovation of a laboratory are often unaware of building codes and architectural standards (1). Although it is the responsibility of the architects and engineers to ensure that the plan meets the appropriate codes and standards, laboratory staff members who are involved in a project should understand these issues sufficiently to avoid making requests that are not in compliance with codes and standards. There are definite and specific limits to the way a laboratory can be built.

Interior Design

The design of laboratories that are part of health care systems often involves meeting organization design standards. In some organizations, the types of materials that are used for construction, color schemes, and many other architectural details are specified from the outset. For some organizations this makes a great deal of sense, as a standard interior design lends a sense of continuity throughout the organization. In many instances it also reduces construction and renovation costs, as the organization can purchase materials in large quantities. For other organizations, however, it makes little sense to adopt interior design schemes developed for one part of the organization for a laboratory. Moreover, not all design schemes are successful, so an organization should not be reluctant to abandon one scheme in favor of a better one. At all costs, one should avoid trendy interior design schemes, as they are less likely to please a large number of employees, become dated more quickly than do more traditional design schemes, and tend to be more expensive. It should always be remembered that one might have to live with a laboratory or office for many years.

Technology

Much of the same logic that applies to interior design also applies to the use of advanced technology in a laboratory. Laboratorians, architects, engineers, and designers are all enamored with technology, and there is a strong temptation to use the most advanced technology in a new laboratory or when renovating an existing one (5). Nonetheless, one should assess carefully the cost of advanced technology versus less advanced but proven technology, evidence that advanced technology has been used successfully in comparable laboratories, and any evidence of long-term durability. Advanced technological products may or may not be better than traditional ones, but the newer they are, the less experience there is with them. Moreover, some advanced technology products are trendy and should therefore be avoided for the same reasons that trendy interior design schemes should be avoided. There are reasons why traditional built-in laboratory casework continues to be used widely and why certain commercial products and vendors have been around for decades.

SPECIFIC DESIGN ISSUES

Laboratory Layout

The laboratory layout is crucial for achieving the goals set forth in the introduction to this chapter. The overall layout of a clinical laboratory is determined by the types of services provided, the numbers and types of specimens that are processed, the physical constraints of the building, and the resources that are available for the construction or renovation project. A laboratory that provides only patient care services has needs that are different from those of one engaged in teaching and/or research. Busy clinical laboratories must be efficient to maximize specimen throughput and to minimize test TAT. Laboratories that support teaching and research missions also need to be efficient but must have the space and facilities to support the broader missions. Decisions regarding the overall layout of the laboratory should be made first and should be immutable during the design process.

Most CMLs are divided into sections according to the way that specimens are handled in those laboratories (e.g., a bench devoted to urine cultures, another to blood cultures, and so on) and/or by discipline (e.g., a mycology section). There are obviously many different ways to organize a laboratory. As noted above, the best approach is to use a generic design so that equipment, staff, and processes can be moved as necessary. Areas that require biosafety level 3 conditions cannot be easily moved, so the laboratory staff should take extra care in choosing a location for these areas.

Specimen Preparation Area

All CMLs need an area to receive and process specimens. This area should be located near the laboratory entrance so that other laboratory staff and couriers do not need to enter the rest of the laboratory to drop off specimens. The area should have ample benchtop space for receiving specimens, for any necessary equipment, and for the handling of specimen requisitions. This area should have one or more class II biological safety cabinets (BSC) to accommodate all initial specimen processing. All necessary information technology (IT) infrastructure and telecommunications equipment should be provided. This area should have a sink for handwashing and for performing Gram's stain and other direct examinations. Some laboratories place a microscope in this area for reading stains, whereas others place microscopes in a separate area of the laboratory. A refrigerator should be located in the specimen preparation area to hold specimens and media. Some laboratories do not have separate incubators for the specimen processing area, opting to place inoculated media directly in the main laboratory incubators, but unless specimens can be placed in these incubators quickly, a holding incubator should be located in the processing area.

The specific design of a specimen preparation area needs to be tailored to the number of specimens that are received, as well as to the types of microbiologic tests that are performed. There is no single design that is optimal for all laboratories. For this reason, and because this is often one of the busiest areas of any microbiology laboratory, the specimen preparation area should receive emphasis during the design phase of the project. The perspective and wishes of experienced technologists are especially important here. Function should drive design.

General Laboratory Bench Space

Work Areas

Many laboratories are constructed on the basis of U-shaped modules or linear benches. Modules typically measure 10 by 10 ft and can accommodate two or three persons. Advantages to the modular approach include minimized foot traffic in work areas, generous countertop and storage space, corner space that is available for computer workstations (corners otherwise are wasted space), an increased sense of privacy for workers, and use of less floor space for aisles and corridors. Advantages to the linear bench approach include ease of cleaning, ease of moving about the laboratory, a subjective sense of less clutter, easier location of large pieces of equipment such as incubators and refrigerators, ease of moving modular casework, and lower design, construction, and renovation costs. Some laboratories use a combination of modules and linear benches. In many cases, the physical layout and constraints of the building determine which approach is best for a given laboratory.

The space surrounding workbenches should be sufficient to accommodate ample waste containers, both for paper and for biohazardous waste. It is inefficient for laboratory staff and housekeepers to empty trash containers more often than is necessary. Ample space should be provided so that aisles are unobstructed and free of clutter, yet aisles that are too wide merely waste space.

The laboratory should contain space for storing completed cultures, stock cultures, reference books, and teaching materials (e.g., parasitology slides). It is most efficient for completed cultures and reference materials to be located close to workbenches. The principle applies to frozen, lyophilized, or viable cultures.

Casework

Laboratory casework can be built-in (custom) or modular. The type of casework selected for a laboratory is, to a large degree, determined by the size of the laboratory. It is expensive to install custom built-in casework in a large laboratory; vendors are able to give better pricing for installing modular furniture in larger facilities. Thus, larger laboratories almost always find it to be more economical to buy commercial modular furniture. Smaller laboratories, on the other hand, may find the opposite to be true. Before making a decision, one needs to be familiar with the advantages and disadvantages of each type of casework.

Built-in casework typically is less expensive to install, can be made from a wider variety of building materials to suit individual tastes, can be very strong, and is durable. It is not unusual to see built-in laboratory casework that is decades old but is still functional and aesthetically pleasing. The disadvantages to built-in casework are that it cannot be moved easily, repairs tend to be more difficult and expensive, modifications (e.g., adding additional power and communication lines) also are more difficult and expensive, and, for the most part, it cannot be reused once it is disassembled. From an interior design standpoint, it has the disadvantage of requiring users to live with the aesthetic choices of predecessors and those characteristic of previous eras.

Modular furniture has been, in part, designed to overcome the disadvantages associated with built-in casework. Specifically, some parts of it can be moved easily, repairs and replacements are easy, modifications are easy and less expensive, and entire units can be disassembled and moved to another location. The converse, however, is equally true: many of the advantages associated with built-in casework are not found in modular furniture. Specifically, modular furniture tends to be more expensive (unless one is buying large quantities, decreasing the unit cost), there is a smaller selection of building materials and colors to suit individual aesthetic tastes, and it tends to be less strong and durable.

It should be noted that one of the main advantages to modular furniture, the ability to move it around, tends to be overstated. This is because the stanchions that support the casework are bolted to the floor, plumbing and drains for sinks are not easily moved, and there are practical limits to moving the electrical power supply and IT and telecommunication cables. In fact, with some commercial modular casework systems, only the cabinets, shelves, and countertops can be moved, and even they can be moved only within the confines of the supporting stanchions.

Air, Gas, and Vacuum Supplies

Most modern laboratories have little need for compressed air, gas, and vacuum supplies, except as needed for specific purposes. Use of open flames should be prohibited, so there is no need for a flammable gas supply. Most health care facilities do not have central systems to supply the types of gases used in microbiological incubators. These should be included within the laboratory facility.

A generous amount of space should be allocated for incubators, refrigerators, freezers, floor-model centrifuges, and floor-model diagnostic equipment (e.g., continuous-monitoring blood culture systems). Electrical power, water supply, telecommunications ports, and other features necessary to support such equipment should be part of the design. The load capacity of the facility should be such that it can support the weight of this equipment, particularly if most of it is to be located in one part of the laboratory.

Special Laboratory Bench Space

Anaerobic Bacteriology

The anaerobic bacteriology capacities needed by most hospital-based CMLs can be accommodated by countertop jars. For larger laboratories, or those with a special interest in anaerobic bacteriology, sufficient space should be allocated to accommodate an anaerobic chamber and gas supply. Countertop models have replaced most of the floor-model anaerobic tents that once were common in laboratories. Some gas-liquid chromatographs require a dedicated exhaust system for discharged gases. Most of the other equipment needed for anaerobic bacteriology does not require special design features.

Mycobacteriology, Mycology, and Virology

An important design requirement for many mycobacteriology, mycology, and virology laboratories is that the facility should meet biosafety level 3 criteria, as described in the following section, in order for the staff to safely handle pathogenic microorganisms such as *Mycobacterium tuberculosis*. In addition to biosafety issues, these laboratories require special equipment not used elsewhere in the CML, such as refrigerated centrifuges, special diagnostic equipment, and the incubators required to hold specimens at various temperatures. Equipment for performing tissue cultures may also be needed. Mycology and mycobacteriology laboratories must have a certified class II BSC.

Biosafety Level 2 and 3 Conditions

Most routine clinical microbiology procedures can be performed safely under biosafety level 2 conditions (15). Even though biosafety level 2 conditions can be met without use of a BSC, use of a BSC protects laboratory workers from laboratory-acquired infections and protects specimens from contamination (8, 16). Moreover, laboratory staff do not always receive sufficient information with specimen requisition forms to know which specimens are likely to contain pathogens that are risky to process (3). Therefore, it is strongly recommended that all specimen processing be done in a class II BSC. Isolates or cultures that are known or likely to contain high-risk microorganisms should be processed only in a class II BSC, particularly fungal and mycobacterial cultures (3, 15). If possible, positive blood and cerebrospinal fluid cultures should also be processed in a class II BSC. Adequate space, power, and ventilation should be provided for each BSC.

Biosafety level 2 conditions are met largely via use of standard microbiological practices (15). In addition to these practices, biosafety level 2 conditions require that (i) laboratory personnel have specific training in handling pathogenic agents, (ii) personnel be directed by competent scientists, (iii) laboratory access be limited when work is being conducted, (iv) extreme precautions be taken in handling contaminated sharp items, and (v) procedures likely to generate infectious aerosols or splashes be conducted in either a class II BSC or other physical containment equipment (15).

Biosafety level 3 conditions include all of the requirements for biosafety level 2 conditions plus special facilities, equipment, and procedures for handling "pathogenic and potentially lethal agents" (15). For those CMLs that do not have the facilities specified for biosafety level 3 conditions, routine microbiologic procedures should be done using biosafety level 2 conditions. Biosafety level 3 practices and protective equipment should be used when handling agents that pose a risk of serious or lethal infection. The specific requirements needed to meet biosafety level 3 conditions are given in reference 15. In brief, these include (i) limited laboratory access; (ii) written policies and procedures for handling agents; (iii) adequate training, proficiency, and competency for handling agents; (iv) use of a class II BSC for handling highly infectious agents; (v) use of adequate face and respiratory protection for procedures done outside a BSC; and (vi) written policies and procedures for handling spills. The facility requirements are given in reference 16. In brief, these include (i) a separate area with access through two sets of self-closing doors; (ii) sealed floors, walls, and ceilings to facilitate decontamination; (iii) a waste disposal system that is available within the area; (iv) a ducted air system that draws clean air from outside the area, with all of the exhaust air (i.e., none of the air is recirculated) discharged to the outside; and (v) the use of HEPA filters in the exhaust of BSCs, in vacuum lines, and in equipment or devices that may produce aerosols or splashes (e.g., centrifuges).

Molecular Microbiology

As for the general microbiology laboratory, planning for a molecular diagnostics laboratory should optimize workspace flexibility, since this technology is rapidly changing and new equipment, methods, and applications will be available in the next few years (6, 12, 14). Prior to establishment of molecular testing, thought must be given to the type of services planned. For example, if the laboratory staff plans to perform only Food and Drug Administration-approved and commercially available kit-based assays, then minimal space and equipment may be all that is needed. Alternatively, if the laboratory staff plans to develop and perform in-house assays, then a significantly greater investment in space, equipment, and technical expertise will be required. In addition, consideration must be given to the specific assays that will be performed, as space and equipment needs vary considerably for different types of assays.

If the laboratory staff plans to perform only commercial amplification kit-based assays, bench space will be needed to accommodate a hood-type work enclosure with a UV light source, an amplification and detection apparatus(es), and a few other pieces of equipment, such as a water bath and a microcentrifuge. As little as 15 linear feet of bench space is needed. To reduce the risk of contamination, two geographically separated work areas are needed. Most commercial kit-based assays include methods to prevent carryover amplification, and automated equipment also may reduce the possibility of carryover, both of which minimize the amount of space that is needed. According to the manufacturers of some commercial diagnostic molecular assays, certain assays can now be performed in a single area and with only minimal need for special handling. Experience will show whether this is an acceptable approach, but it is likely that as the technology of molecular diagnosis changes, there will be a decreasing need for separate areas. As this occurs, laboratories will be able to fully integrate diagnostic molecular assays with the rest of the CML.

Development, validation, and performance of in-house methods that involve amplifying nucleic acids require a more elaborate laboratory. A minimum of 500 ft^2 is required for a small molecular diagnostics laboratory, with three separate workspaces. The three work areas include one for reagent preparation, one for specimen preparation, and a third for amplification. General analysis of DNA or RNA requires an assortment of basic laboratory equipment, as well as specialized equipment. Sources of high-grade deionized water, wet ice, and dry ice are needed, as is an autoclave. A fume hood and storage cabinet for solvents and flammables are also needed. Access to a darkroom is advantageous.

The reagent preparation area is the cleanest of the three work areas and can be located in a separate room, in a hood with the fan turned off to minimize aerosolization, or in an enclosed countertop hood or box. This area is used for the preparation of the master mix and other necessary reagents. To minimize contamination, patient samples, prepared DNA, and amplified products must never be brought into this area. Since equipment can become contaminated with DNA, it is imperative that dedicated equipment be used and stored within this area. Reagents should be stored in a refrigerator free of DNA (and especially amplicons), and disposable items such as tubes, tips, and gloves should also be stored in a manner to prevent their contamination. Staff should wear a designated clean laboratory coat along with clean gloves; staff should leave transportable items (e.g., pens, tape, and scissors) in this area.

The second area is the specimen preparation area. In this area, tubes containing the aliquoted master mix should be brought for the addition of nucleic acid; these tubes should not be returned to the reagent preparation area. Specimen preparation activities can be performed in the open laboratory on a bench but might also be performed in an enclosed space such as a benchtop hood or containment box. After nucleic acid is added to the reaction tubes in this area, the tubes are sealed and placed in the thermocycler for amplification. When removed, the tubes should be taken unopened to the third area for product detection.

The third area is the most contaminated of the three work areas. Laboratory staff should take extreme caution to ensure that laboratory coats, gloves, tube racks, and other equipment are never moved back into the reagent or specimen preparation areas. Some laboratories have opted for separate areas for a darkroom/gel electrophoresis room, a room for processing of radioactive samples, and a room for other functions.

Work Flow

Efficient laboratories are designed so that specimens flow in one direction. Traditional microbiologic testing follows a pathway of specimen receipt and plating, incubation, isolate identification, antimicrobial susceptibility testing, and result reporting. Because this sequence is by its nature linear, CMLs benefit from unidirectional work flow. Unidirectional work flow is also important in molecular microbiology laboratories. In this case, the issue is not so much one of efficiency but rather one of minimizing the risk of amplicon carryover and specimen contamination. While much of this can be accomplished by designing unidirectional work processes, the laboratory facility must accommodate the needs of those processes.

Laboratory Storage

Storage space should be adequate but not excessive. Insufficient storage space makes for a cluttered laboratory; unused storage space makes for a dusty one. Short-term storage capacity should meet the daily needs of the staff and no more. Long-term storage of supplies should be in the main laboratory storage room; there is no reason for long-term storage in the CML itself. Storage space, whether on shelves, in overhead cabinets, or in drawers, should be designed so that workers have most of what they need during the day within arm's reach. Storage space should be designed so that it can be cleaned easily.

Incubators, Refrigerators, and Centrifuges

CMLs should have ample space for large floor-model incubators, refrigerators, and centrifuges. Sufficient aisle space should be allocated next to large units so that the aisle remains unobstructed when a door is opened. Adequate electrical power should be incorporated into the area housing these units, as well as any necessary gas or water supplies. The location of these units should help maximize laboratory efficiency and ensure a unidirectional work flow. As a general rule, a refrigerator and centrifuge should be located in the specimen processing area. Although some CMLs have adopted the principle of scattering incubators and refrigerators throughout the laboratory, with the rationale of minimizing the distance from workbenches, a more efficient use of space is to have a separate area in the laboratory for these large pieces of equipment. This is because these units almost always are wider than countertops are deep, so locating them throughout a laboratory requires the aisles to be wider than would otherwise be necessary. Because the additional space in the other parts of the aisle is not needed, a significant amount of floor space will be wasted.

Handwashing, Water Supply, and Plumbing

The CML should have sufficient sinks to accommodate staining, waste disposal, and handwashing. To prevent laboratory-acquired infections (3, 16), handwashing sinks should be designed so that they can be operated with knee or foot controls. One sink should be located near the laboratory entrance to facilitate handwashing by staff and visitors as they leave the laboratory. Drainpipes should be able to handle the types and volumes of liquid waste generated within the laboratory.

Plumbing is expensive to install at any time but especially so once laboratory construction is completed. In addition, the plumbing and water supply cannot be moved easily in

modern buildings, as one must drill through a concrete floor and then work in the ceiling space of the floor below. This space typically is filled with heating, ventilating, and air-conditioning (HVAC) systems, lighting, IT and telecommunications cables, and electrical power lines, making it difficult and expensive to work there. Such a project is also disruptive to the occupants of the floor below. Therefore, the water supply and plumbing needs should be assessed carefully during the design phase of the project.

Countertops

Countertops at workbenches should be 24 in. deep, with work areas no more than 4 to 5 ft wide. A working space of these dimensions is the most efficient because the entire space is within arm's length for most workers. There should be additional space to each side for storage and equipment. Some countertops will need to be deeper to accommodate countertop-model equipment. Heavy pieces of equipment should be on freestanding tables designed to accommodate the necessary weight. Centrifuges should also be on freestanding tables to minimize the transfer of vibrational forces to adjacent countertops. Sensitive analytical balances should be on freestanding tables. Most modular casework systems include tables that are of the same length and width as the individual countertop pieces and which can be integrated into the overall laboratory layout.

The height of workbenches can be that of a desk (29 to 30 in.) or a counter (36 in.). While there are advantages to both heights, use of one or the other is largely a matter of personal preference. Modular furniture can be adjusted to either height, whereas built-in casework obviously is fixed at one height.

Countertops should be made of materials that can be cleaned and disinfected easily, are durable, and can be replaced or repaired as needed. Most countertops in CMLs do not need to be acid resistant. Countertops that are stain resistant are desirable. Countertops adjacent to sinks should be constructed of water-resistant materials so that repeated exposure to water does not damage them. Lighter colors tend to show stains more but have the benefit of making the laboratory brighter. Practical issues aside, colors generally are selected on the basis of interior design needs and personal preference.

Electrical Power Supply

Electrical outlets should be liberal in number and in excess of the current need. These are easily installed during construction, but upgrading the electrical power supply can be expensive and difficult once construction is completed. One important task during the design phase of the project is to perform a comprehensive audit of the electrical needs of the laboratory, particularly as related to 110- versus 220-V power supplies. The need for emergency power supplies should also be assessed, as this requires a separate electrical power supply. Only critical pieces of equipment should be on the emergency power supply. Critical equipment should be wired into a central alarm system so that the appropriate persons can be notified of any power failures.

HVAC

The HVAC system in a clinical laboratory must be designed so that the laboratory meets the necessary biosafety level while maintaining a constant ambient temperature within a narrow range. Thus, the design of an HVAC system is a challenging task for architects and mechanical engineers. Moreover, once installed, an HVAC system cannot be easily modified: it is one of the most expensive parts of construction, it resides in a relatively inaccessible space, and changing the HVAC in one area of a building may have effects in other areas of the building.

Installing an HVAC system is one of the more expensive parts of laboratory construction. Because revising an existing HVAC system can be expensive or impractical, it is imperative that the design and installation be done correctly. Balancing the airflow in a CML is challenging: (i) CMLs must have lower air pressure than in adjacent areas; (ii) air temperatures in a CML must be maintained within a narrow range; (iii) exhaust air from a CML cannot recirculate into the building; and (iv) the HVAC system must accommodate special needs for odor control. Although it is desirable to use special plenums that draw off odors from workbenches (which is of particular concern in the specimen processing area, parasitology section, anaerobic bacteriology section, and autoclave room), these can exhaust large amounts of air from the CML and make balancing the airflow difficult. For all these reasons, adjustments to the HVAC system may be necessary after the laboratory has been occupied.

IT and Telecommunications

General Considerations

The modern clinical laboratory is highly dependent upon IT and telecommunications systems. Modern telecommunications systems are complex and need to support telefax units, sophisticated telephone systems, videoconferencing, and other types of telecommunications. In the same way, modern IT systems need to meet both current and anticipated needs such as the introduction of new versions of operating systems, databases, and other applications. The IT infrastructure should accommodate the larger institution's (e.g., hospital's) health care information system, the laboratory information system, the facility intranet, and the Internet. Ideally, the IT infrastructure should be designed for high-speed access, have redundant data storage and processing capacity, and have sufficient types and numbers of workstations to accommodate changing IT needs. Because the most efficient laboratories are paperless, it is important that the IT infrastructure support this type of environment.

For many laboratory staff members, and certainly for clerical and administrative staff, most of the day is spent using a computer. The computer workstation has become the focus of most offices and many laboratories. A robust IT system supports a variety of applications that are of immediate benefit to the laboratory staff. Any modern laboratory should have the infrastructure needed to support a modern IT system. In particular, the cable plant should be generic in design and have a fiber-optic backbone with a fiber-optic cable to each outlet in the laboratory. This cable architecture accommodates current IT needs but will also facilitate future transition to voice and video capability. There should be redundancy in the network infrastructure. IT and telecommunications systems should be designed so that information retrieval is limited to those who are authorized to access patient records. The design and installation of the IT and telecommunications systems in a laboratory should be done in close collaboration with the information services within the institution. Once systems have been installed, it is expensive or impractical to make changes to the IT and telecommunications infrastructure.

Wireless Networks

The increased use of devices such as wireless-capable laptop computers as workstations, personal digital assistants for data storage and retrieval, pen tablets, and "smart" cellular telephone systems makes it necessary for health care facilities to have wireless networks. The primary advantage of wireless systems is that users can receive and transmit data signals from any location rather than from only those locations that are cabled for network access. Wireless systems can also be used for dispatch and communication for employees who, by the nature of their work, need to be contacted at sites removed from traditional workstations. This type of system is often a Voice over Internet Protocol (VoIP) system, which operates much like a cell phone but works only over the wireless network in a facility. Another wireless application is the use of radio frequency identification, which can be used to tag and track items by using a simple web-based interface.

Offices and Administrative Support

Office space should be located close to but not within the laboratory. Offices should be sufficient to support the managerial, administrative, research, and teaching functions of the laboratory. Just as it makes little sense for a clinical laboratory to be located off-site, it makes equally little sense for the office space that ostensibly supports a laboratory to be in a remote location. Successful clinical and faculty recruitment and retention often hinge on adequate office space. Compared with other parts of a health care facility, office space is inexpensive to build, maintain, and renovate. It is in the long-term interests of the institution to make the modest investment needed for this part of the laboratory.

The process of designing offices should follow the same principles as those that guide the design of a clinical laboratory: offices should be private, quiet, efficient, and pleasant places to work or to visit. Although some institutions have opted for an open office plan where low modular barriers separate offices, open offices do not provide the privacy necessary for counseling employees or for discussing confidential matters, nor are they quiet. Offices should be equipped with the office equipment, IT and telecommunications infrastructure, and other features that are necessary to make them efficient. Many offices that are associated with clinical laboratories are too small to be pleasant places to work or to visit. Although offices should be sufficiently large, offices that are too large waste space, which never is in excess. As a general rule, individual offices should be no smaller than 100 ft^2. Traditional furniture is less efficient than modular furniture; office size should be adjusted according to the type of furniture that is used. Laboratory directors and supervisors should have offices that are large enough to accommodate several persons during meetings. Offices of up to 200 ft^2 are not unreasonable for directors.

Work Environment

Ergonomic considerations should receive emphasis in laboratory design. Casework, drawers, shelves, keyboard trays, lighting, and space should all be designed according to ergonomic standards. Both ceiling and task lighting should be abundant and well placed. Signs, bulletin boards, and other means of communication should be placed where laboratory staff and visitors can easily see them. A modest investment in the ergonomic and aesthetic properties in the laboratory will go a long way toward making it a pleasant and efficient workplace.

Safety and Security

As stated in the introduction, above all other considerations, a laboratory should be a safe place. For general safety considerations, this can be achieved by ensuring that the laboratory meets all building codes and architectural standards related to safety. For safety considerations related to the practice of clinical microbiology, this can be achieved by designing the laboratory to meet biosafety level 2 criteria and, where necessary, biosafety level 3 criteria.

Clinical laboratories must meet the safety and security requirements of accrediting and regulatory bodies. Eyewash stations, safety showers, sprinkler systems, fire extinguishers and blankets, fire alarms, spill control kits, and emergency power and lighting should be included as specified by building codes. Water supplies should have either backflow preventers or vacuum breaker devices to prevent the inadvertent contamination of potable water supplies.

The laboratory should be designed so that it can be secured during off-hours, or when staffing is minimal. Institution-specific security concerns will guide the need for more comprehensive security assets such as security cameras, restricted access, or electronic monitoring of access.

Cleaning and Waste Handling

A clinical laboratory should be designed so that it can be cleaned easily. All surfaces should be made of materials that are easily cleaned and disinfected. Carpet should never be used as a floor material in a clinical laboratory. To facilitate cleaning, floors should be kept free of clutter; there is no excuse for using floors in a clinical laboratory for storage space.

The laboratory design should accommodate the handling of the large amounts of biohazardous waste that are generated each day. There should be adequate space for waste containers within the laboratory, for biohazardous waste as well as standard waste. Large bins for both types of waste should be located outside the laboratory but nearby. Large pieces of floor-model equipment should be permanently housed on wheels so that they can be moved easily for cleaning. A housekeeping closet should be located close to the laboratory to facilitate daily cleaning.

Some laboratories maintain an on-site autoclave for medium preparation or decontamination of wastes. Some smaller autoclaves are self-contained, but larger autoclaves require steam lines that must be taken into account early in the design process.

THE DESIGN AND CONSTRUCTION PROCESS

Predesign Phase

Beginning the Process

Architecture, engineering, and construction are complex disciplines. Just as one cannot expect a clinical microbiologist to be familiar with the terminology, processes, and regulations of those disciplines, one should not expect an architect, engineer, or contractor to be familiar with the terminology, processes, and regulations that guide the day-to-day operations of a clinical laboratory. Thus, perhaps the most important skill needed to initiate and complete a design or renovation project is communication. Successful design, construction, and renovation projects are characterized by a commitment to education and communication. This is of particular importance when any of the parties who

are involved in the project have little or no prior experience in designing or renovating a laboratory. It is not unusual to meet architects, engineers, or contractors who have no experience with clinical laboratories, just as it is not unusual to meet laboratorians who have never been involved with a laboratory construction or renovation project. It is also not unusual to meet architects, engineers, or contractors who have experience with research or industrial laboratories but not clinical laboratories. Experience with the former does not necessarily translate into expertise in the latter. To facilitate communication and education, it is important to have a clear understanding of the experience and expertise of all the parties who will be involved with the project.

Laboratory Representative

The first step in designing a laboratory is for the laboratory administration to appoint a person who will be the spokesperson throughout the design and construction process. Such an appointment should not be made or taken lightly; this is a significant commitment of time and energy that will extend over one or more years. The laboratory administration must give this person the time needed to meet the commitment, as well as the responsibility and authority to make many decisions, often on short notice. Because so many persons are involved in construction and renovation projects, because projects take so long to complete, and because the staff must live with the outcome for many years, the laboratory representative must have a clear understanding of budget constraints, space allocations, laboratory operations, and hospital and clinic operations. There must be clear lines of communication and authority. The laboratory representative should be selected carefully on the basis of communication skills, experience in laboratory administration, and decision-making ability. This person need not be the laboratory director, although the director generally is in a better position to negotiate for necessary resources than is a senior technologist.

Budget and Space Allocations

The next step in designing a laboratory is to assess, realistically and accurately, the current and future needs of the laboratory, as well as the resources required to meet those needs. Both new and renovated laboratories should be designed to accommodate future expansion of test volume and type. This includes planning for office space, specimen receiving and processing, benches, informatics, communications, and the introduction of automation and future test technologies.

The next step is to obtain realistic design and construction budgets. Hospital laboratory construction is expensive. Nonetheless, fiscal constraints should not interfere with designing and building a safe and efficient laboratory. Along the same lines, realistic allocations of space must be done prior to the design phase. It is expensive and wasteful to design a laboratory and then to be faced with redesigning all or part of it to accommodate changes in the budget or space allocations. An adequate investment to do the project right in the first place is the most economical approach to providing a functional laboratory.

Part of developing a realistic and accurate budget is to decide whether to build a new laboratory or to renovate an older one. This obviously involves decisions about the health care facility as a whole. Estimating the costs of new construction (whether for a new building or expansion of an existing one) is generally straightforward. In contrast, estimating the costs of renovations is challenging. First, original construction drawings may not be available or may no longer be accurate due to changes made after the original construction was completed. Thus, the architect and engineer may have limited knowledge of how the laboratory was constructed or modified. Second, laboratories built according to past architectural standards and building codes may not meet current standards and codes. Third, older facilities are likely to contain asbestos in insulating material and/or floor tiles, as well as lead-based paint. Abating either material is a complex and expensive process. Fourth, older buildings were often built with fixed ceilings and walls, complicating changes in lighting, the HVAC system, IT and telecommunications cabling, plumbing, and the electrical power supply. Fifth, some older buildings do not have the load capacity to support heavy pieces of equipment. Last, construction in older buildings often reveals unexpected issues that must be addressed, adding to the cost and complexity of the project. It is for these and other reasons that it may cost more to renovate an existing building than it would to build a new one.

Design Phase

Several general principles should guide the design phase (10). First, the greater the investment in planning, the more likely a project will be completed on time and under budget. Second, users will be happy with the results of a project only if they believe that they have been able to make significant contributions to it. Third, there need to be clear lines of accountability and communication. Fourth, user expectations should be expressed clearly and unequivocally at the outset; no architect or engineer should be expected to make major changes midway in a project. Last, logic and common sense should guide decisions throughout the process (5).

The first task for the architect, designer, and engineer (the design team) is to understand who the laboratory representative will be, who will have authority to make decisions, and the limits of the budget and space allocations. The next task is for them to learn the needs of the laboratory. These tasks should be undertaken in this order; it is important for the design team to know the constraints of the project before the design phase begins. Once the design team has completed these tasks, the knowledge gained can be used to develop realistic and workable design plans. As the design phase continues, it is for the most part largely one in which the design team works with the laboratory staff to accommodate the needs of the laboratory within the constraints of the project. In many cases, there will be insufficient space or funds to accommodate the needs of the laboratory; in cases such as this, either the laboratory administration must acquire the necessary space or funds or the project will need to be scaled back.

Once preliminary design plans have been drafted and agreed upon, the laboratory staff must address the myriad of details necessary to design a functional laboratory. Every conceivable detail should be thought of and addressed. Nothing should be taken for granted. It is far less expensive and easier to remove features from a design plan than it is to add them at a later point, particularly during the construction phase. The latter process, known as change orders, is one of the most common reasons for construction projects to go over budget. An even thornier problem is the feature that should have been included but cannot be added at any cost once construction has begun.

After final construction documents have been drafted and approved, bids will be requested from general contractors.

Any necessary specifications should be included in the request-for-bids document. Once a general contractor has been selected, that contractor will recruit subcontractors to provide services that the general contractor does not provide. The last step prior to construction is for the institution and general contractor to obtain all necessary building permits and other legal documents needed for the project.

Construction Phase

For new laboratory construction, the construction phase generally proceeds for some time before the laboratory representative needs to be involved. The point at which the laboratory representative needs to work closely with the contractor is when the contractor begins to install walls, electrical power, IT and telecommunications cables, plumbing, and casework. From this point on, the laboratory representative will be called upon to make many unexpected decisions, often on short notice, and to clarify ambiguities in construction documents. It is expensive to make changes at this point in the process, but necessary changes should be made at this time rather than years later, when costs are even higher and the disruption in the laboratory makes the changes impractical.

Renovation projects vary in the amount and types of construction that are needed. Many projects require minimal demolition and few changes to the HVAC, plumbing, electrical power supply, and telecommunications and IT systems. In projects such as these, many of the changes are in casework only, or are more cosmetic in nature, and can be accomplished while the laboratory continues to operate. For more extensive renovations, the required demolition makes it necessary to gut the laboratory, in which case the project more closely resembles that of constructing a new laboratory.

Postconstruction Phase

The postconstruction phase is one of the most important, yet often neglected, phases in the process. Almost all construction projects have a warranty, usually for a short period, and the postconstruction phase provides the opportunity to use the warranty to correct things that were not completed or were done incorrectly. The laboratory staff should monitor carefully everything associated with the project, record their findings, and communicate them to the general contractor at the earliest possible time. The longer one waits, the more difficult it becomes for the contractor to return to the site to correct problems. Once the warranty has expired, then it becomes expensive to fix something that could have been done earlier at no cost.

SUMMARY

The construction of a new clinical laboratory, or the renovation of an existing one, is an excellent opportunity to improve the operations and efficiency of a clinical laboratory. It also offers the laboratory staff an opportunity to assess existing laboratory processes and to modify them. A successful construction or renovation project is characterized by the investment of large amounts of time in planning and designing the new facility, good communication between all of the parties involved in the project, a logical and common-sense approach to the project, and realistic expectations. Done well, a new or renovated laboratory facility will accommodate the needs of the laboratory staff for many years.

RESOURCES

Some general resources are listed in the references (1, 2, 5, 9, 10). The reader who needs additional information should consult The American Institute of Architects (http://www.aia.org/). The Institute can provide additional information, including a list of architects in a given area who have experience in designing laboratories and other health care facilities.

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Laboratory Consultation, Communication, and Information Systems

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4

The flow of information to and from clinical laboratories was revolutionized more than 30 years ago by the introduction of the first computerized laboratory information system (LIS) (2, 13). The initial rationale for a computerized LIS was purely financial; namely, the LIS enabled more efficient billing for laboratory services. Shortly thereafter, improved reporting of textual and numerical test results was enabled by the evolving feature set. Clinical microbiology laboratories began reaping important benefits when it became possible to issue culture and complex antimicrobial susceptibility reports via the LIS.

LISs continue to thrive in the clinical microbiology laboratory today. These laboratories furnish vital information to health care providers, especially those who manage patients with infectious diseases. Laboratory test results essential for the diagnosis and treatment of infections can be reported in a clear and logically presented manner via the LIS. Additional information that promotes optimum patient management, such as guidance for specimen collection, interpretation of test results, and suggestions for additional testing, can also be added (16). Clinical microbiologists today are brokers of critical information that strongly benefits patient care (20). The goal of this chapter is to review the state of information management in clinical microbiology laboratories today, emphasizing the central role of the LIS.

HEALTH INSURANCE PORTABILITY AND ACCOUNTABILITY ACT OF 1996

A significant event that greatly affects the management of health care information in the United States was the passage of the Health Insurance Portability and Accountability Act of 1996 (HIPAA). HIPAA has had an influence so enormous that it is appropriate to begin this chapter with a description of its impact. One of its primary purposes is to maintain the security and privacy of information found in patient medical records. The standards also address the mechanisms by which information can be coded and exchanged electronically. This exchange includes the distribution of laboratory test data to clinicians, insurers, and patients.

HIPAA governs the manner in which patient-specific health information can be generated, disseminated, and stored in the United States (26). A related regulation intended to assist in the implementation of HIPAA was also promulgated and is entitled *Standards for Privacy of Individually Identifiable Health Information* (the Privacy Rule) (21). It was crafted by the Department of Health and Human Services and became effective on 14 April 2001. Most health care institutions and health care plans were obligated to comply with the Privacy Rule by 14 April 2003. Small health care plans were able to delay compliance until 14 April 2004. Although the provisions of HIPAA and the Privacy Rule do not apply to health care organizations outside of the United States, they establish standards for handling patient information that are relevant to and worthy of consideration by governments in all countries.

The Privacy Rule characterizes the safeguards that health care providers must take to protect the privacy of health information. It establishes standards that protect the medical records and other personal health information of patients. It provides patients more control over their own health data and defines limits on the use and distribution of health records by other entities. It declares that patients are entitled to examine and obtain copies of their own health records and to request that mistaken information be corrected. Patients are also at liberty to find out what disclosures of their health information have been made and to whom. The Privacy Rule holds violators of the standards accountable for their actions by the imposition of civil and criminal penalties. It does, however, permit disclosure of patient-specific health information to public health authorities in order to protect the general public health.

Patient-specific data produced by clinical microbiology laboratories in the United States fall under the jurisdiction of HIPAA. It does not matter whether the information is verbal, written, or electronic. The information may be distributed during the preanalytical, analytical, or postanalytical phase of testing and may be advisory, instructional, results oriented, or interpretive. Clinical microbiologists are expected to deliver this information to health care providers in a timely and comprehensible fashion without violating the tenets of HIPAA.

BUILDING THE MICROBIOLOGY COMPONENTS OF LISs

The LISs in use today offer many tools that support the communication of information between clinical microbiology laboratories and health care providers. In order to capitalize on these tools, the individuals responsible for building the

microbiology component of the LIS must thoroughly understand microbiology testing. Ideally, these individuals should be clinical microbiologists with a strong interest in and commitment to building their module of the LIS. If this arrangement is not possible, the builders of the microbiology module should request and receive close guidance from clinical microbiologists during system configuration and validation testing.

In an increasing number of health care facilities, laboratory tests are no longer ordered directly in the LIS by laboratory personnel but instead are requested by clinicians using a computerized order entry system with an electronic interface with the LIS. Under this circumstance, clinical microbiologists must have major input during the selection and formatting of information that will be seen by those ordering microbiology tests in any of these accessory systems. This may require clinical microbiologists to insist that they participate in the order entry information system building and/or validation process. Too often, laboratory personnel are overlooked when teams are assembled for this purpose.

The ordering of microbiology tests and the viewing of microbiology test results are different from those in other laboratory disciplines. Specimens for microbiology testing are collected from a variety of body sites, and there must be a provision in the ordering information system to inform the laboratory from whence specimens were collected. Because the quality of microbiology specimens often deteriorates with time, the time of specimen collection should be documented during the ordering process. Microbiology test results are usually nonnumerical and consist of complex composites of text and tables of antimicrobial susceptibility data. In some instances, initial microbiology test results prompt the automatic ordering of "reflex" tests (e.g., the ordering of antimicrobial susceptibility tests when culture results are positive).

Test Codes

As they are built into the LIS, all laboratory tests are assigned codes (mnemonics) and code translations. It is advantageous if a rigorously applied convention is used during the code assignment process. Logically devised coding conventions promote the remembrance of codes by LIS users. Most systems permit codes of up to six characters in length and allow codes to contain both alphabetical and numerical characters. Although many laboratories depend on alphabetical codes abstracted from actual test names (e.g., URICUL for urine culture and BLOCUL for blood culture), some laboratories use predominantly numerical coding schemes to match codes in other institutional information systems and to eliminate code conflicts among tests with similar names.

A laboratory test may have more than one name in general use (e.g., syphilis serology and the rapid plasma reagin [RPR] test). It is advisable that cross-references to commonly used synonyms for a test be identified during test building so that user-initiated searches for different names for a test will link to the same test code. Otherwise, LIS users will lose patience when they are unable to find a test that they wish to order and they will order it inappropriately as a miscellaneous test.

Entering Results While Ordering Tests

Some LISs allow or even require that results for one or more components of a test or battery be entered during the ordering process. This is often true for microbiology cultures, in which the specimen description is one of the test or battery components. When specimen descriptions are predictable for tests (e.g., a pharyngeal swab for a pharyngeal culture), it is helpful if the LIS can automatically answer the specimen description with a default specimen code. This streamlines the ordering process and eliminates ordering errors. Of course, default specimen description codes should be overridable when appropriate.

TABLE 1 Sample LIS test and battery names and their pending texts[a]

Name	Pending text
Aerobic blood culture	Results in <5 days
CSF culture and Gram stain	Results in <72 h
Fungal culture	Results in <21 days
GC culture	Results in <72 h
Bordetella pertussis culture	Results in <7 days
Urine culture	Results in <24 h
CMV culture	Results in <48 h (M–F)
Res Vir antigen panel	Results in <24 h
Group A *Streptococcus* detection	Results in <2 h
RPR	Results in <24 h

[a] Abbreviations: GC, *Neisseria gonorrhoeae;* CMV, cytomegalovirus; Res Vir, respiratory virus; M–F, Monday through Friday.

Pending Text

Some LISs can be configured to display informative text automatically while test results are pending (Table 1). Examples of "pending text" that might be used with microbiology tests include the expected turnaround time for test results, a telephone number to call if there are questions regarding the test, or a notice that a reflex test will be ordered if the result is positive. The pending text ceases to display once test results have been entered into the LIS.

Text Code Dictionaries

Perhaps the largest effort during the building of the microbiology section of an LIS is in preparing the text code dictionaries. Several dictionaries must be built, including those for microbiology text codes, microbiology method codes, growth medium codes, workload codes, antimicrobial susceptibility codes, specimen description codes, and microorganism name codes.

During the creation of the microbiology dictionaries, an important decision must be made regarding the assignment of microorganism name codes. The use of a consistently applied scheme that is understood by microbiology laboratory personnel simplifies the entry of culture results, since the correct codes for most microorganisms can be determined "on the fly." One coding convention in common use is to take the first letter of the microorganism's genus name and the first several letters of the species name (Table 2). A limitation of this system occurs when codes for different microorganisms turn out to be identical (e.g., ECOLI for both *Escherichia coli* and *Entamoeba coli*). Another system used by some laboratories is to take the first three letters of the genus name and the first three letters of the species name (assuming that the LIS permits six-letter codes). In this case, the mnemonic for *E. coli* the bacterium would be ESCCOL and that for *E. coli* the protozoan would be ENT COL. However, problems still occur in assigning codes for microorganisms like *Oligella ureolytica* and *Oligella urethralis* (OLIURE in both instances). Any coding system utilized will encounter its own set of problems, and thus a secondary

TABLE 2 Sample microorganism name codes and their translations

Microorganism code	Translation
AACTI	*Actinobacillus (Haemophilus) actinomycetemcomitans*
ABAUM	*Acinetobacter baumannii*
ABIOSP	*Abiotrophia (Granulicatella)* sp. (nutritionally variant *Streptococcus*)
ABOVI	*Actinomyces bovis*
ABSISP	*Absidia* sp.
ACANSP	*Acanthamoeba* sp.
ACAVI	*Aeromonas caviae*
ACHRSP	*Achromobacter* sp.
ACIDSP	*Acidiminococcus* sp.
ACINSP	*Acinetobacter* sp.

system should be used when codes specified by the primary convention are ambiguous (e.g., use ECOLI for the bacterium and ENCOLI for the protozoan).

Some LISs permit the creation of group codes for microorganisms that enable users to refer to a group of related microorganisms simultaneously. Generally, group members share a property in common. Examples of group codes might be ANA for anaerobes, GNB for gram-negative bacilli, MOLD for filamentous fungi, and VIRUS for viruses. Rational assignment of microorganisms to groups should be done by individuals with an understanding of both the microbiology laboratory and the planned uses for microorganism group codes in the LIS.

Antimicrobial Susceptibility Batteries

The creation of antimicrobial susceptibility batteries is an essential part of building the microbiology section of an LIS. This undertaking is preceded by defining antimicrobial agent tests that yield numerical results or text codes indicating whether isolates are susceptible, intermediately susceptible, or resistant. Groups of these tests are then assembled to correspond to batteries of antimicrobial agents reflective of the reporting wishes of the laboratory. Separate batteries are usually defined for different categories of microorganisms, like gram-negative bacilli, gram-positive cocci, anaerobes, gram-negative urinary tract isolates, streptococci, and enterococci, among others. In some cases, a separate battery should be created for a single microorganism species (e.g., *Streptococcus pneumoniae*) if that is consistent with accepted local practices (e.g., standards promulgated by the Clinical and Laboratory Standards Institute [CLSI] in the United States) (10). When an isolate belongs to more than one isolate category (e.g., a urine culture isolate of *Escherichia coli* belongs to both the gram-negative bacilli and gram-negative urinary tract isolate categories), then the category that describes the isolate more specifically should take precedence (gram-negative urinary tract isolate battery in this example).

The group of antimicrobial agents assigned to each battery should be based on input from clinicians (particularly infectious diseases physicians), from the microbiology laboratory, and from the recommendations published by the CLSI (10). Many LISs permit the recording and storage of results for a large group of antimicrobial agents but selective reporting of only a limited group of results (e.g., those for agents present in the hospital formulary). As a cost containment initiative, some LISs enable the cascading of results so that a result for an expensive or potentially toxic agent is displayed only if the result for a less expensive or less toxic alternative is resistant (e.g., a ceftriaxone result for *Escherichia coli* is reported only if the cefazolin result is resistant). The decisions of which results to report under different situations should be based on a consensus of the groups mentioned above.

Before antimicrobial susceptibility results can be reported, specific text codes (antimicrobial susceptibility codes) must be defined for the standard designations of susceptible, intermediately susceptible, and resistant (e.g., S for susceptible, I for intermediately susceptible, and R for resistant). Some LISs offer the option of defining additional antimicrobial susceptibility codes that can be used creatively for crafting more functional antimicrobial susceptibility reports. For example, codes can be placed in antimicrobial susceptibility reports to remind technologists to take specific actions. A code like NOINTP can be used to indicate that testing of a particular antimicrobial agent versus a specific microorganism has not been standardized by the CLSI. A code like ESBL can be in place for expressing results for ceftazidime versus *Escherichia coli, Klebsiella pneumoniae, Klebsiella oxytoca,* and *Proteus mirabilis* when the MIC is >1 μg/ml. This code would remind technologists to check isolates for extended-spectrum beta-lactamase production before final reports are issued.

Sophisticated antimicrobial susceptibility reports that include cost information and recommended dosages for antimicrobial agents can be created by some LISs. Reports containing relative or actual cost information reduce pharmacy expenditures by encouraging clinicians to select less expensive antimicrobial agents that still show excellent in vitro activity. Reports with dosage recommendations are particularly helpful in hospitals where medical student, intern, and resident training is taking place.

Billing for Laboratory Tests

The driving force that led to the development of the first LIS was a desire to capture laboratory test billing more efficiently than could be accomplished manually. Because billing transactions could be initiated automatically upon test ordering or upon specimen receipt, it was no longer necessary to complete paper charge tickets that were manually transcribed into ledgers or stand-alone electronic billing systems (23).

Microbiology billing can be more complicated than that in other laboratory sections due to the automatic ordering of follow-up tests (e.g., a laboratory-initiated order for an antimicrobial susceptibility test when a clinically significant microorganism is detected by culture). Such reflex orders may follow initial culture orders by several days, raising the possibility in the United States of difficult-to-reimburse late charges if the billing transactions are handled improperly. Fortunately, most LISs are sophisticated enough to handle billing for laboratory-initiated orders in a manner satisfactory to payers for laboratory services.

Current procedural terminology (CPT) codes were first devised by the American Medical Association in 1966. Among the 8,736 codes in the current 2006 listing is a small group applicable to microbiology testing. Fee-for-service reimbursement for laboratory testing is dependent upon inclusion of the appropriate CPT code(s) in bills for laboratory services rendered. The construction of test and battery billing maintenance in LISs includes indicating the applicable CPT codes so that these critical bits of information can be forwarded electronically to the hospital billing information

system. Although significant improvement in the description of microbiology CPT codes has occurred in recent years, enough ambiguity yet remains that individuals with microbiology expertise should be involved in entering CPT codes into the LIS.

Most vendors have fashioned their LIS microbiology modules so that billing for the most frequent reflex test, antimicrobial susceptibility testing, occurs automatically without manual intervention. The billing transaction includes the appropriate CPT code for sending to the hospital billing information system. It is also feasible with some LISs to use the same automatic billing feature to charge for other reflex tests, such as Western blotting for human immunodeficiency virus type 1 (HIV-1) antibody-positive specimens and confirmatory fluorescent treponemal antibody testing for RPR-positive specimens. In these situations, the reflex test can be built in a manner similar to an antimicrobial agent battery. Each band on a Western blot strip, for example, could be defined as an antimicrobial agent test, and unique susceptibility result codes could be defined to indicate the presence or absence of each antibody band. Apart from the automatic billing feature, this approach offers the benefit of querying results from these other reflex tests using LIS microbiology report functionality.

Microbiology laboratories are allowed to bill additionally for specific activities performed during specimen processing or identification of isolates (Table 3). Examples include the grinding of tissue prior to culture inoculation and the performance of three or more biochemical tests to identify a culture isolate. Since these activities apply only to certain specimens or cultures, they need to be billed on an ad hoc basis. This is accomplished via technologist entry of charges into the LIS as the charges are incurred. Alternatively, charges can be entered as a group when final culture results are issued.

LIS Work Sheets

Individual work sheets (or work lists) are used to group similar laboratory tests within the LIS, providing a convenient way to divide the laboratory workload among technologists. Individual work sheets can be printed by technologists at the beginning of a work shift to obtain lists of specimens or cultures that require their attention that day. Work sheets also can be printed at the end of a work shift to double-check that specimens or cultures were not overlooked. The statuses of tests assigned to individual work sheets can be monitored by calling up standard LIS reports. Examples of these reports are those that provide lists of pending test results and overdue test results.

Microbiology tests are usually assigned to work sheets that correspond to laboratory workbenches, such as the blood culture bench, the respiratory culture bench, and the urine culture bench. For this reason, individuals who understand how the microbiology laboratory is organized should have a voice in assigning tests to work sheets.

Some LISs permit the definition of group work sheets, which are groups of individual work sheets that have characteristics in common (Table 4). Group work sheets are of utility to microbiologists with supervisory responsibilities that span more than one workbench. If the microbiology laboratory staff includes separate section supervisors responsible for bacterial testing, fungal testing, and virologic testing, group work sheets can be defined that enable each section supervisor to monitor the statuses of tests in his or her domain. Similarly, an all-encompassing group work sheet can be defined that includes all of the microbiology individual work sheets. By calling up this work sheet, the laboratory supervisor or director can monitor all tests being worked on in the laboratory.

Work sheet maintenance may include the option of defining the number of days following specimen receipt at which results from tests assigned to individual work sheets are considered overdue. For the overdue log to be functional, the results of tests assigned to individual work sheets must become overdue after the same period of time. It would be impractical to place routine blood cultures (incubated for 5 days) and fungal blood cultures (incubated for 21 days) on the same work sheet if the overdue log is going to be used.

LIS Rules

LIS rules enable laboratories to eliminate errors, automate certain tasks, and minimize manual data entry (3, 30). Rules can apply preanalytically to guide the ordering of tests based on the patient's age, the patient's diagnosis, or previous results in the patient's laboratory file. They also can be used to identify unnecessary test orders to eliminate wastage of reagents and technologist time. Rules can be used during the analytical phase of testing to flag questionable antimicrobial susceptibility results or to remind technologists to report critical test results by telephone. Rules can be triggered

TABLE 3 Examples of manually billed microbiology tests[a]

Bill code	Name	CPT code(s)
M8011H	Bacterial ID (aerobic) × 1	87077
M8012H	Bacterial ID (aerobic) × 2	87077 × 2
M8013H	Bacterial ID (aerobic) × 3	87077 × 3
M8016H	Bacterial ID (anaerobic) × 1	87076
M8031H	Yeast ID × 1	87106
M8035H	Mold ID × 1	87107
M8041H	Viral ID × 1	87253
M8056H	ID by gas-liquid chromatography	87143
M8025H	ID by nucleic acid probe	87149
M8058H	ID by pulsed-field gel electrophoresis	87152
M8051H	Serogrouping by agglutination × 1	87147
M8061H	Concn for AFB smear or culture	87015
M8071H	Mycobacterial ID	87118
M8076H	Macroscopic ID (helminth)	87169
M8077H	Macroscopic ID (arthropod)	87168
M8081H	Tissue homogenization	87176

[a] Abbreviations: ID, identification; AFB, acid-fast bacillus.

TABLE 4 Sample group work sheet codes and names

Work sheet code	Work sheet name
BLOOD	Blood cultures
CSF	CSF cultures
MISCEL	Miscellaneous cultures
RESPIR	Respiratory cultures
URINE	Urine cultures
ANA	Anaerobic cultures
MYCOB	Mycobacterial cultures
MYCOL	Mycology cultures
SEROL	Serologic tests
PARA	Parasitology exams
MICRO	Microbiology group
VIROL	Virology group
MICVIR	Microbiology-virology group

postanalytically to provide clinicians with predefined interpretive comments that clarify the clinical significance of test results and recommend additional testing to consider. The creation of LIS rules suitable for microbiology testing requires individuals who understand both the laboratory and the capability of the rules engine.

LIS rules usually are defined as "if-then" statements. An "if" statement specifies the condition that triggers a rule. A "then" statement describes the actions to be taken when an "if" condition is met. An example of a commonly employed microbiology rule is as follows: if a *Staphylococcus aureus* isolate is resistant to oxacillin ("if" statement), then the susceptibility results for all beta-lactam antimicrobial agents should be reported as resistant ("then" statement). Sophisticated rules engines can process more complex "if-then" statements that include multiple conditions linked by operators such as "and," "or," "greater than," "less than," and "equal to."

Rules should be organized into one or more hierarchies. One hierarchy should define the points at which rules should be executed. This is important because when two or more rules apply in temporal proximity to one another, the corresponding actions should be taken in a logical sequence. For example, separate rules that call for ordering a "clean catch" urine culture for patients older than 18 years and then conditionally reporting a fluoroquinolone susceptibility result if the culture result is positive need to be evaluated in the correct order. The execution point for the rule concerning patient age is at the ordering stage and that for the rule regarding susceptibility testing is at the results stage. The logical order for executing these rules is (i) order rule and (ii) results rule, with the results rule being applied only if the order rule has already been executed. This sequence prevents the results rule from causing a fluoroquinolone susceptibility result to be reported for a patient of less than 18 years of age.

A second hierarchy should determine the action sequence when two or more applicable rules have the same execution point. Examples here are (i) a rule that prevents the ordering of duplicate rotavirus antigen tests for the same patient within 24 h and (ii) a rule that adds a rotavirus antigen test to a culture order for stool specimens collected by the emergency department from patients of less than 12 months of age between 1 January and 30 April. Many rules engines in this situation would execute the rules in the order they were created. Logically, the laboratory's intent is for the rule that prevents the ordering of a second rotavirus assay to take precedence over the rule that automatically orders a rotavirus test.

INFORMATION SHARING DURING THE PREANALYTICAL PHASE OF TESTING

During the preanalytical phase of testing, clinicians wish to select tests that are likely to provide diagnostically useful or therapeutically important information. Clinical microbiologists can be extremely helpful during this phase by supplying information that assists in appropriate test selection.

Laboratory Test Catalog

An important way in which clinical microbiologists benefit patient care is by assisting clinicians in selecting tests to order in a rational manner. This assistance can be offered by fielding telephone queries. However, a more effective method that is available at all times is to develop a catalog of tests that is comprehensive, information rich, and easily accessed.

The laboratory test catalog should be a dynamic document. When new diagnostic tests become available, whether they are performed in-house or not, the list of tests available to clinicians should be updated. Strong consideration should be given to supplying more than just the names of tests. Specimen requirements (including minimum quantity, collection container, and transport conditions), expected turnaround times for test results, and charges for tests are among the relevant bits of information that should be included (Table 5).

Many commercial laboratories publish catalogs on paper that are distributed to ordering sites throughout their customer base. Some laboratories prepare catalogs in a loose-leaf format that enables easy updating as new tests are added

TABLE 5 Sample catalog information about CSF culture and Gram stain[a]

Category	Information
1. Name of test	CSF culture and Gram stain
2. Synonyms or alternate names	CSF C/S; Culture, CSF
3. Specimen type(s)	CSF, ventricular fluid, or subdural fluid
4. Minimum specimen volume	1 ml
5. Specimen container	Sterile, leak-proof container
6. Transport instructions	Bring to laboratory immediately
7. Information needed	None
8. Special requirements	Send tube no. 2 of 4
9. Test schedule, approx TAT	Daily, 72 h
10. Laboratory section	Microbiology
11. Prior approval or notice	Not needed
12. Requisition	Microbiology
13. Storage instructions	Refrigeration (4°C)
14. Reflexive order conditions	Antimicrobial susceptibility test if pathogen recovered
15. CPT code(s)	87070, 87205
16. Comments	Gram stain automatically performed
17. Price	$x.xx

[a] Abbreviations: C/S, culture and sensitivity; TAT, turnaround time.

and old tests are eliminated. Interactive electronic catalogs offer even more advantages. Electronic catalog contents can be quickly searched by using key words or phrases to locate desired information. They can be modified easily and as often as necessary. They can include links to electronic journal articles, textbooks, and resources found on the Internet that contain more information about the tests.

Laboratory Requisitions

Historically, laboratory requisitions have been sheets of paper containing a list of tests from which clinicians can select their orders. The requisitions are delivered to the laboratory accompanied by appropriately labeled specimens, and laboratory personnel then enter the orders into the LIS (if one is in use). With advances in information system technology, an increasing number of hospitals are now utilizing electronic order entry systems in which caregivers place their own orders and the orders are sent electronically to the LIS. Simultaneously, specimen labels are generated at the ordering location and are placed on specimen containers in the presence of the patient to minimize the likelihood of mislabeled specimens. When the labeled specimens arrive in the laboratory, their receipt is recorded in the LIS. Bar-coding technology enables specimen receipt recording and the subsequent tracking of specimens in the laboratory to be accomplished quickly and accurately by wanding the specimen labels with a bar code scanner.

The use of paper requisitions is decreasing. Their biggest drawback is that these requisitions are inflexible when it comes to the addition or removal of tests from the list of choices. However, when they are necessary they can still be used effectively with a little foresight. They should be printed in small batches that last only a few months. When new batches are ordered, the requisition contents should be reviewed and modified where necessary. There should be a location on the requisition where missing test names can be handwritten, and the date the requisition was printed should be evident to enable efficient removal of outdated requisitions from ordering locations.

Electronic requisitions found in computerized order entry systems are much more flexible than paper requisitions. These requisitions can be accessed through the LIS, the hospital information system, an order entry module with an interface with the LIS, or an Internet-based order entry system. The list of tests can be modified quickly whenever there is a change, and there is no worry of exhausting the supply of requisitions. As with paper requisitions, there must be a capacity for ordering tests that are missing from the electronic list. That is generally accomplished through the use of a "miscellaneous" test category in which test specifics (e.g., test name and specimen description) are entered during the ordering process. When a significant number of requests for the same miscellaneous test are received by the laboratory, a practice should be in place for incorporating the test onto the electronic requisition.

The use of the Internet for ordering laboratory tests has been escalating in frequency because it is possible to order from computer workstations located anywhere in the world (27). All that is needed on the ordering computer are Internet browsing software and authorized access to the password-protected ordering system. A concern over this manner of order communication is the possibility of unauthorized electronic eavesdroppers' acquiring sensitive patient information. Sophisticated means of encrypting the information stream exist to protect patient confidentiality and achieve HIPAA compliance.

INFORMATION MANAGEMENT DURING THE ANALYTICAL PHASE OF TESTING

Although information flows predominantly from clinicians to the laboratory in the preanalytical phase of testing and vice versa during the postanalytical phase of testing, the analytical phase of testing finds the laboratory seeking information from other internal and external sources. The clinical microbiology laboratory, in particular, depends heavily on access to repositories of information during the analytical phase of testing. That is because much of the work is visual or decision table oriented. The information received from these sources is then acted upon by individuals in the laboratory.

Procedure Manuals

Laboratory regulatory agencies require all laboratories to maintain up-to-date procedure manuals that are accessible to workers at all times that testing is in progress. Most laboratories rely on traditional procedure manuals comprising pages of information stored in loose-leaf binders. The loose-leaf format lends itself well to the preparation of photocopies for simultaneous use at multiple workbenches and the replacement of outdated procedures with newer versions. A more modern approach is the conversion of paper procedure manuals into a series of online documents that are stored in a word-processing format (e.g., x.doc), portable document format (e.g., x.pdf), or Internet browser format (e.g., x.htm) (25). Such documents are easily modified and can be printed on paper, if desired. Documentation of annual review can be accomplished efficiently and unequivocally via electronic signature. Color photographs to illustrate procedural steps can be embedded in the documents. Perhaps the greatest advantage is that the entire procedure manual can be searched rapidly by using key words or phrases to quickly locate a particular procedure or group of procedures. The CLSI publishes a frequently revised guideline for the preparation of paper or electronic laboratory procedure manuals (approved guideline GP2-A5) that most laboratories follow (6).

Image Libraries

Photographs are indispensable to the accurate identification of many microorganisms, particularly those in the fungal and parasitic groups. Photographs of gross colonies and microscopic morphologies of a variety of microorganisms can be maintained in image repositories that are useful in differentiating similar microorganisms from one another. Every clinical microbiology laboratory should have a collection of photographs to which technologists can refer during their work. The repository may consist of photographs in a textbook, images in a text atlas, or 35-mm slides maintained in the laboratory.

One of the spin-offs of current information system technology has been a growing reliance in the laboratory on digital images instead of images recorded on film (24, 29). High-resolution images can be stored on inexpensive magnetic and optical media and can easily be manipulated and enhanced with computer software. Many laboratories have taken advantage of the dramatic reductions seen recently in the cost of image-capturing hardware (e.g., scanners and cameras) and are building their own image libraries. Images can be placed on servers located anywhere in the world and be viewed and/or downloaded nearly instantaneously over the Internet (4).

Tables of Microbiological Data

Laboratory identification of many microorganisms is based on a comparison of their physiologic and biochemical test properties with those described in reference databases. This comparison can be accomplished manually with a side-by-side assessment or via flowchart analysis. Many microbiology laboratories still identify microorganisms through analysis of information found in tables and flowcharts in textbooks, journal articles, and government documents. This approach is laborious and time-consuming but has the advantage of including human judgment in the decision-making process.

The use of tables to identify microorganisms relies upon determining the best fit between the properties of an unknown microorganism and those of a group of microorganisms belonging to the same species. The tables usually indicate the percentages of species members that exhibit positive results for particular tests. Although this approach is effective when test results for an unknown microorganism closely match those for an established species, it may yield inaccurate identifications when test results are dissimilar to those for known species.

The same information found in the identification tables described above can be converted into computerized databases and then quickly and accurately compared with the properties of an unknown microorganism. This method has been used for many years in a proprietary fashion by the manufacturers of some commercially available systems for microbial identification. The sophisticated analysis includes weighting of key properties and pattern recognition to derive best-fit identifications. The likelihood of an identification and the degree of a microorganism's dissimilarity from similar microorganisms can also be calculated.

It is also possible for individual clinical microbiologists to convert their own identification tables and those present in the public domain into queriable databases. This can be accomplished by using off-the-shelf database software programs to prepare such databases manually. The same software can then be used to define queries that identify the microorganisms included in the database whose biochemical test results best match those for an unknown microorganism.

Flowchart Algorithms

Flowchart algorithms are another approach to microorganism identification that has been used for many years. This approach differs from the use of tables in that identification test results with the greatest discriminating power are placed at the top of the decision tree and are assigned greater weight than results further down the flowchart. Although the early decisions in the flowchart algorithm are often based on unambiguous test results, the decisions made further down in the flowchart may be based on more ambiguous test results or on test results that have lower discriminating abilities.

Laboratory Work Cards

More than that in any other area of laboratory medicine, accurate microbiology testing depends on a reliable flow of information between technologists assigned to different work shifts or work days. That is because microbiology testing of many specimens (especially cultures) cannot be completed in a single work shift and more than one technologist may be involved in the testing. Accordingly, laboratories must have a mechanism in place for sharing information among technologists that concisely communicates the status of testing. Some form of microbiology work card is the solution for many laboratories. Some microbiology laboratories, even those with an LIS, still employ paper work cards that are maintained at each workstation. Technologists record the work performed daily on individual work cards. Information is documented in the form of handwritten standardized codes, short phrases, or check boxes that are understandable by coworkers. Work cards for completed tests undergo supervisory review and are stored for at least 2 years per regulatory requirement.

Almost all LISs can be configured to record microbiology culture workup information on electronic templates that are accessed by individuals working on the same cultures at later times (Table 6). Information is entered onto the electronic work card in the form of text codes or short strings of free text. Entries on the work card are generally organized by isolate or by culture medium and then by chronological order within each of these categories. The benefits of paperless entry include guaranteed legibility, easy supervisory review of work, and automatic indexing of workup data to simplify data queries later. The electronic work card also can be configured to standardize culture workups and accrue workload data for productivity analyses.

Instrument Interfaces

The proliferation of semiautomated and automated instruments for laboratory testing has greatly increased the volume of data transfer between instruments and LISs. Information transfer can be accomplished through manual transcription or automatic communication of data via electronic interfaces.

Transfer of information to and from instruments that do not have an electronic interface with an LIS is accomplished by manual entry. The number of keystrokes required for this activity can be limited on the LIS side through the use of text codes or keyboard mapping to insert commonly used words, phrases, or even blocks of text. Because of the human component of information entry, transcription errors are an ever-present threat and the fidelity of data entry should be constantly monitored by supervisory review.

Far easier and much more reliable than manual transcription is the automatic transfer of information between instruments and LISs (8, 9). This transfer can be accomplished through the use of scripted or electronic interfaces. A scripted interface is in essence a very fast electronic typist. It transcribes information from an instrument to the LIS in the same manner that a technologist would—but much faster and more accurately. The electronic interface is more complicated in that it converts a stream of data from one system into a format understandable by the other system. Batch

TABLE 6 Sample work card entries for *S. pneumoniae* blood culture isolate

Work card prompt	Response
GRST (Gram stain)	GPCPSM (gram-positive cocci in pairs)
CAT (catalase)	NEG (negative)
PHON (telephone report)	DONE (completed)
OPT (optochin)	POS (positive)
BESC (bile esculin)	NEG (negative)
NACL (6.5% NaCl tolerance)	NEG (negative)
BDIL (broth dilution MIC)	DONE (completed)
PURPLT (purity plate)	AOK (pure culture)
SAVE (save isolate on slant)	DONE (completed)

interfaces send data on demand, and dynamic interfaces send data as soon as they become available. Unidirectional interfaces transfer information in a single direction, either from the LIS to an instrument (download) or from an instrument to the LIS (upload). Bidirectional interfaces are more costly and complicated to set up since data must be able to flow in either direction.

The decision as to which type of interface is more suitable for a microbiology instrument depends on the timeliness with which the data are needed, the traffic capacity (bandwidth) of the interface, and the cost-benefit ratio of having data flow in one or both directions. Interfaces that download specimen demographic information from an LIS to an instrument are almost always desirable because they eliminate the need for transcribing required information from one system to another. Interfaces that upload data from an instrument to an LIS are valuable when the data sets are large and contain information that will be reported directly to clinicians (e.g., microbial identification and antimicrobial susceptibility data). When the instrument data are only preliminary in nature and require follow-up work at the laboratory workbench (e.g., positive blood culture results from an automated blood culture instrument), justifying the expense of a bidirectional interface is more difficult.

Inventory Management

Inventory management modules are available for some LISs and are intended to help organize and monitor the utilization of laboratory supplies (7). Some modules also assist with equipment maintenance activities. These systems generate reports regarding ordering patterns for laboratory supplies and can print equipment maintenance logs. They also provide helpful data for cost analysis studies based on supply utilization. If a record of the usage of supplies can be entered into one of these modules, the module can provide a real-time indication of inventory. Some inventory modules also track expiration dates of supplies by lot number. They alert laboratory personnel when additional supplies should be ordered and may even produce paper or electronic order requisitions for submission to the purchasing department. When new supplies are received, inventory levels can be updated in the module. If supplies are not received when expected, the module can remind laboratory personnel to contact the vendor to determine the reason.

Quality Control

The quality control modules found in many LISs aid clinical microbiology laboratory managers by streamlining the entry and review of quality control data. They provide data entry screens and reports that are customized to the requirements of the laboratory. They offer reminders to the laboratory when scheduled quality control data have not been entered. They also send warnings to the laboratory when quality control results are out of range and require the entry of the remedial action taken. When out-of-range quality control results are entered, the individual entering the results is alerted immediately. The LIS then waits for either replacement of the out-of-range results or acknowledgment that the out-of-range results are correct as entered. Acknowledgment that out-of-range quality control results are correct qualifies the results for an exception report that should be reviewed daily by supervisory personnel.

Most LISs are able to plot numerical quality control data on a LIS-defined Levey-Jennings control chart, evaluate the data, and notify users when results are "out of control." A Levey-Jennings control chart is a graphical display of quality control data that indicates the expected mean, the ± 2 standard deviation boundary around the mean, and the ± 3 standard deviation boundary around the mean. The assumption is that if the test method is stable and being performed correctly, current quality control results should exhibit a statistical distribution similar to that of past quality control results. Results outside the ± 2 standard deviation boundary should be encountered no more than 5% of the time, and results beyond the ± 3 standard deviation boundary should be seen no more than 1% of the time. When quality control results fall within the specified boundaries, the test is "in control," the results are acceptable, and patient results can be reported. When quality control results are outside the specified boundaries, the test is out of control, the results are unacceptable, and patient results cannot be reported. The CLSI criteria for evaluating antimicrobial susceptibility quality control data are based on principles similar to those used by Levey-Jennings charts.

Many LISs are also able to evaluate numerical quality control results according to rules developed by Westgard many years ago (31). The data examined may be limited to a single control or may include the results from several controls. An example of a Westgard rule violation that applies to a single control is as follows: a single quality control result falls outside the acceptable range, defined as ± 2 standard deviations from the mean. An example of a rule infraction that involves results from several controls is as follows: four consecutive quality control results are more than 1 standard deviation away from the mean in the same direction. The quality control module in most LISs can be set to apply any of the Westgard rules that have been defined within the system. The rules that will be applied usually can be individualized according to the quality control tests being performed.

Quality Assurance

Generic quality assurance rules can be applied automatically to test results to enhance the recognition of potential problems. The rules listed below frequently are "hard coded" in the LIS software used by laboratories. Additional site-specific quality assurance rules can be defined with the aid of the rules engine described earlier in this chapter.

1. Normal-value checking. Most laboratory regulatory agencies mandate that test results be accompanied by the expected values for the test ("normal range"). Expected values for these tests may be expressed as a numerical threshold or a range for quantitative tests (e.g., expected antistreptolysin O titer, <200 IU/ml), or expected results may be expressed as a text code for qualitative tests (e.g., expected HIV-1 antibody result, "negative"). Inclusion of the expected value or result aids clinicians in interpreting the clinical significance of test results. Most LISs provide a mechanism for defining age-specific, gender-specific, and even species-specific expected values for test results. The expected values are incorporated automatically by the LISs into the test report. Results that are outside the normal value range qualify for a daily quality assurance report. In the microbiology laboratory, normal-value checking generally is limited to nonculture testing.

2. Comparison of current results with past results. Virtually all LISs have the capability of comparing current test results for a patient to those from the same test last performed on the same patient within a defined period of time. Sequential results that exhibit a user-defined significant difference trigger a "delta check" flag. It is the laboratory's responsibility to investigate flagged results to confirm that

the sequential specimens were collected from the same patient or that testing irregularities did not occur during the generation of either result. Once confirmation is achieved, the flagged results can be released for viewing by clinicians. Results that elicit a delta check flag qualify for a daily quality assurance report. In the microbiology laboratory, delta checking is limited typically to nonculture testing.

3. Critical-value checking. An unfortunate type of medical error originating in the clinical laboratory is the failure to notify clinicians of critical test results in a timely fashion as mandated by hospital policy. Almost as important as reporting the critical result itself is documenting that prompt clinician notification has occurred. Critical test results usually are defined as results that require immediate medical interventions of a life-saving nature. Examples from the microbiology laboratory include positive cerebrospinal fluid (CSF) Gram stain results and positive blood culture results. Through the use of LIS-defined automatic flagging of critical test results or the use of a rules engine to analyze laboratory data to identify critical results, the LIS can be an important safeguard against these types of medical errors (14, 15, 17). Results that are deemed critical qualify for a daily quality assurance report.

4. Comparison of smear and culture results. Some LISs are able to perform some quality assurance checks that are unique to the clinical microbiology laboratory. One that is often available is comparison of Gram stain and culture results. When Gram stain findings suggest that microorganisms of a particular morphology are present, the LIS will alert users if the culture results do not include a microorganism with that morphology. Deviations between Gram stain and culture results qualify for a daily quality assurance report.

5. Detection of "bug-drug" antimicrobial susceptibility result inconsistencies. Some LISs allow users to define expected antimicrobial susceptibility results for specific antimicrobial agents versus particular microorganisms. When results differ from the expected, a message displays requesting the technologist to review the result and either change it or file it manually. Acceptance of an unexpected result qualifies the result for a daily quality assurance report.

INFORMATION SHARING DURING THE POSTANALYTICAL PHASE OF TESTING

Communication of information to clinicians from the microbiology laboratory is especially vital during the postanalytical phase of testing, for it is this information that is the basis for patient management decisions. The mode of communication may be verbal, written, or electronic, and the information often consists of more than just test results.

Test Results Reporting

The most frequent information passed on to clinicians during the postanalytical phase of testing consists of the test results themselves. When it comes to microbiology testing, some results are released in their entirety all at once and others are issued sequentially as data become available. Examples of the former situation include results from antigen detection tests, nucleic acid probe tests, and certain microscopic assays. Instances of the latter sort include negative antigen detection results that require confirmation by a more sensitive method and culture results accompanied by a Gram stain report and/or antimicrobial susceptibility data. LISs can report initial microbiology results in preliminary reports and then release final reports once all testing is completed.

Some LISs calculate the transport time for microbiology specimens, which is then displayed in the final report. The specimen transport time is the elapsed time between collection and receipt of specimens by the laboratory, although in some external order entry systems the specimen collection time may actually be the test ordering time. Clinical microbiologists should understand the meaning of transport time as calculated by their LISs before relying on these data during investigation of specimen transport problems.

Another important time point in the processing of microbiology specimens for culture is the plating (setup) time—especially when the laboratory inoculating culture media are remote from the specimen collection site. Some LISs now display the plating time in culture reports to identify specimens that may have experienced processing delays. The plating time is also used by these LISs in calculating the elapsed time for "no growth update" reports (e.g., a preliminary blood culture report might read, "No growth after 18 hours of incubation").

Microscopic examination and culture results are usually displayed in separate sections of the LIS report. Stained-smear results typically are found near the top of the report, since they are available first and acted upon more immediately than culture results. Culture findings frequently evolve with time as one or more isolates are detected and then identified in a stepwise process. For example, a culture that is positive for *Haemophilus influenzae* may be reported initially to contain "pleomorphic gram-negative coccobacilli consistent with *Haemophilus* sp.," then to contain "presumptive *Haemophilus* sp.," and finally to contain "*Haemophilus influenzae* not typeable" as more information is learned about the isolate.

Improved taxonomic techniques during the past 30 years have led to the reclassification of many microorganisms into different or newly created genera and species. While microorganism name changes are a nuisance for laboratory personnel as they are forced to adjust to new nomenclature, they can create serious confusion among clinicians trying to interpret the significance of culture results. The LIS can be an effective tool in keeping clinicians up to date with microorganism name changes. By way of illustration, it is no more difficult for an LIS to issue a culture report as "3+ growth of *Rhizobium (Agrobacterium) radiobacter*" than as "3+ growth of *Rhizobium radiobacter.*" The former report is helpful to clinicians more familiar with the older taxonomic designation.

Interpretive Reports

One of the more innovative ways that clinical microbiology laboratories can benefit patients is through provision of interpretive reports that accompany test results. This is especially true for tests that yield complex results, like HIV-1 genotyping. Virtually all LISs enable clinical microbiologists to supplement test results with comments that were created and stored previously or with free text comments crafted in real time while test results are being reported. Libraries of prewritten comments can be maintained in the LIS and incorporated into reports individually or strung together as a group of comments when the situation warrants.

Supplementation of Microbiology Test Data with Digital Images

While still an uncommon practice, the potential for enhancement of microbiology reports with digital images is very real. Inclusion of digital photographs of Gram stains, acid-fast stains, ova and parasite preparations, or any other microscopic finding is technologically feasible today. Similarly,

photographs of subjectively read test results such as those for group A streptococcal antigen, *Cryptococcus neoformans* antigen, RPR, and many others could also be stored in the LIS for quality assurance purposes. Data from objectively read tests, such as optical density readings from enzyme immunoassays, are commonly stored in the LIS already. It would seem that a more compelling argument could be made for recording visual evidence of subjectively read test results. The precedent for storing digital images has already been set by several commercially available pathology and diagnostic imaging information systems. Many offer optional image storage repositories maintained on separate servers. Pathology and radiology reports can include hypertext links that lead users to photographs stored on the image server. In this way, clinicians have the opportunity to view for themselves the images that led to the pathologist's or radiologist's findings. The same could be true for clinical microbiology results.

Antimicrobial Susceptibility Reports

Antimicrobial susceptibility results, in many circumstances, are more valuable to clinicians postanalytically than are culture results. Hence, these data must be displayed in a fashion that promotes unequivocal understanding of the results so that correct therapeutic decisions can be made. Most LISs enable laboratories to display antimicrobial susceptibility data in a variety of formats, including linear, columnar, and tabular displays. Most enable easy addition or deletion of antimicrobial agents in predefined batteries and also allow the addition of practical information to reports, such as CLSI-recommended comments pertaining to antimicrobial susceptibility test results. In some instances, the report can be customized to show antimicrobial agent dosage information, route of administration, achievable levels of antimicrobial agents in the blood and urine, and the cost of antimicrobial agent therapy.

Virtually all LISs enable the entry of qualitative (S, I, and R) or quantitative (MIC or inhibitory zone diameter) susceptibility data. If quantitative data are entered, the LIS can refer to its own tables of user-entered interpretive criteria (obtained from the CLSI or other official agencies) to convert the data into clinician-friendly qualitative results. The interpretive criteria must be updated frequently by laboratory personnel as important changes are made on an annual basis.

The authors of the CLSI *Performance Standards for Antimicrobial Susceptibility Testing* have recommended carefully worded statements that can be added to susceptibility reports to aid clinicians in the correct interpretation of susceptibility results (10). Such comments can be added via manual insertion of free text, manual insertion of blocks of stored text, or automatic insertion of blocks of stored text after recognition of an appropriate situation by the LIS rules engine.

Nonculture Test Results Reporting

The development of rapid, CLIA-waived, point-of-care assays for diagnosis of infectious diseases has led to an increase in the numbers of nonculture microbiology tests being performed. Most of these tests do not fit into the traditional microbiology battery of microscopic examination, culture findings, and antimicrobial susceptibility results. They are more similar to tests performed in other sections of the clinical laboratory and thus need to be defined in the LIS in a manner different from that for cultures. If such tests are not defined as microbiology tests, it is essential that the report include a description of the specimen, information that is not always part of the test reports from other sections of the clinical laboratory.

Delivery of Test Results to Clinicians

There are many mechanisms by which microbiology test results can be delivered to clinicians. The requirements that all mechanisms have in common are that the results must reach clinicians

1. In a timely manner. Results must be available to clinicians within a time frame that enables appropriate interventions to be made.
2. In a legible and understandable format. Results must be easily read and presented in unambiguous language so that suitable action can be taken.
3. In a form that is free of typographical or transcriptional errors. Systems must be in place that reduce the release of misleading or erroneous laboratory data.

The most frequent means for distributing laboratory test results is still the paper report. Many LISs are set up to print paper reports daily that are then distributed by couriers to clinicians and added to the patient's medical record. Some LISs print the patient's entire up-to-date laboratory file each day with the expectation that reports from previous days will be discarded. Other LISs print only the patient's "new laboratory activity" so that daily reports are added to previously printed reports already in place in the clinician's office or the patient's medical record.

Interim reports show the status of current laboratory activity for each patient selected. Reports can be limited to new activity since the last interim or cumulative report was printed, or they may be printed for all activity that took place over a specified date range. The test results in interim reports are usually displayed in reverse chronological order, with no further sorting available. Some LISs permit interim reports to be called up for groups of patients according to the ordering physician or the patients' location.

Cumulative reports contain results of patient tests grouped by the type of test or the specimen collection site. Examples of cumulative report headers include "Bacterial Cultures," "Fungal Cultures," and "Nucleic Acid Probes" when sorting is by test categorization and "Blood Cultures," "Urine Cultures," and "Genital Nucleic Acid Probes" when sorting is by specimen collection site. It should be the responsibility of the clinical microbiology laboratory leadership, with input from clinicians, to decide the style of cumulative report that is more suitable for a particular hospital. Within each group of tests under a cumulative report header, test results usually are sorted in reverse chronological order. Most LISs can call up cumulative reports by patient, ordering physician, or patient location. Most hospitals depend on postdischarge cumulative reports to serve as the official record of laboratory test results.

Generation of Ad Hoc (User-Defined) Reports

There are occasions in which none of the hard-coded LIS reports available to the laboratory contain the information needed for a specific purpose. Yet, every LIS stores a wealth of useful data elements within tables that may not be accessible via running preset reports (1). Most LISs offer optional modules that enable users to perform user-defined queries of the LIS database. The queries may be made with proprietary LIS-specific tools, or if the LIS data are stored in an open database connectivity (ODBC)-compliant relational database, queries may be made with ODBC-compliant off-the-shelf software (e.g., Microsoft Access or Business Objects

Crystal Reports). Such queries generally are intended to assemble data for a retrospective review of a particular data set. For example, if one wished to obtain a list of all patients during the previous 5 years who had positive blood cultures with simultaneous white blood cell counts of greater than 18,000 per mm^3, an ad hoc query would be the easiest manner to collect the data.

Once the ad hoc query is defined and run, the output report is stored in the LIS or on the user's workstation, usually as a delimited text file, spreadsheet file, or database file. From here the report can be printed on paper or, if resident on the LIS, can be downloaded to a personal computer via a serial port connection to the LIS or via a "file transfer protocol" across the hospital computer network. The ad hoc report file in a personal computer can be opened using standard spreadsheet or database software. Spreadsheet tools (e.g., sorting, filtering, and pivot tables from Microsoft Excel) can be used to analyze the data (22). Database tools (e.g., Microsoft Access and Business Objects Crystal Reports) can be used to perform further queries or configure attractive displays of the data.

Paperless Reports

The trend toward operating clinical laboratories as paperless entities is gathering momentum. Under this paradigm, LIS reports are not printed on paper but instead are printed to files stored on magnetic or optical disk media. Such reports are stored in easily accessible formats, are readily distributed to authorized individuals, and are quickly retrievable for review at any time. If it is necessary to obtain a paper report, the report file can be sent electronically to a printer.

Although an increasing number of hospitals are encouraging clinicians to view laboratory results via a clinician portal rather than in the LIS, all LISs provide on-demand viewing of laboratory test results online. LIS inquiry functions enable the review of test results for specimens collected on a particular date or range of dates or for a single test or group of tests performed in a particular section of the laboratory. Some LISs enable viewing of serial quantitative data in graphical format so that numerical trends are more easily spotted.

For those hospitals that expect clinicians to order laboratory tests and view laboratory results in a clinician order entry-results-viewing portal, an interface must be built between the clinician portal and the LIS. The advantage to this approach is that clinicians can be trained to perform a wide variety of functions (e.g., ordering and viewing laboratory results, ordering and viewing diagnostic imaging results, ordering pharmaceuticals, and viewing patient vital signs and progress notes) on a single information system. This strategy fits in nicely with the tendency in U.S. hospitals toward greater provider interaction with information systems. The major disadvantage is that the format for displaying laboratory test results may not be as feature rich and well organized in a clinician portal as it would be in an LIS.

Electronic Delivery of Reports

When the LIS is a node on the hospital local-area or wide-area computer network, it becomes feasible to transmit report files electronically to workstation nodes and printers on the network. Some LISs possess report scheduling capability by which preset or ad hoc reports can be run at designated daily, weekly, monthly, quarterly, or annual intervals, printed automatically to files, and then sent to workstations or groups of workstations within the network. In fact, a growing number of laboratories no longer print patient discharge cumulative reports on paper. Alternatively, they print the reports to a file and send the file via the hospital local-area network to the health information management department (medical records) for inclusion in the patient's electronic medical record.

Another emerging trend among laboratories is granting access to laboratory test results via the Internet (11). Authorized individuals are able to log in to an Internet information server where an up-to-the-minute copy of the laboratory test result database resides. Users then can view results over a secure, encrypted channel from anywhere in the world.

Another recent technological development is the ability to transmit laboratory test results via a wireless connection to hand-held devices such as alphanumeric pagers, digital telephones, and personal data assistants. Although still in its infancy, this methodology has the potential to replace the telephoning of critical laboratory reports to clinicians, if a means for acknowledging receipt of the report can be implemented. One can also envisage daily downloading of interim reports for a clinician's patient list to a hand-held device. It remains to be seen whether the complex laboratory data found in microbiology reports can be transmitted and viewed effectively on these hand-held devices.

Verbal Delivery of Reports

Verbal delivery of laboratory results, in almost every instance, is an adjunct to conveying results by other means. Verbal reporting very often is the method of choice for initial reporting of critical values. In the microbiology arena these may be preliminary results, such as the Gram stain morphologies of microorganisms growing in blood cultures, or they may be final results such as positive results for a test to detect cryptococcal antigen in CSF. When noncritical laboratory results are delivered verbally, it is often in situations in which clinicians telephone the laboratory because they do not have access to the standard means of results distribution. This method of reporting microbiology results must be carried out with caution since it is very easy for complicated results to be confused or misunderstood. Reading back of verbally communicated critical reports has become the norm in the United States. The other common context for verbal discussion of test results is during the course of a consultation over results released previously in another manner.

Consultations Concerning Laboratory Reports

Passive consultations from the laboratory perspective are those in which clinicians contact the laboratory for advice in interpreting test results. Such consultations concerning microbiology results should be handled by the laboratory director or the supervisor, one of whom should be reachable for this purpose at all times. Active consultations are those in which laboratory directors or supervisors seek out clinicians to discuss the clinical significance of test results and suggest additional tests that should be considered. Although active consultations require commitment and effort on the part of the laboratory staff, the return on the investment is easily appreciated in terms of improved patient care.

An underutilized method of communication between the microbiology laboratory and clinicians is the addition of information signed by the laboratory director to patient medical records. This information can be of great assistance to clinicians in the understanding of test results and can provide valuable suggestions for follow-up testing. The advantages of this route of information sharing are that the clinical

microbiologist's observations can be read at the convenience of clinicians and that the information is located in the same place in the medical record as key information from other clinical services.

Most hospitals and regulatory agencies require that the medical staff bylaws specify the individuals who are authorized to place information in patient medical records. It is the responsibility of the microbiology laboratory director and his or her colleagues in laboratory medicine to ensure that they have the necessary authorization if this is a desired practice.

Preparation of Periodic Antibiogram Reports

A much-appreciated service provided by many clinical microbiology laboratories is the distribution of cumulative institutional antimicrobial susceptibility data reports to clinicians (28). These reports generally are prepared on an annual basis but may be offered more frequently if warranted. In an attempt to standardize the contents of antibiogram reports, the CLSI has issued document M39-A2, entitled *Analysis and Presentation of Cumulative Antimicrobial Susceptibility Test Data* (5). This document recommends methods for recording and analyzing antimicrobial susceptibility data for epidemiologically significant microorganisms. To avoid biasing the data, the standard recommends including only the first isolate of a particular species from a patient per analysis period and including only isolates derived from cultures performed for diagnostic purposes. To improve the statistical validity of the data, the standard urges limiting the report to organisms tested 30 or more times during the analysis period.

At the time of this writing, most LIS vendors are still determining how they will help laboratories comply with the recommendations in the CLSI M39-A2 document. In the meantime, clinical microbiologists should consider downloading their antimicrobial susceptibility data to a personal computer and then preparing their cumulative antimicrobial susceptibility reports with the aid of spreadsheet or database software that enables compliance with the CLSI document (Table 7).

TABLE 7 Sample pivot table from Microsoft Excel showing antimicrobial susceptibility data for *S. pneumoniae* tested during calendar year 2005

Culture battery	(All)
Collection month	(All)
Collection year	2005
Order location	(All)
Order physician	(All)
Specimen description	(All)
Gender	(All)

Drug	Result (%)		
	S	I	R
Ceftriaxone (CSF)[a]	68	10	22
Ceftriaxone (other)[a]	81	17	2
Chloramphenicol	100		0
Clindamycin	88	2	10
Erythromycin	59	2	39
Levofloxacin	100	0	0
Penicillin	45	25	30
Trimethoprim-sulfamethoxazole	55	4	41
Vancomycin	100		0

[a] CSF, isolate recovered from cerebrospinal fluid; other, isolate recovered from specimen other than cerebrospinal fluid.

Submission of Laboratory Results to External Repositories

Jurisdictional regulations (e.g., those issued by local, county, state, or national agencies) mandate that hospitals and/or laboratories report positive test results for diagnosis of certain infectious diseases to public health authorities. Traditionally, this reporting has been accomplished via written notification that is either mailed or faxed to the responsible authority. Some LISs are beginning to include software tools that make it easier for clinical microbiology laboratories to comply with reporting regulations (19). Automatic faxing of reports to designated telephone numbers is now a feature of selected LISs. This capability is being extended by some LISs to enable submission of HIPAA-compliant encrypted reports via e-mail and the Internet.

Another emerging phenomenon in some hospitals is a joint venture between clinical microbiology laboratories and infection control departments to export data in a secure fashion to a commercial external data warehouse where the data are analyzed on a fee-for-service basis to detect epidemiologically significant trends. Reported experience has indicated that such a program does enable identification of problem areas in the hospital where infection control intervention might be useful (12).

SUMMARY

LISs have enabled tremendous progress to be made in the dissemination of information from the clinical microbiology laboratory to providers of health care (18). Laboratory test data are delivered more efficiently and accurately than ever before. Other information that is imperative to the ordering of correct tests, the collection of appropriate specimens, and the interpretation of test results can be communicated effectively. The role of these systems in the daily practice of clinical microbiology will only continue to increase in importance. It behooves all clinical microbiologists to become more knowledgeable about and comfortable with information systems, for they are perhaps the most powerful tool available to us in our new role as information brokers.

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General Principles of Specimen Collection and Handling

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5

In terms of the effectiveness of the laboratory, nothing is more important than the appropriate selection, collection, and handling of a specimen for microbiologic diagnosis. When specimen collection and management are not priorities, the laboratory can contribute little to patient care. Consequently, all members of the medical staff involved in this process must understand the critical nature of ensuring specimen quality. It is the responsibility of the laboratory to provide complete and accurate specimen management information in a form that can be easily incorporated into the procedure manual of those health care workers (i.e., nurses and other allied nursing personnel) who have primary responsibility for the collection of specimens. The information provided should address safety, selection, collection, transportation, acceptability, and labeling. This chapter provides an approach for developing a policy for proper collection and handling of specimens destined for analysis in the clinical microbiology laboratory for adult and pediatric patients. Special emphasis is given to and more details are provided for pediatric specimens in this chapter because of the unique character of this patient population and the special procedures often required for obtaining appropriate specimens. Details of specimen management can be found in the relevant chapters for each major group of microorganisms covered in this Manual (bacteriology, chapter 20; virology, chapter 80; mycology, chapter 116; parasitology, chapter 133).

Appropriate specimen management, or the lack of it, affects patient care in several very important ways (19). It is the key to accurate laboratory diagnosis that directly affects patient care and patient outcome; it influences therapeutic decisions; it affects hospital infection control and prevention, patient length of stay, and overall hospital costs; it plays a major role in laboratory costs; and it clearly influences laboratory efficiency. For these reasons, every laboratory should develop a rational, sound, and relevant specimen management policy and enforce it as strictly as possible.

SAFETY

Biosafety at the laboratory bench is of primary concern to laboratorians. Health care workers may be unaware of the potential etiologic agent(s) residing in the specimen being transported to the laboratory. Policies designed to protect laboratory and other personnel from accidental exposure to these agents must be in place. Most microbiology laboratory texts, including chapter 8 of this Manual, have sections on laboratory procedures that should contain safety information related to specimen management. Specific reference material on biosafety should be available in every microbiology laboratory. The reference materials available in the laboratory could include *Biosafety in Microbiological and Biomedical Laboratories,* 4th ed. (69) and *Biosafety in the Laboratory: Prudent Practices for the Handling and Disposal of Infectious Materials* (52).

In general, laboratorians should comply with the following policies for safety in specimen management:

1. Wear gloves, gowns, and, where appropriate, masks and/or goggles when collecting specimens (17).
2. Use leak-proof specimen containers and transport the containers within a sealable, leak-proof plastic bag with a separate compartment for paperwork (18).
3. Never transport syringes with needles to the laboratory. Instead, transfer the contents to a sterile tube or remove the needle with a protective device, recap the syringe, and place it in a sealable, leak-proof plastic bag (60).
4. Do not transport leaking specimen containers to the laboratory or process them. Notify the physician or the responsible nurse of the leaking container and explain the potential compromised nature of the results if processing is continued; ask for a repeat specimen. If a new specimen is submitted, autoclave and discard the leaking one (50). If another specimen cannot be obtained, e.g., needle aspirates, body fluids, or bone marrow, work with the existing specimen container within a biological safety cabinet.

SELECTION AND COLLECTION OF THE SPECIMEN

Before a specimen is collected for analysis, the specimen or the collection site must be selected and must represent a location of active disease. Even careful collection methods will produce a specimen of little clinical value if it is not obtained from a site where the infection is active. Some of the common sites of infection where ready sources of contamination reside include the bladder, where urethral organisms and those from the perineum may easily contaminate the urine specimen; blood, which is not infrequently contaminated by commensal flora from the venipuncture site; the endometrium, which may contain commensal vaginal

flora; fistulas, which may contain organisms from the gastrointestinal tract; the middle ear, a specimen from which will be contaminated with flora of the external auditory canal if a swab is used to collect the specimen; the nasal sinus, which may contain nasopharyngeal flora; and sites of subcutaneous infections and superficial wounds, which are commonly contaminated by skin and mucous membrane flora.

General specimen selection and collection guidelines should include the following:

1. Avoid commensal contamination from indigenous flora, whenever possible, to ensure a sample representative of the infectious process (9, 50, 60). Specimens from many sites of infection may contain an etiologic agent that would be considered part of the normal flora in a healthy host. This "background noise" of normal flora (i.e., from skin, membranes, and the respiratory tract) could interfere with the interpretation of culture results as well as overgrow and obscure the true agent of disease.
2. Select the correct anatomic site from which to obtain the specimen and collect the specimen by the proper technique and with the proper supplies, as described in the tables of this and subsequent chapters of this Manual.
3. Optimize the capture of anaerobes from specimens by using the proper precautions, procedures, and supplies; biopsy or needle aspirates are the specimens of choice, while anaerobic swabs are the least desirable (35, 50). Never refrigerate specimens for anaerobic culture but, rather, maintain them at room temperature (32).
4. Collect adequate volumes; insufficient material may yield false-negative results.
5. Place the specimen in a container designed to promote the survival of suspected agents and to eliminate leakage and potential safety hazards.
6. Label each specimen container with the patient's name and identification number, source, specific site, date, time of collection, and initials of the collector (21).

The collection of specimens with swabs may or may not be the method of choice for the collection of a particular specimen for microbiologic analysis (37, 50). It is critical that specimen collectors know the appropriate device and method for the collection of samples. Swab tips for specimen collection are usually made of cotton, Dacron (a polyester), or calcium alginate. Most come with a plastic shaft, although swabs with wooden shafts are available. The swabs with wooden shafts are generally not recommended for routine specimen collection because they may contain toxic products and could inactivate herpes simplex virus and interfere with some *Ureaplasma* identification methods. Cotton-tipped swabs are less popular today because they may contain fatty acids that could interfere with the survival of some bacteria and *Chlamydia* spp. However, most nonfastidious bacteria are not affected if cotton-tipped swabs are used. Cotton-tipped swabs are also suitable for the collection of specimens from the vagina, cervix, or urethra for the detection of *Mycoplasma*. Dacron- and rayon-tipped swabs have a wide range of uses including the collection of specimens for the detection of viruses and can facilitate the survival of *Streptococcus pyogenes*. Calcium alginate-tipped swabs can be toxic for lipid-enveloped viruses and some cell cultures as well as for some strains of *Neisseria gonorrhoeae* and *Ureaplasma urealyticum*. These are useful for the collection of specimens for *Chlamydia* spp. (37). Newer tips of polyurethane foam are finding wide acceptance.

Swabs with flexible wire shafts and small tips are recommended for use for the collection of nasopharyngeal specimens, including sampling for *Bordetella pertussis*, and male urethral specimens for diagnosis of gonorrhoea. Specimens on plastic-shafted swabs that are labeled by the specimen collector as "nasopharyngeal" are not likely to be true representatives of nasopharyngeal flora and may actually contain representatives of the nasal or throat flora (50).

TRANSPORTATION

1. All specimens must be promptly transported to the laboratory, preferably within 2 h (35). If processing is delayed, specimens collected for the detection of bacterial agents may be stored under specified conditions (see chapter 20).
2. In general, do not store specimens for bacterial culture for more than 24 h. Viruses, however, usually remain stable for 2 to 3 days at 4°C (37, 38).
3. Optimal transport of clinical specimens, including specimens for anaerobic culture, depends primarily on the volume of material obtained. Submit small amounts within 15 to 30 min of collection; biopsy tissue may be maintained for up to 20 to 24 h, if stored at 25°C in an anaerobic transport system (35).
4. Otherwise, surgical or biopsy specimens are usually stored at 4°C for up to 1 week or per laboratory policy.
5. Environmentally sensitive organisms include *Shigella* spp. (which should be processed immediately), *Neisseria gonorrhoeae, N. meningitidis,* and *Haemophilus influenzae* (which is sensitive to cold temperatures). Never refrigerate spinal fluid, genital, eye, or internal ear specimens (50). Storage conditions for some specimens and agents are summarized in Table 1.
6. Transportation of clinical specimens and transportation of infectious substances from one health care facility or laboratory to another, regardless of the distance, requires strict attention to specimen packaging and labeling instructions (17, 18, 37). Materials for transport must be labeled properly and packaged and protected during transport. The courier vehicles must also be marked and designated as carrying biologic agents. Any clinical specimen, including swabs, scrapings, body fluids, or tissues, that is known or reasonably expected to contain a pathogen is classified as an *infectious substance*.

For specific packaging and shipping instructions, one can refer to a number of sources; the most comprehensive instructions are described by the Department of Transportation in 49 *Code of Federal Regulations* (http://hazmat.dot.gov/ or http://www.iata.org). Several areas of packaging and shipping are of extreme importance, and one must ensure that everyone involved in packaging and shipping (including courier activities) is current on the specific regulations, including the legal responsibilities of the laboratory as a "shipper"; the proper use of certified packaging (only packaging that has been certified by the United Nations for infectious substances can be used); the proper use of necessary package labeling and markings; and the proper completion of the required documentation. Frequent referral to the appropriate websites is recommended in order to ensure that compliance with the latest recommendations is accomplished.

Bacterial and Fungal Specimen Transport

Containers for specimen transport and directions on how to use them are often available from the laboratory. The potential etiologic agent suspected in the patient dictates the specific collection method and transport system that will support the viability of the agent. Specimens for fungal cultures should not be collected with a swab because of the

TABLE 1 Storage conditions for various transport systems and suspected etiologic agents[a]

Preservative or medium type	Specimens held at 4°C	Specimens held at 25°C
No preservative	Autopsy tissue, bronchial wash, intravenous catheter, CSF (viral agent), lung biopsy, pericardial fluid, sputum, urine (all)	CSF (bacterial agents), synovial fluid
Anaerobic transport media		Abdominal fluid, amniotic fluid, anaerobic cultures, aspirates, bile, cul-de-sac material, deep lesion material, IUD for *Actinomyces* sp., lung aspirate, placenta (delivery by cesarean section), sinus aspirate, tissue (surgery), transtracheal aspirate, urine (suprapubic aspirate)
Direct inoculation of media		Corneal scraping, blood cultures, RL or BG plates for *Bordetella* spp., JEMBEC plates for *Neisseria gonorrhoeae,* vitreous humor
Aerobic transport media[b]	Burn wound biopsy, *Campylobacter* spp., ear (external), *Shigella* spp., *Vibrio* spp., *Yersinia* spp.	Bone marrow, *Bordetella* spp., cervix, conjunctiva, *Corynebacterium* spp., ear (internal), genital cultures, nasopharynx, *Neisseria* spp., *Salmonella* spp., upper respiratory tract specimens

[a] Abbreviations: BG, Bordet-Gengou; IUD, intrauterine device; JEMBEC, John E. Martin biological environmental chamber; RL, Regan-Lowe medium.
[b] Stuart's medium, charcoal-impregnated swabs originally formulated for *N. gonorrhoeae* transport; Amies medium, modified Stuart's medium that incorporates charcoal in medium instead of in the swab; Cary & Blair medium, similar to Stuart's medium but modified for fecal specimens, with the pH increased from 7.4 to 8.4.

potential interference of the swab fibers with direct microscopic examination of the specimen. Swabs are acceptable for use for the collection of specimens for the detection of suspected yeast infections, however. Most specimen containers must be sterile since the presence of contaminating flora from nonsterile containers may lead to errors in culture interpretation. Containers for feces need not be sterile but should be clean containers with tight-fitting lids. If there is a question as to whether a specimen container should be sterile for a specific specimen, assume that it should be sterile.

Other useful products and devices include sterile, screw-capped containers for collecting urine or sputum specimens. The containers should be prepared and packaged for patient use, with directions, including illustrations, that can be understood by patients. Biopsy and tissue specimens may also be placed into these sterile cups, although "biopsy" samples may tend to be smaller than "tissue" samples. To keep these tissues moist, one may add a small amount of nonbacteriostatic saline to the cup rather than wrap the tiny tissue specimen in gauze. Sterile petri dishes or special envelopes can be used to transport hair, skin, or nail scrapings to the mycology laboratory. Commercial transport devices for *N. gonorrhoeae* such as the JEMBEC (John E. Martin biological environmental chamber) system with CO_2 tablets may provide better results than CO_2-containing bottles, especially for transport by courier. The bottles may not have consistent amounts of CO_2, and improper manipulation during inoculation causes a loss of the atmosphere.

As with bacterial specimens, fungal specimens for culture should be placed into sterile containers and transported to the laboratory promptly. For skin and nail scrapings, cleansing of the site with 70% alcohol is required prior to specimen collection. Nail scrapings for submission to the laboratory must be collected from the deeper, infected portion of the nail, and the initial superficial scrapings should be discarded because they will likely be contaminated. A UV lamp (Wood's lamp) is helpful when selecting infected hair since some dermatophytes will fluoresce. Details can be found in chapter 116. Most other specimens, including blood, other sterile body fluids, and urine, respiratory, fecal, and tissue specimens, are collected and submitted as described elsewhere for bacterial or mycobacterial specimens.

Virus, Rickettsia, Chlamydia, and Mycoplasma Transport

The methods and media used for the transport of bacteria are inappropriate for the transport of viruses and chlamydiae. Viral transport media (VTM) prevent drying, maintain viral viability during transport, and prevent the overgrowth of contaminating bacteria. Many of the formulations contain either Eagle's minimum essential medium or Hanks' balanced salt solution, along with fetal bovine serum or bovine serum albumin (BSA). VTM may be prepared in-house or purchased commercially. There is little evidence in the literature that one VTM is better than another. However, in virtually all cases where a specimen is submitted for viral analysis, the specimen should be selected and collected in a manner appropriate for the target organ (37).

Liquid-based transport systems contain a protein (BSA, gelatin, or fetal bovine serum) and a combination of antimicrobial agents in a buffered solution. Tissue for viral analysis may also be placed into this type of medium. A phosphate-buffered sucrose-containing transport system (2SP) may be used for virus and chlamydia transport. The antimicrobial agents present in the 2SP are not inhibitory to *Chlamydia* spp.

A transport system containing human newborn foreskin fibroblasts is commercially available and useful for recovery and early detection of cytomegalovirus and herpes simplex virus. This cell system has a limited shelf life and is useful only for viruses that grow in fibroblasts.

If specimens should arrive in the laboratory having been inappropriately placed into Stuart's or Amies bacterial transport systems, the swabs may be transferred into one of the systems of liquid VTM.

Recovery of rickettsia seems to be enhanced if glutamate is present in a sodium-free, buffered salt solution. A sucrose-phosphate-glutamate transport medium containing BSA is often used to transport rickettsiae, mycoplasmas, and chlamydiae (37). Manufacturers of nucleic acid probes, amplification systems, or enzyme immunoassay (EIA) antigen detection systems often recommend or supply specific transport media and swabs for the collection and transport of specimens to be tested in their systems.

SPECIMEN ACCEPTABILITY OR REJECTION CRITERIA

At times, specimens arriving in the laboratory may have been improperly selected, collected, or transported. This is essentially the equivalent of a specimen being out of control. This out-of-control process must receive the same attention as does an out-of-control identification method or susceptibility test; there must be a corrective action. Processing and reporting results for these specimens to the physicians may provide misleading information that can lead to misdiagnosis and inappropriate therapy. Consequently, the laboratory must adhere to a strict policy of specimen acceptance and rejection.

Listed below are several examples of situations in which specific laboratory policies must be formulated and enforced to ensure specimen quality:

1. No label. Do not process, but immediately contact the submitting physician or nurse. For specimens obtained by noninvasive means (urines, sputums, or throat specimens), have a new specimen submitted. For specimens obtained by invasive procedures (needle aspirates, body fluids, or tissues), process the specimen only after directly consulting with the physician who obtained the specimen and/or the patient's physician. Note the problem on the report, complete with an incident report and documentation of the corrective action taken.
2. Prolonged transport. Do not process, but alert the submitter and request a repeat specimen for specimens obtained by noninvasive means. Note the problem on the patient's report: "Received after prolonged delay." For specimens obtained by invasive procedures (needle aspirates, body fluids, or tissues), directly contact the patient's physician and process as in situation no. 1 above.
3. Improper or leaking container. Do not process. Immediately call the submitter and request a repeat specimen, where appropriate. Note the problem on the patient's report and the corrective action taken. For specimens obtained by invasive procedures (needle aspirates, body fluids, or tissues), directly contact the patient's physician and process as in situation no. 1 above.
4. Specimen unsuitable for request (e.g., request for anaerobic culture for a specimen transported aerobically). Do not process. Contact the submitter, clarify the test request, and indicate the discrepancy. Request a proper specimen for the test requested.
5. Duplicate specimens on the same day for the same test request (except blood and tissue). Do not process. Place the specimen in the proper preservative at the correct storage temperature. Call the submitter and indicate the duplication. Note the problem on the report.

There may be instances in which a given specimen must be processed even though its quality is compromised, e.g., a difficult or unusual case, and then only after a consult between the patient's physician and the laboratory director. Table 2 lists specimens that provide little, if any, clinical information; processing of these specimens should be discouraged.

TABLE 2 Specimens to be discouraged due to questionable microbial information

Specimen type	Alternative or comment
Burn, wounds (swabs)	Submit tissue or aspirate
Colostomy, discharge	Do not process
Decubiti (swabs)	Submit tissue or aspirate
Foley catheter tip	Do not process
Gangrenous lesion (swab)	Submit tissue or aspirate
Gastric aspirates of newborns	Do not process
Lochia	Do not process
Periodontal lesion (swab)	Submit tissue or aspirate
Perirectal abscess (swab)	Submit tissue or aspirate
Varicose ulcer (swab)	Submit tissue or aspirate
Vomitus	Do not process

Sterile body fluids may be submitted from patients with serious or life-threatening illness and must be handled quickly and appropriately. The decision of whether to centrifuge the fluid and culture the specimen on agar media or in blood culture bottles must be incorporated into the laboratory protocol. Table 3 lists some management suggestions for handling sterile body fluids (8).

While the above discussion has focused more on general policy issues surrounding specimen management, the details of specimen management for adults are covered in subsequent and appropriate sections of this Manual. Infants and children represent an important patient population that is often overlooked in specimen management discussions, and there are many instances in which specimens from this patient group require special methods for selection, collection, and transport. The section that follows provides perspective and guidance on these issues.

SPECIMEN MANAGEMENT ISSUES FOR PEDIATRIC PATIENTS

Collection of specimens from pediatric populations may be influenced by factors not encountered when dealing with adults. The types of disease as well as the anatomic areas primarily affected by the infectious agent may differ. Recognition of the critical differences inherent in the collection of specimens for microbiological assays from infants and children aids in optimizing the detection of pathogens from this patient population. Table 4 summarizes the salient features of specimen management in this special population.

The volumes of specimen available for testing vary according to the age and size of the child. Limited volumes are especially pertinent to the collection of blood, urine, cerebrospinal fluid (CSF), other sterile fluids, and tissue samples submitted for culture. Multiple phlebotomies performed for a variety of diagnostic tests can affect the volume of blood collected for culture due to the concern of critical volume depletion in infants and smaller children. The diagnosis of certain types of bacterial meningitis may be hindered by the smaller volumes of CSF available, coupled with the problems inherent in performing lumbar puncture in this age group. The average total volume of CSF in children and

TABLE 3 Specimen management of sterile body fluids other than blood and CSF[a]

Fluid	Collection container	Concentration	Stain	Comment
Amniotic	Anaerobic tube	No	Gram stain	
Culdocentesis	Anaerobic tube	No	Gram stain	
Dialysis effluent	Isolator tube, urine cup, or Bx2	Centrifuge or filter	Gram stain or AO (low detection rate)	<100 leukocytes/ml is normal; use one-third of filter for one of three media
Pericardial	B and/or anaerobic tube	Cytospin from tube	Gram stain from cytospin	Few leukocytes in normal fluid
Peritoneal (ascites)	Bx2 (10 ml) + anaerobic tube	Cytospin from tube	Gram stain from cytospin	<300 leukocytes/ml is normal
Pleural (effusion, transudate, thoracentesis, empyema)	Anaerobic tube	Cytospin from tube	Gram stain from cytospin	>5 ml needed for fungi; none to a few leukocytes is normal; many leukocytes are found with empyema
Synovial	B + anaerobic tube	Cytospin from tube	Gram stain from cytospin	A few leukocytes is normal

[a]The information in this table is from reference 8 and reprinted with permission of Elsevier. Abbreviations: B, blood culture bottle; Bx2, aerobic and anaerobic blood bottles; AO, acridine orange stain. Cultures and stains can be done from any cytospin sediment.

infants is approximately 40 to 90 ml, while the volume range for adults is 90 to 150 ml. Lastly, infants and children not only excrete smaller volumes of urine but are also often unable to void on command. In some cases, <1.0 ml of urine may be available for testing.

The greatest challenge, therefore, for laboratories processing specimens from pediatric patients is making the most of the limited amounts of specimens received for culture. Although the procedures for the collection of many specimen types mirror the protocols used for adult patients, some important differences exist.

Blood Samples for Cultures

Use of proper skin disinfection techniques is even more important in children prior to collection of blood for culture since only one bottle collected during a 24-h period may be available for culture, making the categorization of contaminant versus pathogen difficult to assess. Due to the low incidence of anaerobic bacteremia, routine inoculation of an anaerobic blood culture bottle is not warranted. Although the collection of blood from infants and children by venipuncture is achievable by skilled phlebotomists, venous access in pediatric inpatients is often through peripheral or central venous catheters, which eliminates the need for attempts to gain peripheral venous access. Such lines are used primarily for administration of fluids and therapeutic drugs and are infused with heparin to inhibit clotting when not in use. When the catheter is used to obtain blood cultures, the line must be adequately disinfected and flushed of all inhibitory substances before the specimen is obtained. Since volume depletion in the patient is a concern, the amount of fluid to be discarded, as well as the amount of blood available for testing, is limited. The procedure utilized most often, the discard method, is based on the amount of fluid flushed from the line on the weight and size of the child (39). Minimal discard volumes for infants, for example, are in the range of 0.3 to 1.0 ml (59). In response to the decreased volumes of blood that can be obtained from pediatric patients for culture, blood culture bottles that contain approximately 20 ml of broth and that accommodate an inoculation volume of up to 4 ml are available. The smaller volume of broth allows for a close approximation of the recommended blood-to-broth ratio necessary to diminish the effect of growth inhibitors.

Cerebrospinal Fluid

The primary reason for collection of CSF is for the diagnosis of acute bacterial or viral meningitis or CSF shunt infections. Lumbar puncture is sometimes difficult in an infant or child. A specimen obtained by lumbar puncture that yields only blood is indicative of a failure to access the subarachnoid space (23). If the CSF initially contains a small amount of blood but clears as additional fluid is collected, a repeat lumbar puncture is not required. If only a small amount of fluid is retrieved from the patient due to age and size, CSF containing clotted blood is sometimes sent for culture pending a repeat lumbar puncture. The clot is homogenized prior to plating, and an acridine orange fluorescent stain of a direct smear of a cytospin preparation is recommended to facilitate rapid detection of potential pathogens. Gross blood in the specimen may obscure the visualization of organisms when stained by Gram's method.

Ventricular shunts are used for drainage in patients who overproduce CSF. Ventricular shunt malfunctions in both children and adults are associated with infection and/or disconnection or obstruction of the catheter (26, 54). The majority of shunt infections are acquired at the time of shunt placement and are associated with organisms usually considered skin flora, such as *Staphylococcus epidermidis* or *Propionibacterium acnes* (12, 67).

Specimens for Detection of Otitis and Sinusitis

Acute otitis media is also a common pediatric disease (11, 53). Uncomplicated otitis media does not require confirmation by culture; however, a persistent infection may require retrieval of fluid from the middle ear via tympanocentesis for identification of the specific pathogen causing the infection. A swab specimen of the external auditory canal is unsuitable for diagnosis of acute otitis media or otitis media with effusion. Potential contamination of purulent drainage with resident flora may interfere with accurate analysis of the culture.

Uncomplicated sinusitis is often treated empirically on the basis of the patient's clinical presentation (71). Specimens for culture may be obtained from patients with chronic sinusitis refractory to therapy. Bilateral cultures are recommended (71). Secretions from the region of the maxillary ostium are sampled with a swab under direct vision; however, unless the specimen is obtained very carefully, interpretation of culture

TABLE 4 Requirements for pediatric specimen collection

Specimen type and source	Collection	Comments
Blood for culture		
Peripheral	As for adults. Withdraw a volume of ≥0.5 ml and inoculate into the blood culture bottle.	Collection of larger volumes will aid in retrieval of low concentrations of circulating organisms. Inoculation of one aerobic bottle is usually sufficient.
Peripheral catheter (see comment)	Disinfect the venipuncture site. Insert the catheter and attach a T-connector with syringe to the catheter and withdraw the blood for culture.	A blood culture may be obtained when a peripheral catheter is inserted.
Indwelling central venous catheter	After disinfection, disconnect the extension tubing or cap from the catheter hub. Disinfect the hub. Withdraw a minimum volume of blood and discard. Attach a second sterile syringe and withdraw an additional ≥0.5 ml for culture.	Accidental aspiration of heparin may inhibit the growth of bloodborne pathogens; therefore, flush the catheter with heparin or saline.
Implantable device (for therapy administration)	Disinfect the skin site. Insert a Huber needle through the skin into the apparatus; follow the procedure for collection of blood from a central venous catheter described above.	These devices may rarely be used for vascular access for the retrieval of blood for culture.
CSF		
Lumbar puncture	As for adults.	Difficulties are often encountered in patient positioning and restraint; in addition, there are limitations to the volume of fluid retrievable.
Ventricular shunts	As for adults.	Correct labeling of shunt fluid is important because organisms considered "contaminants" from lumbar punctures may be significant pathogens in ventricular shunt infections.
Dermatologic specimens		
Bacterial and viral cultures		
Pustule or vesicular lesions	Disinfect surface and allow to dry. Unroof the pustule. Aspirate fluid for culture and then insert swab and rotate vigorously to collect fluid and cells from the advancing margin.	Specimens that contain no inflammatory cells are from the superficial areas of the lesion and new specimens must be collected. Viral pathogens are best retrieved from the base of a lesion.
Petechiae, purpura, ecthyma gangrenosa	With a scalpel blade, vigorously scrape the outer margin of the lesion.	A Gram stain of petechiae material may give an indication of meningococcal infection.
Fungi	For a dry lesion, scrape the lesion with a scalpel, glass slide, or toothbrush. For moist lesions, use a Dacron swab. For hair, use scissors and forceps. For nails, scrape with a scalpel. Place scraping directly onto fungal medium or place in a sterile container and send for culture.	A small toothbrush is useful for collection of scrapings.
Specimens for detection of scabies	Disinfect the area and allow to dry. Apply a single drop of mineral oil to the papule and abrade the infested area with a sterile scalpel. Transfer skin scrapings to a sterile container or microscope slide with coverslip for transport to the lab.	Place the microscope slide with coverslip in a secure holder so that the coverslip is not dislodged during transit.
Feces		
Bacterial and viral cultures	As for adults. A rectal swab showing feces is suitable for bacterial or viral culture.	Devices that fit into the toilet bowl or techniques such as lining a diaper with plastic wrap facilitate retrieval of feces for testing.
C. difficile toxin	As for adults.	Toxin-producing strains of *C. difficile* may be normal in some infants <2 years of age. Interpret a positive toxin result for individuals in this age group with caution.

(Continued on next page)

TABLE 4 *(Continued)*

Specimen type and source	Collection	Comments
Ovum and parasite examination	As for adults. Submit feces-coated rectal swabs only for antigen detection EIAs, not routine ovum and parasite examinations.	See bacterial and viral cultures. The volume of preservatives present in commercial ovum and parasite collection and transport tubes should be adjusted to retain the recommended stool-to-fixative ratio of 3:1.
Pinworms	Use a commercial paddle sampling device or place the adhesive side of a cellophane tape strip onto a microscope slide. Peel back the tape to expose the adhesive side of the tape. While holding the slide against an applicator, press the tape firmly against the perianal skin. Replace the tape back over the slide and press the adhesive side onto the slide.	The applicator stick provides a safe backing for the glass slide while gentle pressure is applied to the skin for collection of the specimen with the adhesive. Do not use "invisible" or "magic" tape.
Gastric aspirates (may not provide clinically relevant data)	A premeasured length of lubricated catheter is passed gently into the mouth or nasopharynx and is continued through the esophagus into the stomach. The contents are aspirated and place in a sterile container for immediate transport to the lab. If no gastric secretions are obtained, a lavage of sterile distilled water is collected for specimen processing.	Three consecutive early-morning, fasting specimens are preferred for mycobacterial culture, but infants may not be able to provide such a sample. Collect the aspirate as long after the last feeding as possible. Environmental mycobacterial species may appear in aspirated formula. Neutralization of the specimen must occur upon arrival in the lab.
Genital specimens	Use a small-tipped Dacron swab with a flexible smooth wire. The specimen of choice for a prepubertal female is a vaginal swab or washing. Collect a urethral swab from prepubertal males.	STDs in prepubertal girls involve the vagina as opposed to the cervix. Specimens from adolescents are the same as those collected from adults.
Ear specimens		
Otitis (otitis media)	Cleanse the external auditory canal with an antiseptic. Using an otoscope, insert a 1-ml tuberculin syringe with a 3.5-in 22-gauge spinal needle bent at a 30° angle through the tympanic membrane and aspirate the fluid in the chamber into a sterile vial or syringe.	Needle aspiration of fluid (tympanocentesis) is the recommended method for obtaining a specimen. A purulent discharge from a ruptured membrane can be collected for culture by using a sterile swab.
Respiratory specimens		
Bronchoalveolar lavage specimens	As for adults.	Specimens from unsheathed catheters may contain contaminating oropharyngeal flora. In infants and younger children, <10 ml is often retrieved. If >10 ml is collected, centrifuge the sample prior to plating.
Protected brush specimens	As for adults.	
Nasal specimens	Insert a sterile swab at least 1 cm into the opening of the anterior nares.	Used primarily for surveillance of methicillin-resistant *Staphylococcus aureus* or to assess upper respiratory tract colonization in children with immunologic defects.

(Continued on next page)

TABLE 4 Requirements for pediatric specimen collection *(Continued)*

Specimen type and source	Collection	Comments
Nasal washes	Aspirate approximately 4 ml of sterile saline into a 1-oz tapered rubber bulb. Tip the patient's head back approximately 70 degrees and insert the bulb into the nostril until it is occluded. Squeeze the bulb to dispense the saline, hold for a few seconds and then release to collect the secretions. Dispense the specimen into a sterile container and transport to the lab as soon as possible.	A nasal wash or nasal aspirate (see below) is often cited as the preferred specimen type for collection of respiratory secretions for culture for either viruses or *Bordetella pertussis* and direct smear examination and in pediatric patients. Transport specimens for viral cultures on ice.
Nasopharyngeal aspirate	Attach a sterile suction catheter to a (mucus) trap and introduce the end of the catheter into the nasopharynx until resistance is encountered. Withdraw the catheter 1–2 cm and apply suction to aspirate the sample. Dispense the specimen into a sterile container and immediately transport it to the lab.	See nasal wash. Transport specimens for viral cultures on ice.
Nasopharyngeal swab specimens	Insert the swab into the nasopharyngeal cavity to the point of resistance and then gently rotate it. Place the swab into an appropriate transport medium and send to the lab immediately.	Young children and infants require use of a swab with a small-tip circumference such as a calcium alginate or small-tip Dacron swab. Transport specimens for viral cultures on ice.
Throat swab specimens	Tilt the child's head back and ask the child to open the mouth as wide as possible. Carefully insert the sterile swab(s) into the oral cavity and sample the surfaces of the back of the throat and tonsils.	In children, the major pathogen of bacterial pharyngitis is group A streptococci (GAS). If a rapid screen of a throat swab specimen for GAS is performed, collect two swabs to do a culture to confirm negative screening findings or to rule out a possible false positive caused by a member of the *Streptococcus anginosus* group. Avoid touching other areas of the oral cavity to prevent specimen contamination with oropharyngeal flora.
Tracheal aspirate	After oxygenation of the patient, attach a sterile suction catheter to a (mucus) trap and introduce the end of the catheter into the trachea until resistance is encountered. Withdraw the catheter 1–2 cm and apply suction to aspirate the sample.	Although grading systems for assessment of the quality of pediatric tracheal aspirates have been proposed, careful evaluation is required prior to their implementation.
Transtracheal aspirate	As for adults.	
Sputum specimens	As for adults.	Since children are often unable to produce sputum, tracheal aspirates are more often collected in pediatric populations.
Specimens for detection of viruses	Specimens of choice are similar to others in this table (45).	Rectal swabs are acceptable for detection of rotavirus antigen.
Specimens for detection of *Chlamydia*	Conjunctival and/or nasopharyngeal swabs are appropriate for neonatal screening.	See genital specimens above. Vaginal (females) or urethral (males) swabs are required for chlamydia culture for the determination of sexual abuse.

results may be hindered by the presence of contaminating flora. Needle aspiration of the sinus is recommended for definitive diagnosis of the etiologic agent of infection (49).

Respiratory Specimens

For the diagnosis of group A streptococcal pharyngitis (10, 14), collection of two pharyngeal swabs is optimal for performance of both a rapid antigen detection assay and culture (one swab for each assay). A single swab shared for both methods may reduce the sensitivity of the antigen detection assay due to a reduction in the concentration of organisms available after culture inoculation (10). Although the reported sensitivities of some rapid assays may suggest that a confirmatory culture may be eliminated in the event that the screen is negative,

careful consideration must be given to the possible implications of an undetected infection in some children (10). The collection of nasopharyngeal swab specimens, washes, or aspirates for culture and/or detection of antigens of respiratory viruses and *Bordetella pertussis* is satisfactory (4, 47, 48). Again, collection of more than one swab increases the chance of isolate detection. Transport of swabs in a suitable holding medium is necessary to ensure organism viability if delays are anticipated between the time of collection and specimen receipt by the laboratory. Due to the small diameter of the nasal passages in some infants and children, coupled with inflammation of the nasal mucosa during the infection, specimen collection with a swab is sometimes more difficult and thus provides an inadequate sample.

A sputum specimen for diagnosis of pneumonia is difficult to obtain from children. More commonly, a tracheal or endotracheal tube aspirate is sent for microbiological culture. The utility of endotracheal tube aspirates as predictors of pediatric lower respiratory tract infection is influenced by the role that accumulated secretions within the tube play in the promotion of bacterial colonization (30, 62, 75). A bronchoalveolar lavage or the use of a protected brush will provide a superior specimen for intubated children with clinical evidence of pneumonia (44, 46, 74). Although pediatric bronchoscopes are available, the tubing diameter is sometimes too large for certain pediatric patients. In these situations, a small-diameter catheter is useful for performance of the lavage (2, 43). If an unprotected catheter is used, however, commensal oral flora will reduce the chances for recognition of the true etiologic agent. Aspiration of gastric secretions is performed for infants and children with presumed pneumonia caused by *Mycobacterium tuberculosis* (1, 61). After the patient has fasted overnight, the swallowed respiratory secretions are aspirated from the stomach by using gastric intubation and sent for diagnostic testing (1, 64). Problems with this method include the inability of infants on a feeding schedule to maintain a fasting state prior to specimen collection. Lastly, many pediatric centers provide care for children with cystic fibrosis. Specimens from these patients are periodically sent to the laboratory for surveillance of lower respiratory tract colonization by various potentially pathogenic microorganisms. Recommended specimens for culture of these types of organisms include sputum, tracheal aspirates, or throat swabs (6, 29, 56).

Genital Specimens

Genital specimens are usually collected from pediatric patients for (i) investigation of possible sexual abuse or rape or (ii) diagnosis of premenarchal vulvovaginitis and/or urethritis. Since these specimens are often irretrievable, every effort must be made to process pediatric urogenital specimens for culture. Although the cervix is the specimen source for diagnosis of gonorrhea and chlamydia in adolescent and adult females, detection of these sexually transmitted diseases (STDs) in prepubertal females requires sampling of the vaginal vault (5). A urethral swab sample is collected from prepubescent males. Culture is required for confirmation of both types of infections (5). Antigen detection assays are not acceptable due to the high reported rates of false positivity (33, 72). Likewise, the performance of molecular methods has not been adequately assessed for detection of STDs in children and is not considered admissible in the event of legal proceedings (34).

Urine Specimens

Collection of uncontaminated urine specimens from pediatric patients is a challenge. The acquisition of a clean-catch specimen from older children is hindered by the same problems experienced with adult patients. A urine specimen collected by catheterization is used for all pediatric age groups and, if performed properly, can yield a specimen free of urethral contaminants (4). Although suprapubic aspiration is considered the optimum method for urine collection from infants, the technique is frequently unsuccessful in dehydrated patients (4). Specimens must be transported to the lab within 30 min of collection or stored under refrigeration for no longer than 24 h (50). Urine transport systems containing preservatives are available for adult patients, who characteristically excrete larger volumes of urine. For optimum performance, the urine and preservative must be in the ratio recommended by the manufacturer. At present, no transport system is available to accommodate the lower-volume pediatric urine specimens.

Fecal Specimens

The best clinical predictors of a positive stool culture in children are a combination of persistent diarrhea of >24 h in duration, fever, and either blood in the stool or abdominal pain with nausea and vomiting (20, 45, 57, 61, 66). Many cases of endemic diarrhea occur in children <5 years of age and are caused by pathogens that are endemic to an area, such as rotavirus, shigellae, *Giardia lamblia,* and cryptosporidia (20). Since most diarrheal disease is community acquired, a single stool culture obtained during the first 72 h after admission to the hospital can be used for diagnosis for almost 98% of children with bacterial gastroenteritis (15, 16, 55). Depending on the age of the child, nosocomially acquired diarrheal disease is most often attributed to rotavirus or *Clostridium difficile* (13, 28). Interpretation of positive *Clostridium difficile* toxin assay results for children <2 years of age may be difficult due to intestinal colonization of this group with toxin-producing strains (22, 42, 68). A freshly obtained stool sample is preferable for all fecal assays. A rectal swab is less optimal but is acceptable for recovery of bacterial enteric pathogens, surveillance for multidrug-resistant organisms, and performance of certain antigen detection assays. A rectal swab is not recommended for some detection assays for *Clostridium difficile* toxin. If a delay in transport is anticipated, fecal specimens for either bacterial culture or parasite detection should be placed in an appropriate transport medium or preservative, respectively. In order to maintain the 3:1 recommended ratio of stool to preservative for parasite transport vials, the volume of preservative in the vial may require adjustment prior to inoculation of a small pediatric sample.

Pinworm *(Enterobius vermicularis)* infection is a common ailment of children. After establishment of infection in the colon, the female adult pinworm periodically migrates to the perianal area and deposits her eggs on the skin. Commercial sampling paddles are available for sampling, but cellophane or cellulose tape applied to the perianal skin in the morning, before the patient washes or defecates, enables collection of the eggs for identification.

Specimens from Neonates

The neonatal nursery and intensive care unit pose unique challenges for the microbiology laboratory. The problems inherent in decreased sample amounts available from these tiny patients may be compounded by the unpredictable response to infection displayed by neonates (31, 51). For example, isolates retrieved from the mucous membranes, skin, ear canal, nasopharynx, gastric aspirate, or rectum usually do not match the results of blood, CSF, or tissue cultures (31, 51). Differentiation of colonization versus true infection, therefore,

may be very difficult. Microorganisms are acquired either through transmission in utero, during delivery, or from nosocomial spread via hospital personnel, various medical devices, or environmental sources (36, 63). Infections reported to occur in neonates include sepsis, meningitis, otitis media, diarrhea, osteomyelitis or septic arthritis, conjunctivitis or orbital cellulitis, pneumonia, and various skin infections (7, 24, 27, 31, 51, 70, 73). Congenital infection is most often caused by agents such as *Toxoplasma gondii* or viruses such as herpes simplex virus, cytomegalovirus, varicella-zoster virus, parvovirus, enterovirus, rubella, or hepatitis B virus (25, 31, 41, 51, 65). Collection methods and appropriate specimen types for neonates are similar to those recommended for older infants and children with the exception of blood for culture. Depending on the age of the newborn, recommended sampling sites include the peripheral vein, umbilical artery, and capillary blood (31). Although a minimum of 0.5 to 1.0 ml is most often cited as the recommended amount of specimen to be collected, larger volumes are recommended for optimal recovery of organisms (40, 58).

Viral Specimens

Since smaller specimen volumes are often received from pediatric patients, newer molecular methods may aid in the detection of pediatric systemic or central nervous system viral infections. The antigenemia assay for detection of cytomegalovirus in blood may be impeded by both the smaller specimen volumes and the smaller polymorphonuclear leukocyte concentrations in neutropenic children undergoing transplantation or therapy for oncological problems. Details regarding diagnosis of viral infections can be found in chapter 80 of this Manual.

Dermatologic Specimens

Rashes are a common manifestation of many childhood illnesses. The same techniques are used to sample skin lesions from children and adults. A small, disposable toothbrush is valuable for obtaining scrapings of certain types of dermatophytic fungal lesions and produces fewer traumas than using a scalpel (3). Retrieval of skin samples for detection of scabies often yields no visible organism; however, the distribution of lesions differs between infants and young children and their older counterparts (3).

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Procedures for the Storage of Microorganisms

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6

Long- and short-term preservation of microorganisms for future study has a long tradition in microbiology. Culture collections of microorganisms are valuable resources for scientific research in microbial diversity and evolution, patient care management, epidemiological investigations, and educational purposes. Preserved individual strains of microorganisms serve as permanent records of microorganisms' unique phenotypic profiles and provide the material for further genotypic characterizations. Such reference collections can encompass rare infectious agents unique to an individual or catalog the history of disease caused by common pathogens such as those responsible for community outbreaks.

There are multiple methods for microbial preservation. Effective storage is defined by the ability to maintain an organism in a viable state free of contamination and without changes in its genotypic or phenotypic characteristics. Secondly, the organism must be easily restored to its condition prior to preservation. Microbial preservation methods have been evaluated extensively over the past 50 years, and often, optimal methods for preservation depend on a microorganism's taxonomic classification. Review articles, monographs, and books have been published that provide detailed information about the storage of various types of microorganisms (1, 10, 14, 15, 27). For clinical microbiology laboratories, simple and broadly applied methods are necessary to maintain organisms for short- and long-term recovery. This chapter presents methods that can be used for the storage of bacteria, protozoa, fungi, and viruses.

OVERVIEW OF PRESERVATION METHODS

Short-Term Preservation Methods

Direct Transfer to Subculture

The simplest method for maintaining the short-term viability of microorganisms, most often used for bacteria, is periodic subculture to fresh medium. Although simple, if microorganisms are saved for more than 1 week, this method is potentially labor-intensive, requires extensive laboratory space, and may compromise a microorganism's phenotypic profile. Each transfer to a new subculture increases the likelihood of mutation with undesirable changes in a microorganism's characteristics.

The interval between transfers varies among organisms. Additionally, the rate of mutation is quite variable. Some organisms appear stable indefinitely with repeated transfer, and others may change phenotypic traits after as few as two or three passages. The actual rate of mutation, however, has not been studied using sequencing technology. Issues that must be addressed with direct transfer include the medium to be used, the storage conditions, and the frequency of transfer.

Maintenance Medium

The medium should support the survival of the microorganism but minimize its metabolic processes and slow its rate of growth. Extreme environments should be avoided because microorganisms have the unique ability to adapt through mutation events in order to survive in suboptimal surroundings. A medium with too high a nutrient content will induce rapid replication that requires more frequent transfers. The optimal medium for maintaining microorganisms has not been clearly defined and most likely varies from one genus to another. Media that have been used include distilled water, tryptic soy broth, and nutrient broths (e.g., from Becton Dickinson and Co. and Oxoid Ltd.), all of which may be used with or without cryopreservatives.

Storage Conditions

Many laboratories store organisms, most often bacteria, for short periods on routine agar media at the workbench. Cultures kept in this fashion are subject to drying. A better method is to transfer organisms into screw-top test tubes and to store them in an organized location away from light and significant temperature changes. To prevent drying, caps can include rubber liners, or film can be wrapped over the top of the tube before or after the cap is screwed on. Storage at lower temperatures (5 to 8°C) slows metabolic processes and maintains viability for longer periods.

Frequency of Transfer

There is no set protocol for the frequency of transfer since storage conditions, media used, and types of microorganisms vary among laboratories. Individual laboratories should conduct studies for each category of microorganism to determine acceptable intervals between transfers under their conditions used for storage. Such studies would involve performing subcultures at scheduled times until the laboratory identifies an acceptable interval between transfers at which

a microorganism can reliably and reproducibly be recovered. (When transfers are performed, 5 to 10 representative colonies should be used to avoid the possibility of introducing an altered genotypic or phenotypic characteristic.)

Quality Control Procedures

Although it is not necessary with each transfer, the status of the specimen should be assessed periodically. Ongoing viability, stability of phenotype, microorganism identity, and the rate of contamination of specimens should be determined and noted in a log.

Immersion in Oil

An alternative to capping tubes is to add a layer of mineral oil to the top of the specimen. Many bacteria and fungi can be stored for periods of up to 2 to 3 years by this method, and transfers are not needed as frequently. Microorganisms are still metabolically active in this environment, and mutations can still occur. Contamination of the specimen can occur if the mineral oil is not adequately sterilized.

Mineral oil should be medicinal-grade oil with specific gravity of 0.865 to 0.890 (e.g., from Roxane Laboratories or Becton Dickinson and Co.). For sterilization, it should be heated to 170°C for 1 to 2 h in an oven (10). Autoclaving is not considered acceptable.

To prepare the specimen, an inoculum of 5 to 10 colonies of the microorganism should be placed on an agar slant or in tubed broth media. Once growth is identified, a layer of mineral oil at least 1 to 2 cm deep is added, and the agar must not be exposed to air. As with the simple transfer method, tests for viability should be performed to determine the optimal transfer schedule that will ensure microorganism recovery. Transfers will be less frequent than those of microorganisms stored without oil; however, oil is more difficult to add to vials and to clean up in the event of spills.

Freezing at −20°C

Refrigeration or freezing in ordinary freezers at −20°C may be used to preserve microorganisms for periods longer than those that can be accomplished by repeated transfers. Viability may be maintained for as long as 1 to 2 years for specific microorganisms, but overall, damage caused by ice crystal formation (15) and electrolyte fluctuations (10) results in poor long-term survival. The medium used for storage appears to be important, since preservation times vary from a few months to 2 years depending upon which medium is used (12, 15, 17). Modern self-defrosting freezers with freeze-thaw cycles must be avoided because cyclic temperature fluctuation will destroy the microorganism.

Drying

Although most microorganisms do not survive drying, molds and some spore-forming bacteria may be dried and stored for prolonged periods. Soil can be used as a storage medium if it is autoclaved and air dried. Soil should be autoclaved for several hours on two successive days. It is then transferred into sterile glass tubes. A 1-ml suspension of the microorganism is inoculated into the tube, and the tube is left open to air dry before being closed with a sterile stopper. The sample is stored in a refrigerator (10). Although potentially effective, soil is not a standardized, defined, and consistent product for use over long periods. Instead, commercial silica gel can be used in small cotton-plugged tubes after being heated in an oven to 175°C for 1.5 to 2 h (15), with moderately successful recovery of fungi. Alternatively, a suspension of 10^8 microorganisms can be inoculated onto sterile filter paper strips or disks. The paper is dried in air or under a vacuum and is placed in sterile vials. These vials can be stored in the refrigerator for up to 4 years, and then single strips or disks can be removed as needed (10). This method is commonly used for quality control organisms.

Storage in Distilled Water

Most organisms do poorly in distilled water, but some survive for prolonged periods. Many fungi and *Pseudomonas* sp. survive for several years in distilled water at room temperature (15, 21). McGinnis et al. found that with the exception of fungi that do not easily sporulate, 93% of yeasts, mold, and aerobic actinomycetes can be easily and inexpensively preserved this way (20).

Long-Term Preservation Methods

Whereas the methods described above may be used to store microorganisms for periods of up to a few years, ultralow-temperature freezing and freeze-drying (lyophilization) are recommended for long-term storage. Although the initial investment in ultralow-temperature freezers and lyophilization may be costly, these methods are less labor-intensive over time, require less laboratory space (e.g., a cryovial versus broth or agar media), and reduce the chances of mutation events. Of course, mutations may still occur, and this phenonomen was recently observed in *Staphylococcus aureus* strains that lost the *mec*A gene during long-term preservation at −80°C (30). Similar to those with other preservation methods, survival rates after freeze-drying vary with species. Evaluating microorganisms over a 10-year period, Miyamoto-Shinohara et al. found that survival rates after freeze-drying for *Brevibacterium* sp. and *Corynebacterium* sp. approached 80% whereas those for *Streptococcus mutans* decreased to 20% after 10 years (21).

Ultralow-Temperature Freezing

Microorganisms can be maintained at temperatures of −70°C or lower for prolonged periods. Systems for achieving these temperatures include ultralow-temperature electric freezers and liquid nitrogen storage units. With either system, unwanted heating can occur due to the loss of electrical power or liquid nitrogen. Close observation of the system and an adequate alarm mechanism are essential since any increase in temperature will reduce viability. In the event that the temperature does rise, restoring power and returning to the target storage temperature as quickly as possible are essential. The presence of a cryopreservative such as glycerol may reduce the risk to microorganisms upon short exposure to higher temperatures (22). If thawing does occur, there are no guidelines for rapid restoration of the storage condition. Refreezing of the sealed vials as described below may be considered.

Storage Vials

Storage vials must be able to withstand very low temperatures and maintain a seal for their contents. Plastic (polypropylene) or glass (borosilicate) tubes may be used. Plastic vials with screw tops and silicone washers are much easier to use than glass vials that must be sealed with a flame and then scored and broken open. Several commercial suppliers stock acceptable vials (e.g., Fisher Scientific Products, VWR Scientific, Wheaton Science Products, and Becton Dickinson and Co.). Vials come in a variety of sizes. Half-dram vials are available from several suppliers and can be conveniently packaged in a 12-by-12 grid so that 144 vials are stored in one box or layer.

Cryoprotective Agents

To protect microorganisms from damage during the freezing process, during storage, and during thawing, cryoprotective agents are often added to the culture suspension. Whereas most bacteria, fungi, and viruses survive better with such additives, studies have shown that cryoprotective agents will significantly damage others. The reader is referred to detailed references for specifics (Table 1) (1, 15). Rapid freezing without additives may still be acceptable for the long-term survival of protozoa, although freeze-drying may be preferred.

There are two types of cryoprotective agents: those that enter the cell and protect the intracellular environment and others that protect the external milieu of the organism. Glycerol and dimethyl sulfoxide (DMSO) are most often used for the former; sucrose, lactose, glucose, mannitol, sorbitol, dextran, polyvinylpyrrolidone, polyglycol, and skim milk are used for the latter. Combinations of agents as well as detergents (e.g., Tween 80 and Triton WR 1339), other carbohydrates (e.g., honey), and calcium lactobionate have also been used. The most universal cryoprotectant is DMSO; however, the optimal cryoprotectant often varies with the microorganism. For example, glycerol appears to be best suited for the preservation of bacteria. A current and comprehensive review of protectant additives used in the cryopreservation of microorganisms is provided by Hubalek (16).

TABLE 1 Common procedures for preservation of microorganisms

Organism group	Storage method	Cryopreservative	Storage temp (°C)	Storage duration (yr)
Gram-positive bacteria	Transfer	None	Room temp	0.2–0.3
	Immersion in mineral oil	None	4	0.6–2
	Freezing	Sucrose, glycerol	−20	1–3
	Ultralow-temp freezing	Skim milk, sucrose, glycerol	−70 to −196	1–30
	Lyophilization	Skim milk, sucrose	4	30
Streptococci	Freezing	Skim milk	−20	0.2
	Ultralow-temp freezing	Skim milk	−70 to −196	0.2–1
	Lyophilization	Skim milk	4	0.5–30
Mycobacteria	Freezing	Skim milk	−20	3–5
	Ultralow-temp freezing	Skim milk	−70 to −196	3–5
	Lyophilization	Skim milk	4	16–30
Gram-negative bacteria	Transfer	None	Room temp	0.1–0.3
	Immersion in mineral oil	None	4	1–2
	Freezing	Sucrose, lactose	−20	1–2
	Ultralow-temp freezing	Sucrose, lactose, glycerol	−70 to −196	2–30
	Lyophilization	Skim milk, sucrose, lactose	4	30
Spore-forming bacteria	Transfer	None	Room temp	0.2–1
	Immersion in mineral oil	None	4	1
	Drying	None	Room temp	1–2
	Freezing	Glucose	−20	1–2
	Ultralow-temp freezing	Skim milk, glycerol	−70 to −196	2–30
	Lyophilization	Skim milk, lactose	4	30
Filamentous fungi	Transfer	None	4 to 25	2–10
	Immersion in mineral oil	None	Room temp	1–40
	Storage in distilled water	None	Room temp	1–10
	Drying	Soil, silica gel	Room temp	1–4
	Ultralow-temp freezing	Glycerol, DMSO	−70 to −196	2–30
	Lyophilization (sporeformers)	Glycerol, sucrose, DMSO, skim milk	4	2–30
Yeasts	Storage in distilled water	None	Room temp	1–2
	Drying	Nutrient medium	Room temp	1–2
Protozoa	Freezing	Blood, nutrient broth with DMSO or sucrose	−20 to –40	
	Ultralow-temp freezing	Blood, nutrient medium with DMSO or glycerol	−70 to −196	
Viruses	Transfer	Nutrient medium	4	0.5
	Ultralow-temp freezing	SPGA	−70 to −196	1–30
	Lyophilization	SPGA	4	6–10

Glycerol is added at a concentration of 10% (vol/vol), and DMSO is added at 5% (vol/vol). Prior to use, glycerol is sterilized by autoclaving. Once prepared, it can be stocked at room temperature for months. DMSO must be filter sterilized and can be stored in open containers for only 1 month prior to use.

Of the external products, skim milk is the most often used. Dehydrated skim milk is purchased from medical product suppliers (e.g., Becton Dickinson and Co. and Oxoid). It is autoclaved and used in a final concentration of 20% (wt/vol) in distilled water (1). This is double the concentration suggested by the manufacturers if the intent is to make a reconstituted equivalent of regular milk.

Preparation of Microorganisms for Freezing

Microorganisms are inoculated into a medium that adequately supports maximal growth. Cultures are allowed to mature to the late growth or stationary phase before being harvested. Broth specimens are centrifuged to create a pellet of microorganisms. The pellet is withdrawn and resuspended in 2 to 5 ml of broth with the appropriate concentration of cryoprotectant additive. For agar specimens, broth containing the cryoprotectant is placed on the surface of the agar. The surface is scraped with a pipette or sterile loop to suspend microorganisms, and then the broth mixture is pipetted directly into freezer vials. Alternatively, the agar surface can be scraped with a sterile loop. The microorganisms can then be transferred directly into the vial of cryoprotectant and emulsified into a final dense suspension. The volume of the aliquots to be frozen is typically 0.2 to 0.5 ml.

Freezing Method

The American Type Culture Collection (ATCC) recommends slow, controlled-rate freezing at a rate of 1°C per min until the vials cool to a temperature of at least −30°C, followed by more rapid cooling until the final storage temperature is achieved (1). Controlled-rate freezers are required for the initial phase of cooling. Studies in the 1970s showed that uncontrolled-rate freezing may be acceptable for most organisms and is much less expensive or labor-intensive (15). When organisms are stored in liquid nitrogen, however, it is still recommended that vials be placed initially in a −60°C freezer for 1 h and then transferred into the liquid nitrogen. When organisms are stored permanently at −60 to −70°C, the vials can be placed directly into the freezer.

Small glass beads or plastic beads (e.g., from Fisher Scientific Products or Wheaton Science Products) can also be added to storage vials before freezing. The culture suspension will coat the beads, and then individual beads can be removed from storage for reconstitution without thawing the entire sample (8).

Thawing

Damage to microorganisms occurs as they are warmed from the frozen state. Critical temperatures appear to be between −40 and −5°C. Studies suggest that rapid warming through these temperatures improves recovery rates. Stored culture vials should be warmed rapidly in a 35°C water bath until all ice has disappeared (1, 15). Once a vial is thawed, it should be opened and the organism should be transferred to an appropriate growth medium immediately. Great care must be exercised during the thawing phase since rapid temperature changes and resulting air pressure changes inside vials can cause the vials to explode. Protective clothing and eyewear must be worn during this process.

Freeze-Drying (Lyophilization)

Freeze-drying is considered to be the most effective way to provide long-term storage of most bacteria. Better preservation occurs with freeze-drying than with other methods because freeze-drying reduces the risk of intracellular ice crystallization that compromises viability. Removal of water from the specimen effectively prevents this damage. On the other hand, the process of drying causes extensive damage to molds, protozoa, and most viruses. Hence, these microorganisms cannot be stored by this method. Among bacteria, the relative viability with lyophilization is greatest with gram-positive bacteria (sporeformers in particular) and decreases with gram-negative bacteria (15), but overall, the viability of bacteria can be maintained for as long as 30 years. In addition, large numbers of vials of dried microorganisms can be stored with limited space, and organisms can be easily transported long distances at room temperature.

The process combines freezing and dehydration. Organisms are initially frozen and then dried by lowering the atmospheric pressure with a vacuum apparatus. Freeze-drying has been extensively reviewed in the past (14), and the required equipment includes a vacuum pump connected in line to a condenser and to the specimens. Specimens can be connected individually to the condenser (manifold method) or can be placed in a chamber where they are dehydrated in one larger air space (chamber or batch method). Alexander et al. and Heckly have both published detailed descriptions of equipment options (1, 14).

Storage Vials

Glass vials are used for all freeze-dried specimens. When freeze-drying is performed in a chamber, double glass vials are used. In the chamber method, an outer soft-glass vial is added for protection and preservation of the dehydrated specimen. Silica gel granules are placed in the bottom of the outer vial before the inner vial is inserted and cushioned with cotton. For the manifold method, a single glass vial is used. For both methods, the vial containing the actual specimen is lightly plugged with absorbent cotton. The storage vial in the manifold method or the outer vial in the chamber method must be sealed to maintain the vacuum and the dry atmospheric condition. All vials are sterilized prior to use by heating in a hot-air oven.

Cryoprotective Agents

Research concerning cryoprotective agents has been extensively reviewed (14). In general, the two most commonly used agents are skim milk and sucrose. Skim milk is used most often for chamber lyophilization, and sucrose is used most often for manifold lyophilization. Skim milk is prepared by making a 20% (vol/vol) solution of skim milk in distilled water. The solution is divided into 5-ml aliquots and autoclaved at 116°C with care taken to prevent overheating and caramelizaton of the solution. The preparation is then used in smaller volumes as described above for freezing. Sucrose is prepared in an initial mixture of 24% (vol/vol) sucrose in water and added in equal volumes to the microorganism suspension in growth medium to make a final concentration of 12% (vol/vol).

Preparation of Microorganisms for Freezing

As with simple freezing, maximum recovery of organisms is achieved by using microorganisms in the late growth or stationary phase from the growth of an inoculum in an appropriate growth medium. High concentrations of microorganisms are considered to be important. The ATCC recommends a

concentration of at least 10^8 CFU/ml (1), and Heckly suggests a concentration of 10^{10} CFU/ml or higher (15).

Freeze-Drying Methods

In the chamber method, inner vials with the microorganism suspension are placed in a single layer inside a stainless steel container. This container is placed in a low-temperature freezer at −60°C for 1 h. The container is then transferred to a chamber containing dry ice and ethyl Cellosolve (Becton Dickinson and Co.) and covered with a sealable vacuum top, which is connected in sequence to a condenser reservoir also filled with dry ice and ethyl Cellosolve and to a vacuum pump. The vacuum is maintained at a minimum of 30 μm Hg for 18 h. At the same time, the outer vials are prepared by being heated in an oven overnight, filled with silica gel granules and cotton, and placed in a dry cabinet with <10% relative humidity. The freeze-dried inner vials are inserted into the outer vials, and the outer vials are heat sealed. Multiple different strains or species should probably not be processed in the same batch. Cross contamination rates vary from 0.8 to 3.3% when two different microorganisms are placed on opposite sides of the same container and are as high as 8.3 to 13.3% when microorganisms are intermingled (3).

In the manifold method, a rack of individual vials is used rather than a single container. The rack is placed in a dry ice-ethyl Cellosolve bath. After the freezing process, the vials are connected by individual rubber tubes in sequence to the condenser container filled with dry ice and ethyl Cellosolve and to the vacuum pump. As in the method described above, the vacuum is maintained at 30 μm Hg for 18 h and then the individual vials are sealed.

Storage

Individual vials need to be appropriately labeled and sorted. Storage at room temperature does not maintain viability and is not recommended. Storage at 4°C in an ordinary refrigerator is acceptable, but survival may be improved at temperatures of −30 to −60°C (1, 14).

Reconstitution

Care must be taken when opening vials for reconstitution because of the vacuum inside the vial. Safety glasses should always be worn, and vials should be covered with gauze to prevent injury if the vial explodes when air rushes in. Reconstitution should also be conducted in a closed hood to avoid dispersal of microorganisms. The surface of the vial should be wiped with 70% alcohol, and then the top of the glass vial can be scored and broken off or punctured with a hot needle. A small amount (0.1 to 0.4 ml) of growth medium is injected into the vial with a needle and syringe or a Pasteur pipette, the contents are stirred until the specimen is dissolved, and then the entire contents are transferred with the same syringe or a pipette to appropriate broth or agar media. A purity check must be done on each specimen because of the possibility of either cross contamination or mutation during the preservation process.

Procedures for Specific Organisms

Procedures for specific organisms are described below and summarized in Table 1.

Bacteria

All of the material presented in this chapter applies primarily to the preservation of bacteria. Simple transfer, storage under mineral oil, drying, or freezing at −20°C can maintain bacteria for short periods; freezing in ultralow-temperature electric freezers at −70°C or in liquid nitrogen at −196°C or freeze-drying can provide long-term preservation. A summary of the studies of bacterial preservation has been published (15). In general, serial transfer will preserve bacteria for up to a few months, storage under mineral oil or with drying will last 1 to 2 years, freezing at −20°C will preserve bacteria for 1 to 3 years, freezing at −70°C will preserve bacteria for 1 to 10 years, and freezing in liquid nitrogen and freeze-drying will preserve bacteria for up to 30 years (10). For fastidious bacteria such as *Streptococcus pneumoniae*, *Neisseria* spp., and *Haemophilus* spp., the optimal methods are lyophilization and freezing at −70°C by using Trypticase soy broth with glycerol as a preservation medium (23, 26, 31). Stock cultures of quality control microorganisms can be maintained in a cryopreservative suspension for up to 1 year at −20°C or indefinitely at −70°C.

Protozoa

Information concerning the preservation of protozoa is limited, in keeping with the infrequent need for such a process in clinical microbiology laboratories. Variable methods for individual genera are described. In general, freezing appears to be preferred to freeze-drying. All of the following procedures are as described by the ATCC (1).

Acanthamoeba sp., *Leishmania* sp., *Naegleria* sp., *Trichomonas* sp., and *Trypansoma* sp. can be handled as described above for ultralow-temperature freezing with 5% (vol/vol) DMSO as the cryoprotecting agent. These organisms should be stored in liquid nitrogen.

Acanthamoeba sp. and *Naegleria* sp. can also be dried at room temperature onto filter paper. Aliquots of a microorganism suspension (0.3 ml) are pipetted onto the paper in a shell vial and dried in air for 14 days at room temperature and then in a vacuum desiccator for an additional week. The vials are sealed and stored in liquid nitrogen.

Entamoeba sp. is stored frozen at −40°C. Specimens should be suspended in a mixture of growth medium containing 12% (vol/vol) DMSO and 6% (vol/vol) sucrose.

Leishmania sp. may also be prepared by inoculation of the organism into an animal host. At the peak of infection, the spleen is harvested and homogenized in half the final volume of ATCC medium 811 salt solution. Freezing is completed with 10% glycerol as the cryoprotectant.

Plasmodium sp. can be stored from infected blood samples. At the height of parasitemia, blood is obtained and anticoagulated with the following preparation: 1.33 g of sodium citrate, 0.47 g of citric acid, 3.00 g of dextrose, 200 mg of heparin (sodium), and 100 ml of distilled water. The final concentration of anticoagulant added to blood is 10%. To this anticoagulated blood, 30% glycerol in 0.0667 M phosphate buffer is added to a final concentration of 10% (vol/vol) glycerol. Freezing should occur in liquid nitrogen.

Trypanosoma sp. must be harvested from an animal host. At the peak of parasitemia, blood is withdrawn into heparinized tubes and diluted 1:1 in Tyrode's solution (8.0 g of NaCl per liter, 0.02 g of KCl per liter, 0.2 g of $CaCl_2$ per liter, 0.1 g of $MgCl_2$ per liter, 0.05 g of NaH_2PO_4 per liter, 1.0 g of $NaHCO_3$ per liter, and 1.0 g of glucose per liter) with 1 to 5% phenol red added. Then 5% DMSO is added as the cryoprotectant, and the specimen is stored in liquid nitrogen.

Yeasts and Filamentous Fungi

All of the techniques described above have been applied to the storage of yeasts and fungi (5, 10, 15, 27). The individual

method employed depends upon the species to be preserved and whether or not it sporulates.

Subculturing. Subculturing is the simplest method of maintaining living fungi and involves serial transfer to fresh solid or liquid media. Storage is accomplished usually at room or refrigerator temperature. Fungi may be maintained by subculturing for a number of years. Care must be taken to avoid aerosolization and contamination of the laboratory or other specimens.

Storage under oil. Whereas species of *Aspergillus* and *Penicillium* have remained viable under oil for 40 years (27), many species have shown deterioration after 1 to 2 years and must be transferred periodically. Taddei et al. also reported the successful storage and recovery of actinomycetes stored under paraffin oil for 10 to 30 years (28).

Water storage. Many fungi can be stored successfully for prolonged periods in distilled water (21, 24). A simple method is to pipette 6 to 7 ml of sterile distilled water onto 2-week-old culture slants in screw-cap tubes. The spores and fragments of hyphae are dislodged by scraping with the pipette, and the suspension is transferred to a sterile 1-g vial, which is tightly capped and stored at 25°C. Fungi are revived by subculturing 0.2 to 0.3 ml of the suspension to appropriate media (4).

An alternative method is to cut agar blocks from the growing edge of a fungal colony and place them in sterile distilled water in bottles with screw-cap lids (13). The cultures are stored at 20 to 25°C. The fungi are retrieved by removing a block and placing it mycelium side down on growth medium appropriate for that species (27). Contamination (22.8%) is a significant problem with this method (13).

Drying. Drying as described above has been used for fungi. Only 6 of 16 genera of fungi stored in this fashion survived for 4 years (2). The greatest success is reported for sporulating fungi stored in silica gel or in soil (27).

Freezing. Fungi have been successfully preserved by storage in liquid nitrogen by using glycerol or DMSO as cryopreservatives. Broth cultures containing nonpathogenic fungi are disrupted in a Waring blender and suspended in equal parts of DMSO or glycerol to achieve final concentrations of 5 or 10%, respectively. Pathogens should not be disrupted in a mechanical blender because of the potential biohazard associated with aerosolization. *Histoplasma, Paracoccidioides,* and *Blastomyces* species should be frozen in the yeast phase, and *Coccidioides* species should be frozen in the early mycelial phase to minimize exposure of laboratory personnel. Otherwise, procedures for freezing are as described above.

Freeze-drying. Most spore-forming fungi can be preserved by freeze-drying. Cultures to be stored by freeze-drying should be grown on agar or broth media to the point of maximum sporulation (1) and processed as described above. Survival in storage for many years has been demonstrated (6, 25), but this is true only for sporulating organisms. Young vegetative hyphae of fungi do not survive freeze-drying (27).

Viruses

Viruses tend to be more stable than other microorganisms because of their small size and simple structure and the absence of free water. Many viruses can be stored for months at refrigerator temperatures or for years by ultralow-temperature freezing or freeze-drying. Storage at −20°C is not recommended (15, 18). Larger viruses tend to be less stable than smaller ones (11).

Ultralow-temperature freezing is effective in a number of situations. In addition to cryoprotectants described above, sucrose-phosphate-glutamate containing 1% bovine albumin (SPGA) (15, 18) and hypertonic sucrose are particularly effective, the latter for storing labile viruses such as respiratory syncytial virus (19). If ultralow-temperature freezing is employed, the rate of freezing should be as high as possible, using small-volume suspensions (0.1 to 0.5 ml). In addition to freezing of pure isolates, stool specimens known to contain viral enteric pathogens have been maintained at −70 to −85°C for 6 to 10 years with reasonable recovery and no change in the morphological characteristics of astroviruses, small round structured viruses, enteric adenoviruses, rotaviruses, and caliciviruses (32).

Gallo et al. evaluated five types of media for the storage of human immunodeficiency virus-infected peripheral blood lymphocytes and concluded that freezing peripheral blood lymphocytes in RPMI 1640 containing 10% fetal bovine serum and 10% DMSO and storing them at −60°C is acceptable for human immunodeficiency virus isolation (9).

Freeze-drying is probably the optimum method for preserving viruses for extended periods. A detailed review of acceptable procedures has been published (11). Virus suspensions freeze-dried in medium supported with SPGA appear to survive better (15, 29). Lyophilization of polioviruses and other enteroviruses works best when electrolytes are removed by dialysis or ultrafiltration (15).

Select Agents

In response to the Public Health Security and Bioterrorism Preparedness and Response Act of 2002, federal regulations require laboratories that store select agents to register and comply with the standards established by the act (7). A current and complete list of microorganisms considered to be select agents can be found at www.cdc.gov/od/sap. Regardless of the method for long-term preservation, laboratories must register with the Department of Health and Human Services and Centers for Disease Control and Prevention Select Agent Program. In order to minimize risk to public health and safety, select agents must be stored in a highly secured area with restricted access and appropriate safeguards. Only registered individuals who have completed training for handling select agents can access and retrieve these microorganisms from storage. An accurate and current inventory of select agents held in long-term storage must be maintained.

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THE CLINICAL MICROBIOLOGY LABORATORY IN INFECTION DETECTION, PREVENTION, AND CONTROL

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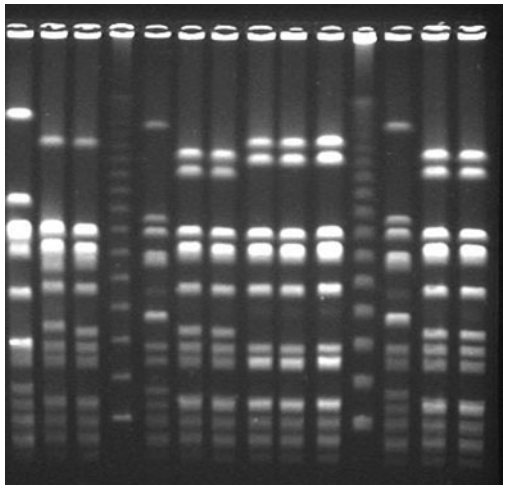
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SmaI digest of *Staphylococcus aureus* on PFGE gel (R. Hollis, University of lowa).

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Decontamination, Disinfection, and Sterilization

ANDREAS F. WIDMER AND RENO FREI

7

Decontamination, disinfection, and sterilization are basic components of any infection control program. Patients expect that any reusable instrument or device used for diagnosis or treatment has undergone a process to eliminate any risks for cross-infection. However, the infection control literature documents many reprocessing failures, including numerous reports of transmission of nosocomial pathogens from contaminated endoscopes (30, 102, 224, 231, 234). Before 1990, it was very difficult to prove a causal relationship between a contaminated device and a subsequent nosocomial infection. Today, state-of-the-art clinical epidemiology supported by molecular typing tools such as pulsed-field gel electrophoresis, PCR, and genome sequencing can enable the hospital epidemiologist to prove a causal relationship between the use of a contaminated device and a consequent infection. Molecular epidemiology has, thus, provided scientific tools that can identify the limitations of the available disinfection and sterilization methods and can provide the impetus to improve reprocessing technologies.

Despite those advances, little research has been done that will lead to major breakthroughs in disinfection and sterilization in the near future. Thus, we believe the key issues instead will be to standardize and optimize our applications of current knowledge. Clearly, more research is needed in this field, but resources for such work have been limited. In fact, most disinfectants were introduced to the market more than 20 years ago and little is known about their modes of action and the mechanisms of resistance. Excellent reviews on this topic are published by Block (34), by McDonnell and Russell (169), and by Russell et al. (200). In addition, few basic procedures in decontamination, disinfection, and sterilization have been tested in randomized clinical trials. In this chapter, we have tried to cite the highest level of evidence available. However, given the dearth of studies, we have often had to cite results of observational studies, animal models, in vitro tests, and expert opinion because higher levels of evidence are not available.

Despite the lack of resources, reprocessing techniques, disinfectants, and general infection control practices have garnered more attention recently than in the past. This is due in part to the increasing frequency of multiresistant bacterial pathogens at a time when pharmaceutical companies have shifted from developing antimicrobial agents to designing drugs for chronic diseases (261). Moreover, new pathogens, such as the viruses causing severe acute respiratory syndrome (SARS) and avian influenza (122) and the prions causing Creutzfeldt-Jakob disease (CJD), and variant CJD (vCJD), are emerging for which there are few if any treatments. Consequently, the medical community needs better knowledge on disinfection and sterilization to prevent the spread of these pathogens.

PRINCIPLES OF TERMINOLOGY, DEFINITIONS, AND CLASSIFICATION OF MEDICAL DEVICES

Background

There is no uniform terminology for disinfection and sterilization, and many problems arise as a result. Many terms are ill defined even within the United States and Europe. In addition, the testing procedures for disinfectants are not as far advanced and well defined as those for testing the MIC for an organism based on the recommendations of the Clinical and Laboratory Standards Institute (formerly NCCLS). However, there currently are efforts to standardize and harmonize the terminology on an international level. For example, International Organization for Standardization (ISO) norms for sterilization were published in 2004. Manufacturers now must provide specific data on how to reprocess their medical devices. In the past, such information was frequently missing in the users' manuals.

Classification of Devices for Reprocessing

Background

The principal goal of disinfection and sterilization is to reduce the numbers of microorganisms on a device to a level that is insufficient to transmit infectious organisms, with a considerable safety margin. The most conservative approach would be to reprocess all items and devices with overkill sterilization. Obviously, not all items must undergo the most vigorous process to eliminate any microorganisms. For example, items such as blood pressure cuffs that are used at nonsterile body sites do not need to be sterilized between patients. In contrast, only sterilization will eliminate any risk of infection from devices used in normally sterile body sites. In some cases, the best choice may be to use disposable items instead of reusable devices, because reprocessing may be more expensive or may not provide the desired level of

safety. The latter may apply to items in contact with the neural tissue of a patient suffering from any form of CJD or with tonsils and other lymphatic tissues of persons with bovine spongiform encephalopathy (BSE) or vCJD (26, 67, 260). Therefore, devices must be classified to allow staff to define the appropriate method for disinfection and/or sterilization for each item. A classification system should balance the potential risks for transmission of infection (e.g., the infectious dose) and the resources available to achieve the necessary or desired level of antimicrobial killing.

The most commonly used classification was proposed by Earle H. Spaulding in 1968 (232). He proposed three categories that are based on a device's potential for transmitting infectious agents: critical, semicritical, and noncritical (Table 1). The Centers for Disease Control and Prevention (CDC) cites this classification in its *Guidelines for Handwashing and Hospital Environment Control* (http://www.cdc.gov/mmwr/preview/mmwrhtml/rr5116a1.htm), and the U.S. Food and Drug Administration (FDA) cites it in its document *Content and Format of Premarket Notification [510(k)] Submissions for Liquid Chemical Sterilants and High-Level Disinfectants* (see http://www.fda.gov/cdrh/index.html). Most infection control professionals worldwide use this classification as well. However, this simple classification does not work perfectly for all devices. Even the definition of sterilization as the absence of any viable microorganisms must be revised to address the prions responsible for CJD and vCJD (187).

Critical Items

Items that enter normally sterile parts of the human body, such as surgical instruments, implants, and invasive monitoring devices (Table 1), are classified as "critical items." Because items classified as critical carry the highest risk for the patient, sterilization is the preferred method for reprocessing of these items. Autoclaving is the method of choice if the device is not heat labile. Alternative sterilization processes that use ethylene oxide or plasma require prolonged times, and the FDA has not approved them for use with instruments that have small, dead-end lumens, which are difficult to sterilize. Liquid sterilization with a glutaraldehyde-based formulation or peracetic acid is acceptable if sterilization by one of the methods mentioned above is not feasible and the formulation and/or automated device has been cleared by the FDA.

Semicritical Items

Semicritical objects come into contact with mucous membranes or nonintact skin and should be free of microorganisms except spores. Intact mucous membranes generally resist bacterial spores but are susceptible to other microorganisms such as vegetative bacteria (e.g., *Mycobacterium tuberculosis*) and viruses (e.g., human immunodeficiency virus [HIV] and cytomegalovirus). Examples of semicritical equipment include anesthesia equipment, respiratory equipment, and endoscopes. These items should be processed with a high-level disinfectant such as glutaraldehyde, stabilized hydrogen peroxide, peracetic acid, or a chlorine compound. Chlorine compounds corrode items and, therefore, are rarely used to disinfect medical devices.

Noncritical Items

Noncritical items, such as bedside tables, crutches, stethoscopes, furniture, and floors, come into contact with intact skin only. Intact skin is a very effective barrier against microorganisms, and therefore, these items and devices do not need to be sterilized. Such items pose a very low risk for direct transmission of pathogens and can usually be cleaned at the bedside or wherever they have been used with a low-level disinfectant. For example, health care workers can disinfect their stethoscopes by wiping the surfaces with alcohol. However, noncritical devices can contribute to the transmission of pathogens by the indirect route. For instance, up to 60% of cultures of the environments near patients colonized or infected with vancomycin-resistant enterococci are positive for this organism (56). Health care workers can contaminate their hands when they touch these surfaces. If they do not practice hand hygiene, they can spread these pathogens

TABLE 1 Spaulding classification of devices

Clinical device	Definition	Example	Infectious risk	Reprocessing procedure	
				FDA classification	EPA classification
Critical device	Medical device that is intended to enter a normally sterile environment, sterile tissue, or the vasculature	Surgical instruments	High	Sterilization by steam, plasma, or ethylene oxide; liquid sterilization acceptable if no other methods feasible	Sterilant or disinfectant
Semicritical device	Medical device that is intended to come in contact with mucous membranes or minor skin breaches	Flexible endoscope	High, intermediate	Sterilization desirable; high-level disinfection acceptable	Sterilant or disinfectant
Noncritical device	Medical device that comes in contact with intact skin	Blood pressure cuff, electrocardiogram electrodes	Low	Intermediate or low level	Hospital disinfectant with label claim for tuberculocidal activity
Medical equipment	Device or component of a device that does not typically come in direct contact with the patient	Examination table	Low	Low-level disinfection, use of sanitizer	Hospital disinfectant without label claim for tuberculocidal activity but with claim for virucidal activity against HIV

TABLE 2 Principles of medical device classification

Classification	FDA regulation	Premarket requirements by the FDA	Proposed classification by the Global Harmonization Task Force[a]	Examples
Class I	Least regulated, requires fewest regulations	None	A	Band-Aid, tongue depressor
Class II	Must meet federal performance standards	Premarket notification [510(k)]	B	Surgical gowns, drapes, scrub sponges
			C	Orthopedic implants
Class III	Implanted and life-supporting or life-sustaining devices are required to have FDA approval for safety and effectiveness	Premarket approval	D	Artificial hearts

[a] Details available at http://www.ghtf.org/sg1/inventorysg1/pd_sg1_n015r22.pdf.

to devices or directly to other patients. Therefore, noncritical items must be decontaminated if they are likely to be contaminated with pathogenic organisms.

The FDA also has developed a classification based on safety considerations and the regulations manufacturers must meet before marketing a device. Medical products are listed as class I to III products (Table 2). Simple products such as a tongue depressor are classified as class I medical products, which must meet very simple requirements before they can be marketed legally. Class II products, such as autoclaves, require premarket notification [510(k)] demonstrating that the device to be marketed is at least as safe and effective as a legally marketed device. Class III devices are those that support or sustain human life and are of substantial importance in preventing impairment of human health (e.g., a pacemaker). Due to the level of risk associated with class III devices, the FDA requires companies to file a premarket approval (PMA) application to obtain marketing clearance (section 515 of the Federal Food, Drug and Cosmetic Act). The PMA application must contain sufficient valid scientific evidence documenting that the device is safe and effective for its intended use (56).

DECONTAMINATION AND CLEANING

In Europe, decontamination basically means cleaning an item to remove organic material, protein, and fat. In the United States, the term describes a cleaning step and any additional step required to eliminate any risk of infection to health care workers while they handle a device without protective attire. The FDA defines the cleaning process as including all steps necessary to remove, inactivate, or contain contamination, beginning immediately after an item has been used for clinical purposes; continuing with the steps to decontaminate, clean, and package a device up to the first step of the sterilization process; and ending with quality control tests.

Regardless of regulations, cleaning is always the initial step of the decontamination process on both continents. In this chapter, we will use the term decontamination to describe the removal of debris, blood, and proteins and most microorganisms. This process usually, but not necessarily, renders the device "safe to handle" by health care workers who are not wearing protective attire. Basic definitions are outlined in Table 3.

The first step in reprocessing used medical devices is for health care workers to prevent debris from drying on the items. Research on prion diseases demonstrates that removal of debris is seriously impaired if the debris is allowed to dry on a medical device (87). Therefore, the reprocessing cycle should start as soon as possible and items should be kept wet if delays in reprocessing are anticipated (115, 179). Cleaning can be done physically or chemically; it can also be done by hand, by sonication, or by use of washers. In the United States, cleaning is frequently performed manually with water and a detergent. In Europe, many countries rely primarily on washers-disinfectors that rinse items with cold water and then with warm water plus a detergent. The cycle is completed with hot water at >90°C. Items such as bedpans and urinals can be cleaned and disinfected by putting the items into a machine, pushing a button, and removing them after a 2- to 5-min procedure.

All sterilization techniques other than steaming have been shown to fail in 1 to 40% of sterilization cycles if residual proteins and/or salts are not removed by a proper cleaning process (11). Even steam sterilization at 134°C for 18 min, recommended by the World Health Organization (WHO) to inactivate prions, can fail to prevent cross-transmission if the device does not undergo a cleaning process (87, 115, 179).

For floors, surfaces, and noncritical items, cleaning with a detergent is sufficient in most situations and a disinfection process contributes little if any additional effect (76). In addition, residual proteins and debris that escape the cleaning process may interfere with disinfectants and even cause them to lose activity (76). In the United States, routine disinfection of environmental surfaces in patient care areas is recommended as an additional safety precaution in case the environment is contaminated with unrecognized body fluids; in Europe, this practice is restricted to intensive care units and emergency rooms (227).

DISINFECTION

Disinfection is the second critical step in reprocessing of medical devices. To be effective, disinfection must be preceded by thorough cleaning and it must be done properly. Staff must check the disinfectant's concentration regularly if it is diluted at the place of use, even if it is diluted with an electronically monitored dilution device. Failures of the valve or other critical parts of the device can result in an insufficient final concentration, which usually cannot be detected by checking either the appearance or the odor of

TABLE 3 Definitions and terms

Term	Standard[a]	Technical-microbiological log CFU reduction	Comment
Sterilization	(Closely monitored) validated process used to render a product free of all forms of viable microorganisms, including all bacterial endospores	$\geq 10^6$ log CFU reduction of the most resistant spores for the sterilization process studied, achieved at the half-time of the regular cycle (ISO 14937)	Prions require an adapted definition because of their high resistance to any form of sterilization
Disinfection	Elimination of most if not all pathogenic microorganisms, excluding spores	No clear-cut defined reduction level; a minimum estimate is $\geq 10^3$ log CFU reduction of microorganisms, excluding spores; log unit reductions of 4 to 5 commonly achieved for devices; these are estimates, because there is no international standardization	Some high-level disinfectants achieve microbial reduction, including level of reduction of spores similar to sterilization, if long incubation times and/or temperatures of >25°C are applied; this is called liquid sterilization by sterilants
Decontamination	Reduction of pathogenic microorganisms to a level at which items are "safe to handle" without protective attire	Elimination of debris and proteins by cleaning and/or a disinfection or sterilization process; in Europe, the process is restricted to cleaning only, which achieves a minimum reduction of ≥1 log CFU; most cleaning processes achieve ≥ log CFU reductions of 3 to 5; these are estimates, because there is no international standardization	Manual and/or mechanical cleaning with water and detergents or enzymes is a prerequisite before disinfection or sterilization; in Europe, this term is used for cleaning of items; in the United States, it defines the process that makes an item "safe to handle"; it may include a cleaning process but also a disinfection or even a sterilization process; the U.S. term "decontamination" refers to the health care workers' safety; in Europe, the term is used for the item only
Antisepsis	Patient related: disinfection of living tissue or skin Health care worker related: reduction or removal of transient microbiological flora	Preoperative skin preparation with an alcohol-based iodine compound Hand washing: (scrub) reduction of ≥1 log CFU Hand disinfection (rub-in): reduction of ≥2.5 log CFU	Antiseptic agents are handled as drugs by the FDA

[a] Examples for standards are as follows: ethylene oxide sterilization (industrial facility use, ISO 11135; health care facility use, ANSI/AAMI ST 41) and moist-heat sterilization (industrial facility use, ISO 11134; health care facility use, ANSI/AAMI ST 46).

the disinfectant. Many manufacturers provide test strips to check for the appropriate concentration. Of note, numerous outbreaks have occurred when staff members have not followed appropriate protocols. For example, *Klebsiella oxytoca* caused an outbreak after an infection control committee allowed staff members to decrease the concentration of a glutaraldehyde-based surface disinfectant because they did not like the odor. The outbreak stopped after staff members resumed use of the disinfectant to the recommended concentration (190). An outbreak of 58 cases of *Mycobacterium xenopi* infection occurred when instruments used for discovertebral operations were rinsed with tap water after they were disinfected (22).

Principles and Antimicrobial Activities of Compounds

The antimicrobial spectrum of disinfectants varies. Low-level disinfectants destroy lipid-enveloped viruses such as HIV and most vegetative bacteria (Fig. 1) (35), but many disinfectants, including alcohol, are ineffective against nonlipid or small viruses such as poliovirus. For example, isopropyl alcohol has little activity against poliovirus, but >90% ethanol is very active (247). Intermediate-level disinfectants are effective against nonlipid or small viruses, such as poliovirus, and high-level disinfectants are effective against mycobacteria (Table 4).

The antimicrobial spectra of disinfectants are tested differently from those of antimicrobial agents. Microbiology laboratories that test disinfectants must know the special methods needed to accurately assess the activities of disinfectants. In fact, MICs are of little help because the goal of disinfection is to kill rather than inhibit the growth of microorganisms. In contrast to sterilization curves, killing curves for disinfectants are not linear and the rate of log killing decreases as the inoculum concentrations decrease (i.e., as the number of CFU per milliliter decreases). Therefore, a 3-log-unit killing is more easily achieved with disinfectants if the inoculum is large, e.g., 10^8 CFU, than if

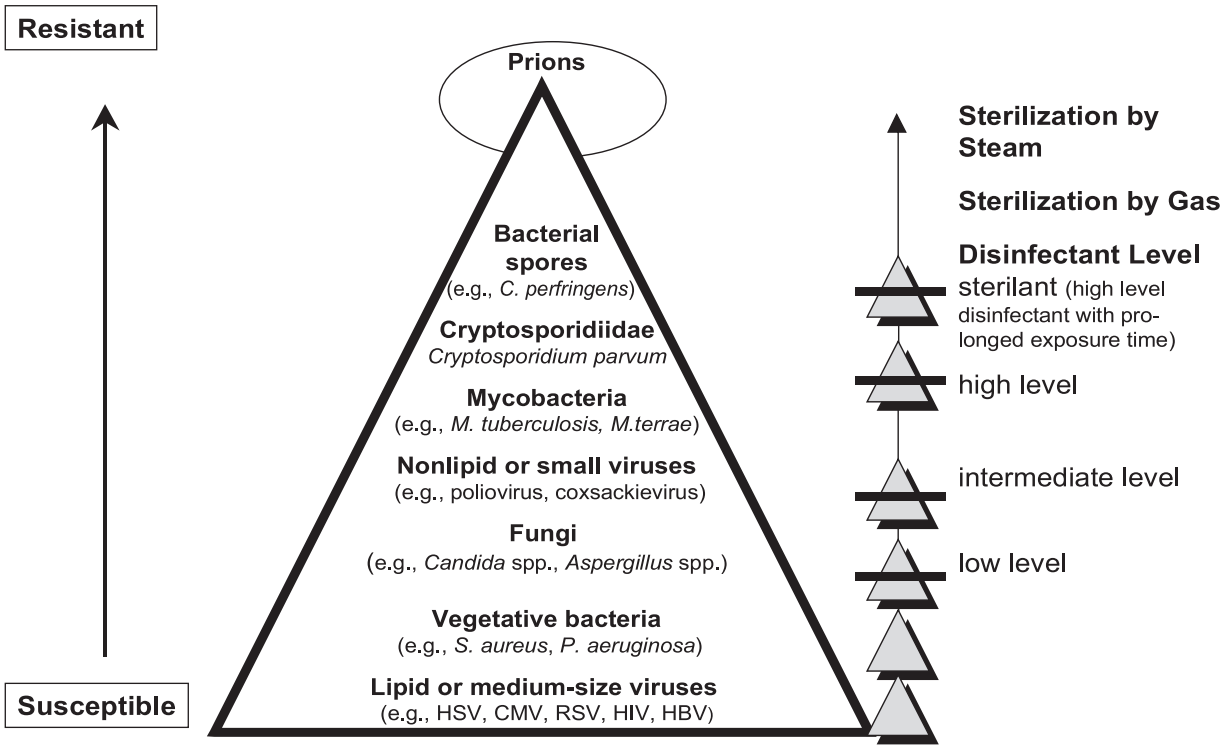


FIGURE 1 Microorganisms' resistance to disinfectants. HSV, herpes simplex virus; CMV, cytomegalovirus; RSV, respiratory syncytial virus; HBV, hepatitis B virus.

the inoculum is 10^4 CFU. Most disinfectants must be inactivated before they are incubated in media or plated because bacteria do not grow in the presence of very low concentrations of a disinfectant (inhibitory effect). However, if the compound is inactivated, bacterial growth can be demonstrated. Like antimicrobial agents, some disinfectants display a postexposure effect on bacterial growth. The postexposure effect has been quantified for a variety of disinfectants. Alcohols in general have little, if any, postexposure effect, but chlorhexidine, octenidine, and chloramine delay regrowth after exposure for several hours (35).

The FDA requires that the microbicidal efficacy of liquid chemical sterilants and high-level disinfectants be assessed in three different types of tests before these products can be legally marketed in the United States.

1. Potency testing incorporates the Environmental Protection Agency (EPA)'s test requirements for registration of germicides, such as the Association of Official Analytical Chemists' (AOAC) sporocidal test, tuberculocidal test, and use dilution tests for *Staphylococcus aureus* ATCC 6538, *Salmonella enterica* serovar Cholerasuis ATCC 10708, and *Pseudomonas aeruginosa;* EPA virucidal tests for viruses including poliovirus type 2 and herpes simplex virus; and FDA-recommended tests, such as total killing and end-point analysis and comparison of survivor and predicted curves. (Note that in Europe, disinfectants should have been tested by the methods defined by established or proposed European Norms [EN] such as EN 1040 [bactericidal activity] and EN 1275 [fungicidal activity]).

2. Simulated-use testing involves testing the disinfectant under artificially created worst-case scenarios to determine how long instruments need to be in contact with the disinfectant if cleaning failed and the instruments are still contaminated with substantial organic matter and microbes. The instruments are contaminated with an organic load and appropriate test microorganisms (the organism depends on the level of disinfection being claimed); the conditions of the artificially contaminated devices represent worst-case postcleaning conditions before exposure to the germicide.

3. "In-use" testing involves cleaning medical devices used for clinical purposes according to a facility's operating procedures.

As noted above, the FDA includes a tuberculocidal test in its test procedures. This test does not account for the effect of cleaning before devices are disinfected. Devices are treated with 2% horse serum (proteinaceous load) and with 10^5 to 10^6 CFU of *Mycobacterium terrae* or equivalent nontuberculous mycobacteria. Under these conditions, a device would need to be immersed in a disinfectant (e.g., 2.4% alkaline glutaraldehyde) for >45 min at >25°C for complete tuberculocidal killing. However, Rutala and Weber demonstrated that proper cleaning eradicates at least 4 log units of microorganisms (209), and Hanson et al. showed that cleaning bronchoscopes before disinfection removes all detectable contaminants, with an up to 8-log-unit reduction in the viral load (114). Therefore, Rutala and Weber recommend that the FDA accept a standardized cleaning protocol followed by a 20-min immersion at 20°C with an FDA-approved disinfectant as adequate to kill mycobacteria (209). The EPA maintains an updated list of registered low-level and intermediate-level disinfectants (http://www.epa.gov/oppad001/chemregindex.htm) and the FDA maintains a list of approved high-level disinfectants and sterilants (http://www.fda.gov/cdrh/ode/germlab.html) on their websites.

TABLE 4 Overview of common disinfectants[a]

Germicide(s)	Use dilution	Level of disinfection	Active against:						Important characteristics									Applications in hospitals
			Bacteria	Lipophilic viruses	Fungi	Small or hydrophilic viruses	*Mycobacterium tuberculosis*	Bacterial spores	Shelf life of >1 wk	Corrosive/ deleterious effect	Residue	Inactivated by organic matter	Skin irritant	Eye irritant	Respiratory irritant	Toxic	Environmental concerns	
Glutaraldehyde	2–3.2%	High/CS	+	+	+	+	+	+	+	−	+	−	+	+	+	+	−	Endoscopes
Hydrogen peroxide	3–25%	High/CS	+	+	+	+	+	±	+	±	−	±	+	+	−	+	−	Contact lenses
Chlorine	100–1,000 ppm free chlorine	High	+	+	+	+	+	±	+	+	+	+	+	+	+	+	±	Selected semicritical devices
Isopropyl alcohol	60–95%	Intermediate	+	+	+	±	+	−	+	±	−	±	±	+	−	+	−	Small-area surfaces
Glucoprotamine	1.5–4%	Intermediate	+	+	+	+	+	−	+	−	−	−	+	+	−	−	−	Diagnostic instruments
Phenolic compounds	0.4–5% aqueous	Intermediate	+	+	+	±	+	−	+	−	+	−	+	+	−	+	+	Surgical instruments
Iodophors	30–50 ppm free iodine	Intermediate	+	+	+	+	±	−	+	±	+	+	±	+	−	+	−	Medical equipment
Quaternary ammonium compounds	0.4–1.6% aqueous	Low	±	+	±	−	−	−	+	−	+	+	+	+	−	+	−	Disinfection in food preparation areas and floors

[a] Data from references 34, 89, 201, and 247 and from the *Laboratory Biosafety Manual*, World Health Organization, Geneva, Switzerland, 1983 (268a). Abbreviations: CS, chemical sterilant; +, yes; −, no; ±, variable results. Efficacy of the disinfectants is based on an exposure time of less than 30 min at room temperature. Spores require prolonged exposure times (up to 10 h) unless used with a washer-disinfector at higher temperatures.

Definitions and Terms (Adapted from FDA and EPA Definitions)

Since the FDA regulates the most critical part of disinfection and sterilization, FDA definitions are used throughout the chapter unless stated otherwise. The most important definitions are given in Table 5.

Guidelines for Choosing a Disinfectant

Rutala published guidelines for the selection and use of disinfectants and recommendations on the preferred method for disinfection and sterilization of patient care items (62, 201, 202). The CDC recently issued guidelines for environmental infection control in health care facilities including recommendations for cleaning and disinfection (227).

When choosing a disinfectant, staff members should review its effectiveness against the expected spectra of pathogens (Tables 4 and 5) to ensure that it is adequate for the intended purpose. In addition, the staff must ensure that the disinfectant is compatible with the devices it is intended to disinfect and that devices that are immersed longer than recommended will not be damaged. The latter factor is important because staff members may forget to remove instruments (e.g., during weekends or night shifts). Prolonged exposure to a disinfectant may damage the instrument. Staff members should also consider the toxicity, odor, compatibility with other compounds, and residual activity of disinfectants (Table 6). It is prudent to contact colleagues already using a disinfectant before introducing it in a health care facility. The experience of health care professionals at different institutions can be helpful, allowing the staff to learn about problems such as interactions with detergents, unexpected coloring and odors, and employees' responses to the change. We have found that employees often complain bitterly after a disinfectant has been changed. Moreover, a new disinfectant used on environmental surfaces may interact with those used in the past and temporarily release unpleasant odors. Written infection control standards describing how to care for environmental surfaces can help staff members avoid combining incompatibile equipment and disinfectants. Once staff members have identified a product that meets a facility's needs, only strong evidence from good studies should lead a facility to replace it with a new product (e.g., one that has improved activity or works faster).

Disinfection by Heat Versus Immersion in Germicides

Disinfection by heat has become much more common than in the past and has replaced disinfection with germicides for many applications in European health care facilities, including our institution, the University Hospital Basel, Basel, Switzerland (225). The advantages of these devices are obvious: (i) the processes are automated and are monitored and documented in a manner similar to that for sterilization, (ii) microogranisms have not developed resistance to the processes, and (iii) the cost per load is probably less than that for germicides. In addition, studies by Gurevich et al. (111) indicate that pasteurization with a germicide is more effective than pasteurization without a germicide. However, washers include a cleaning process with an average reduction of 4 log units, coupled with heat disinfection (5-log-unit killing; washer-disinfectors such as the AMSCO Reliance 430 achieve an inactivation factor of >5 log units [111, 139]), resulting in a total reduction of 8 to 9 log units. This surpasses any international requirements for high-level disinfection.

Thermal disinfection has several disadvantages. First, the cost to purchase and install the equipment is much higher than that for systems using a germicide. Second, considerable power is needed to heat the water. Third, some nonspore-forming microorganisms such as enterococci resist temperatures of up to 71°C for 10 min. Thus, recommendations such as those in the United Kingdom (the Department of Health requires 65°C for 10 min, 71°C for 3 min, or 80°C for 1 min) may not be adequate for these organisms (43).

Medical washer-disinfectors that are intended to clean, low- and intermediate-level disinfectants, and dry surgical instruments, anesthesia equipment, hollowware, and other medical devices are exempt from the premarket notification procedures described by FDA in subpart E of part 807 of the chapter subject to §880.9. The ISO provided standards for these processes in preapproval ISO norm 15883 (prEN ISO 15883), which defines the standards for disinfection of washer-disinfectors by heat with and without the addition of disinfectants (http://www.iso.org). The ISO has not defined a temperature at which these devices must work but rather allows manufacturers to choose a temperature in a given range at which their devices should operate.

In the United States, hot-water pasteurization is generally performed at 77°C for 30 min (36), but few scientific data

TABLE 5 FDA and EPA definitions of important terms

Term	Definition
Germicide	Agent that destroys microorganisms; the prefixes of terms with the suffix "-cide" (e.g., virucide, fungicide, bactericide, sporicide, and tuberculocide) indicate which microorganisms the germicide kills
Sterilant (chemical)	Chemical germicide that achieves sterilization
High-level disinfectant	Germicide that when used according to the labeling kills all microbial pathogens except large numbers of bacterial endospores
Intermediate-level disinfectant	Germicide that when used according to the labeling kills all microbial pathogens except bacterial endospores
Low-level disinfectant	Germicide that when used according to the labeling kills most vegetative bacteria and lipid-enveloped and medium-size viruses; such disinfectants are regulated by the EPA
Minimum effective concentration	Minimum effective concentration of a liquid chemical germicide that achieves the microbicidal activity claimed by the manufacturer
Cleaning (or precleaning)	Removal of foreign material (e.g., organic or inorganic contaminants) from medical devices as part of a decontamination process

TABLE 6 Overview of common antiseptic compounds[a]

Compound(s)	Antiseptic effect on[b]:					Rapidity of action	Residual activity[b]	Typical concn(s) (%)	Affected by organic matter	Safety for humans
	Gram-positive bacteria	Gram-negative bacteria	Viruses	Fungi	*Mycobacterium tuberculosis*					
Alcohols	+++	+++	++[c]	+++	+++	15–30 s	None	70–95	?[e]	Drying, flammable
Chlorhexidine	+++	++	++	+	++	Min	+++	4, 2, 0.5 in alcohol	Minimally	Leads to ototoxicity and keratitis
Iodophors	+++	++	++	++	++	Min	+	10, 7.5, 2, 0.5	Yes	Causes skin irritation
Octenidine[d]	+++	+++	++	++	++	Min	+++	0.1	Minimally	Limited data on safety
PCMX	++	+	+	+	+	Min	++	0.5–3.75	Minimally	Appears to be safe
Triclosan	++	++	++	±	+	Min	+++	0.3–1.0	Minimally	Appears to be safe

[a] Data from references 149, 189, and 264.
[b] ± poor; +, fair; ++, good, +++, excellent.
[c] Ethanol at >95% is highly effective against viruses; isopropanol has limited effectiveness against small and nonlipid viruses.
[d] Not available in the United States.
[e] Conflicting data.

support the use of a particular temperature. prEN ISO 15883 introduces the A_0 concept, which is based on the fact that a defined temperature will generate a predictable lethality effect against microorganisms. Corresponding exposure temperatures and time periods that achieve high-level disinfection can be calculated by assuming the presence of particularly heat-resistant microorganisms in numbers in excess of those likely to be encountered on the medical devices to be processed. prEN ISO 15883 introduces the term A_0 for moist-heat disinfection (thermal disinfection). The A_0 value of a moist-heat disinfection process denotes the lethality effects expressed in terms of the equivalent time in seconds at a temperature of 80°C delivered by the process to the medical device with reference to microorganisms possessing a z value of 10. Given a predefined A_0, equivalent killing of microorganisms is achieved if the following formula is followed: $A_0 = \sum 10^{(80-T)/Z}\Delta t$, where T is temperature in Celsius and t is time in seconds. An A_0 value of 600, which can be achieved at 80°C over 10 min, 90°C over 1 min, or 70°C over 100 min, is the minimum requirement for noncritical medical devices (273, 274). An A_0 value of at least 3,000, which can be achieved by exposure to hot water (e.g., at 90°C; the medical device must tolerate this temperature for >5 min), must be employed for medical devices contaminated with heat-resistant viruses such as rotavirus and hepatitis B virus. An A_0 value of at least 3,000 is also appropriate for high-level disinfection of all semicritical devices. The test procedure based on the A_0 concept has been highly reproducible and found to be suitable to test washer-disinfectors (273, 274).

Overview of Commonly Used Disinfectants for Devices

Glutaraldehyde

Among aldehydes that exhibit biocidal activity, including glyoxal, *ortho*-phthalaldehyde (OPA), succinaldehyde, and benzaldehydes, glutaraldehyde and formaldehyde are the most extensively studied aldehydes. In-depth reviews may be found elsewhere (21, 198, 201). Glutaraldehyde is the predominant commercially available aldehyde. Because it has potent and broad-spectrum microbiocidal activity and is compatible with many materials (including metal, rubber, and plastic), glutaraldehyde is often regarded as the high-level disinfectant and chemical sterilant of choice in many health care facilities. Glutaraldehyde-based formulations are most commonly used for high-level disinfection of medical equipment such as endoscopes, transducers, dialysis systems, and anesthesia and respiratory therapy equipment (201).

The mechanism of action is complex and is related to the alkylation of sulfhydryl, hydroxyl, carboxy, and amino groups in the cell wall, cell membrane, nucleic acids, enzymes, and other proteins of microorganisms. The biocidal activities of glutaraldehyde solutions are dependent on a variety of variables, such as pH, temperature, concentration at the time of use, the presence of inorganic ions, and the age of the solution (21). Aqueous solutions of glutaraldehyde are usually acidic and are not sporicidal in this form. Therefore, they need to be activated by adding an alkalinizing agent. These activated solutions, however, rapidly lose their activity because glutaraldehyde molecules polymerize at an alkaline pH. Therefore, the shelf life of such solutions is limited to 14 days unless the manufacturer recommends otherwise. To overcome this problem, some manufacturers have developed novel formulations with longer shelf lives (e.g., activated dialdehyde solutions containing 2.4 to 3.5% glutaraldehyde with a maximum reuse life of 28 days).

The activities of disinfectants increase as the temperature rises. Among eight disinfectants tested, glutaraldehyde was found to be the chemical most strongly affected by temperature (99). Some stable acid glutaraldehydes may be used at temperatures of 35 to 55°C at concentrations below 2%. Glutaraldehyde retains its activity in the presence of organic matter.

A standard 2% aqueous solution of glutaraldehyde buffered to pH 7.5 to 8.5 is bactericidal, tuberculocidal, sporicidal, fungicidal, and virucidal. It rapidly kills both gram-negative and gram-positive vegetative bacteria. Longer exposure times are required to inactivate spores and mycobacteria. Spores of *Bacillus* and *Clostridium* spp. are generally destroyed by 2% glutaraldehyde in 3 h, whereas spores of *Clostridium difficile* are eliminated more rapidly (206). In contrast, *Cryptosporidium parvum* oocysts remain viable and infectious after 10 h in a 2.5% glutaraldehyde solution (268). Several investigators have questioned glutaraldehyde's ability to inactivate mycobacteria. For example, Rubbo et al. (197) demonstrated that glutaraldehyde inactivates *Mycobacterium tuberculosis* more slowly than alcohols, formaldehyde, iodine, and phenol. Ascenzi (21) showed in the quantitative suspension test that 2% glutaraldehyde kills only 2 to 3 log units of *Mycobacterium tuberculosis* in 20 min at 20°C. Similarly, Collins (66) reported that glutaraldehyde cannot completely inactivate a standardized suspension of *Mycobacterium tuberculosis* within 10 min. Nontuberculous mycobacteria such as *Mycobacterium avium*, *Mycobacterium intracellulare*, and *Mycobacterium gordonae* are more resistant to inactivation than *Mycobacterium tuberculosis* (65). These and other data suggest that 20 min (at 20°C) is the minimum exposure time needed to reliably inactivate tuberculous and nontuberculous mycobacteria by using 2% glutaraldehyde, provided that the contaminated item has been thoroughly cleaned before disinfection (131, 201). Glutaraldehyde-resistant mycobacteria have been isolated from endoscope washers-disinfectors (107, 251) (see "Endoscopes" below).

The virucidal activity of glutaraldehyde extends to the nonenveloped (hydrophilic) viruses, which are generally more resistant to disinfectants than are the enveloped (lipophilic) viruses. Numerous viruses were documented to be inactivated, including HIV, hepatitis A virus, hepatitis B virus, poliovirus type 1, coxsackievirus type B, yellow fever virus, and rotavirus (21, 141).

Disadvantages of glutaraldehyde include the fact that it coagulates blood and can fix proteins and tissue to surfaces (167, 201). In addition, glutaraldehyde has a pungent and irritating odor and its vapor at the level of 0.2 ppm irritates the eyes, throat, and nose. Health care workers exposed to glutaraldehyde can develop allergic contact dermatitis, asthma, rhinitis, and epistaxis. Measures that may minimize employee exposure include covering immersion baths with tight-fitting lids, improving ventilation, and using ducted exhaust hoods or ductless fume hoods with vapor absorbents, personal protective equipment, and appropriate automated machines for endoscope disinfection (12, 201). Due to dilution, glutaraldehyde concentrations commonly decline during use in manual and automatic baths used for endoscopes (167). Test strips should be used to ensure that the glutaraldehyde concentration has not fallen below 1 to 1.5%. Equipment disinfected with glutaraldehyde and rinsed inadequately has caused serious clinical complications including proctocolitis (colonoscopes; 79, 262); and keratopathy (ophthalmic instruments; 48). Because the infectivity of prions can be stabilized when instruments are treated with formaldehyde before they are autoclaved (48), aldehydes are no longer recommended for disinfecting endoscopes in some European countries (e.g., France; see "Bovine Spongiform Encephalopathy and Variant Creutzfeldt-Jakob Disease" below).

ortho-Phthalaldehyde

The 0.55% OPA solution has been approved as a high-level disinfectant by the FDA and by agencies in other countries. However, different countries or areas have set different exposure times for a 0.55% solution of OPA at 20°C to achieve high-level disinfection: 12 min in the United States, 10 min in Canada, and 5 min in Europe, Asia, and Latin America.

Compared with glutaraldehyde, OPA has several advantages: (i) it does not require activation, (ii) it is compatible with many materials (i.e., similar to glutaraldehyde), (iii) it is more stable during storage and can be reused as well at a pH range as wide as 3 to 9, (iv) it has low vapor properties, (v) its odor is barely perceptible, and (vi) it is more rapidly mycobactericidal than glutaraldehyde in vitro and has good activity against glutaraldehyde-resistant strains at longer exposure times (93). However, 0.5% OPA is slowly sporicidal and does not inactivate all spores within 270 min of exposure (255). In addition, OPA stains proteins, skin, clothing, and instruments. OPA vapors may irritate the respiratory tract and eyes. At present, the effects of long-term exposure and safe exposure levels are not well defined. Therefore, OPA must be handled with appropriate safety precautions (i.e., by using gloves, fluid-resistant gowns, and eye protection) and it must be stored in containers with tight-fitting lids.

If additional studies corroborate OPA's advantages, this compound may replace glutaraldehyde for many uses, especially endoscope disinfection. The new agent appears to be particularly useful in washer-disinfectors, where glutaraldehyde-resistant mycobacteria have emerged (251, 255).

Formaldehyde

Formaldehyde and its condensates are reviewed in depth elsewhere (192). Formaldehyde in aqueous solutions or as a gas has been used as a disinfectant and sterilant for many decades. Its use in the health care setting, however, has sharply decreased for several reasons. The irritating vapors and pungent odor produced by formaldehyde are apparent at very low levels (<1 ppm). In addition, allergy to formaldehyde is fairly common. Moreover, the Occupational Safety and Health Administration in the United States and the Health and Safety Executive of the United Kingdom indicated that formaldehyde vapors may be carcinogenic. Thus, the Occupational Safety and Health Administration limits the 8-h time-weighted average exposure in the workplace to a concentration of 0.75 ppm. Elevated levels of occupational exposures have been found among workers in dialysis units and gross anatomy laboratories (8). Consequently, formaldehyde and formaldehyde-releasing agents are used infrequently in health care institutions, despite this agent's broad-spectrum microbicidal activity. In fact, formaldehyde has been largely replaced by peracetic acid as an agent for disinfecting hemodialysis equipment and water dialysate tubing systems. Paraformaldehyde vaporized by heat is used to decontaminate biological safety cabinets.

Chlorine and Chlorine-Releasing Compounds

Due to its hazardous nature, chlorine gas is rarely used as a disinfectant. Among the large number of chlorine compounds commercially available, hypochlorites are the most

widely used disinfectants. Hypochlorite has been used for more than a century and remains an important disinfectant. Rutala and Weber published an extensive review of uses for inorganic hypochlorite in health care facilities (210), and Karol reviewed the potential hazards and significant benefits of chlorine use (139).

Aqueous solutions of sodium hypochlorite are usually called household bleach. Bleach commonly contains 5.25% sodium hypochlorite, or 52,500 ppm of available chlorine; a 1:10 dilution of bleach provides about 300 to 600 mg of free chlorine per liter. Alternative chlorine-releasing compounds frequently used in health care facilities include chloramine-T, sodium dichloroisocyanurate tablets, and chlorine dioxide. Demand-release chlorine dioxide is an extremely reactive compound and must be prepared at the point of use. It is used primarily to chlorinate potable water, swimming pools, and wastewater. In Europe, commercial chlorine dioxide preparations are available to disinfect instruments.

In aqueous solutions, all chlorine compounds release hypochlorous acid, the most likely active compound. The mechanism of microbicidal action of hypochlorous acid has not been fully elucidated, but it inhibits key enzymatic reactions within cells and denaturates proteins. Lowering the pH or raising the temperature or concentration increases its antimicrobial efficacy. Chlorine compounds have broad antimicrobial spectra including, at higher concentrations, bacterial spores and *Mycobacterium tuberculosis*. Therefore, hypochlorite can be used as a high-level disinfectant for semicritical items.

Concentrations of 100 ppm of available chlorine inactivate vegetative bacteria and viruses in 10 min. Suspension tests document that both enveloped and nonenveloped viruses, including HIV, hepatitis A and B viruses, herpes simplex virus types 1 and 2, poliovirus, coxsackievirus, and rotavirus, are inactivated (210). In one study, a concentration of 100 ppm of chlorine eliminated 99.9% of *Bacillus subtilis* endospores in 5 min (267). However, endospore-forming bacteria, mycobacteria, fungi, and protozoa usually are less susceptible to chlorine than other microorganisms, and high concentrations of chlorine (1,000 ppm) are required to completely destroy them. Despite this limitation, sodium hypochlorite solutions (500 and 1,600 ppm) have been reported to decrease *Clostridium difficile* environmental contamination and to terminate an outbreak of infections caused by this organism (134). *Cryptosporidium* oocysts are particularly resistant to chlorine. These oocysts remain infective for several days in swimming pool water containing recommended chlorine concentrations; because of their small size, they may not be removed efficiently by conventional pool filters. Outbreaks of *Cryptosporidium* infections have been associated with drinking water and swimming pools (16). Of note, chloramine-T and sodium dichloroisocyanurate seem to have lower sporicidal activities than does sodium hypochlorite.

Hypochlorite is fast acting, nonstaining, nonflammable, and inexpensive. However, its use is limited because it is corrosive, inactivated by organic matter, and relatively instable. Sodium hypochlorite can injure tissues; however, such injury occurs rarely in health care facilities (210). Inhalation of chlorine gas may irritate the respiratory tract, resulting in a cough, dyspnea, and pulmonary edema or chemical pneumonitis. The potential carcinogens trihalomethanes have been detected in chlorine-treated water, and high levels of trihalomethanes can be detected when hospitals hyperchlorinate their water systems (118).

Chlorine compounds have other important disadvantages. Blood or other organic matter substantially inactivates hypochlorites and other chlorine compounds. Consequently, items used for patient care and environmental surfaces must be cleaned before hypochlorite is used. In addition, the presence of a biofilm (e.g., in the pipes of a water distribution system) also reduces the efficacy of chlorines significantly. Moreover, the free available chlorine levels in solutions can decay to 40 to 50% of the original concentration after the container has been opened for one month. Therefore, concentrations higher than those established in laboratory experiments should be used in practice. Loss of free chlorine can be minimized if the solutions are dilute and alkaline, kept and used at room temperature, and stored in closed opaque containers.

Depending on the concentrations employed, sodium hypochlorite is used in hospitals as a high-level disinfectant for selected semicritical devices (e.g., dental equipment and mannequins used for cardiopulmonary resuscitation training), as an intermediate-level disinfectant (e.g., hemodialysis equipment), and as a low-level disinfectant for environmental surfaces and hydrotherapy tanks. For example, the CDC recommends that health care workers use a 1:100 dilution (5,000 ppm) of hypochlorite to decontaminate spills of blood and certain other body fluids (53). Because chlorine can be inactivated by blood and other organic material, a full-strength solution or a 1:10 dilution will be safer unless the surface is cleaned before it is disinfected (62, 88, 258). Household bleach also can be used to disinfect table tops and incubators, to clean spills in laboratories, or to disinfect syringes used by drug addicts if sterile disposable syringes are not available (54).

At low concentrations, chlorines (usually about 0.5 ppm of free chlorine) are used to chlorinate the drinking water. Hyperchlorination of institutional water systems has controlled epidemics caused by *Legionella pneumophila* (118) but also corrodes the water distribution system (118). Stabilized solutions of chlorine dioxide appear to be less toxic and more efficacious than chlorine for controlling growth of legionellae (113). A growing number of municipal water treatment plants in the United States are using monochloramine as a residual disinfectant. Chloramination of drinking water has several advantages over the use of free chlorine, including decreasing the risk of Legionnaires' disease at the municipal level or in individual hospitals (144). However, outbreaks of *Cryptosporidium* infections have occurred in cities that use chloramines in their drinking water.

Hydrogen Peroxide

Hydrogen peroxide, a strong oxidizer, is used for high-level disinfection and sterilization. It produces destructive hydroxyl free radicals that attack membrane lipids, DNA, and other essential cell components. Although the catalase produced by anaerobic and some aerobic bacteria may protect cells from hydrogen peroxide, this defense is overwhelmed by the concentrations used for disinfection (154). Generally, a 3% hydrogen peroxide solution is rapidly bactericidal, but it kills organisms with high cellular catalase activity (e.g., *Staphylococcus aureus* and *Serratia marcescens*) less rapidly. Surprisingly, 3% hydrogen peroxide was ineffective against vancomycin-resistant enterococci (164, 223).

Spores are more resistant than vegetative bacteria to hydrogen peroxide. For example, a 3% solution of hydrogen peroxide destroyed 10^6 spores in six of seven exposure trials that were 150 min long; a 10% solution always was successful in 60 min (257). Higher concentrations of hydrogen peroxide (17.7 and 35.4%) killed *Bacillus subtilis* spores in 9.4 and 2.3 min, respectively (152). In a recent investigation,

10% hydrogen peroxide was the most active of the seven chemical disinfectants tested against *Bacillus subtilis* spores (218). However, other investigators found that the sporicidal activity of hydrogen peroxide was lower than those of peracetic acid and chlorine (10). Hydrogen peroxide's sporocidal activity can be enhanced by increasing the concentration or the temperature or by using it in conjunction with ultrasonic energy, ultraviolet radiation, and some chemical agents such as peracetic acid (162, 170, 247).

A 0.3% solution of hydrogen peroxide inactivates HIV in 10 min (162), and a 3% concentration inactivates rhinovirus in 6 to 8 min at 37°C (170). However, a 6% solution did not inactivate poliovirus at 1 min (247).

Hydrogen peroxide does not coagulate blood and does not fix tissues to surfaces. In fact, it may enhance removal of organic material from equipment. Hydrogen peroxide has a low toxicity for humans. It decomposes into oxygen and water, and therefore, it is environmentally safe. It is neither carcinogenic nor mutagenic. Concentrated solutions may irritate the eyes, skin, and mucous membranes. Hydrogen peroxide can be destroyed easily by heat or enzymes (catalase and peroxidases). Stabilized solutions can be used for high-level disinfection of semicritical items. However, one must consider the corrosive effects of hydrogen peroxide on copper, zinc, and brass in using this disinfectant (201). The FDA has approved commercial products containing either 7.5% hydrogen peroxide alone or combinations with peracetic acid as liquid sterilants and high-level disinfectants for processing of reusable medical and dental devices (http://www.fda.gov). Concentrations of 3 to 6% are used to disinfect ventilators, soft contact lenses (3% for 2 to 4 h) (125), and tonometer biprisms (153, 154, 201). Vaporized hydrogen peroxide is also used for plasma sterilization (see below).

Despite its limited toxicity, hydrogen peroxide can damage human tissues. Patients exposed to endoscopes contaminated by residual hydrogen peroxide have developed pseudomembrane-like enterocolitis (pseudolipomatosis) (216). In addition, patients who were exposed to tonometer tips disinfected with hydrogen peroxide and rinsed improperly suffered corneal damage (153). Use of hydrogen peroxide to clean wounds and to develop dental regimens remains controversial (154).

Peracetic Acid

Peracetic acid (or peroxyacetic acid) is a more potent germicidal agent than hydrogen peroxide and was the most active agent in several in vitro studies (9, 219). Concentrations of $<1\%$ are sporicidal even at low temperatures. The mechanism of action of peracetic acid has not been fully elucidated, but it is likely to be similar to that of hydrogen peroxide and other oxidizing agents. Peracetic acid remains effective in the presence of organic matter. At low concentrations it is considerably less stable than hydrogen peroxide; preparations with appropriate stabilities have been developed and are commercially available. Peracetic acid corrodes steel, galvanized iron, copper, brass, and bronze, and it attacks natural and synthetic rubbers. In addition, concentrated solutions can seriously damage eyes and skin. Furthermore, some investigators have raised concerns about the potential toxicity of the combination of peracetic and acetic acids (117). Feldman et al. reported that mortality rates in freestanding dialysis facilities that reprocess dialyzers with peracetic and acetic acid are higher than those in facilities that discard dialysis filters or used formaldehyde for reprocessing (86). To date, investigators have not determined whether the higher death rate is caused by the disinfectants or is associated with other practices at the facilities or with patient risk factors.

Nevertheless, because peracetic acid has powerful germicidal activity and does not produce toxic residues, peracetic acid is very attractive for use in health care settings, most frequently in combination with hydrogen peroxide to disinfect hemodialyzers. The FDA lists several commercial products containing a combination of peracetic acid and hydrogen peroxide as high-level disinfectants and chemical sterilants. The use of peracetic acid for chemical sterilization of instruments and endoscopes (STERIS System 1) is discussed below.

Alcohols

For centuries, the alcohols have been appreciated for their antimicrobial properties. Alcohol is defined by the FDA as having one of the following active ingredients: ethyl alcohol, 60 to 95% by volume in an aqueous solution, or isopropyl alcohol, 50 to 91.3% by volume in an aqueous solution. Ethyl alcohol (ethanol) and isopropyl alcohol (isopropanol) are the alcoholic solutions most often used as surface disinfectants and antiseptic agents in health care institutions because they possess many qualities that make them suitable both for disinfection of equipment and for antisepsis of skin. They are fast acting, minimally toxic to the skin, nonstaining, and nonallergenic. Alcohols evaporate readily, which is advantageous for most disinfection and antisepsis procedures. The uptake of alcohol by intact skin and the lungs when alcohol is used topically is negligible. Alcohols have better wetting properties than water due to their lower surface tensions, which along with their cleansing and degreasing actions make alcohols effective skin antiseptics. Alcoholic formulations used to prepare the skin before invasive procedures should be filtered to ensure that they are free of spores, or 0.5% hydrogen peroxide should be added (193). Alcohols are also excellent products for intermediate-level and low-level disinfection of small, clean surfaces, equipment, and the environment (e.g., rubber stoppers of medication vials, stethoscopes, and medication preparation areas).

Alcohols have some disadvantages. If alcoholic antiseptics are used repeatedly, they may dry and irritate the skin. Therefore, preparations for hand disinfection should contain emollients (see "Hygienic Hand Disinfection" below). Moreover, alcohols may damage rubber, certain plastic items, and the shellac mountings of lensed instruments after prolonged and repeated use (201). Moreover, alcohols are flammable (one should consider the flash point) and, thus, must not be used on large surfaces, particularly in closed, poorly ventilated areas. Alcohols cannot penetrate protein-rich materials. Therefore, a spray or a wipe with alcohol may not disinfect a surface contaminated with blood or other body fluids that has not been cleaned first.

The exact mechanism by which alcohols destroy microorganisms is not fully understood. The most plausible explanation for the antimicrobial action is that they coagulate (denaturate) proteins (e.g., enzymatic proteins), impairing specific cellular functions (150). Ethyl and isopropyl alcohols at appropriate concentrations have broad spectra of antimicrobial activities that include vegetative bacteria, fungi, and viruses. In fact, their antimicrobial efficacies are enhanced in the presence of water, with optimal alcohol concentrations being 60 to 90% by volume.

Alcohols (i.e., 70 to 80% ethyl alcohol) rapidly (i.e., 10 to 90 s) kill vegetative bacteria, such as *Staphylococcus aureus*, *Streptococcus pyogenes*, *Enterobacteriaceae*, and *Pseudomonas aeruginosa,* in suspension tests (193). Isopropyl alcohol is

slightly more bactericidal than ethyl alcohol (150) and is highly effective against vancomycin-resistant enterococci (223). It also has excellent activity against fungi, such as *Candida* spp., *Cryptococcus neoformans*, *Blastomyces dermatitidis*, *Coccidioides immitis*, *Histoplasma capsulatum*, *Aspergillus niger*, and dermatophytes and mycobacteria, including *Mycobacterium tuberculosis*. However, alcohols generally do not destroy bacterial spores. In fact, fatal infections due to *Clostridium* spp. occurred when alcohol was used to sterilize surgical instruments.

Both ethyl and isopropyl alcohols inactivate most viruses with a lipid envelope (e.g., influenza virus, herpes simplex virus, and adenovirus). However, several investigators found that isopropyl alcohol has lower virucidal activity against naked, nonenveloped viruses (201). In the experiments by Klein and DeForest, 2-propanol, even at 95%, could not inactivate the nonenveloped poliovirus type 1 and coxsackievirus type B in 10 min (141). In contrast, 70% ethanol inactivated these enteroviruses (141). Neither 70% ethanol nor 45% 2-propanol killed hepatitis A virus when their activities were assessed on stainless steel disks contaminated with fecally suspended virus. Among 20 disinfectants tested, only 3 reduced the titer of hepatitis A virus by greater than 99.9% in 1 min (2% glutaraldehyde, sodium hypochlorite with >5,000 ppm of free chlorine, and a quaternary ammonium formulation containing 23% HCl) (166). Bond et al. (35) and Kobayashi et al. (143) demonstrated that 2-propanol (70% for 10 min) or ethanol (80% for 2 min) made human plasma contaminated with hepatitis B virus at high titer noninfectious for susceptible chimpanzees (143). Both 15% ethyl alcohol and 35% isopropyl alcohol (162) readily inactivate HIV, and 70% ethanol rapidly inactivates high titers of HIV in suspension, independent of the protein load. However, the rate of inactivation decreased when virus was dried onto a glass surface and high levels of protein were present (249). In a suspension test, 40% propanol reduced the rotavirus titer by at least 4 logs in 1 min (147) and both 70% propanol and 70% ethanol reduced the release of rotavirus from contaminated fingertips by 2.7 log units. In comparison, the mean reductions obtained with liquid soap and an aqueous solution of chlorhexidine gluconate were 0.9 and 0.7 log units, respectively (17).

Phenolics

Since Lister's pioneering use of phenol (carbolic acid) as an antiseptic, a large number of phenol derivatives (or phenolics) have been developed and marketed. Phenol derivatives originate when one of the hydrogen atoms on an aromatic ring is replaced by a functional group (e.g., an alkyl-, benzyl-, phenyl-, amyl-, or chlorogroup). The three phenolics most commonly used as constituents of disinfectants are *o*-phenylphenol, *o*-benzyl-*p*-chlorophenol, and *p-tert*-amylphenol. The addition of detergents to the basic formulation results in products that clean, dissolve proteins, and disinfect in one step. Phenolics at higher concentrations act as gross protoplasmic poisons, penetrating and disrupting the bacterial cell wall and precipitating the cell proteins (180). Lower concentrations of these compounds inactivate cellular enzyme systems and cause essential metabolites to leak from the cell.

Phenol compounds at concentrations of 2 to 5% are generally considered bactericidal, tuberculocidal, fungicidal, and virucidal against lipophilic viruses (180). However, the manufacturers' efficacy claims have generally not been verified by independent laboratories or the EPA (201). A collaborative study by Rutala and Cole documented that randomly selected EPA-registered phenolic detergents or quaternary ammonium compounds do not consistently meet the manufacturers' bactericidal label claims (203). Phenolics tested by the AOAC use dilution method at the recommended use dilution failed to kill *Pseudomonas aeruginosa* in 33 to 78% of laboratories. However, extreme variability of test results has been observed among laboratories testing identical products (203).

Phenolics at in-use dilutions are not lethal to bacterial spores. Terleckyi and Axler found that a 2% phenolic kills a wide spectrum of clinically important fungi except *Aspergillus fumigatus* (242). Although 5% phenol inactivated both lipophilic and hydrophilic viruses, Klein and DeForest found that 12% *o*-phenylphenol was effective only against lipophilic viruses (141). Similarly, other investigators demonstrated little or no virucidal effect of a phenolic against coxsackievirus type B4, echovirus type 11, or poliovirus type 1 (178). Martin et al. showed that a 0.5% commercial phenolic formulation (2.8% *o*-phenylphenol and 2.7% *o*-benzyl-*p*-chlorophenol) inactivated HIV (162) but that another commercial product containing phenolics at a final concentration of 1% did not completely inactivate cell-associated HIV suspended in blood (81). A phenol-based preparation (14.7% phenol diluted 1:256 in tap water) and a bleach dilution (800 ppm of available chlorine) reduced rotavirus numbers similarly and interrupted transfer of virus from disks to fingerpads (222).

Phenolic compounds are relatively tolerant of anionic and organic matter. They are absorbed by rubber and plastics and leave a residual film, which may irritate skin and tissues. *p-tert*-butylphenol and *p-tert*-amylphenol have been reported to depigment skin. Although differences between the various compounds exist, phenolics are degraded in wastewater at a lower rate than other germicides, which limits their use in Europe.

Phenolic germicidal detergent solutions may be used for intermediate-level and low-level disinfection of surgical instruments and noncritical patient care items. These compounds are also appropriate for decontaminating the hospital environment, including laboratory surfaces. They should not be used to disinfect bassinets and incubators because they can cause hyperbilirubinemia in infants (201).

Quaternary Ammonium Compounds

A wide variety of quaternary ammonium compounds (quats) with antimicrobial activities have been introduced in the past decade. Some of the compounds used in health care settings are benzalkonium chloride, alkyldimethylbenzyl ammonium chloride, and didecyldimethyl ammonium chloride. Quats are cationic surface-active detergents, which appear to kill microorganisms by disrupting cell membranes, inactivating enzymes, and denaturating cell proteins (171). However, they have limited antimicrobial spectra. Products sold as hospital disinfectants are not sporicidal and are generally not tuberculocidal or virucidal against hydrophilic viruses. Scientific investigations using the AOAC use dilution method have not reproduced the bactericidal and tuberculocidal claims made by the manufacturers (204). Consequently, health care workers should be suspicious of the claims on labels and of results from in-house evaluations that have not been verified by an independent laboratory. The germicidal activity may have been overestimated because the compounds tested were incompletely inactivated. In this case, the bacteriostatic (inhibitory) activity rather than the bactericidal activity is measured (171). The antimicrobial spectra of quats may be improved by combining them with amines and biguanides or by using them at higher temperatures in washing machines.

Several outbreaks of infections have been associated with quat solutions contaminated in use by gram-negative bacteria such as *Pseudomonas* spp. or *Serratia marcescens* or by *Mycobacterium abscessus* (90, 177, 244). The contaminated solutions were used as antiseptics on skin and tissue and as disinfectants on patient care supplies and equipment (e.g., cardiac catheters and cystoscopes). In fact, microbiology laboratories use the quat cetrimide in selective media to isolate *Pseudomonas aeruginosa*.

Quats have other disadvantages. Genes conferring resistance to quats have been detected in 6 to 42% of *Staphylococcus aureus* isolates collected in Japan and Europe (165). Organic matter, anionic detergents (soaps), and materials such as cotton and gauze pads can reduce the microbicidal activities of quats. Despite these limitations, quats are nonstaining, odorless, noncorrosive, and relatively nontoxic. They are excellent cleaning agents, but sticky residue may build up on surfaces. On the basis of their limited antimicrobial spectra, they should be used in hospitals only for environmental sanitation of noncritical surfaces such as floors, furniture, and walls (201).

Other Germicides of Interest

Glucoprotamine, the conversion product of L-glutamic acid and cocopropylene-1,3-diamine, possesses a broad antimicrobial spectrum that includes vegetative bacteria, mycobacteria, fungi, and enveloped viruses (77, 173). A clinical study examining used specula from a gynecologic clinic demonstrated that the product killed >6 log units of vegetative bacteria excluding spores (263). The manufacturer's data sheets indicate good compatibility of the compound with humans, the environment, and various materials. A commercial product, available in Europe, can be used to disinfect instruments and endoscopes.

Peroxygen compounds have proven efficacy against bacteria, bacterial spores, fungi, and a broad spectrum of viruses. A 1% concentration of a new commercial formulation containing peroxygen achieved a 10^5-fold increase in killing of *Bacillus subtilis* in 2 to 3 h in the absence of blood, but killing was poor in the presence of blood (64). Moreover, several investigators have found that peroxygen has poor mycobactericidal activity (45, 107). In addition to other applications, these compounds may be suitable for disinfecting laboratory equipment and workbenches.

Superoxidized water is prepared at the point of use by the electrolysis of NaCl solution, which generates hypochlorous acid and a mixture of radicals with strong oxidizing properties (175). Freshly generated solutions rapidly destroy bacteria including spores and mycobacteria, fungi, and viruses in the absence of organic loading (229). A commercial adaption of this process (i.e., Sterilox) has been marketed in Europe since 1999 and recently was approved by the FDA (see "Endoscopes" below) (175). Because Sterilox solutions are unstable, they should be used only once for high-level disinfection. Some investigators have claimed that superoxidized water is compatible with instruments and that it does not damage the environment, irritate the respiratory tract and skin, or corrode metal. However, others have reported that superoxidized water damages flexible endoscopes. Further studies are needed to explore the use of this new disinfectant in clinical settings.

Metals such as copper and silver ions inactivate a wide variety of microorganisms (217). Although further work is required to explore their use in health care, they currently are used to disinfect water and to prevent infections associated with medical devices (e.g., intravascular catheters impregnated with silver sulfadiazine). For example, copper-silver ionization systems are successfully used to minimize legionella colonization in water systems (237).

Surfacine is a new, silver-based surface germicide that may be applied to inanimate or animate surfaces. Surfacine immediately eliminates microorganisms from surfaces and also has long-term residual activity (44, 212). This novel antimicrobial coating might be suitable for a wide range of applications including preventing microbial contamination of medical devices, if further studies confirm the promising preliminary data.

Specific Issues

Cleaning and Disinfecting Surfaces and Floors

In general, the environment is not a primary reservoir for nosocomial pathogens. However, in some cases, such as respiratory syncytial virus (112) and the SARS coronavirus (97), environmental contamination may be important. The CDC's recent guidelines for environmental infection control in health care facilities recommend using an EPA-registered hospital detergent-disinfectant designed for general housekeeping purposes in patient care areas, especially in intensive care units, operating rooms, and emergency rooms, where blood, body fluids, or multidrug-resistant organisms may have contaminated surfaces (227). A one-step process is adequate in most areas, but a rinse step is necessary in nurseries and neonatal intensive care units, especially if a phenolic agent is used (270). Products with quats allow staff members to clean and disinfect in one step, but residual quats may leave surfaces sticky and smeared. Other products may require a two-step approach, a cleaning step and a disinfection step, doubling the human resources needed to do the work. "High-touch" surfaces (e.g., doorknobs, bedrails, and light switches) should be disinfected more frequently than "low-touch" surfaces. A simple detergent is adequate for cleaning surfaces for other patient-care areas and in nonpatient-care areas. Cleaning with a detergent is much more important than adding a disinfectant to the solution. In fact, several studies found that adding a disinfectant does not prolong the reduction in bacterial loads on surfaces (76). Routine disinfection of environmental surfaces is necessary in all areas housing patients in contact isolation (e.g., patients infected with methicillin-resistant *Staphylococcus aureus* [MRSA]). A recent study indicates that twice-daily disinfection is necessary to control an outbreak with vancomycin-intermediate *Staphylococcus aureus* (73).

Emergence of Resistance to Biocides

Microorganisms rarely become resistant to disinfectants. However, frequent use of sublethal concentrations of disinfectants can select for resistant strains (19, 37, 253). Mechanisms of resistance include acquisition of resistance plasmids, changes in the cell membrane (e.g., chlorhexidine in *Pseudomonas stutzeri*), capsule formation (*Klebsiella* spp.), and activation of the *norA* efflux pump *(Staphylococcus aureus*). A large proportion of household soaps now contain antibacterial agents (up to 45% in one study), which may increase the probability that resistant bacteria will emerge (183). Multiple outbreaks have been associated with soaps containing antibacterial agents such as chlorhexidine, hexetidine solution, and chlorxylenol (19, 37, 253). However, the concentrations of biocides used in the health care setting are much higher than the minimum biocidal concentrations in vitro. Therefore, resistance has not become a major problem in the clinical setting to date. Readers desiring more information

about disinfectants and antiseptics (34, 89) and resistance to these agents should read several excellent articles (34, 89, 157, 228).

Inactivation of Emerging Pathogens and Antibiotic-Resistant Bacteria

New and emerging pathogens such as vCJD prions, noroviruses, SARS coronavirus, avian influenza virus, hypervirulent *Clostridium difficile*, Panton-Valentine leukocidin-producing *Staphylococcus aureus*, and gram-negative rods producing extended-spectrum β-lactamases or metallo-β-lactamases threaten the public health. Only limited data exist regarding the susceptibility of emerging pathogens to commonly used disinfectants or sterilants. Surrogate microbes have been studied for some pathogens. Examples include feline calicivirus for noroviruses, vaccinia virus for variola virus, and *Bacillus atrophaeus* (formerly *Bacillus subtilis*) for *Bacillus anthracis* (259). Other infectious agents that cannot be evaluated by standard testing procedures (e.g., hepatitis C virus) have been tested by alternative methods, such as PCR. With the exception of prions, there is no evidence that emerging pathogens are less susceptible to approved standard disinfection and sterilization procedures than are comparable classical pathogens. (Inactivation of prions, including those associated with vCJD, is discussed below.) In particular, there are no data demonstrating that disinfectants used at recommended contact conditions and concentrations are less effective against antimicrobial-resistant bacteria than against antimicrobial-susceptible bacteria (213). Standard disinfection and sterilization procedures for patient care equipment as recommended in guidelines and in this chapter are adequate to disinfect or sterilize instruments or devices contaminated with blood and other body fluids (213).

Other than one peroxygen compound, hospital disinfectants registered by the EPA do not have specific claims for activity against noroviruses. Because noroviruses are nonenveloped, most quats do not have significant activity against them. Phenolic-based preparations have been found to be active in vitro against a surrogate virus from this group. However, concentrations two- to fourfold higher than those recommended by the manufacturers for routine use may be required. In the event of a norovirus outbreak, the CDC recommends using a hypochlorite solution (minimum chlorine concentration of 1,000 ppm) to decontaminate hard, nonporous, environmental surfaces (http://www.cdc.gov/ncidod/hip/gastro/norovirus.htm).

SARS coronavirus and avian influenza virus are inactivated by sodium hypochlorite and a commercially available peroxygen compound (148); phenolic compounds and quats are less effective. A sporicidal germicide is required to efficiently eliminate *Clostridium difficile* spores. In a recent study, glutaraldehyde (2%), peracetyl ions (1.6%; equivalent to 0.26% peracetic acid), and acidified nitrite demonstrated biocidal activity against *Clostridium difficile* spores (269). Hypochlorite-based disinfectants have been used, with some success, to disinfect environmental surfaces in areas with ongoing transmission of *Clostridium difficile*. Recent nosocomial outbreaks of *Clostridium difficile* infections caused by virulent strains suggest that hospitals may need to focus more on environmental cleaning and disinfection (168).

Decontamination in the Event of Biological Terrorism

If a biological agent is released, environmental decontamination measures may be necessary to decrease the risk of spreading the disease. A decontamination agent should be effective against possible pathogens and readily available at a reasonable cost. Therefore, sodium hypochlorite (household bleach) is usually recommended, especially if bacterial spores are involved. This agent is well suited for various decontamination procedures in the laboratory and health care settings. In addition, it may be used to decontaminate protective equipment and clothing worn by first responders and decontamination workers.

Smallpox virus does not survive long in the environment but may remain viable for extended periods under favorable conditions. CDC guidelines recommend incinerating items that are not needed or cannot be decontaminated, sterilizing items in an autoclave or an ethylene oxide sterilizer, decontaminating spaces and rooms with vaporized paraformaldehyde (or use of an Amphyl fogger), and soaking equipment or wiping down surfaces with a 5% aqueous solution of a phenolic germicidal detergent (http://www.bt.cdc.gov/DocumentsApp/Smallpox/RPG/GuideF/Guide-F.pdf). Because contaminated clothing can spread the virus to personnel, bed linens and clothes must be autoclaved or laundered in hot water supplemented with bleach (119). Disinfectants that are used for standard hospital infection control, such as sodium hypochlorite and quaternary ammonium compounds, decontaminate surfaces effectively (119). Only vaccinated personnel should perform the decontamination procedures.

Bacillus anthracis spores are extremely stable and can remain viable for decades in the environment (129). The CDC recommends that laboratory staff use a 1:10-diluted hypochlorite solution when addressing spills and items and surfaces contaminated with *Bacillus anthracis* (http://www.bt.cdc.gov/Agent/Anthrax/LevelAProtocol/Anthracis20010417.pdf). Decontamination of a building or of large areas contaminated with anthrax spores is extremely difficult. Spotts Whitney et al. have summarized the literature on the inactivation of *Bacillus anthracis* spores (233). *Clostridium botulinum* and its spores are killed by a 1:10 dilution of sodium hypochlorite. Heat (>85°C for 5 min) or 0.1 M sodium hydroxide (contact time, 20 min) inactivates the toxin (20). Persons with direct exposure to powder or liquid aerosols containing *Franciscella tularensis* should wash their body and clothing with soapy water (74). In the circumstances of a laboratory spill or intentional release, environmental surfaces can be decontaminated with a 1:10-diluted hypochlorite solution. After 10 min, a 70% alcohol solution can be used to further clean the area and reduce the corrosive action of the bleach (74). *Yersinia pestis* does not survive long outside the host. The WHO estimated that a plague aerosol would be effective and infectious for 1 h. Thus, areas exposed to aerosols of *Yersinia pestis* do not need to be decontaminated (128).

Equipment or environmental surfaces contaminated with the agents causing Ebola hemorrhagic fever, Marburg hemorrhagic fever *(Filoviridae)*, Lassa fever, and related infections (those with *Arenaviridae* and *Bunyaviridae*) should be disinfected by using a suitable registered hospital disinfectant or a 1:100 dilution of a hypochlorite solution. Surfaces grossly soiled with vomitus or stool should be disinfected with a 1:10 dilution of a hypochlorite solution. If possible, serum samples used for laboratory tests should be pretreated with heat inactivation at 56°C and polyethylene glycol *p-tert*-octylphenyl ether (Triton X-100). Treatment with 10 μl of 10% Triton X-100 per 1 ml of serum for 1 h reduces the titers of hemorrhagic fever viruses in serum samples. If treatment with Triton X-100 is not feasible, heat inactivation alone may reduce infectivity somewhat (52).

Medical, public health, and laboratory responses to the release of organisms and toxins that pose a risk to national security (i.e., variola major virus [smallpox virus], *Bacillus anthracis*, *Clostridium botulinum* toxin, *Francisella tularensis*, *Yersinia pestis*, and certain filoviruses and arenaviruses) are discussed in chapter 9 of this Manual and in numerous publications (20, 74, 119, 128, 129). The CDC published guidelines for the management of patients with suspected viral hemorrhagic fever (52) and recently posted updated guidelines at http://www.cdc.gov/ncidod/hip/BLOOD/VHFinterimGuidance05_19_05.pdf.

Endoscopes

Reprocessing of endoscopes is probably the most challenging reprocessing task in health care. Multiple outbreaks have been associated with insufficient reprocessing techniques or defects in endoscopes (Table 7). However, ample data indicate that a sufficient level of safety can be achieved even with manual disinfection of endoscopes if the guidelines are followed strictly (163). Flexible endoscopes have intricate, sophisticated small parts that are difficult to clean, which must be cleaned before they can be disinfected because organic material such as blood, feces, and respiratory secretions interfere with disinfection (68). A large study of several centers in the United States found that 23.9% of specimens from the internal channels of 71 gastrointestinal reprocessed endoscopes grew $\geq 10^6$ CFU of bacteria and that 78% of the facilities did not sterilize all biopsy forceps (135). Other studies have documented that up to 40% of the institutions do not follow published guidelines for endoscope disinfection (12, 84, 104) and that reuse of disposable endoscopic accessories is common in the United States. These items frequently are not sterilized, and reprocessing protocols are not standardized. Therefore, reused disposable items might be a source of cross-transmission (61, 68).

Currently, most high-level disinfectants approved by the FDA for reprocessing of endoscopes contain >2% aldehyde with or without peracetic acid (http://www.fda.gov/cdrh/ode/germlab.html). However, aldehydes should be used only after completion of the cleaning cycle because they may stain prions to the instruments. Endoscopes, which are semicritical items, must be immersed in ≥2% glutaraldehyde for ≥20 min to achieve the necessary level of disinfection. These parameters are sufficient to kill ≥3 log units of mycobacteria, the most resistant vegetative bacteria. However, glutaraldehyde-resistant mycobacteria have been identified (107). Several authors have raised concerns that *Clostridium difficile* may not be fully inactivated by standard reprocessing procedures, but transmission of *Clostridium difficile* by contaminated endoscopes has not been reported to date. Moreover, cryptosporidia withstand several hours of exposure to glutaraldehyde (268) but do not survive on dry surfaces (191).

The glutaraldehyde concentration in commercial cleaner-disinfectors can decrease by more than 50% after 2 weeks, which may promote the emergence of resistant bacteria (251). Higher concentrations of glutaraldehyde (3.2% instead of 2%) appear to be safe for endoscopes and achieve the required ≥3-log-unit killing with a higher margin of safety than that achieved with the standard concentration (7). OPA and peracetic acid plus hydrogen peroxide can be used to disinfect endoscopes. Because the latter might corrode some endoscopes, reprocessing staff should ensure that the manufacturer of the endoscope approves this disinfectant for reprocessing.

Automated washer-disinfectors specifically for endoscopes were developed, in part, to reduce the work needed to reprocess endoscopes and to decrease the risk of human

TABLE 7 Outbreaks and pseudo-outbreaks associated with contaminated endoscopes or instruments for minimally invasive procedures

Microorganism(s)	No. of cases	No. of deaths	Yr of publication	Problem identified	Type of outbreak	Reference(s)
Klebsiella pneumoniae, Proteus vulgaris, Morganella morganii	11	0	2005	Loose port of the bronchoscope's biopsy channel	Mixed	57
Pseudomonas aeruginosa	16	0	2005	Defective biopsy forceps	Mixed	68
Pseudomonas aeruginosa	3	0	2004	Probable defective endoscope	Infections	91
Pseudomonas aeruginosa	39	3	2003	Loose biopsy port cap in the bronchoscope	Infections	234
Pseudomonas aeruginosa	18	0	2001	Improper connection to liquid sterilization device	Mixed	230
Mycobacterium xenopi	58	0	2001	Inappropriate disinfection of microsurgical instruments, tap water rinse after disinfection	Infections	22
Pseudomonas aeruginosa	11	2	2000	Failure of washer-disinfector, purchased without expert advice, poor maintenance	Infections	211, 224
Pseudomonas aeruginosa, mycobacteria	29	0	1999	Problems related to the use of STERIS System 1 processor	Mixed	15
Hepatitis C virus	2	0	1997	Cleaning, immersion	Infections	46
Mycobacterium tuberculosis	2	0	1997	Cleaning, immersion	Infections	174
Mycobacterium tuberculosis (multidrug resistant)	5	1	1997	Cleaning, immersion	Infections	3
Pseudomonas aeruginosa	23	0	1996	Failure of washer-disinfector	Pseudo-outbreak	32
Nontuberculous mycobacteria	4	0	1992	Failure of washer-disinfector	Pseudo-outbreak	108
Multiple microorganisms	377	7	1993	Cleaning, immersion, use of tap water, poorly designed washer-disinfector	Infections	231 (review)

errors during manual reprocessing. These machines rinse the endoscopes, clean them in several steps, and run a full-cycle disinfection process. The time endoscopes are exposed to disinfectants is set by the machine and cannot be shortened, as it can be by busy staff members who are manually reprocessing endoscopes.

However, endoscope washers can become contaminated with pathogenic bacteria. For example, one study found gram-negative bacteria and/or mycobacteria in 27% of cultures of specimens obtained before the final alcohol rinse and in 10% of cultures of specimens obtained thereafter. In the same study, 37 and 27% of the manually disinfected endoscopes remained contaminated at the same time points (92). In 1992, Olympus recalled its 835 model endoscope washers because the design allowed the internal tanks and tubing to become colonized by waterborne organisms such as *Pseudomonas* spp. (recall no. Z-039/040-2 by the FDA). In 1999, the CDC reported three outbreaks related to the STERIS System 1 (15). This device is supposed to sterilize the endoscopes, but they must first be cleaned manually (42). Table 7 summarizes outbreaks related to endoscopes, including those related to contaminated washer-disinfectors.

Newer washer-disinfectors should continuously monitor the pressure in all channels to detect debris blocking the channels, provide adapters for all types of endoscopes, use an appropriate disinfection process with an FDA-approved disinfectant, use filtered water or sterile water for rinsing, and have a built-in automatic disinfection process. These washer-disinfectors can help staff members trace problems by monitoring and documenting the disinfecting process in a manner similar to that used by autoclaves.

To avoid problems, knowledgeable staff should review currently marketed machines before purchasing a washer-disinfector to ensure that the one they choose is appropriate for their needs (23). To facilitate this process, the FDA recommends that the manufacturer provide a list of all brands and models of endoscopes that are compatible with the washer-disinfector and highlight limitations associated with processing of certain brands and models of endoscopes and accessories. Preferably, the manufacturer should identify endoscopes and accessories that cannot be reliably reprocessed in the device (negative list). In addition, health care workers should be trained to use the equipment and monitored subsequently to ensure that they follow the protocol exactly. Although not mandatory, it is prudent to regularly culture the rinse water of washer-disinfectors for pathogens such as *Pseudomonas* spp. and *Mycobacterium* spp. to identify problems before clinical cases occur. However, outbreaks may occur despite negative routine culture results (191, 268).

If washer-disinfectors recycle water, residual glutaraldehyde may remain on the endoscopes. Manual reprocessing is more prone to leave residual glutaraldehyde on endoscopes than are automated washer-disinfectors (83). Thus, endoscopes that are manually disinfected should be thoroughly rinsed to remove any residual disinfectant, specifically glutaraldehyde. Patients exposed to residual glutaraldehyde can develop colitis (79, 262).

Reprocessed endoscopes should be stored vertically (to facilitate drying) in a cabinet (to protect them from dust and secondary contamination). Reprocessed endoscopes that are stored for days or weeks probably should be reprocessed again before use or, alternatively, the channels should be rinsed with 70% alcohol that is filtered to remove spores if this agent is compatible with the instrument. However, the necessity of these precautions has not been established.

Guidelines for infection prevention and control in flexible endoscopes have been updated (12) and should be consulted before choosing of a method and/or disinfectant for reprocessing. The following checklist adapted from the FDA recommendations may help staff members reprocessing endoscopes avoid errors (http://www.fda.gov/cdrh/safety/endoreprocess.html).

1. All staff members must comply with the manufacturer's instructions for cleaning endoscopes.
2. Determine whether your endoscope is suitable for reprocessing in an automatic washer-disinfector, which is the preferred method.
3. Compare the reprocessing instructions provided by the endoscope and washer-disinfector manufacturers and resolve any conflicting recommendations.
4. Follow the instructions provided by the manufacturers of the endoscopes and the chemical germicides.
5. Consider drying endoscopes with alcohol.
6. Monitor adherence to the protocols for reprocessing of endoscopes.
7. Provide comprehensive, intensive training for all staff members who reprocess endoscopes; keep records of persons attending training.
8. Label endoscopes sent for repairs as "contaminated equipment for repair."
9. Implement a comprehensive quality control program.

An updated list of sterilants and high-level disinfectants approved by the FDA in a 510(k) with general claims for processing of reusable medical and dental devices can be found on the FDA website (http://www.fda.gov/cdrh/ode/germlab.html). Rutala and Weber have reviewed these substances (211, 212). Of note, more than 20% of all damage to endoscopes is associated with disinfecting agents. Therefore, staff members who reprocess these items must ensure that the instruments and the disinfectant are compatible (61). A new norm (prEN ISO 15883-4) will soon be adapted for washer-disinfectors designed for thermolabile endoscopes.

Dental Equipment

Critical and semicritical dental instruments should be sterilized; if they will not be used immediately, they should be packaged before they are sterilized. All high-speed dental handheld pieces should be sterilized routinely between patients. Handheld pieces that cannot be heat sterilized should be retrofitted to attain heat tolerance; if this is not feasible, they should not be used. The adequacy of sterilization cycles should be verified by periodically (e.g., at least weekly) including a biological indicator with the load. This recommendation is rarely followed in Europe (109). In fact, 33% of British dental practices do not have a policy on general disinfection and sterilization procedures and only 3% own a vacuum autoclave (24).

Environmental contamination can be a problem in dental offices. For example, *Legionella* spp. can contaminate the air-water syringes and high-speed outlets in dental units. Moreover, Piazza et al. found that about 6% of samples from workbenches, air turbine handheld pieces, holders, suction units, forceps, and dental mirrors were positive by PCR for hepatitis C virus (184). Therefore, infection control issues, particularly in regard to hepatitis C and B viruses, may be more important in dentistry than has been appreciated previously.

The CDC and the American Dental Association (ADA) have published guidelines for infection control in dental settings (1, 55). The ADA recommends that metal and porcelain equipment be immersed in glutaraldehyde or exposed to

this disinfectant, that removable dentures and acrylic or porcelain be disinfected with iodophors or chlorine compounds, and that wax rims or bite plates be disinfected with a spray containing iodophors. Additional information can be found on the website of the ADA (http://www.ada.org/prof/resources/positions/doc_policies.pdf).

Disinfectants for Living Tissue

Compounds that disinfect living tissue are frequently called antiseptic agents. They must meet many more requirements than compounds used to disinfect inanimate surfaces, e.g., floors. In addition, some of the agents are considered drugs and, thus, are regulated by the FDA. The antimicrobial spectra of commercially available agents are summarized in Table 6. The choice of an agent should not be based only on the desired effect but, like that of antimicrobial agents, on side effects. Antiseptics rarely cause serious side effects, and most agents on the market have excellent safety profiles. Nevertheless, health care workers must remember that these agents can cause side effects such as anaphylactic shock in patients who have contact with chlorhexidine (82, 182).

Hygienic Hand Washing and Hand Disinfection

Hand hygiene is the single most important infection control measure (40). However, it remains difficult to motivate health care workers to perform this simple procedure faithfully (78). The CDC has published detailed guidelines on hand hygiene (40), and in 2006, the WHO launched a global effort to improve hand hygiene in health care facilities (http://www.who.int/patientsafety/events/05/HH_en.pdf). In-depth reviews have been published by several authors (137, 245, 264).

Microorganisms on the hands can be classified into three groups (186): (i) transient flora, which are contaminants taken up from the environment; (ii) resident flora, which are permanent microorganisms on the skin (264); and (iii) infectious flora. Resident bacteria, most of which are on the uppermost level of the stratum corneum, have low pathogenicity and infection rates, and persons with normal immune systems who do not have implants or foreign bodies rarely acquire infections with these organisms. The density of resident bacteria on the skin ranges between 10^2 and 10^3 CFU/cm^2, and these resident bacteria limit colonization with more pathogenic microorganisms (i.e., they contribute to colonization resistance). During their daily work, health care workers can contaminate their hands with pathogens. If they do not practice good hand hygiene, they can transmit these organisms to susceptible patients. Several studies indicated that pathogens such as *Staphylococcus aureus* (127), *Klebsiella pneumoniae* (2), *Acinetobacter* spp., *Enterobacter* spp., and *Candida* spp. can be found on the hands of >20% of health care workers. Moreover, numerous epidemics have been traced to health care workers' contaminated hands (41, 215, 221, 266, 272).

The goal of hand hygiene outside the operating room is to eliminate the transient flora without altering the resident flora. Hand washing for 15 and 30 s kills 0.6 to 1.1 and 1.8 to 2.8 log units, respectively (196). However, health care workers are very busy and frequently wash their hands for less than 10 s, which is insufficient to kill the transient flora (245, 264). One major advantage of the alcohol-based hand rubs is that performing hand hygiene with these products takes about 25% of the time required for hand washing (245, 264). Moreover, compliance with hand-washing procedures does not exceed 40% even under controlled study conditions (40). However, recent studies have shown that compliance with the use of the alcohol-based hand rubs exceeds that with hand washing (185). Furthermore, other studies have demonstrated that rubbing one's hands with an alcohol-based hand rub kills bacteria and most viruses more effectively than hand washing with a medicated soap (27, 194). Of note, investigators have not determined whether the level of killing is associated with the efficacy of preventing nosocomial infections.

Alcohol-based hand rubs have several other practical advantages for hand hygiene over washing with soap and water. Dispensers for the alcohol-based products are less expensive than sinks and can be installed at locations that are more convenient for health care workers. Furthermore, unlike sinks (172), the dispensers have not been associated with outbreaks.

Given the numerous advantages of these products, the CDC's current hand hygiene guidelines recommend that health care facilities consider introducing alcohol-based hand rubs as the primary mode of hand hygiene (40). Most health care institutions in northern Europe now use an alcohol-based hand rub for many indications for which hand washing was previously the standard of care. At the University Hospital Basel, the use of an alcohol-based hand rub has replaced hand washing in >85% of opportunities for hand hygiene, provided that the hands are not visibly soiled (A. Trampuz, N. Lederray, and A. F. Widmer, *Program Abstr. 41st Intersci. Conf. Antimicrob. Agents Chemother.*, abstr. K1335, 2001). Also at the University Hospital Basel, dispensers for alcohol-based hand rub are available between all beds and at each nurse's desk; two dispensers are available at each bed in intensive care units. Among 4,500 health care workers, we have not identified a single case of documented allergy to the alcohol-based hand rub, resulting in an incidence density of <1:45,000 person-years. Dermatitis may occur, most frequently related to insufficient use of emollients and ointments.

In the United States, health care facilities should consult with the fire marshal before installing dispensers because many states have laws that prohibit placing multiple containers in emergency exits and halls. However, there are no published reports of fires caused by these products in Europe and such events are also very rare in the United States (39).

Surgical Hand Washing (Scrub) or Surgical Hand Disinfection (Rub-In)

In contrast to hand hygiene outside of the operating room, the surgical hand scrub aims to eliminate both transient and resident flora so that if the surgeon's gloves are punctured or torn, the bacteria from his or her hands do not contaminate the surgical site. Tiny holes are observed in ≥30% of surgeons' gloves after operations, even when high-quality gloves are used. Cruse and Foord found that the incidence of surgical site infection is three times higher if the surgeon's gloves are punctured than if they are intact after the procedure (5.7 and 1.7%, respectively) (71). An experimental study demonstrated that the level of bacterial leakage through pin holes ranges between 10^3 and 10^4 CFU (94). Moreover, a persistent antimicrobial effect is required after washing or disinfection to limit bacterial regrowth underneath the gloves (96). Thus, antiseptic preparations intended for use as surgical hand preparations are evaluated for their abilities to reduce the number of bacteria released from hands (i) immediately after scrubbing (immediate activity), (ii) after surgical gloves have been worn for 6 h (persistent activity), and (iii) after numerous applications over 5 days (cumulative activity). Immediate and persistent activities are considered the most important. Guidelines in

the United States recommend that agents used for surgical hand preparation should significantly reduce the number of microorganisms on intact skin, contain a nonirritating antimicrobial preparation, have broad-spectrum activity, and be fast acting and persistent. Agents such as chlorhexidine that have a prolonged postexposure effect are preferred because of this theoretical advantage, but there are no data from controlled clinical trials proving that the incidence of surgical site infections is lower when chlorhexidine is used.

Alcohol-based surgical rubs have several advantages over traditional surgical scrubs. Alcoholic preparations are more effective than any medicated soap for the surgical scrub, and they do not alter the skin as much as chlorhexidine washes do. Moreover, the water supply in an operating room may harbor *Pseudomonas* spp. that may contaminate the hands of surgical personnel after they perform their surgical scrub (31). Brushes, which are used during a surgical scrub, may do more harm than good, and they should be used only to clean the fingernails, not to clean the skin. Given the advantages of the alcohol-based preparations, the presurgical scrub has been replaced in many European countries by the alcohol-based surgical rubs (245), and the WHO's guidelines recommend the surgical hand rubs (WHO guidelines are available at http://www.who.int). Alcoholic gels are frequently promoted but are significantly less effective than liquids and should not be used in the operating room (146). A very rapid protocol (1.5 min) for the surgical hand rub has been recently proposed and was rapidly accepted by surgeons at the University Hospital Basel (138).

Of note, both routine hand hygiene and surgical hand preparations must balance removing unwanted bacteria from health care workers' hands and maintaining the integrity of the health care workers' skin because damaged skin is more likely than normal skin to become colonized with pathogenic organisms. Therefore, either hand hygiene products should contain emollients or health care facilities should provide moisturizing hand lotions, which do not damage latex, for their staff so their skin does not become dry, cracked, and irritated.

Presurgical Skin Disinfection

The aim of skin disinfection is to remove and kill rapidly the skin flora at the site of a planned surgical incision. However, currently available antiseptics do not eliminate all microorganisms at the incision site. In fact, coagulase-negative staphylococci can be isolated frequently even after three applications of agents such as iodine-alcohol to the skin (98).

The FDA defines a skin disinfectant as a "fast acting, broad-spectrum and persistent antiseptic-containing preparation that significantly reduces the number of microorganisms on intact skin" (14). Spore-free alcohols are well suited for this purpose, but they lack persistent activity; iodine is frequently added for this purpose (100). Povidone-iodine continuously releases free iodine that results in a prolonged antmicrobial effect. However, for a short-term procedure, alcoholic preparations can be sufficient for skin preparation.

Before a patient's skin is prepared for a surgical procedure, the skin should be free of gross contamination (i.e., dirt, soil, or any other debris) (160). Although preoperative showering has not been shown to reduce the incidence of surgical site infections, this practice may decrease bacterial counts and ensure that the skin is clean (195). The antiseptics used to prepare the skin should be applied by using sterile supplies and gloves or a no-touch technique, moving from the incision area to the periphery (160). The person preparing the skin should use pressure because friction increases the antibacterial effect of the antiseptic. For example, alcohol applied without friction reduces bacterial counts by 1.0 to 1.2 log CFU compared with 1.9 to 3.0 log CFU when friction is used. In comparison, alcoholic sprays have little antimicrobial effect and produce potentially explosive vapors (156).

Common Antiseptic Compounds

Alcoholic Compounds

The reader is referred to "Alcohols" above. As outlined above, alcohol is the most important skin disinfectant. Alcohols used for skin disinfection before invasive procedures should generally be free of spores to avoid any contamination. Although the risk of infection is minimal, the low additional cost for a spore-free product is justified. One study indicated that isopropyl alcohol from a commercial hand rub may be absorbed through the dermis, violating religious beliefs of some health care workers (246). However, the WHO resolved this issue in the guidelines that they will publish in 2006.

Chlorhexidine

Chlorhexidine gluconate, a cationic bisbiguanide, has been widely recognized as an effective and safe antiseptic for nearly 40 years (75, 189). Chlorhexidine formulations are extensively used for surgical and hygienic hand disinfection (see the discussion above). Other applications include preoperative showers (or whole-body disinfection), antisepsis in obstetrics and gynecology, management of burns, wound antisepsis, and prevention and treatment of oral disease (e.g., plaque control, pre-and postoperative mouthwash, and oral hygiene) (75, 189). When chlorhexidine is used orally, its bitter taste must be masked and it can stain the teeth. Intravenous catheters coated with chlorhexidine and silver sulfadiazine are used to prevent catheter-associated bloodstream infections (159).

Chlorhexidine is most commonly formulated as a 4% aqueous solution in a detergent base. However, alcoholic preparations have been demonstrated in numerous studies to have better antimicrobial activity than detergent-based formulations (151). Bactericidal concentrations destroy the bacterial cell membrane, causing cellular constituents to leak out of the cell and cell contents to coagulate (75). Chlorhexidine gluconate's bactericidal activity against vegetative gram-positive and gram-negative bacteria is intermediately rapid. In addition, chlorhexidine provides persistent antimicrobial action that prevents the regrowth of microorganisms for up to 6 h. This effect is desirable when a sustained reduction in microbial flora reduces infection risk (e.g., during surgical procedures). Chlorhexidine has little activity against bacterial and fungal spores except at high temperatures. Mycobacteria are inhibited but are not killed by aqueous solutions. Yeasts and dermatophytes are usually susceptible, although the fungicidal action varies with the species (74). Chlorhexidine is effective against lipophilic viruses (e.g., HIV, influenza virus, and herpes simplex virus types 1 and 2), but viruses such as poliovirus, coxsackievirus, and rotavirus are not inactivated (75). Unlike that of povidone-iodine, blood and other organic material do not affect the antimicrobial activity of chlorhexidine significantly (155). However, inorganic anions and organic anions such as soaps are incompatible with chlorhexidine and its activity is reduced at extreme acidic or alkaline pHs and in the presence of anion- and non-ion-based moisturizers and detergents.

Microorganisms can contaminate chlorhexidine solutions (181), and resistant isolates have been identified. For

example, Stickler found chlorhexidine-resistant *Proteus mirabilis* after chlorhexidine was used extensively over a long period to prepare patients for bladder catheterization (235). Chlorhexidine resistance among vegetative bacteria was thought to be limited to certain gram-negative bacilli (such as *Pseudomonas aeruginosa*, *Burkholderia* [*Pseudomonas*] *cepacia*, *Proteus mirabilis*, and *Serratia marcescens*) (236). However, genes conferring resistance to various organic cations, including chlorhexidine, have been identified in *Staphylococcus aureus* clinical isolates (165, 176).

Chlorhexidine has several other limitations. When absorbed onto cotton and other fabrics, it usually resists removal by washing. If a hypochlorite (bleach) is used during the washing procedure, a brown stain may develop (75). Long-term experience with the use of chlorhexidine has demonstrated that the incidence of hypersensitivity and skin irritation is low. However, severe allergic reactions including anaphylaxis have been reported previously (82, 271). Although cytotoxicity has been observed with exposed fibroblasts, no deleterious effects on wound healing have been demonstrated in vivo. There is no evidence that chlorhexidine gluconate is toxic if it is absorbed through the skin, but ototoxicity can occur when chlorhexidine is instilled into the middle ear during operative procedures. High concentrations of chlorhexidine and preparations containing other compounds (e.g., alcohols and surfactants) may damage eyes (239).

Iodophors

Iodophors essentially have replaced aqueous iodine and tincture as antiseptics. Iodophors are chemical complexes of iodine bound to a carrier such as polyvinylpyrrolidone (povidone) or ethoxylated nonionic detergents (poloxamers). These complexes gradually release small amounts of free microbicidal iodine. The most commonly used iodophor is povidone-iodine. Its preparations generally contain 1 to 10% povidone-iodine, which is equivalent to 0.1 to 1.0% available iodine. The active component appears to be free molecular iodine (I_2). A paradoxical effect of dilution on the activity of povidone-iodine has been observed. As the dilution increases, bactericidal activity increases up to a maximum and then falls (105). Commercial povidone-iodine solutions at dilutions of 1:2 to 1:100 kill *Staphylococcus aureus* and *Mycobacterium chelonae* more rapidly than do stock solutions (28). *Staphylococcus aureus* can survive a 2-min exposure to full-strength povidone-iodine solution but cannot survive a 15-s exposure to a 1:100 dilution of the iodophor (28). Thus, iodophors must be used at the dilution stated by the manufacturer.

The exact mechanism by which iodine destroys microorganisms is not known. Iodine may react with microorganisms' amino acids and fatty acids, destroying cell structures and enzymes (105). Depending on the concentration of free iodine and other factors, iodophors exhibit a broad range of microbicidal activity. Commercial preparations are bactericidal, mycobactericidal, fungicidal, and virucidal but not sporicidal at the dilutions recommended for use. Prolonged contact times are required to inactivate certain fungi and bacterial spores (201). Despite their bactericidal activities, povidone-iodine and poloxamer-iodine solutions can become contaminated with *Burkholderia (Pseudomonas) cepacia* or *Pseudomonas aeruginosa*, and contaminated solutions have caused outbreaks of pseudobacteremia and peritonitis (29, 69). In fact, *Burkholderia cepacia* survives for up to 68 weeks in a povidone-iodine antiseptic solution (13). The most likely explanation for the prolonged survival of these microorganisms in iodophor solutions is that organic or inorganic material and biofilm may mechanically protect the microorganisms.

Iodophors are widely used for antisepsis of skin, mucous membranes, and wound sites. A 2.5% ophthalmic solution of povidone-iodine is more effective and less toxic than silver nitrate or erythromycin ointment for prophylaxis against neonatal conjunctivitis (ophthalmia neonatorum) (130). In some countries, povidone-iodine alcoholic solutions are used extensively for skin antisepsis before invasive procedures (18). Iodophors containing higher concentrations of free iodine may be used to disinfect medical equipment. Solutions designed for use on the skin should not be used to disinfect hard surfaces because the concentrations of the antiseptic solutions are usually too low for this purpose (201).

The risk of side effects, such as staining, tissue irritation, and resorption, is lower for iodophors than for aqueous iodine. Iodophores do not corrode metal surfaces (105). However, a body surface treated with an iodine or iodophor solution may absorb free iodine. Consequently, increased serum iodine levels (and serum iodide levels) have been found in patients, especially when large areas were treated for a long period (105). For this reason, hyperthyroidism and other disorders of thyroid functions are contraindications for the use of iodine-containing preparations. Likewise, iodophors should not be applied to pregnant and nursing women or to newborns and infants (50). Because severe local and systemic allergic reactions have been observed, iodophors and iodine should not be used for patients with allergies to these preparations (256).

Iodophores have little if any residual effect. However, for a limited time they may have residual bactericidal activity on the skin surface, because free iodine diffuses into deep regions and also back to the skin surface (105). The antimicrobial efficacy of iodophors is reduced in the presence of organic material such as blood.

Triclosan and PCMX

Triclosan (Irgasan DP-300 or Irgacare MP) has been used for more than 30 years in a wide array of skin care products, including hand washes, surgical scrubs, and consumer products. A review of its effectiveness and safety in health care settings has been published previously (132). A concentration of 1% has good activity against gram-positive bacteria, including antibiotic-resistant strains, but less activity against gram-negative organisms, mycobacteria, and fungi. Limited data suggest that triclosan has a relatively broad antiviral spectrum, with high-level activity against enveloped viruses, such as HIV type 1, influenza A virus, and herpes simplex virus type 1. However, the nonenveloped viruses prove more difficult to inactivate. Clinical strains of *Staphylococcus aureus* with low-level resistance to triclosan have been identified, and the clinical significance of this remains unknown (238). Triclosan is added to many soaps, lotions, deodorants, toothpastes, mouthrinses, commonly used household fabrics, plastics, and medical devices. Moreover, the mechanisms of triclosan resistance may be similar to those involved in antimicrobial resistance (6) and some of these mechanisms may account for the observed cross-resistance of laboratory isolates to antimicrobial agents (63). Consequently, concerns have been raised that widespread use of triclosan formulations in non-health care settings and products may select for biocide resistance and even cross-resistance to antibiotics. However, environmental surveys have not demonstrated an association between triclosan usage and antibiotic resistance (199).

Triclosan solutions produce a sustained residual effect against resident and transient microbial flora that is minimally affected by organic matter. Numerous studies have not identified toxic, allergenic, mutagenic, or carcinogenic potential. Triclosan formulations may help control outbreaks of MRSA infections when the formulations are used for hand hygiene and for bathing of patients (132). However, some MRSA isolates have reduced triclosan susceptibility. Triclosan formulations are less effective than 2 to 4% chlorhexidine gluconate when used for surgical scrub solutions; properly formulated triclosan solutions may be used for hygienic hand washing.

PCMX (chloroxylenol) is an antimicrobial used in handwashing products. It is available at concentrations of 0.5 to 3.75%. Its properties are similar to those of triclosan. Nonionic surfactants may neutralize PCMX.

Octenidine

Octenidine dihydrochloride is a novel bispyridine compound, which is an effective and safe antiseptic agent. The 0.1% commercial formulation compares favorably with other antiseptics with respect to antimicrobial activity and toxicologic properties. In vitro and in vivo, it rapidly kills both gram-positive and gram-negative bacteria as well as fungi (101, 226). Octenidine is virucidal against HIV, hepatitis B virus, and herpes simplex virus. Similar to chlorhexidine, it has a marked residual effect. No toxicologic problems have been found when the 0.1% formulation was applied according to the manufacturer's recommendations. The colorless solution is a useful antiseptic for mucous membranes of the female and male genitals and the oral cavity (25), but its bad taste limits its use orally. In a recent observational study, the 0.1% formulation was highly effective and well tolerated for the care of central venous catheter insertion sites (243). The results of this study have also been supported by a randomized controlled clinical trial (M. Dettenkofer and A. F. Widmer, *Abstr. Eur. Congr. Clin. Microbiol. Infect. Dis.*, 2006, Nice, France, abstr. O147). Octenidine is not registered for use in the United States.

STERILIZATION

Principles, Definitions, and Terms

As outlined in Table 3, sterilization is not a relative term but defines the complete absence of any viable microorganisms, including spores. However, this absence cannot be proved by current microbiologic techniques (133). Therefore, sterilization can be defined as a closely monitored, validated process used to render a product free of all forms of viable microorganisms, including all bacterial endospores. To test the ability of sterilization systems to meet the latter definition, manufacturers developed a worst-case scenario that allows the process (log killing) to be quantified and estimates the probability of process failure. Large safety margins were included in this test, which is based on the assumption that items are heavily contaminated with spores, soil, and proteins. Note that although these conditions are used for the testing, in clinical practice items that are heavily soiled should not be sterilized and such a scenario would represent a critical failure of the reprocessing cycle. Any device undergoing sterilization first must undergo an appropriate cleaning process.

A manufacturer must demonstrate that a sterilizer is effective against a wide range of clinically important microorganisms before it can be approved by the FDA. In addition, proof of efficacy must be performed with organisms (usually bacterial spores) that have been shown to be the most resistant to the new technology. A validated and reliable biological indicator must be developed, and studies must establish that sterility will be consistently achieved when critical process parameters operate within a defined range. This assures the operator that as long as there is no operational error or equipment failure, sterility is achieved.

Several guidelines are essential documents for staff members who need to understand reprocessing and sterilization of medical devices. The ISO 14937 document provides general criteria for characterizing a sterilizing agent and for the development, validation, and routine control of a sterilization process for medical devices. ISO 11134 (moist heat) and ISO 11135 (ethylene oxide) documents describe the standards for use of these methods of sterilization in the industrial setting in the United States. The American National Standard Institute/Association for the Advancement of Medical Instrumentation published adaptations of these standards for health care facilities: standard 46 (moist heat) and standard 41 (ethylene oxide) (Table 3). In Europe, EN 550, EN 554, and EN 285 define the standards for steam and ethylene oxide sterilization. ISO 14161 provides guidance that staff can use when selecting and using biological indicators and when interpreting the results of these tests. ISO 17664 specifies which information medical device manufacturers must provide so that the medical device can be processed safely and will continue to function properly. Readers are referred to other references for additional information about sterilization (34, 106, 133).

Monitoring

Any sterilization process must be monitored by mechanical, physical, chemical, and facultatively biological methods. Before routine use, the performance of the machine should be validated with the most difficult load used at the institution to ensure that the process is safe. In addition, a printout of the physical parameters (e.g., temperature and pressure) during sterilization should be kept for documentation purposes. In 1963, Bowie and Dick determined that if residual air remained in a sterilizer after the vacuum phase and there was only one package in the chamber, the air would concentrate in that package (38). They developed the Bowie-Dick test to determine whether steam penetration and air removal occurred successfully. This test does not provide information about the sterilization process.

Chemical indicators placed on items in a sterilizer change color if they are exposed to adequate temperatures and exposure times. They are inexpensive, easy to use, and provide a visual indication that the item has been exposed to the sterilization process. Good clinical indicators are able to identify a sterilizer failure. However, some are too sensitive, giving false-positive results (205, 208), which may cause unnecessary recalls of adequately sterilized items. Less sensitive chemical indicators do not detect small deviations in the process.

Biological indicators are the best monitors of the sterilization process. If the spores in commercially available standard biological indicators do not grow during an appropriate incubation period, the results indicate that the process was able to kill $\geq 10^6$ CFU. For flash sterilization, the Attest Rapid Readout biological indicator detects the presence of a spore-associated enzyme, α-D-glucosidase, and permits the staff to assess the efficacy of sterilization within 60 min (252). Staff members should investigate positive biological indicators because they can provide the only indication that something is wrong with the sterilization process (51).

An important question is whether a load can be distributed before the final results of the biological indicator are available (i.e., parametric release). The Joint Commission on Accreditation of Healthcare Organizations standard allows the use of appropriate chemical indicators without routine use of a biological indicator. A common approach is to use the sterilized items if the physical and chemical parameters of adequate sterilization were met without waiting for the culture results from the biological indicators.

In Europe, routine use of biological indicators is not required if the sterilizer has undergone testing by a validation procedure used for industrial steam sterilization (EN 285, EN 550, EN 554, or EN 556). Most sterilizers in European hospitals probably do not meet these very strict requirements (250), and consequently, biological indicators are used regularly to ensure that they are working properly. These industrial standards for validation of steam sterilization will be implemented in health care organizations, but this change is controversial because of the associated expenses. The future is likely to involve parametric release with regular validation and/or commissions of the equipment. Legal issues will probably determine the outcome of this discussion, and lawyers are likely to accept nothing but a zero risk. However, the goal of a zero risk for contamination in central sterilization services will probably contribute to excessive health care costs. Therefore, standards for sterilization should exclude a risk for contamination after the reprocessing cycle but should avoid steps that are performed only for legal reasons.

Packaging, Loading, and Storage

Items that are clean and dry should be inspected and then wrapped and packaged (or put in containers) before sterilization. Wrappers should allow steam or gas to penetrate into the package but should protect the items from recontamination after sterilization. Items wrapped only in muslin can become contaminated when they are handled after steam sterilization (265). Items should be labeled with information such as expiration date, type of sterilization, and identification code so that items can be traced.

Steam Sterilization

The most reliable method of sterilization is one that uses saturated steam under pressure. It is inexpensive, nontoxic, and very reliable.

Steam penetrates fabrics, and its inherent safety margin is much higher than that of any other sterilization technique. Therefore, it should be used whenever possible. Obviously, other techniques must be used for heat-sensitive items. Steam irreversibly coagulates and denaturates microbial enzymes and proteins. Three parameters are critical to ensuring that steam sterilization is effective: the amount of time the items are exposed to steam, the temperature of the process, and the level of moisture. Unlike time and temperature, the moisture condition in the autoclave cannot be directly determined. The D value determines the time required to kill 10^6 CFU of the spores most resistant to the sterilization process under study. Devices or instruments must reach the desired temperature, which is not necessarily identical to the temperature displayed on the autoclave's gauge. A drop of only 1.7°C (3°F) increases the time required to sterilize an item by 48%. Without moisture, a temperature of 160°C is required for dry-heat sterilization. Dry air does not provide steam for condensation, and the heat transfer to objects is slower than it is when moisture is present. Pressurized steam quickly transfers energy to the sterilizer load and causes more rapid denaturation and coagulation of microbial proteins. In addition, pressurizing the steam allows one to achieve dry 100% saturated steam. Thus, there is no mist that may cause the packaging and/or the items to become wet.

Residual air in the autoclave interferes with the sterilization process. The amount of air within the sterilizer can be estimated by comparing the chamber pressure with the saturated steam pressure calculated from the average chamber temperature. A measured pressure greater than the calculated saturated pressure indicates the presence of residual air in the chamber. Such monitoring devices are common in the United Kingdom.

Several types of autoclaves are available: gravity displacement steam sterilization, prevacuum steam sterilization, and steam flash-pressure pulsing steam sterilization autoclaves. The sterilization process is less consistent in gravity displacement steam sterilizers than in the other sterilizers (70). For example, gravity displacement autoclaves are more likely than the other systems to leave residual air in the chamber before the steam is introduced. Prevacuum sterilizers resolved part of this problem and cut the cycle time in half. However, the effectiveness of sterilization still can be compromised by small leaks (1 to 10 mm Hg/min) in the sterilizer (133). The most current technology is the steam flash-pressure pulsing steam sterilization technique, in which air leaks do not decrease the effectiveness of the process. This method nearly eliminates the problem of air in the chamber and reduces the thermal lag upon heating of the load to the desired exposure temperature (70).

The process of sterilization has several cycles: conditioning, exposure, and drying. Common cycles for steam sterilization in prevacuum or flash-pressure pulsing steam sterilizers are 121°C for 15 min (121°C for 30 min in a gravity displacement sterilizer) and 132°C for 4 min (see the FDA addendum to the sterilizer guidance, issued 19 September 1995 [http://www.cenorm.org]). EN 554 requires steam sterilizers to provide this temperature throughout the entire chamber within a narrow margin (0 to +3°C).

Flash sterilization is an emergency process used, for example, after a surgical instrument, which is needed immediately, is dropped during a procedure (158). Unwrapped devices are placed in a sterilizer (usually in the operating suite and sometimes without a biological indicator) and exposed to pressurized steam for 3 min. The autoclaves employed are gravity displacement sterilizers that have the problems mentioned previously. If health care workers are in a hurry, they may not clean the item properly, which will prevent proper sterilization. In addition, because the items are not wrapped, they can be contaminated easily when they are transported to the operating room. Even properly wrapped sterile items can become contaminated if they are transported several times (265). Moreover, some patients have been injured by items that were flash sterilized (214). Therefore, flash sterilization is controversial, and several investigators have suggested that it should be used only in emergency situations when no other device is available. Flash sterilization should not replace standard sterilization protocols (85) and should not be used to save time in sterilizing items by the standard methods or to avoid purchasing extra instrument sets (160). Because documentation takes time, staff members often do not document appropriately when they use flash sterilization. Consequently, if something goes wrong, tracing the sterilized items may be impossible.

Ethylene Oxide Gas

Temperature- and/or pressure-sensitive items have been sterilized traditionally with ethylene oxide in a standard gas.

Ethylene oxide inactivates all microorganisms, including spores, probably by an alkylation process. *Bacillus subtilis* bacterial spores are among the most resistant, and therefore, these are used as a biological monitor for this process. A new rapid-readout ethylene oxide biological indicator will indicate an ethylene oxide sterilization process failure by producing fluorescence, which is detected in an autoreader within 4 h of incubation at 37°C, and a color change related to a change in the pH of the growth media within 96 h of continued incubation (212).

The process of sterilizing items with ethylene oxide begins by adding nitrogen gas to remove air or by evacuating the chamber. Items are then exposed to ethylene oxide at 55°C (130°F). Six variable but interdependent parameters—gas concentration, vacuum, pressure, temperature, relative humidity, and time of exposure—must be controlled when ethylene oxide is used. The gas concentration cannot be measured on line, limiting the extent of monitoring. Therefore, the concentration should be validated as outlined by ISO 11135.

Ethylene oxide sterilization has several disadvantages; it is useful only as a surface sterilizer because it does not reach blocked-off surfaces. In addition, ethylene oxide is flammable, explosive, and carcinogenic to laboratory animals and it requires special safety precautions. Moreover, items sterilized by ethylene oxide must be aerated for approximately 12 h to remove any traces of the gas. Thus, the entire process takes >16 h, but modern ethylene oxide sterilizers can run at shorter cycles. Furthermore, toxic residues can be trapped in the wrapper or the items. Polyvinyl chloride and polyurethane absorb ethylene oxide readily and require long periods to dissipate the oxide. The wrapper should be a barrier against recontamination after sterilization, but it also can prevent ethylene oxide from reaching the item. Therefore, only materials with documented ethylene oxide penetration and dissipation properties should be used as wrappers.

The future of ethylene oxide in sterilization is limited, mainly due to its toxicity. However, no currently available technology, including plasma sterilization (see below), can replace sterilization with ethylene oxide entirely. In addition, sterilization with ethylene oxide does not fail as frequently as sterilization with plasma when residual proteins and/or salts are present on the items (11).

Plasma Sterilization

Low-temperature plasma is produced in a closed chamber with a deep vacuum, an electromagnetic field, and a chemical precursor (hydrogen peroxide or a mixture of hydrogen peroxide and peracetic acid). The resulting free radicals, the chemical precursors, and the UV radiation are thought to rapidly destroy vegetative microorganisms, including spores.

Sterrad

The Sterrad 100 sterilizer, the first plasma sterilizer for use in health care facilities, has been on the market in Europe since 1990 and in the United States since 1993. In August 1997, the Sterrad 100 system was approved to sterilize certain surgical instruments with long lumens, such as those used in urologic, laparoscopic, and arthroscopic procedures, including instruments with single stainless steel lumens of ≥3 and <400 mm in length. The Sterrad 100S has since replaced the Sterrad 100. The Sterrad 100S adds one sterilization cycle and, thereby, fulfills the requirement to kill 10^6 CFU of spores at the half point of the cycle. A smaller device, the Sterrad 50, has been independently tested for efficacy (207). Other sizes, e.g., the large Sterrad 200, approved by the FDA in 2003, can sterilize small lumens (single stainless steel lumens with an inside diameter of 1 mm or larger and Teflon or polyethylene lumens with an inside diameter of 6 mm or larger). The new Sterrad NX system, approved by the FDA in April 2005, is the fastest low-temperature hydrogen peroxide gas plasma sterilizer yet. This system employs a new vaporization system that removes most of the water from the hydrogen peroxide, improving diffusion of peroxide into lumens. Consequently, a broad range of instruments, including single-channel flexible endoscopes, can be processed within 38 min. In 2001, the FDA cleared biological indicators suitable for plasma sterilization.

Regardless of the model, the basic steps are the same. Medical instruments are placed in the sterilization chamber, a strong vacuum is created in the chamber, and a solution of 59% hydrogen peroxide and water is automatically injected from a cassette into the sterilization chamber. The solution vaporizes and diffuses throughout the chamber, surrounding the items to be sterilized. Radiofrequency energy is applied to create an electric field, which in turn generates the low-temperature plasma, inducing free radicals. The combination of the diffusion pretreatment and plasma phases sterilizes the item while eliminating harmful residuals. At the end of the cycle, the radiofrequency energy is turned off, the vacuum is released, and the chamber is filled with filtered air, returning it to normal atmospheric pressure.

Plasma sterilizers have several disadvantages. First, materials that absorb too much hydrogen peroxide (e.g., cellulosics and some nylons such as those from connectors, cables, and insulators), materials that catalytically decompose hydrogen peroxide (e.g., copper and nickel alloys from electrical wire, solder, and surgical instruments), and materials that react with hydrogen peroxide such as organic dyes (colored anodized aluminum) and organic sulfides of solid lubricants in endoscopic devices cannot be sterilized in a Sterrad. Second, the cassettes required to run the device and the special nonmuslin wrappers are relatively expensive.

Low-Temperature Sterilization by Ozone

The 125L ozone sterilizer uses medical-grade oxygen, water, and electricity to generate ozone within the sterilizer to provide an efficient sterilant without producing toxic chemicals or using high temperatures. (It runs at 25 to 35°C.) Ozone forms when oxygen is submitted to an intense electrical field that separates oxygen molecules into atomic oxygen (O), which in turn combines with other oxygen molecules (O_2) to form triatomic oxygen (O_3), or ozone, providing a sterility assurance level of 10^6 in approximately 4 h. At the end of the cycle, the oxygen and water vapor safely vent directly into the room. The sterilization chamber has a capacity of 125 liters. Processed medical instruments require no aeration at the end of the sterilization cycle. Medical devices are packaged in a TS03 sterilization pouch or in anodized aluminum sterilization containers. The TS03 OZO-TES'P' self-contained biological and chemical indicators should be used to evaluate the machine's performance. An ozone sterilizer can be installed as a free-standing unit or recessed behind a wall. These devices are used primarily in Canada. They are approved by the FDA, but few health care facilities in the United States use them.

Liquid Sterilization

The FDA approved the STERIS System 1 in 1988, but this system is not considered a sterilizer in Europe (72). The machine is designed to sterilize immersible devices, including flexible endoscopes, with 35% liquid peracetic acid (an

FDA-approved sterilant that is sporicidal [123, 126], supplemented with buffering, anticorrosion, wetting, and surface-active agents). Peracetic acid is automatically diluted with sterile filtered water, and the items are exposed for 12 min. The entire sterilization process takes approximately 30 min at ca. 50°C. Items can be used immediately after the process is completed and do not need to be aerated.

Clinical studies of the STERIS System 1 have been performed with bronchoscopes, hysteroscopes, colonoscopes, and rigid endoscopes (42, 254). Independent efficacy tests demonstrated some failures (42). Exposure time and temperature are monitored electronically, and conductivity is used as a surrogate marker for peracetic acid concentration. However, the machine can complete its cycle normally and print a report stating that the concentration of peracetic acid was in the normal range when it was run intentionally without peracetic acid (161). Commercially available spores can be used for monitoring sterilization (145), but false-positive test strips can occur, related to improper use of the clip that attaches the test strips (110). Other disadvantages of this system include the high cost of purchasing and using the equipment, which is considerably greater than the cost of purchasing and using systems for high-level disinfection with glutaraldehyde (95). In addition, the device does not clean the items. Thus, the cleaning step adds to the overall time of reprocessing the items. The STERIS System 1, like all other nonsteam sterilizers, cannot meet the requirements for sterilization if residual debris and/or proteins are present on the items.

Hot Air Sterilization

Hot air sterilization is not a state-of-the-art technology, but it is still used in many countries. However, the distribution of dry heat to the instruments requires long exposure times. Temperatures of >185°C resinify paraffin, destroying instruments' lubrication, and higher temperatures are corrosive, resulting in loss of hardness. Therefore, hot air sterilization has largely been replaced by better, safer, and faster technologies.

REUSE OF SINGLE-USE DEVICES

Current FDA policy states that the responsibility for the safety and performance of reprocessed single-use devices lies with the reprocessor, not the original manufacturer. The FDA considers the hospital to be the manufacturer of a single-use device if the device has been resterilized. Therefore, the reprocessor must ensure that the reprocessed items are sterile and are not contaminated with toxic substances such as endotoxins or residual ethylene oxide and that the product's integrity, composition, and function are essentially identical to those of a new product. Most hospitals cannot afford to generate appropriate data on the quality and performance of reprocessed single-use items. In addition, if a manufacturer changes the product, the reprocessor needs to redo the analyses before the device can legally be marketed after reprocessing (management-of-change guidelines). The FDA published a final guidance (see the website for details: http://www.fda.gov/cdrh/ohip/guidance/1333.html and http://www.fda.gov/cdrh/ohip/guidance/1408.html).

Some institutions resterilize items that have not been used on patients but that, for instance, were dropped and/or had the packages damaged. Even this approach can be problematic. For example, the FDA published an alert documenting that the quality of an implant that was originally sterilized with ethylene oxide and then resterilized with steam was impaired by the reprocessing method (see http://www.fda.gov/cdrh/steamst.html for details). In addition, the quality, product integrity, and performance of many reprocessed plastic or rubber products are unknown. Moreover, the FDA does not allow health care facilities that send equipment and supplies to a reprocessing company to transfer full responsibility to that company (see the full text at http://www.fda.gov/medwatch/safety/1997/device.htm). Furthermore, if a hospital reprocesses a single-use device, the hospital is responsible for ensuring that the device complies with all applicable FDA labeling requirements, even if the device is exempt from the premarket requirements. If the hospital does not ensure that the device complies with FDA labeling requirements, the device is misbranded and the hospital may be considered in violation of section 301(k) of the Food, Drug and Cosmetic Act. As of 14 August 2001 and 14 February 2002, the FDA enforces premarket filing requirements for reprocessed class II devices (i.e., moderate-risk devices such as a cardiac mapping catheter used to map electrical activity of the heart; http://www.fda.gov/cdrh/comp/guidance/1168.pdf) and marketing clearance requirements. Many issues are not yet resolved. The FDA has set a prioritization scheme (http://www.fda.gov/cdrh/reuse/1156.pdf).

In many countries throughout the world, health care facilities reprocess single-use items (sometimes illegally) because resources are limited and this activity may be the only way to provide patients with access to state-of-the-art health care. In fact, a commercial reprocessor in Germany legally reprocessed >4 million single-use items without any serious reported side effects, saving between 30 to 50% of the cost of new items. Thus, we believe that new reprocessing technologies using washer-disinfectors coupled with highly effective low-temperature sterilizers can kill all microorganisms, even in narrow lumens such as cardiac catheters. We also believe that with the expertise of infection control personnel, health care facilities can provide the desired level of microbiological and toxicological safety. However, they probably cannot ensure that the design and function of the devices are still adequate. Thus, in the United States and countries with similar regulations on quality assurance programs, reuse of single-use devices may not be cost-effective. In addition, organizations that sell used single-use devices to patients and/or insurance companies as new devices will encounter legal and ethical issues. Because devices change and new devices are introduced, it is difficult for health care facilities and reprocessing companies to do the studies needed to document that reprocessing is safe, thus making it difficult to reprocess single-use devices on a large scale. However, financial restriction may change the current belief that patients will not accept reprocessed single-use devices. The reader should consult the FDA website and experts in the field before considering reprocessing of single-use devices.

BOVINE SPONGIFORM ENCEPHALOPATHY AND VARIANT CREUTZFELDT-JAKOB DISEASE

CJD has been identified on all continents and is thought to occur worldwide. The incidence of CJD is estimated to be about 1 case per 10^6 persons per year. Most cases of CJD are sporadic; <10% of CJD cases may be related to a genetic autosomal dominant predisposition, and some nosocomial cases are related to the use of contaminated tissue or contaminated human growth hormone. It is generally accepted that eating BSE agent-contaminated meat is the cause of vCJD (121, 188). The vCJD has brought about a major medical and economic crisis in Europe (49, 140, 188).

By 1 January 2006, 154 cases were reported from the United Kingdom. Cases that fulfill the new WHO case definition (21 May 2001) also have been reported from France (14 cases), Canada (1 case), Spain (1 case), Ireland (1 case), and Italy (1 case). The peak of the epidemic was in the year 2000 (28 cases in the United Kingdom), falling to 5 cases in the United Kingdom in December 2005. In the United States, one patient who was a former resident of the United Kingdom has been diagnosed with vCJD and the first case of BSE in cattle was identified in 2003. However, more cases may occur because 37 tons of "meals of meat or offal" that were "unfit for human consumption" was sent from the United Kingdom to the United States in 1997, well after the government banned imports of such risky meat (59). As of 2006, no curative therapy is available for CJD or vCJD; however, several approaches have been investigated with limited success (103).

The agent causing vCJD is not a classic microorganism but an altered prion protein (5, 33, 187, 220). Its origin remains obscure, but the BSE agent from cattle is most probably responsible for the vCJD in humans. In the 1980s a step in tallow extraction from rendered carcasses was eliminated, allowing some tissue infected with scrapie to survive the process and allowing the infectious agent to be recycled as cattle-adapted scrapie or BSE. The animal food was no longer sterilized at 134°C for 20 to 30 min but, rather, was pasteurized before being fed to animals whose carcasses, with encased spinal cords and paraspinal ganglia, were later processed legally into hot dogs, sausages, and precooked meat patties (47). Investigators postulate that a high incidence of scrapie in sheep and a large proportion of sheep in the mix of carcasses that was rendered for livestock feed may explain why the incidence of BSE in British cattle was more than 10 times higher than that in cattle in any other European country. In the United Kingdom, the number of people exposed to potentially infective doses through food may be extremely high.

vCJD has a different clinical presentation and occurs at a much younger age than CJD (60). The mean age at death from vCJD is 28 years; only 6 of 90 patients died at the age of 50 years or older (248). Two hypotheses that may explain why the age group of 50 and under is most affected are that the incubation period is shorter in the young than in the elderly and that the young are more susceptible to infection. In addition, all patients genotyped so far are homozygous for methionine on codon 129 of the prion protein gene. It is postulated that heterozygous individuals may have much longer incubation times before vCJD becomes evident. Therefore, asymptomatic carriers may pose a risk for transmission if they undergo routine surgery and instruments are not reprocessed by a prion-safe program.

Patients suffering from vCJD harbor large numbers of prions in their tonsils and spleens before they have signs, symptoms, and pathological findings from the disease. In contrast, patients with sporadic CJD suffer from spongiform encephalopathy long before the prion spreads into muscles and lymphoid tissue (120). Consequently, France has developed very strict precautions. For example, the United Kingdom required that all tonsillectomies be performed with disposable instruments. In 2002, this practice was discontinued because serious complications arose when disposable instruments were used.

The fact that the vCJD prion agent is found in lymphoid tissue and tonsils indicates that prions are not restricted to neural tissue (103). Studies of sheep naturally infected with scrapie demonstrate that the infectious agent first appears in lymphatic tissue of the tonsils and gastrointestinal tract, suggesting that the oral route may be the principal mode of transmission. In addition, numerous studies underline the importance of B cells in transmission of the BSE agent (142). Lymphatic organs typically show early accumulation of prions and B cells, and follicular dendritic cells are required for efficient neuroinvasion. The actual entry into the central nervous system probably occurs via peripheral nerves, and the prion accumulates in neural tissue once inflammation of the lymphoid tissue is in progress (116).

Experimental evidence from animal models indicates that blood can contain prion infectivity, which suggests a potential risk for transmissible spongiform encephalopathy transmission via proteins isolated from human plasma (124). Three cases of probable transmission by blood transfusion raise more concern about the safety of the blood supply (4). In the United States, beginning in August 1999, persons who resided in or traveled to the United Kingdom for a total of 6 months from 1980 through 1996 have been deferred from blood donation, as have persons who received bovine insulin derived from cattle in the United Kingdom. Recently, both the American Red Cross and the FDA announced new, expanded deferrals for travel and residence in the United Kingdom and other European countries (58) and they are conducting a look back to consider recipients who received potentially contaminated blood. The United Kingdom no longer collects plasma from its inhabitants and, as a further precautionary measure, has instituted leukocyte reduction (removal of white blood cells) for blood transfusions.

Previously, problems with reprocessing of instruments used on patients with CJD were limited to invasive instruments that came into contact with neural tissue, predominantly instruments used in neurosurgery. However, as noted above, vCJD is highly lymphotropic, so that any instruments used on lymphoid tissues may be contaminated with prions (142). As outlined above, appropriate reprocessing of surgical items includes cleaning, disinfection, and sterilization. Aldehydes enhance the resistance of prions and abolish the inactivating effect of autoclaving (48). Therefore, aldehydes are no longer recommended for disinfecting surgical instruments in Europe before they have undergone a thorough cleaning process. In France, aldehydes are no longer used for endoscope reprocessing, despite evidence that peracetic acid may stain prions as well (136). Small resistant subpopulations of infective prions may survive autoclaving at 132 to 138°C. These resistant subpopulations are not inactivated by reautoclaving, and they have biological characteristics that differentiate them from the main population (240). The worst-case scenario is that the agent for vCJD might become self-replicating when it contaminates surgical instruments. Therefore, prions challenge reprocessing techniques like never before.

The minimum requirements for decontamination procedures and precautions for materials potentially contaminated with either the agent that causes CJD or the agent that causes vCJD remain unknown. However, it is clear that dry heat (160°C for 24 h), formaldehyde sterilization, and standard steam sterilization do not sterilize prion-contaminated items (80). The scientific uncertainties and lack of data do not allow agencies like national health departments, the WHO, or the CDC to formulate guidelines that are completely evidence based, and this explains why various countries have taken different approaches to addressing issues of reprocessing of instruments. In January 2001, the British government spent the equivalent of $300 million to improve reprocessing techniques in Central Sterilization Services and required the use of disposable instruments for tonsillectomies. The French Public Health Office published their recommendations on 14 March 2001. They require all

surgical instruments with potential exposure to lymphatic tissue, the central nervous system, or the eyes to be soaked in sodium hypochlorite for 1 h or NaOH for 1 h and sterilized at 134°C for 18 min. If instruments do not tolerate this aggressive approach, they must be cleaned twice, treated with various chemicals such as peracetic acid, iodophores, 3% sodium dodecyl sulfate, or 6 M urea, and autoclaved at 121°C for 30 min. Since 2002, Switzerland requires all surgical instruments to be sterilized at 134°C for 18 min. The background of the Swiss recommendation is that the usual rendering process for carcasses, which was discontinued, resulted in only a 1-log-unit reduction of the infectious particles (241). Therefore, a reduction in the number of infectious particles may suffice to stop transmission. The CDC recommends that instruments exposed to potentially prion-contaminated items to be autoclaved for 1 to 1.5 h at 132 to 134°C, immersed in 1 N sodium hydroxide for 1 h at room temperature, or immersed in 0.5% hypochlorite sodium (at least 2% free chlorine) for 2 h at room temperature. (See the CDC website for further information: http://www.cdc.gov/ncidod/dvrd/vcjd/index.htm.) However, these recommendations are not based on what is known about the agent of vCJD.

High-risk patients are patients with suspected CJD and their family members, patients treated with pituitary extracts, and patients who received cornea transplants. In addition, items should be considered contaminated with prions if a brain biopsy for the diagnosis of CJD is requested. Instruments used in such procedures should be discarded or placed under quarantine until the histopathological diagnosis is known. The incidence of vCJD in the United Kingdom is decreasing rapidly, indicating that current reprocessing techniques suffice. However, knowledge about this topic is increasing rapidly over time and our current understanding may be shown to be false in the future (26). In May 2005, British officials published an excellent assessment of the risk for contaminating surgical instruments with prions (http://www.dh.gov.uk/assetRoot/04/11/35/42/04113542.pdf). The key observation in this report is that on average 0.2 mg of protein remains on surgical instruments despite "standard cleaning and disinfection," which is sufficient to cause an experimental case of CJD. Therefore, more research and new methods of cleaning and disinfection are needed for surgical instruments. The reader is referred to the websites of the CDC, the FDA, and the WHO to obtain the most recent updates on this topic.

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Prevention and Control of Laboratory-Acquired Infections*

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8

Laboratory biosafety describes the active, assertive, evidence-based process that laboratorians use to prevent microbial contamination, infection, or toxic reaction as they actively manipulate live microorganisms or their products, thereby protecting themselves, other laboratory workers, the public, and the environment (43). The goals of a laboratory safety program are to prevent laboratory-acquired infections in workers and to prevent accidental releases of live agents that may endanger humans, animals, and plants. Laboratory safety programs involve all aspects of the laboratory cycle, starting before the specimens or microorganisms arrive in the facility, including the processes used to handle specimens or organisms in the laboratory (e.g., the proper use of reagents, materials, and equipment and the safe storage and transport of specimens and organisms), and culminating when specimens or cultures are terminally sterilized or pathogenic microorganisms are destroyed. Laboratories must train their personnel to use safe practices and must develop methods for monitoring work practices.

EPIDEMIOLOGY

Infections are usually characterized as laboratory acquired in retrospect, and these assessments are often based on the assumption that the only likely exposure occurred while the person was in a laboratory or on the finding that a source of exposure outside the laboratory cannot be identified. Consequently, trivial laboratory events may be considered possible exposures (30, 37). However, one must appreciate that the total laboratory testing cycle begins well before the sample actually reaches the laboratory (the preanalytic phase of laboratory testing) and that personnel can be exposed while they collect and transport samples. In the past, infections acquired while personnel collected samples were considered to be laboratory acquired if the samples were collected solely for a laboratory investigation. Currently, infections acquired by phlebotomists through needle stick injuries are routinely considered to be laboratory-acquired infections (24), but infections acquired by phlebotomists when they inhale droplet nuclei (e.g., those from chicken pox) as they collect samples in patients' rooms are not included in this category (23).

Microbiology laboratories run by pioneers including Pasteur and Koch were active by 1840 to 1860. The first known laboratory-acquired infection, Mediterranean fever, was reported in 1899 (11). Sulkin and Pike conducted the first systematic study of laboratory-acquired infections when they surveyed 5,000 American laboratories in 1951 (22). Sulkin extended this study in 1961 and 1965, and Pike extended it further in 1976. In total, Sulkin and Pike cited 3,921 laboratory infections between 1930 and 1974, with a mortality of 164 (4.1%). These infections were reported from research facilities (2,307; 58.8%), diagnostic facilities (677; 17.3%), industries producing biological products (134; 3.4%), and teaching facilities (106; 2.7%); 697 (17.8%) did not have a source specified.

The results of four surveys performed in the United Kingdom between 1971 and 1991 indicated that within clinical facilities, the most infections were identified among workers in the microbiology laboratory, followed by those in the autopsy service (23). Over the 20-year period, the number of infections reported each year dropped more than 80%, from 104 to only 17. It would be tempting to conclude that this decline indicates that laboratories are becoming safer; however, there are no active monitoring or surveillance programs that capture the true number of accidents and infections. Thus, even comprehensive listings of laboratory-acquired infections are incomplete (52).

LABORATORY SAFETY AND PERSONAL ATTRIBUTES

Phillips conducted a matched-case control study of 33 laboratory workers who experienced laboratory-associated injuries over a 2-year period and found no differences in ages, lengths of employment, years of formal education, use of glasses, use of prescription medications, off-the-job accidents, or driving records for person with injuries and those without injuries (45). However, persons in the group who had accidents were significantly more likely to have had a laboratory accident or a laboratory infection before the 2-year study period and were significantly more likely to have a low opinion of laboratory safety programs. When the conditions surrounding the accidents were examined, 36% of accidents were found to occur when the employee was working too quickly, either just before lunch or at the end of the day. In 30% of accidents, the employee acknowledged breeching safety regulations. In summary, in this study attitudes and work habits were important

*This chapter contains information presented in chapter 9 by Andreas Voss and Eric Nulens in the eighth edition of this Manual.

contributing factors to laboratory accidents. Similar results linking attitudes and behaviors with poor safety practices continue to be found.

Over time, laboratorians have learned that even conventionally accepted practices can result in serious infection. Mouth pipetting, marking blood spots, transporting samples to the laboratory in corked or sheathed sharps, recapping needles, and eating or smoking in the laboratory were at one time common practices in properly run medical laboratories. All of these practices are now known to be risky and are prohibited. Other common practices, such as examining bacterial culture plates with an eyeglass or sniffing plates to help identify organisms, are now controversial (9). Thus, to maintain and improve laboratory safety, staff members must review and critique current practices diligently and be open to changing their procedures as appropriate.

RISK-BASED CLASSIFICATION OF MICROORGANISMS

The international community has developed a common risk classification scheme for microorganisms that can help laboratories determine the best laboratory practices and environmental requirements . Group 1 biological agents are unlikely to cause human disease. Group 2 biological agents can cause human disease and may be a hazard to workers but are unlikely to spread to the community; effective prophylaxis or treatment is usually available. Group 3 biological agents cause severe human disease and are a serious hazard to workers; they may spread to the community, but effective prophylaxis or treatment is usually available. Group 4 biological agents cause severe human disease and are a serious hazard to workers; they may present a high risk of spreading to the community, and effective prophylaxis or treatment is usually not available. A partial list of microorganisms by category is provided in Table 1.

Different countries usually classify organisms consistently. However, there are some organisms that are classified differently by different countries. These differences can affect a laboratory's certification, and laboratories that send agents domestically or internationally must know the local and international requirements.

Conventionally, laboratory behavior and practices have been matched to the level of risk associated with expected hazards. (See Table 2 for practices considered to be appropriate for handling of samples with group 2 agents.) Recently, microbiologists have begun to rely less on a rigid classification of organisms when designing laboratory practices and rather to mix different levels of containment and safety practices to match the risk posed by each organism. Thus, in working with a particular organism, the best laboratory practice may be to use biosafety level 2 containment with a higher level of safety practices. Moreover, laboratorians should not treat all situations as equal. Rather, they must consider biosafety to be a

TABLE 1 Risk-based classification of microorganisms[a]

Group	Bacterium(a)	Virus	Fungus(i)	Parasites
1	No clinical organisms			
2	*Bacillus* species (not *Bacillus anthracis*)	Adenovirus	*Cryptococcus* species	All clinically important parasites
	Clostridium species	Calicivirus	*Candida* species	
	Corynebacterium diphtheriae	Coronavirus (not Co-V)	All dermatophytes	
	Escherichia coli	Herpesvirus	*Aspergillus* species	
	Enterobacteriaceae	Influenza virus		
	Mycobacteria other than *Mycobacterium tuberculosis*			
	Staphylococcus species			
	Streptococcus species			
3	*Bacillus anthracis*	Lymphocytic choriomeningitis Hantaan virus	*Coccidioides immitis*	
	Brucella species	St. Louis encephalitis virus	*Blastomyces dermatitidis*	
	Coxiella burnetii	Japanese encephalitis virus	*Histoplasma capsulatum*	
	Francisella tularensis	Western equine encephalitis virus	*Paracoccidioides braziliensis*	
	Mycobacterium tuberculosis	West Nile encephalitis virus		
	Mycobacterium avium	SARS coronavirus		
		Prions		
4		Lassa virus		
		Marburg virus		
		Ebola virus		
		Herpes simiae virus		

[a] Based on reference 29a. This table is presented as a guide only. It should not be considered to be either complete or consistent with all jurisdictions. Specific national requirements may differ from the information presented in this table.

TABLE 2 Standard microbiology practices for all laboratories (biosafety level 2 and higher)[a]

1. A documented safety manual must be available for all staff.
2. Personnel must receive training on the potential hazards associated with the work involved and the necessary precautions to prevent exposure to infectious agents.
3. Eating, drinking, smoking, storing either food or personal belongings, and applying cosmetics are not permitted.
4. Mouth pipetting is prohibited.
5. Long hair is to be tied back or restrained.
6. Access to laboratory and support areas is limited to authorized personnel.
7. Doors to working areas in laboratories must not be left open.
8. Open wounds and cuts should be covered with waterproof dressings.
9. Laboratories are to be kept clean and tidy.
10. Protective laboratory clothing, properly fastened, and footwear with closed toes and heels must be worn by all personnel, visitors, trainees, and others working in the laboratory.
11. Eye and face protection must be used where there is a known or potential risk of exposure to splashes or flying objects.
12. Gloves must be worn for all procedures that may involve direct skin contact with biohazardous material. Gloves are to be removed when a laboratory task is completed and before leaving the laboratory.
13. Protective laboratory clothing must not be worn in nonlaboratory areas.
14. If known or suspected exposure occurs, contaminated clothing must be decontaminated before laundering.
15. The use of needles, syringes, and other sharp objects should be limited strictly. Needles should not be bent, sheared, recapped, or removed from the syringe; they should be promptly placed in a puncture-resistant sharps container for disposal.
16. Hands must be washed with an appropriate antiseptic soap or rubbed with an alcohol-based hand gel after gloves have been removed, before leaving the laboratory, and at any time after handling materials known or suspected to be contaminated.
17. Work surfaces must be cleaned and decontaminated with a suitable disinfectant at the end of the day and after any spill of potentially biohazardous material.
18. Contaminated materials and equipment that are removed from the laboratory for servicing or disposal must be appropriately decontaminated.
19. Autoclaves used for decontamination should be regularly monitored with biological indicators.
20. All contaminated materials must be decontaminated before disposal or reuse.
21. Leak-proof containers are to be used to transport infectious materials.
22. Spills, accidents, breaches of containment, or exposures to infectious materials must be reported immediately to the laboratory supervisor.
23. An effective rodent and insect control program must be maintained.

[a] Adapted from references 29a and 56a.

dynamic rather than a static interaction. Factors that influence risk include the specific biohazardous agents, the sample volumes, the concentrations of the agents in the samples, the most likely routes of exposure, the workload, host factors (e.g., immunosuppression or pregnancy), the complexity of the task, and the knowledge and experience of the worker.

ASSESSING RISK AND HAZARDS

To plan strategies for improving safety, the staff first must recognize risk factors, potential weak points in processes, and possible solutions. Regular laboratory audits can also help the staff recognize specific problems, and sometimes these problems can be solved by implementing or reactivating established guidelines. A failure modes and effects analysis can help the staff assess current approaches and plan improvements.

BIOSAFETY AND CLINICAL LABORATORY DESIGN

Persons designing laboratories, including the containment equipment and facilities, must know the classifications of microorganisms flowing into a laboratory. For research laboratories, where the microorganism load is known, the process of matching risk and containment is straightforward. However, in clinical laboratories, the contents of samples are usually unknown and specimens may contain microorganisms across the spectrum of classification. That being said, most isolates recovered from clinical samples are classified as biosafety level 1 or 2; thus, most clinical laboratories must be able to provide containment level 2 (Table 3). Laboratories that process viral cultures or investigate mycobacterial cultures should be designed to accommodate level 3. Laboratories that may handle exotic pathogens in risk group 4 must have high-containment level 4 facilities.

In the wake of the 2001 terrorist attacks in New York and the anthrax mail scares that happened shortly afterwards, microbiologists have become aware that laboratory biosafety measures are a component of bioterrorism defense (18, 35, 49, 53). Specific funding has been allocated for the construction of new national and regional biocontainment laboratories. Laboratories with increased levels of containment and security are useful not only for addressing possible bioterrorism and biosecurity issues but also for handling specimens from epidemics of emerging infections, such as severe acute respiratory syndrome (SARS) and pandemic influenza. These laboratories must control access to their facilities, systematize procedures for specimen receiving and disposal, develop incident reporting and emergency response plans, provide security for stored agents, and if appropriate, track specimens accurately and develop trace-back systems (40, 48, 49). Caution needs to be taken to ensure that the needs for confidentiality and information containment do not interfere with the open communications necessary for laboratory safety and public health (37).

TABLE 3 Laboratory design requirements for biosafety level 2 laboratories[a]

1. A space separated from public areas by lockable doors
2. Laboratory doors with appropriate signage
3. Door openings of sufficient size to allow safe passage of equipment
4. Work surfaces that are nonabsorptive and scratch, stain, chemical, and heat resistant
5. A means of treating waste before disposal must be readily available.
6. Windows designed to prevent ingress of flying insects
7. Separate spaces for street and laboratory clothing
8. Ready access to hand-washing sinks
9. Emergency showers and eye-washing stations
10. Biosafety cabinets and other primary containment equipment (recommended)
11. Hand-washing sinks that can be operated without hands (recommended)
12. An air supply of 100% outside air with no recirculation (recommended)

[a] Adapted from references 29a and 56a.

SAFETY EQUIPMENT AND THE CLINICAL LABORATORY

Splashguards

Splashguards, the minimum level of safety equipment, should be made of cleanable, clear glass or plastic and should be large enough to protect workers from gross splashes. Memos and procedure descriptions should not be taped onto splashguards because they prevent workers from seeing what they are doing. Splashguards can be effective barriers provided that they are appropriately placed with respect to both workflow and the workers' heights. Staff members can use splashguards as an appropriate alternative to biosafety cabinets when opening vacuum blood tubes.

Biosafety Cabinets

Biosafety cabinets can protect the laboratory worker and the laboratory environment from splashes and aerosols and also protect samples from becoming contaminated (55, 56). Class 1 cabinets have an open front and are under negative pressure with respect to the laboratory; these cabinets exhaust their air through a HEPA filter, and the exhausted air usually is returned to the work area. Class 2 cabinets increase the level of safety by including a HEPA-filtered downward-flow air curtain, which increases the degree of separation between the air in the room and the air in the cabinet. Class 2 cabinets may exhaust air back to room air (Class 2A) or through an exhaust system to the air outside the building (Class 2B). Class 2B cabinets can be further subclassified based on additional features. Class 3 cabinets are completely enclosed and provide gas-tight containment. They are accessible only through glove ports. Class 3 cabinets provide the most suitable containment for working with exotic pathogens. Because class 1 and class 2A units exhaust air into the laboratory, they should not be used to handle volatile chemicals and reagents. Because biological safety cabinets often have open heating elements or flames and may not have anticorrosive ductwork, they should not be used as alternatives to chemical fume hoods.

All safety cabinets must be tested and certified by a qualified person on a regular basis to ensure that they maintain their required face velocity and negative pressure. That being said, even properly maintained and certified cabinets can malfunction and put staff members at risk if equipment and materials are improperly placed inside the cabinet. If the cabinet is overcrowded or equipment is stacked against the front or back grill, airflow will be disrupted and contaminated air may backwash out the front of the unit.

Chemical Fume Protection

A fume hood is a mechanically ventilated, partially enclosed workspace where harmful volatile chemicals and reagents can be handled safely. The primary function of a fume hood is to contain and remove gases and vapors. Most fume hoods use ducts and a fan to capture heat and airborne chemical contaminants, transport them out of the work area, and discharge them into the atmosphere outside the building. Chemical fume hoods differ from biosafety cabinets in that they are usually ducted, must be constructed of noncombustible materials, and must be explosion proof. Nonducted, or recirculating, fume hoods cannot be used in the laboratory to contain volatile chemicals (17). Laboratories that work with specific highly corrosive reagents or chemicals such as perchloric acid or with radioisotopes need fume hoods designed to accommodate these agents.

Fume hoods must be placed in rooms with sufficient airflow that is properly balanced so that the air entering the fume hood is replaced and backwash into the room is prevented (17). Chemical fume hoods should be tested regularly for face velocity and for containment with the ASHRAE 110 tracer gas test. Face velocity alone is not a valid indication of containment (17).

Centrifuges

The safety centrifuge was first described in 1975 (28). However, accidental contamination of laboratories and personnel continues to occur because staff members do not use centrifuges properly (19, 27). Rotors can become contaminated if plastic centrifuge tubes crack or distort while being spun (27). If the O-ring seals on containers and rotors do not create a good seal, organisms can be aerosolized (29). Thus, centrifuges must be maintained properly (2) and must be used properly. Laboratories should maintain a log for each centrifuge that includes the rotor serial number, the speed in revolutions per minute, the spin duration, the time of use, and the operator's name for each use (2). Basic safety procedures for centrifuges include having workers (i) keep a centrifuge's lid closed while the centrifuge is operating, (ii) stay with a centrifuge until the full operating speed is attained and the machine is running without vibrating, (iii) stop immediately and check the load balances if the centrifuge vibrates, (iv) check swing-out buckets for clearance and support, (v) use a noncorrosive product to clean and disinfect rotors and cups after each use, (vi) report all spills and breakage to the laboratory safety officer, and (vii) clean spills as soon as aerosols have settled.

Sharps Protection

Scalpels, needles, broken glass, and other sharps commonly cause injuries and laboratory-2 acquired infections (7). To the extent possible, staff members either should not use sharps by hand or should use safety barriers (25). Because sharps may be contaminated with infectious or cytotoxic agents or both, they should be discarded in sharps containers.

Sharps containers minimize injuries and transmission of potentially harmful agents if they are readily accessible and appropriately used. Sharps containers used in medical laboratories should be designed specifically for sharps such as needles, syringes with needles, blades, clinical glass, and other items capable of causing cuts or punctures (25). Sharps containers should be leak proof and puncture resistant and should not degrade in autoclaves, either require no assembly or be easy to assemble, have a designated fill line, be appropriately labeled, and be available in a variety of sizes. Within this framework, manufacturers can implement a variety of designs. Sharps containers should resist toppling over and should be durable enough to withstand being dropped onto a hard surface (6). Containers that are not resistant to penetration or compression put staff members at risk. Tin cans or other containers should not be used in lieu of containers designed specifically for sharps.

Sharps containers must be labeled prominently with the universal biohazard symbol. In addition, sharps containers for sharps contaminated with cytotoxics must display the cytotoxic hazard symbol. The international color code is yellow for biohazardous medical sharps and red for cytotoxic medical waste including contaminated sharps.

Staff members should never force sharps into a container, and they should not fill containers to more than three-quarters of their maximum capacity to avoid accidents from overfilling. Once filled, sharps containers should be securely and irreversibly closed for containment. The containers should be sterilized in an autoclave and then disposed of in accordance with local requirements. Containers with sharps contaminated with cytotoxic or prion proteins must be incinerated.

Most sharps containers are designed for single use only. However, some commercial units that can be reused are now available. These containers must be transported to a central disposal unit where they can be opened safely, emptied, decontaminated, and redistributed.

REQUIREMENTS OF THE INTERNATIONAL ORGANIZATION FOR STANDARDIZATION

The International Organization for Standardization Technical Committee 212 has developed documents that medical laboratories should use to improve their performance, including ISO 15189:2003, *Medical Laboratories—Particular Requirements for Quality and Competence* (32c), and ISO 15190:2003, *Medical Laboratories—Requirements for Safety* (32d). In those countries where laboratories are certified or accredited according to International Organization for Standardization requirements, these documents are considered essential standards.

ISO 15190:2003 states that the laboratory's management is responsible for the safety of all employees and visitors to the laboratory and that ultimate responsibility rests with the laboratory director. The laboratory must identify an appropriately trained and experienced laboratory safety officer to assist the laboratory director and managers with safety issues. The laboratory safety officer must have the authority to stop activities that are deemed unsafe. The laboratory safety officer is responsible for designing and maintaining the laboratory safety program and for monitoring its effectiveness. The International Organization for Standardization requires that a laboratory safety program include a laboratory safety manual, safety audits (see Table 4 for audits required by ISO 15190:2003), inspections, risk assessments, and safety records. For further details, ISO 15190:2003 is available through the International Organization for Standardization website (www.iso.ch) or the Clinical and Laboratory Standards Institute (www.clsi.org).

TABLE 4 Laboratory safety audits required by ISO 15190:2003 (32d)

Health and safety policy
Written procedures that include safe work practices
Safety-oriented education and training of staff
Safety-oriented supervision
Use and maintenance of hazardous materials and substances
Health surveillance
First-aid equipment and services
Accident and illness investigations
Health and safety committee review
Records and statistics on accidents and near misses
Review of safety program
Regular site safety inspections

SAFETY PREPAREDNESS AND THE MATERIAL SAFETY DATA SHEET

Every laboratory should have a readily accessible written plan that staff members can use in case of emergencies or accidents. Laboratories can use published guidelines (25) as they prepare their own plan. In addition, up-to-date Material Safety Data Sheets (MSDS) for all chemicals and microorganisms; equipment and materials for containment of spills, including personal protective equipment, absorbent, and disinfectant (bleach or accelerated hydrogen peroxide); and personnel trained in first aid should be readily available, and their locations should be known by all staff members. Moreover, staff members should know the location of emergency equipment, including showers and eye-washing stations; should know routes for evacuation in case of a fire or a spill; and should practice evacuating regularly. Showers and eye-washing stations should be tested regularly to ensure that they will function when needed. A regular internal safety audit program should assess whether staff members are prepared to deal with accidents.

MSDS for chemicals should be obtained from the supplier, or may be available from a variety of commercial and free Internet sites. A list of MSDS for microorganisms is available through the Public Health Agency of Canada website (http://www.phac-aspc.gc.ca/msds-ftss/).

It is beyond the scope of this chapter to address the specifics of the medical management following accidents that involve infectious agents. That being said, laboratories should follow several steps if exposures occur. Staff members should (i) report every accident or injury, including those that are seemingly trivial (30), to the appropriate safety officer or supervisor; (ii) clean scratches and puncture wounds immediately; (iii) seek medical attention quickly, especially if it is recommended by first-aid attenders or occupational health advisors (5); and (iv) give the microorganism's MSDS to the clinician evaluating the injury if the likely or probable

agent is known. In addition, staff members should investigate each incident to learn the details of the accident and to identify its root causes and recommend preventive measures. Subsequently, staff members should audit compliance with the recommendations to ensure that they have been incorporated into practice in a timely manner.

HAND HYGIENE AND THE USE OF PERSONAL PROTECTIVE EQUIPMENT

Hand Hygiene

Hand washing and hand antisepsis are the most useful techniques for preventing transmission of microorganisms and for protecting staff from laboratory-acquired infections (14). Hands can become contaminated when staff members collect samples, handle sample containers, handle contaminated equipment, or touch sample storage units.

Nonmedicated detergent-based soap products and water do not disrupt normal skin flora but can reduce the number of transient hand flora, including both bacteria and viruses (14, 54). The efficacy of hand washing with such products is directly related to the duration of the procedure. Plain soaps can dry and irritate skin. Plain soaps may be appropriate in public washrooms but are not appropriate in the clinical laboratory. A variety of antimicrobial products are available, including products containing chlorhexidine, triclosan, iodophors, and others. Staff members should consider issues such as the types of organisms processed by the laboratory and also characteristics of each product (e.g., the fragrance, consistency, and propensity to irritate and dry skin) when selecting products for use in the laboratory.

Waterless, alcohol-based hand hygiene products can be rapid and convenient alternatives to hand washing with water and antimicrobial soap products, especially when a sink with running water is not immediately accessible (38, 54). The efficacy of alcohol-based products may be reduced when staff members' hands are soiled or are contaminated with spore-forming organisms or nonenveloped viruses (54). Staff members should wash their hands with an antimicrobial soap and with water when their hands are visibly soiled or are contaminated with proteinaceous materials or with materials that have a high microbial load.

Gloves

Gloves can be an important barrier within the laboratory, provided that they are used appropriately (25). Clearly, gloves prevent damage when hands are exposed to heat, cold, and toxic materials. For example, staff members should use insulated gloves when they take materials out of −70°C freezers, when they work with liquid nitrogen, and when they remove materials from autoclaves. General purpose utility gloves (kitchen or rubber gloves) provide ample protection for cleaning of biological spills and for decontamination. Utility gloves can be cleaned and reused. They should be examined regularly for cracks, tears, and peeling, and damaged utility gloves should be discarded. Staff members handling chemical solvents, toxic chemicals, and dyes should not use utility gloves but rather should use chemical-resistant gloves that should be available in all laboratories that handle chemical solvents. Disposable gloves of latex, vinyl, or nitrile can effectively reduce exposure risk, especially for handling blood, body fluids, and excrement. These gloves are particularly important because staff members may be unaware of the abrasions on their hands (36). In specific settings, staff members may need gloves that are elbow or shoulder length.

Disposable gloves do not prevent needle stick injuries, but double gloving can reduce the volume of blood or body fluids carried by a needle. Cotton undergloves may provide more protection from sharps injuries than a second vinyl or latex glove. In the morgue, staff members may need to wear chain mail gloves. Gloves provide an important protective barrier; however, they may also be a source of harm. Vinyl, latex, or cotton undergloves can reduce contact irritation that some staff members note when using rubber gloves. If staff members wear gloves for long periods, moisture can damage the skin on their hands (16, 20, 47). Surveys of dentists who wear gloves for 6 h daily indicate that many, especially young women with preexisting eczema, develop glove intolerance including glove-related mucous membrane irritation such as conjunctivitis, rhinitis, and asthma (57).

Gloves are easily torn. In-use durability studies indicate that vinyl gloves may tear as often as 40% of the times they are used, depending upon the presence of powder and the length of the user's fingernails (36). Latex gloves are more durable but may cause atopic reactions.

Staff members must remove their gloves at the end of a task or when the task is interrupted to prevent environmental contamination and transmission of pathogenic organisms. Indeed, persons wearing contaminated gloves can contaminate the equipment (42) and can also transmit organisms that cause serious infections (46). Moreover, gloves reduce microbial contamination of the hands but do not prevent it entirely. Therefore, staff members must still use hand hygiene when they remove gloves. Phlebotomists often wear gloves while collecting specimens, but this may not be essential if the risk of exposure to blood and body fluids is sufficiently low or if gloves of an appropriate size or material are not readily available. Regardless of whether phlebotomists do or do not wear gloves while collecting specimens, they should always remove their gloves between patients and wash their hands or use an alcohol-based hand gel.

IMMUNIZATION

Mental alertness and good laboratory practices are the most important aspects of laboratory safety, but immunizations are also an important source of protection. Immunizations may not prevent infections, but usually they protect persons against serious illness. All laboratory staff, including pregnant women, should have a complete primary immunization with tetanus and diphtheria toxoids and should receive a booster every 10 years (4). Laboratory workers who have direct contact with patients and those with chronic pulmonary or cardiovascular disease or other chronic illnesses including diabetes mellitus and renal dysfunction or who are immunocompromised should receive the influenza immunization annually. All staff members with possible occupational exposure to human blood and body fluids should receive the hepatitis B vaccine.

Cases of meningococcal illness possibly linked to laboratory exposure have been identified (3, 13, 21). Thus, microbiologists who are routinely exposed to meningococci, especially if these organisms may be aerosolized, should consider receiving meningococcal immunization. Laboratorians working with specific pathogens and in specific situations should consider additional immunizations such as a human diploid cell rabies vaccine, typhoid vaccine, and vaccinia vaccine (39, 41). In the past, *Mycobacterium bovis* BCG vaccination was considered of value for health care workers; however, it is no longer recommended as a primary strategy for controlling tuberculosis because the protective efficacy of the vaccine in health

care workers is uncertain (8). Prior immunization with BCG may cause difficulty in the interpretation of tuberculin skin test responses following true infection with *Mycobacterium tuberculosis* (15).

LABORATORY-ACQUIRED HIV INFECTION, HEPATITIS B, AND HEPATITIS C

Laboratory workers are at risk for exposure to hepatitis C virus (HCV), hepatitis B virus (HBV), and human immunodeficiency virus (HIV). However, safeguards introduced into medical laboratories have decreased the risk. For example, after the hepatitis B vaccine was introduced in 1982, the incidence of hepatitis B infection was decreased by more than 95% (25).

According to the Division of Health Care Quality Promotion of the Centers for Disease Control and Prevention (http://www.cdc.gov/ncidod/hip/BLOOD/hivpersonnel.htm), between 1978 and December 2001, only 16 clinical laboratory workers acquired HIV infection occupationally; 17 other persons may have acquired their infections in laboratories. We have no information on the prevalence of hepatitis C in health care workers. However, the National Center for Infectious Diseases estimates that about 2 health care workers out of 100 (1.8%) will become infected with HCV (range, 0 to 10%) after injuries with needles or sharps contaminated with HCV-positive blood (http://www.cdc.gov/ncidod/diseases/hepatitis/c/faq.htm#1h).

Given the publicity about bloodborne pathogens, laboratory directors may assume that their employees understand the epidemiology of these organisms and will take steps to protect themselves. However, recent surveys of health care workers demonstrate that existing and new staff members need to be educated frequently (51).

LABORATORY-ACQUIRED PARASITIC INFECTIONS

In her extensive review, Herwaldt has described 200 cases of laboratory-acquired parasitic infections occurring between 1929 and 1999 (30). Although the distribution of infection-associated pathogens changed from decade to decade, the number of cases identified in each decade (19 to 28) remained relatively constant. Sharps (needle and glass) injuries were common factors, often occurring when workers manipulated research animals or produced blood smears for malaria.

PRIONS

Samples from patients with Creutzfeldt-Jakob disease (CJD) may be submitted to the laboratory for investigation. To date there are no known cases of laboratory-acquired CJD and there is no evidence that laboratorians are at increased risk of developing CJD. That being said, staff members who handle samples should wear gloves and should discard samples as medical waste. No special precautions are required for disposal of body fluids (50).

Prions are extremely difficult to inactivate. Therefore, equipment that has been exposed to tissues, especially those of neurological origin, from patients with CJD should either be disposed of if they do not tolerate heat or autoclaved at 134°C for 18 min (prevacuum sterilizer) or at 121 to 132°C for 1 h (gravity displacement sterilizer). Autoclaving in water may be more effective than autoclaving in its absence (26). Equipment may also be soaked in 1N NaOH for 1 h (26, 34). Work surfaces that may have been contaminated by the tissue should be cleaned with a 1:10 dilution of sodium hypochlorite. Other chemical treatments that have been described as milder alternatives include a combination of proteinase K and sodium dodecyl sulfate (34), proteinase K followed by an alkaline cleaner, and proteinase K followed by treatment in vaporized hydrogen peroxide (26).

Variant CJD is unique among human prion disease because the prion protein accumulates in follicular dentritic cells of lymphoid tissue (31, 33). In the United kingdom, studies of lymphoid tissue have identified the presence of prion protein (PrP) in appendices and tonsils (32). This has led to concern of possible contamination of blood, transplant tissues, and surgical instruments. The presence of long-term asymptomatic carriage and the absence of readily available diagnostic tests and a definitive significance of the findings make it difficult to implement new specific policies and procedures for processing routine samples and cleaning surgical and pathology equipment.

SARS CORONAVIRUS

The outbreak of SARS caused by a coronavirus raised international concern among the infection control and laboratory communities about the hazards of aerosol spread of communicable viruses. Information collected during the epidemic demonstrated that staff members in microbiology laboratories and those who receive and accession respiratory samples, especially via vacuum tube delivery systems, may have been at risk (44). However, the virus could be readily contained (10). In fact, laboratory workers had one of the lowest rates of illness or serological conversion and only one death was suspected to result from a laboratory accident, which occurred in a level 4 facility (44).

To ensure that laboratory workers are safe while working with the SARS coronavirus, guidelines developed in Singapore recommend that staff members in laboratories equipped and designed at biosafety level 2 use practices more consistent with biosafety level 3, such as opening specimen containers and handling the samples only in biosafety cabinets (10). In addition, samples should be centrifuged only in sealed safety buckets that are opened within a cabinet. Finally, N95 respirators should be considered for additional protection.

LABORATORY-ACQUIRED INFECTIONS AND EXTERNAL QUALITY ASSESSMENT

Infections acquired in clinical laboratories are not always associated with clinical samples. Documented clusters of bacterial infections have been associated with samples sent to laboratories for proficiency testing (12), and quality control organisms have contaminated other samples in laboratories (1). Regardless of the source of the specimens, staff members in clinical microbiology laboratories must use appropriate biosafety practices when handling any and all viable microorganisms.

SAFETY AND POINT OF CARE

Medical laboratories are responsible for all laboratory tests, even those performed outside of the laboratory itself. Increasingly, medical laboratories are responsible for point-of-care testing for monitoring of blood glucose, coagulation, and oxygenation. Despite the improved equipment and hygiene protocols, patients continue to acquire hepatitis B and C from

TABLE 5 Electronic information sources and resources

Organization or Publication	Abbreviation	Website
Occupational Safety and Health Administration	OSHA	http://www.osha.gov
Clinical Laboratory Improvement Act	CLIA	http://www.cms.hhs.gov/clia
Code of *Federal Register*	CFR	http://www.gpoaccess.gov/cfr/
Department of Transportation	DOT	http://www.dot.gov
Nonregulatory International Organization for Standardization	ISO	http://www.iso.org
Clinical and Laboratory Standards Institute	CLSI	http://www.clsi.org
American Biosafety Association	ABSA	http://www.absa.org
International Air Transport Association	IATA	http://www.iata.org
Public Health Agency of Canada's MSDS "Infectious Substances"	PHAC	http://www.phac-aspc.gc.ca/msds-ftss/msds12e.html

contaminated point-of-care devices. To prevent transmission of bloodborne pathogens when staff members use these devices, they must adhere strictly to infection control protocols for use and cleaning of this equipment. The International Organization for Standardization final draft international standard ISO/FDIS 22870, *Point-of-Care Testing (POCT) Requirements for Quality and Competence* (32b), provides guidance on quality management for point-of-care testing.

TRANSPORTATION OF SAMPLES

Laboratories are responsible to prevent, to the extent possible, people from being exposed to infectious agents that are present in laboratory samples, including samples being transported to and from the laboratory. The laboratory should ensure that leak-proof containers are available for transporting specimens within a clinic or hospital and that the containers are transported in a secure outer package such as a sealable plastic bag, preferably emblazoned with the international biohazard label. Samples that are transported outside of the facility should be packaged in a secure, firm outer container. The sample container itself should be surrounded with materials that protect it and that also could absorb a spill if the container was damaged. Specimens transported outside the facility, especially by road, rail, water, or air, must be packaged and shipped according to local and federal regulations. Most jurisdictions prohibit transporting samples of infectious agents through the postal service.

Air transport of microorganisms is governed by federal regulations that must comply with the requirements of the International Civil Aviation Organization (ICAO) as adopted by the International Air Transport Association. The ICAO specifies the requirements for packaging and labeling, including the proper shipping name and appropriate UN number, for samples known to contain infectious agents based on the sources of the samples and the likely pathogens contained. Every laboratory that transports samples is required to have at least one person who is certified in knowing the requirements for packaging and transport, including how to complete the shipping documents.

For additional information, staff members can refer to the ICAO's *Technical Instructions for the Safe Transport of Dangerous Goods by Air* (32a) or to federal requirements (http://hazmat.dot.gov/regs/rules.htm).

ELECTRONIC INFORMATION SOURCES AND RESOURCES

Laboratory safety is a primary focus of interest for many organizations. Some of the agencies have the authority to regulate laboratory practices in their specific nation or area of jurisdiction. Nonregulatory sites, including international organizations, can provide instructive materials that aid laboratory staff in improving their safety programs. Table 5 contains a list of sites that provide important current safety information.

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Laboratory Detection of Potential Agents of Bioterrorism*

ROSEMARY HUMES AND JAMES W. SNYDER

9

Since the events of 11 September 2001 and the anthrax release in October 2001, bioterrorism preparedness and response have remained a high priority for the nation. Clinical microbiology laboratories serve as sentinels with the major responsibility to raise suspicion of a possible bioterrorism-associated agent based on the information gleaned from the recovery of a suspicious microbial agent. Consequently, clinical microbiologists must be familiar, through education and training, with the targeted agents of bioterrorism and the application of standardized diagnostic procedures designed to rule out or refer such agents for confirmatory testing. Clinical and public health laboratories must enhance their partnerships and communication to ensure that potential bioterrorism events are detected early. This chapter provides an overview of the most important issues. Other American Society for Microbiology (ASM) publications on this subject include references 15 and 21, the second edition of the *Clinical Microbiology Procedures Handbook* (18), and the ASM website (http://www.asm.org). The Centers for Disease Control and Prevention (CDC) also provides extensive information on bioterrorism preparedness and the agents of concern on its website (http://www.bt.cdc.gov).

HISTORY

There is evidence of germ warfare dating back to the ancient Greeks and Romans, long before individuals understood the germ theory of disease or deliberately tried to produce biological weapons of mass destruction. The U.S. Army and Air Force had an offensive biological weapons research program at Fort Detrick, Md., from 1942 to 1969. The Biological and Toxic Weapon Convention in 1972 resulted in an agreement that development of biological weapons should be stopped worldwide (9, 28). Most Western governments ceased their offensive programs; however, the former Soviet Union activated a clandestine program that was called Biopreparat. Following his defection from the Soviet Union in 1992, Ken Alibeck, Deputy Director of Biopreparat, revealed important details about the program's development of a variety of bioterrorist agents (1, 11).

Immediately following the Gulf War of 1991, the United Nations' inspectors in Iraq found evidence that Saddam Hussein had been building a bioweapons program, and they destroyed all biologic material that was found. Concerned that Iraq had reestablished a program to create weapons of mass destruction, the U.S. government developed extensive smallpox response plans and vaccinated health care workers, first responders, and soldiers in 2003, before the second Gulf War (2). However, UN inspectors did not find any evidence of an active bioweapons program when the regime fell.

Prior to 2001, the most well-known example of bioterrorism in the United States occurred in 1984, when followers of Bhagwan Shree Rajneesh sprayed an aerosolized form of *Salmonella enterica* serovar Typhimurium on salad bars at two local restaurants in Dalles, Oreg., to prevent local citizens from voting in an upcoming election. The resulting outbreak affected 750 people (35).

The threat posed by bioterrorism and the panic caused by such events were demonstrated in the fall of 2001, when 23 cases of anthrax (12 cases of inhalational anthrax and 7 confirmed and 4 suspected cases of cutaneous anthrax) occurred among persons in the District of Columbia, Florida, New Jersey, New York, and Connecticut after an unidentified perpetrator mailed *Bacillus anthracis* spores in letters (5–7, 19, 20, 23, 24, 36). Law enforcement personnel, public health officials, and community health care providers, including clinical laboratories, were inundated by citizens who found powdery substances, letters, packages, and other materials which they were certain contained anthrax spores. More than 125,000 samples were tested, many in areas where there was no evidence of anthrax contamination (27). This outbreak and its aftermath demonstrated that federal, state, community, and institutional bioterrorism readiness plans needed to be updated. Clinical microbiologists have an essential role in these plans; they will likely be the first to detect the etiologic agent(s) and must promptly report their results to the proper authorities. Consequently, clinical microbiologists should have a general understanding of biological terrorism, including the technical and administrative principles that will help them recognize and manage such an event.

GENERAL FEATURES OF BIOTERRORISM

Definitions

Biological terrorism (bioterrorism) is defined as "the intentional use of microorganisms or toxins derived from living organisms to produce death or disease in humans, animals,

* This chapter contains information presented in chapter 10 by James W. Snyder and Alice S. Weissfeld in the eighth edition of this Manual.

or plants." From the public and private health perspective, bioterrorism can be defined as the deliberate release of pathogens or toxins into a civilian population with the intent to cause illness or death (17, 19, 21). Humans, agricultural animals, and plants could be targets of bioterrorism (17, 39).

Biological warfare refers to using biological agents (i.e., microbial pathogens and/or toxins) indiscriminately against masses of people including either military personnel or civilians. The terms biocrime or biothreat are used when a bioterrorist, which may be an individual or a state- or nonstate-sponsored group, targets a specific group or individual. A biocrime is a criminal act in which biological agents are used as weapons, and a biothreat is characterized as a suspected but unconfirmed release of a biological agent(s) (15). The bioterrorism-associated cases of anthrax that occurred in the United States in October and November 2001 are examples of a biocrime.

Bioterrorism events are categorized as either overt or covert. A covert event or attack will not be recognized immediately because of the delay between exposure and onset of illness (i.e., the incubation period). For example, the case of inhalational anthrax in the journalist from Florida in 2001 represented a *covert* event because the etiology was unknown until gram-positive bacilli were seen in the patient's cerebral spinal fluid, but the subsequent anthrax cases were *overt* events because the contaminated letters contained a note announcing the threat. As exemplified in the Florida case, emergency medicine physicians and other primary health care providers, including clinical microbiologists, will most likely identify the first cases in a covert event (8).

Characteristics Suggesting Biological Attacks

Covert attacks pose the greatest challenge to early detection; thus, microbiologists should be aware of the epidemiological and microbiological clues that suggest that an act of bioterrorism or a biocrime has occurred (21; U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID): Biological Warfare and Terrorism Medical Issues and Response Satellite Broadcast, 26 to 28 September 2000). Some key indicators are as follows:

- A single case of a disease is caused by an uncommon agent (e.g., *Burkholderia mallei*, *Burkholderia pseudomallei,* or hemorrhagic fever virus)
- Large numbers of people have unexplained diseases or die unexpectedly
- A disease does not occur naturally in a given geographic area
- The illness is unusual (or atypical) for a given population or age group, or presents in an unusual fashion
- The incidence of a disease (e.g., tularemia or plague) increases substantially above its stable endemic rate
- Unusual deaths or illness among animals precedes or accompanies illness or death in humans
- Isolates from distinct sources at different times or locations have a similar genetic type
- The agent is transmitted in an atypical manner through aerosols, food, or water, suggesting deliberate sabotage

CATEGORIZATION OF BIOLOGICAL AGENTS

Biological agents are classified as pathogens or toxins. The CDC and its affiliated partners developed a targeted (critical) agent list (Table 1) based on (i) the agent's ability to cause mass casualties, be widely disseminated, and be transmitted from person to person and (ii) the public's likely perceptions of a potential intentional release of particular agents. Although few individuals or terrorist groups possess the scientific and

TABLE 1 Critical biological agent categories for public health preparedness

Biological agent(s)	Disease
Category A[a]	
Bacillus anthracis	Anthrax
Clostridium botulinum (botulinum toxins)	Botulism
Francisella tularensis	Tularemia
Yersinia pestis	Plague
Variola major	Smallpox
Filoviruses and Arenaviruses (e.g., Ebola virus, Lassa virus)	Viral hemorrhagic fevers
Category B[b]	
Coxiella burnetii	Q fever
Brucella spp.	Brucellosis
Burkholderia mallei	Glanders
Burkholderia pseudomallei	Melioidosis
Alphaviruses (VEE, EEE, WEE)[c]	Viral encephalitis
Rickettsia prowazekii	Epidemic typhus
Toxins (e.g., ricin, staphylococcal enterotoxin B, *Clostridium perfringens* epsilon toxin)	Toxic syndromes
Chlamydia psittaci	Psittacosis
Food safety threats (e.g., *Salmonella* spp., *Escherichia coli* O157:H7, *Shigella* spp.)	Gastroenteritis, hemolytic uremic syndrome
Water safety threats (e.g., *Vibrio cholerae*, *Cryptosporidium parvum*)	Gastroenteritis
Category C	
Emerging pathogens and potential risks for the future	
Emerging threat agents (e.g., Nipah virus, hantavirus)	

[a] Agents that pose the greatest threat due to their infectiousness relative, ease of transmission, or high mortality.
[b] Agents having a moderate ease of transmission and morbidity with a low rate of mortality.
[c] VEE, Venezuelan equine virus; EEE, eastern equine encephalomyelitis virus; WEE, western equine encephalomyelitis virus.

technical resources needed to weaponize and successfully disperse an agent via aerosol (the most likely mode of dispersal), the targeted agents possess unique advantages that make them suitable for bioterrorism or biocrimes.

The CDC has defined biothreat levels (A to C) to categorize the critical agents on the basis of their threat to national security, their ability to cause morbidity and mortality, and their potential for genetic engineering. Category A organisms are considered to pose the greatest threat to national security because they (i) can be easily disseminated or transmitted from person to person; (ii) cause high mortality; (iii) may afflict large numbers of people, overwhelming the health care and public health systems; (iv) may cause public panic and social disruption; and (v) require special preparations such as enhanced surveillance, enhanced diagnostic capabilities in microbiology laboratories, and stockpiles of medicines and equipment to protect the public's health. Category B agents are moderately easy to disseminate, cause moderate morbidity and low mortality, and challenge the national capacity for surveillance and diagnosis. This category includes pathogens that are foodborne or waterborne (e.g., *Salmonella* species, *Shigella dysenteriae, Escherichia coli* O157:H7, *Vibrio cholerae,* and *Cryptosporidium parvum*). Category C agents are primarily emerging pathogens that could be engineered for mass dissemination in the future (8, 25, 30).

PREPAREDNESS AND RESPONSE TO BIOTERRORISM

The U.S. Department of Homeland Security (DHS) was created in 2002 to prevent and deter terrorist attacks, protect citizens from threats and hazards, and respond to possible attacks. As defined in the 2003 Homeland Security Presidential Directive/HSPD-5, DHS serves as the lead agency in managing and coordinating the national response to acts of terrorism, natural disasters, or other emergencies (http://www.dhs.gov, www.fema.gov/pdf/reg-ii/hspd_5.pdf). The Federal Emergency Management Agency (FEMA), responsible for planning for and responding to disasters and for helping communities mitigate and recover from catastrophes, became part of DHS in March 2003.

A National Response Plan (NRP), National Incident Management System (NIMS), and Interim National Preparedness Goal (NPG) have been developed to provide standards for planning and response. Bioterrorism preparedness has been integrated into all-hazards—chemical, biologic, radiologic, nuclear, explosive (CBRNE)—emergency response planning. Several Homeland Security Presidential Directives establish roles for other federal agencies, including the Department of Health and Human Services (HHS) and the Environmental Protection Agency, in prevention of and response to CBRNE. The Federal Bureau of Investigation (FBI) is the federal agency that leads criminal investigations.

The Department of Defense has created over 32 "weapons of mass destruction civil support teams" (WMD-CST) that help local and state authorities respond to CBRNE domestic incidents of terrorism. The WMD-CSTs identify agents and substances, assess consequences, advise local and state authorities about how to respond, and assist with requests for additional military support. Additional CST teams are being added to provide coverage in every state (http://www.globalsecurity.org/military/agency/army/wmd-cst.htm; http://www.defenselink.mil/news/Jan2004/n01202004_200401201.html).

Professional organizations for the first responder and medical communities have developed numerous training programs and online resources. The Association of Professionals in Infection Control and Epidemiology and the CDC developed a Bioterrorism Preparedness and Response Program for hospitals to use in creating institutional plans for disease surveillance, epidemiologic investigation, rapid laboratory diagnosis, communication, and assessing readiness (12; http://www.apic.org/Content/NavigationMenu/PracticeGuidance/Topics/Bioterrorism/APIC_BTWG_BTRSugg.pdf). In addition, ASM has included on its website a template that clinical microbiology laboratories can use to create a bioterrorism response plan.

Since 2000, HHS, through the CDC, has provided funding to state and city health departments to enhance local emergency preparedness. Public health laboratories have received funding to rebuild infrastructure and to improve their molecular detection capabilities and the biosafety and biosecurity in existing facilities (38). The CDC has also established a strategic national stockpile of supplies necessary for treating persons affected by bioemergencies, including therapeutics (e.g., chemical antidotes, antimicrobical agents, antitoxins, life support medications, and supplies and equipment for intravenous fluids, airway maintenance, and surgical procedures), vaccines, and personal protective equipment. Since 2002, the Health Resources and Services Administration (HRSA) has awarded funds that hospitals and communities have used to improve their capacity to care for mass casualties. Funds from CDC and HRSA have been used to provide training and reference materials for clinical sentinel laboratories and to strengthen communication and partnerships between clinical and public health laboratories (APHL Public Health Laboratory Issues in Brief: Bioterrorism Capacity; http://www.aphl.org/docs/bt_issue_brief_2005_final.pdf).

LRN

In 1999, the CDC, in collaboration with the Association of Public Health Laboratories (APHL) and the FBI, established a model in which clinical and public health laboratories would be linked and integrated into a network called the Laboratory Response Network (LRN). The goal of the LRN is to facilitate rapid detection and confirmatory testing of suspected agents of bioterrorism. As shown in Fig. 1, all laboratories have a defined role and level of responsibility (8, 14, 21, 26). Although the LRN was initially structured to test only

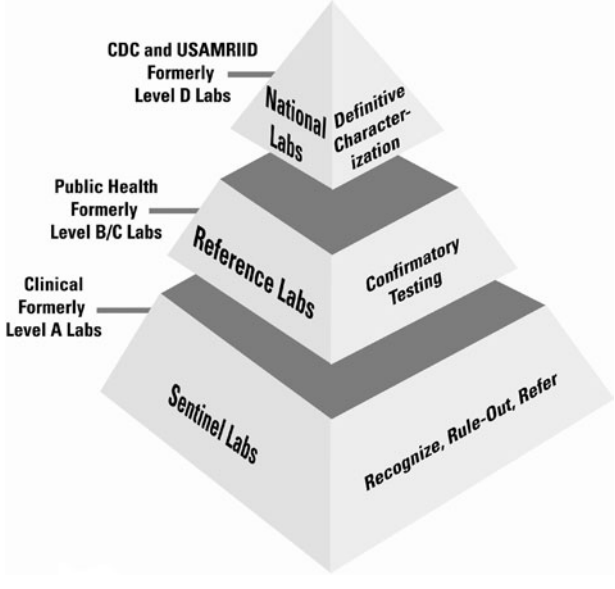


FIGURE 1 Laboratory Response Network (LRN).

clinical specimens for biologic agents, during the anthrax events of 2001, the LRN performed more than one million tests on clinical and environmental samples.

Most hospital and commercial reference laboratories have been classified as sentinel (formerly level A) laboratories. The primary role of sentinel laboratories is to "raise suspicion" when rule-out testing indicates that a targeted agent may be present in a clinical sample and to promptly refer suspicious isolates and specimens to an LRN reference laboratory for confirmatory testing (26, 34). For example, the microbiologists who initially isolated *B. anthracis* from the journalist in Florida were suspicious that the organism was the etiologic agent for anthrax but they could not confirm the species identification or definitively prove that this organism did not cause the infection. Thus, they referred the isolate to the Florida State Department of Health Laboratory, which confirmed the isolate to be *B. anthracis*. Sentinel laboratories are restricted to testing human specimens only and *must* forward or refer all other samples, e.g., environmental and animal, to a reference level (formerly level B/C; also known as confirmatory laboratories) laboratory (31, 34).

The nationwide network of LRN reference laboratories comprises local, state, and federal public health laboratories; food testing, veterinary diagnostic, and environmental testing laboratories affiliated with state and local public health departments and federal agencies; laboratories on military installations; and selected international laboratories. In general, these laboratories have biosafety level 3 (BSL-3) facilities, federally approved equipment, and standardized validated reagents for clinical and environmental testing so that they can respond to biological terrorism and other public health emergencies. Reference laboratories have established functional linkages with law enforcement, including the FBI, and employ chain-of-custody and LRN-approved testing protocols that are consistent with legal evidentiary requirements (4). Reference level laboratories perform rapid tests, such as PCR and fluorescent-antibody tests, to presumptively identify microorganisms or toxins; biochemical tests to confirm the identification of suspect isolates; and antimicrobial susceptibility tests of these isolates. National (formerly level D) laboratories, located at the CDC and USAMRIID, perform definitive characterization of biologic agents and have BSL-4 facilities in which highly infectious agents can be handled safely.

The success of the LRN depends on effective coordination and communication among all the laboratories in the network. Public health and clinical laboratories must establish working relationships in advance so that they can develop integrated response plans and effective lines of communication, exchange and share information, and test the system through exercises and drills. As any biothreat or bioterrorism event unfolds, information will flow from the CDC to public health laboratories, which, in turn, should transmit the information to the sentinel laboratories quickly.

Sentinel Lab Preparedness

To be successful in their role, sentinel laboratory personnel, including the laboratory director, must be aware that they might recover agents of bioterrorism from clinical samples. In addition, they must work with infection control staff and administrative personnel to develop both laboratory and institution-wide response plans. These plans should include (i) information regarding access to the LRN and use of the recommended diagnostic testing protocols; (ii) safety guidelines; (iii) protocols for internal and external communication and notification; (iv) criteria for packaging and transporting infectious substances safely; (v) measures to increase laboratory security; and (vi) telephone numbers for the local and state public health laboratories and the CDC (21, 34). A template for a laboratory response plan is available on the ASM website. If a bioterrorism event exposes a large number of people, clinical and public health laboratories will be overwhelmed with specimens. Thus, laboratory response plans should consider surge capacity needs for personnel and supplies (32).

Because most likely agents of bioterrorism are encountered rarely in clinical settings, laboratory personnel should be trained to recognize the targeted agents and apply the sentinel laboratory standardized testing protocols for ruling out these agents and for referring suspicious isolates (14). These protocols are available at the ASM website (http://www.asm.org/Policy/index.asp?bid=6342). In addition, because any clinical microbiology laboratory could have a sentinel role in detecting potential agents of bioterrorism, CDC, ASM, and APHL jointly recommend that, at a minimum, all sentinel clinical microbiology laboratories (i) meet facility and operational criteria for BSL-2, including the availability and use of a certified Class II biological safety cabinet (37); (ii) have appropriate Clinical Laboratory Improvement Amendments certification; (iii) enroll in proficiency testing (or an equivalent measurement) for Comprehensive Bacteriology and Laboratory Preparedness; and (iv) participate in training and exercises for bioterrorism agent rule-out testing sponsored by a state public health laboratory or other appropriate agency. Laboratories whose physical facilities do not meet these criteria must recognize that they may receive samples containing agents of concern, and thus, their staff should be trained and be familiar with rule-out and referral procedures and biosafety issues. In February 2006, ASM, CDC, and APHL approved a formal definition of sentinel laboratories; it can be found at http://www.asm.org/Policy/index.asp?bid=6342.

It is essential that clinical microbiology laboratories develop a relationship with their local and state health laboratories, many of which provide formal training in laboratory safety, procedures for ruling out targeted biological agents, characteristics of diseases produced by these agents, and packaging and shipping of infectious substances.

Safety

Laboratory personnel must be vigilant in case they isolate one of the targeted agents from what appears to be a routine specimen. Category A and B agents have caused laboratory-acquired infections (29, 31). To reduce the risk that laboratory personnel will be exposed to these agents, flowcharts for LRN sentinel laboratory procedures should be incorporated into standard operating procedures, and physicians should be taught to inform the laboratory when highly infectious agents are in the differential diagnosis (31, 33). If laboratory personnel suspect that a specimen or culture may contain a biothreat-related agent, they should conduct all manipulations in a biosafety cabinet to prevent transmission of the organism. Additional information regarding biosafety is available in chapter 8.

Environmental samples and "unknown packages" may contain very high concentrations of disease-causing agents or toxins, a volatile, toxic chemical, or a radioactive substance; such packages could even be explosive. Powders, in particular, may be highly contaminated and can be aerosolized easily. *Thus, to ensure that personnel and patients are not endangered, sentinel laboratories should not accept or process nonhuman specimens (animal or environmental) but should forward such specimens directly to the state public health laboratory as directed by law enforcement or public health authorities* (31, 34). Public health laboratories have worked with law enforcement

and first responders to develop procedures for evaluating the level of threat posed by environmental samples and to screen these samples for safety (http://www.bt.cdc.gov/planning/pdf/suspicious-package-biothreat.pdf).

Sentinel Laboratory Protocols

A partnership of subject matter experts from CDC, ASM, and APHL developed protocols for LRN sentinel laboratories, which describe a number of the targeted Category A and B agents of bioterrorism (Table 2) and provide standardized methods for ruling out critical agents and referring specimens to LRN reference laboratories. These detailed protocols are published on the ASM website (http://www.asm.org/Policy/index.asp?bid=6342). If sentinel laboratory testing fails to rule out an agent of bioterrorism, all suspicious specimens and isolates should be immediately sent to an LRN reference laboratory, where the specialized tests necessary to definitively identify these organisms (e.g., molecular detection methods, enzyme immunoassays, and direct and indirect fluorescent-antibody assays) are available. Clinical laboratories should maintain a subculture of all suspicious isolates that are forwarded for LRN reference testing until the reference laboratory has completed the definitive identification. Sentinel laboratories do not routinely process specimens following law enforcement chain-of-custody guidelines. However, they may be instructed by law enforcement or public health officials to implement specific procedures if a biocrime is suspected.

To simplify matters for hospital laboratories, the procedures provide practical conventional microbiological methods to rule out four of the most likely agents, *B. anthracis*, *Francisella tularensis*, *Brucella* species, and *Yersinia pestis*, using only eight simple procedures: oxidase, catalase, hemolysis, motility, satelliting, β-lactamase, Gram stain, and urease. The flowcharts from these procedures are reproduced here (Fig. 2 to 5). ASM, CDC, and APHL, as partners in the LRN, recommend that all sentinel laboratories use the LRN sentinel protocols to rule out bioterrorism agents, including *B. anthracis*, rather than using newly described molecular detection assays, new commercial detection and rule-out assays, or tests that will be developed in the near future. If personnel from sentinel laboratories choose to use the latter set of tests, they first must understand the limitations of the assays and the necessary quality control measures and the validation requirements.

TABLE 2 Sentinel laboratory protocols available at ASM website

Bacillus anthracis (anthrax)
Botulinum toxin
Brucella spp.
Burkholderia mallei and *B. pseudomallei*
Coxiella burnetii
Francisella tularensis (tularemia)
Staphylococcal enterotoxin B
Yersinia pestis (plague)
Unknown viruses
Packaging and shipping
Bioterrorism readiness plan for laboratories

Bacillus anthracis

Humans can become infected with *B. anthracis* by handling contaminated materials or consuming undercooked contaminated meat. Infection may also result from inhalation of *B. anthracis* spores from contaminated animal products, or following the intentional release of spores. Human-to-human transmission has not been reported. Three forms of anthrax occur in humans: cutaneous, gastrointestinal, and inhalational. Appropriate specimen types will depend on the disease

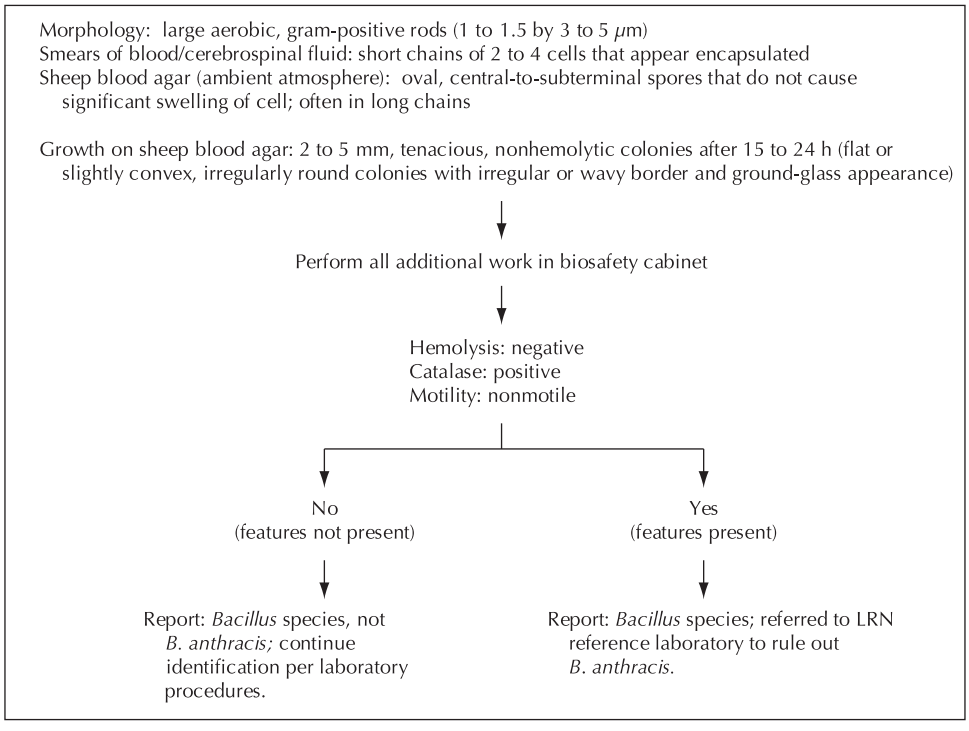


FIGURE 2 Sentinel rule-out testing for *B. anthracis*.

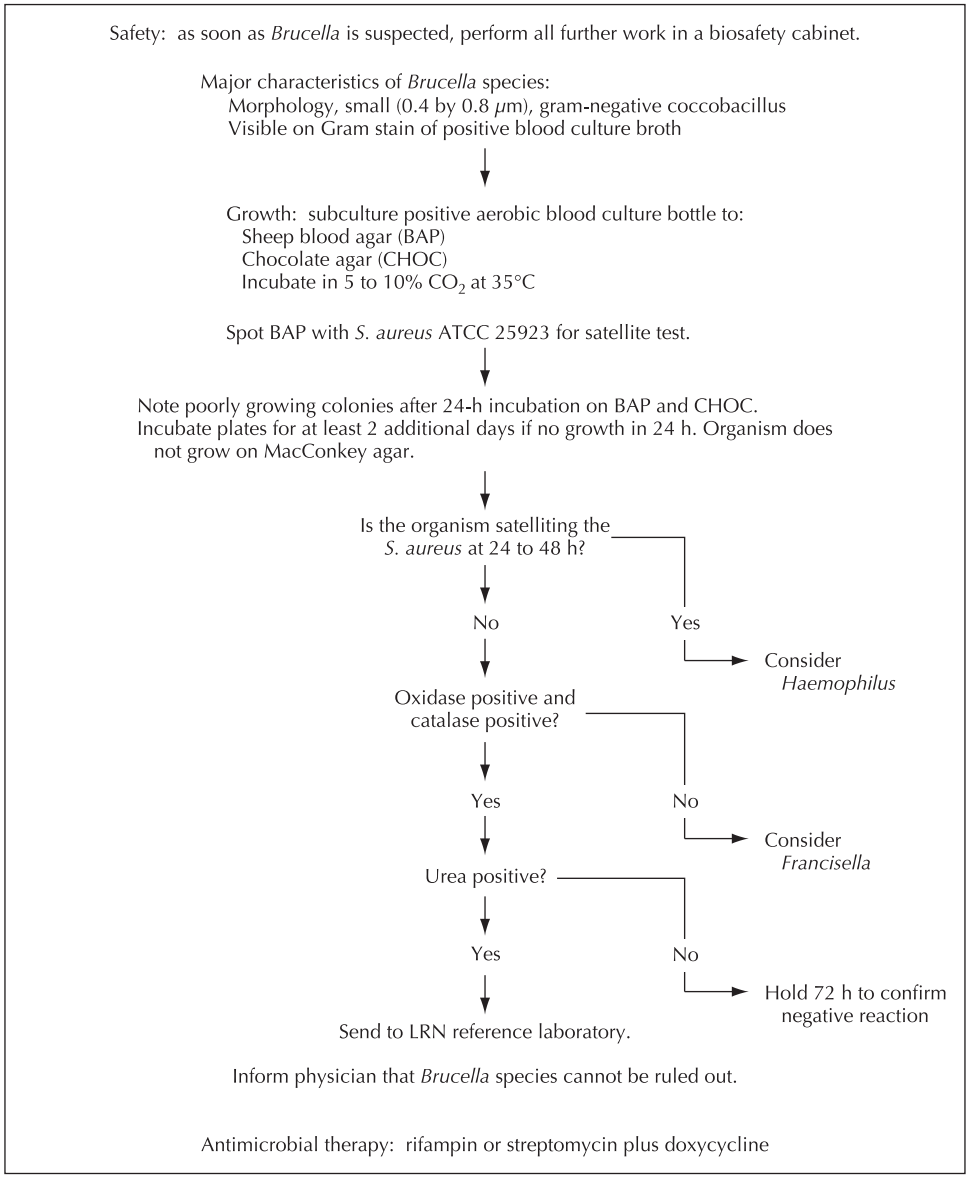


FIGURE 3 Sentinel rule-out testing for *Brucella* spp.

presentation. Clinicians should submit (i) blood, vesicular fluid, swabs from beneath lesions, or tissue/punch biopsy of eschars to diagnose cutaneous anthrax; (ii) stool and blood specimens to diagnose gastrointestinal anthrax; (iii) sputum and blood to diagnose inhalational anthrax; and (iv) cerebrospinal fluid if anthrax meningitis is suspected (15, 22, 29).

B. anthracis grows rapidly, producing 2- to 5-μm flat or slightly convex colonies that have a ground-glass appearance and often have comma-shaped projections from the colony edge (i.e., the "Medusa-head"). *B. anthracis* is nonhemolytic and has a tenacious consistency on blood agar. *B. anthracis* is a large gram-positive rod (1 to 1.5 by 3 to 5 μm). In Gram stains from blood or clinical material, vegetative cells are often encapsulated and appear in short chains. When grown on blood agar or a similar medium, the organism generally appears as long chains of bacilli; it is not encapsulated but may form oval, central-to-subterminal spores. *B. anthracis* is nonmotile and catalase positive. Hemolysis, Gram stain morphology, or motility can be used to rule out this organism when the result provides clear evidence that the isolate is not *B. anthracis* (e.g., a clearly visible zone of beta-hemolysis). In general, a combination of two level A tests is necessary to rule out this organism. Additional information about *B. anthracis* can be found in chapter 32 and in the sentinel laboratory protocol at the ASM website.

Some laboratories might consider using a commercially available, FDA-approved, culture-dependent method, the RedLine Alert (Tetracore Inc., Gaithersburg, Md.; http://www.tetracore.com) as an adjunct to the current sentinel tests (Gram stain, hemolysis, catalase, and motility) for rapid, presumptive identification of *B. anthracis* or ruling out of this organism. (Readers should not infer from this discussion that ASM, APHL, or the CDC endorse this test.) The test, a lateral-flow immunoassay housed in a plastic cassette, was designed

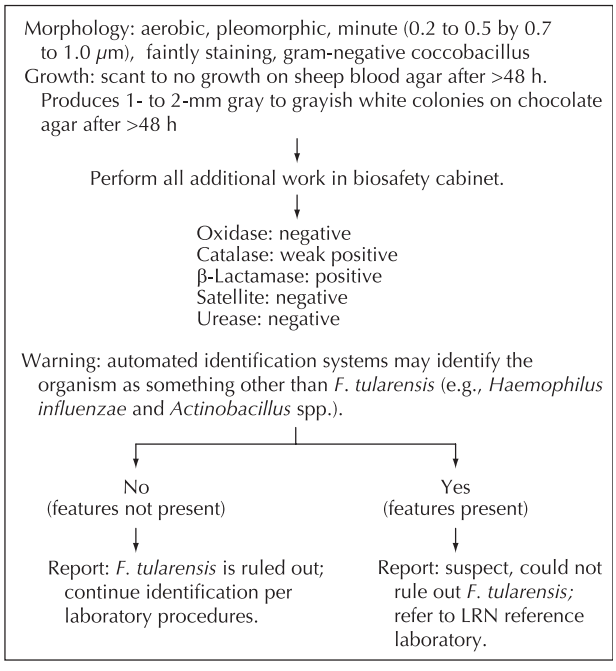


FIGURE 4 Sentinel rule-out testing for *F. tularensis*.

primarily to screen nonhemolytic *Bacillus* colonies cultured on sheep blood agar. The principal target of the test is a cell surface protein commonly found in *B. anthracis* vegetative cells, and the presence or absence of this protein can be used to differentiate *B. anthracis* from other nonhemolytic *Bacillus* colonies. A positive control (lyophilized antigen derived from the nonpathogenic Sterne strain of *B. anthracis*) and reconstitution buffer (Colony Isolation Buffer) are provided in the test kit. A non-*Bacillus* species, such as *Staphylococcus aureus*, or a nonhemolytic *Bacillus* species, such as *B. megaterium*, can be used as external negative controls. The Sterne strain (avirulent; devoid of plasmid pXO2) of *B. anthracis* has been excluded from the Select Agent Rule and can be used as an additional external positive control. Test results are available within 15 min. If the test is positive and the bacterial colony is nonhemolytic, *B. anthracis* cannot be ruled out and the sample must be sent to an LRN reference laboratory for confirmation. To ensure that the test is accurate and performs appropriately, external controls should be performed with each new test lot.

According to the manufacturer, the RedLine Alert test has been extensively evaluated against many strains of *B. anthracis*, non-*anthracis Bacillus* species, and non-*Bacillus* pathogens. The test's limitations include the following: (i) only colonies grown on sheep blood agar should be tested; (ii) hemolytic colonies, including *Bacillus* species, should not be tested; (iii) suspicious powders, other environmental samples, or spore preparations should not be tested; (iv) false-negative results may occur if the amount of specific antigen is below the sensitivity of the test; (v) the test cassette cannot be reused; and (vi) isolates that produce negative results but are suspected of being *B. anthracis* by other criteria (e.g., colonies that are flat or slightly convex and irregularly rounded, with edges that are slightly undulate, have a ground-glass appearance, or exhibit a tenacious consistency) should be forwarded to an LRN reference laboratory for confirmatory testing.

Brucella spp.

Brucellosis is a zoonotic infection; there are four species recognized as causing infection in humans: *Brucella abortus* (cattle), *Brucella melitensis* (goats, sheep, and camels), *Brucella suis* (pigs), and *Brucella canis* (dogs). Infection may occur via enteric, percutaneous, or respiratory exposure. The infective dose for these organisms is very low if acquired via the inhalation route, which makes them a potentially effective bioterrorism agent and also makes them hazardous in the clinical microbiology laboratory. Specimens suspected or known to contain *Brucella* spp. and *Brucella* spp. isolates should be handled in a biologic safety cabinet, and all culture plates

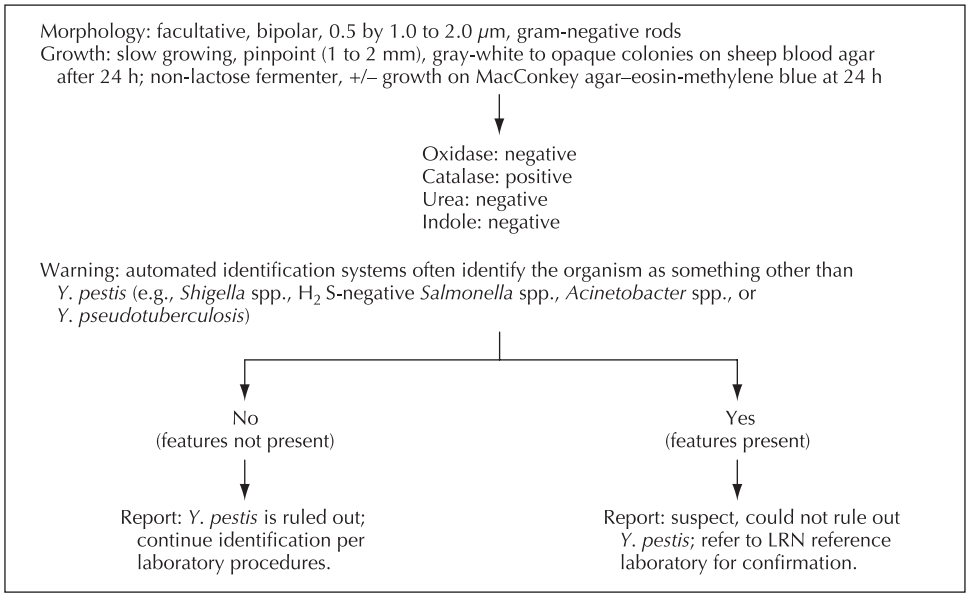


FIGURE 5 Sentinel rule-out testing for *Y. pestis*.

should be sealed. Blood and bone marrow are the best specimens for diagnostic cultures, but spleen, liver, joint fluid, samples from abscesses, and cerebrospinal fluid occasionally yield *Brucella* spp. Serologic studies are available; both acute- and convalescent-phase (>14 days after acute phase) serum samples should be collected (10, 15, 29).

Brucella spp. are tiny (0.5 to 0.7 μm by 1.5 μm), faintly staining gram-negative coccobacilli. Isolation of *Brucella* spp. in blood culture is often delayed, and incubation should be extended for 10 to 21 days if infection with this organism is suspected. On blood or chocolate agar, these organisms produce pinpoint, nonhemolytic, convex, smooth colonies after 48 h. To rule out these organisms, sentinel laboratories should perform the oxidase, catalase, and urea tests, all of which are positive for *Brucella* spp. With the Christensen's tube test, urea hydrolysis can be observed in as early as 15 min of incubation with *B. suis* strains, 1 day for most strains of *B. abortus* and *B. melitensis,* and more than 1 day for some *B. melitensis* strains. Refer to chapter 52 of this Manual and to the sentinel laboratory protocol for additional information.

Burkholderia mallei and *Burkholderia pseudomallei*

Burkholderia mallei is the etiologic agent of glanders, a febrile illness typically seen in horses, mules, and donkeys. Naturally occurring human infection, most often the result of exposure to an infected animal, presents as cutaneous nodules with lymphadenitis or as systemic disease with pneumonia and bacteremia. *Burkholderia pseudomallei* is an environmental organism found in soil and water and is most likely acquired by direct contact with or aerosols from environmental sources. This organism can cause melioidosis, a disease endemic to the tropical regions of the world, which can manifest as an asymptomatic, acute, subacute, or chronic process. Appropriate specimens for diagnosis of glanders and melioidois include blood or bone marrow, sputum or bronchoscopy specimens, material from abscesses, wound swabs, and urine. Acute- and convalescent-phase serum specimens should be collected for serologic diagnosis of *B. pseudomallei*. Currently, no serology test for *B. mallei* is available in the United States. All cultures suspected of containing *B. mallei* or *B. pseudomallei* should be handled in a biological safety cabinet (16).

B. mallei is a small gram-negative coccobacillus, which typically grows in 2 days on sheep blood agar as smooth, gray, translucent colonies, without pigment or distinctive odor. Colonies may or may not grow on MacConkey agar. Additional presumptive identification criteria include these test results: oxidase variable and indole and motility negative; catalase, arginine dihydrolase, and nitrate positive; and resistance to colistin and polymyxin B. Isolates suspected to be *B. mallei* based on colony and Gram stain morphology should be evaluated with these tests.

B. pseudomallei is a small gram-negative rod that often appears on sheep blood agar as small, smooth, creamy colonies in 1 to 2 days, which gradually change after a few days to dry, wrinkled colonies similar to *Pseudomonas stutzeri*. Colonies are neither yellow nor violet pigmented. The organism also grows on MacConkey agar. *B. pseudomallei* often produces a distinctive musty or earthy odor that is very pronounced on opening a petri dish or even opening an incubator door when a positive plate is present. Do not sniff plates, as this can be very dangerous.

B. mallei and *B. pseudomallei* are not in the databases used by some of the commercial identification systems; laboratory staff should consult with the manufacturer to determine whether the system they use includes this information. More details about these agents and their disease spectrum and diagnosis are discussed in chapter 49 and in the sentinel laboratory protocol.

Francisella tularensis

Tularemia is caused by the zoonotic agent *F. tularensis*. In humans, disease can occur in several forms, depending to some extent on the route by which the bacterium enters the body. If used for bioterrorism, the organism would most likely be dispersed via aerosol, resulting in pulmonary or typhoidal disease. Symptoms for both syndromes are often nonspecific. Human-to-human transmission is rare; however, numerous laboratory-acquired infections have been documented. Laboratory staff should manipulate suspicious cultures in a biosafety cabinet using BSL-3 practices; culture plates should be sealed. Appropriate specimens to diagnose pulmonary disease include sputum, bronchial aspirates or washes, and blood (10, 15, 31).

On Gram stain, *F. tularensis* characteristically appears as tiny (0.2 to 0.7 μm by 0.7 to 1.0 μm), pleomorphic, poorly staining, gram-negative coccobacilli. The organism is fastidious, requires cysteine for growth, and grows slowly, producing tiny grayish white colonies after 48 to 72 h. Isolates with characteristic Gram stain and culture morphology that are oxidase negative, catalase weakly positive, beta-lactamase positive, satellite or XV test negative, and urease test negative could be *F. tularensis* (cannot be ruled out) and should be immediately referred to the nearest LRN reference laboratory.

Additional information about other clinical manifestations of tularemia, the modes of transmission, and the organism's characteristics are discussed in chapter 52 as well as the sentinel laboratory protocol.

Yersinia pestis

Plague is a zoonotic disease caused by *Y. pestis*. Humans can acquire plague through the bite of infected fleas, direct contact with contaminated tissue, or inhalation of aerosolized bacteria. Plague presents in one of three clinical syndromes: bubonic, septicemic, or pneumonic. In a bioterrorism event, the agent would most likely be aerosolized, resulting in pneumonic disease, which has a high mortality rate and is highly transmissible from person to person. Appropriate specimens include tracheal or bronchial washes, sputum, blood, and aspirates or biopsy specimens of affected tissues (bubos). Acute- and, if necessary, convalescent-phase sera can be tested for antibodies to *Y. pestis* (3, 15, 29).

Gram stains of specimens and cultures containing *Y. pestis* often reveal plump, gram-negative rods (1 to 2 μm by 0.5 μm) with a bipolar or safety pin appearance. Bipolar staining may be more apparent with Wright stain. This appearance should trigger suspicion of *Y. pestis*. On routine media, pinpoint colonies can appear after 24 h. After incubation for 48 h, the small gray-white to slightly yellow colonies are opaque and produce little or no hemolysis. Under ×4 enlargement, colonies have a raised, irregular "fried egg" appearance, which becomes more prominent as the culture ages. Colonies also can be described as having a "hammered copper" shiny surface. *Y. pestis* grows best at 28°C. Isolates that are catalase positive and oxidase, urease, and indole negative and that exhibit classic Gram stain and growth characteristics suggestive of *Y. pestis* should be referred to an LRN reference laboratory for confirmatory testing. More details about this organism and disease manifestations can be found in chapter 44 and in the LRN sentinel laboratory protocol.

Smallpox (Variola Virus)

The CDC has developed an extensive smallpox response plan and a clinical algorithm to assist physicians as they assess patients and develop differential diagnoses. Varicella (chickenpox) is the infection most likely to be confused with smallpox. Guidelines for specimen collection and laboratory testing for variola, the causative agent of smallpox, and other look-alike diseases are also provided on the CDC website (http://www.bt.cdc.gov/agent/smallpox). If clinicians suspect that a patient has smallpox, the person designated by the facility should contact the public health laboratory and state health officials immediately.

Variola virus can grow and amplify easily in most routine cell lines used for cultivating herpesviruses such as varicella-zoster virus and herpes simplex virus (21, 29). While clinical microbiology/virology laboratories should never attempt to isolate this virus, which requires BSL-4, technologists may neither suspect nor recognize the potential hazard. If technologists manipulate a culture in the routine manner or passage the culture while trying to confirm the etiology of the cytopathic effects, they may increase the risk that personnel will be exposed to smallpox virus. Therefore, laboratory personnel should be familiar with the risks from the most likely viral agents of bioterrorism and understand appropriate safety practices (see the "Unknown Virus" protocol for sentinel laboratories on the ASM website for helpful materials). If a laboratory inadvertently isolates the smallpox virus, the laboratory director should notify both the state health laboratory and the CDC immediately.

Reporting

The sentinel laboratory staff should inform infection control personnel promptly if they identify suspicious isolates. Infection control staff should follow internal chain of command and communication policies for notifying local and state health officials, who are responsible to communicate with law enforcement. The chain of command should be clearly described in each institution's bioterrorism preparedness program (34). There are several reasons why sentinel laboratories should not report possible bioterrorism events externally. First, many potential agents of bioterrorism are found naturally in certain geographic areas and cause sporadic infections. Second, incorrect characterization of a suspect agent can cause public panic, placing undue burdens on public health and law enforcement agencies.

Regulatory Issues

Packaging and Shipping

Sentinel laboratories must refer suspicious isolates to reference laboratories. Therefore, proper packaging and shipping of infectious agents is one of their important functions (refer to chapter 5, "General Principles of Specimen Collection and Handling"). Cultures and clinical specimens known or suspected to contain infectious substances must be packaged according to domestic and international regulations on dangerous goods and infectious substances promulgated by the U.S. Department of Transportation (DOT), the International Air Transport Association, and the Canadian Transportation of Dangerous Goods Regulations. Recently various regulatory authorities have attempted to harmonize the requirements; updated regulations are published periodically. Anyone, including microbiologists, who ships infectious substances is required to complete formal training, become certified in the application of these regulations, and be familiar with current regulations. Formal training is available through the U.S. DOT and most commercial suppliers of approved packaging containers (see the DOT's and the commercial suppliers' websites).

Select Agent Program

In accordance with provisions of Public Law 107-188, the "Public Health Security and Bioterrorism Preparedness Response Act of 2002," the CDC and the U.S. Department of Agriculture Animal and Plant Health Inspection Service (USDA-APHIS) regulate the possession, use, and transfer of biological agents and toxins that could pose a severe threat to public health and safety (13). The CDC maintains a list of select agents that threaten humans, and the USDA-APHIS maintains a list of organisms that threaten animals; together they maintain a shared (overlap) list of organisms, primarily "high consequence" zoonotic agents that threaten humans and animals (42 CFR Part 73, 7 CFR Part 331, and 9 CFR Part 121). The Select Agent regulation requires all entities, including individuals, government agencies, universities, research institutions, and commercial facilities that possess, use, or transfer biologic agents and toxins that pose a significant threat to public health, to register with either CDC or USDA depending on the agents they possess. As a condition of registration, each entity must designate a Responsible Official to ensure that staff members comply with the regulation and to develop and implement a written security plan. Persons with access to select agents must submit required forms for a security risk assessment through the U.S. Attorney General, Department of Justice.

As described in 42 CFR Part 73.5, clinical or diagnostic laboratories that acquire a select agent from a specimen presented for diagnosis or verification are exempt from the regulation, provided that (i) laboratory personnel transfer or destroy the select agent within 7 days after the organism's identification has been confirmed; (ii) the agent is secured against theft, loss, or release during the period between identification and transfer or destruction; and (iii) laboratory personnel report the select agent to the CDC or USDA as defined in the regulation (usually within 7 days; viral hemorrhagic fever viruses, variola virus, and *Y. pestis* must be reported immediately). The laboratory must document that the organism was transferred or, if the organism was destroyed, the method of destruction and must maintain these records for 3 years. Additional exemptions apply to clinical and diagnostic laboratories that acquire a select agent as part of a proficiency testing exercise, provided that the agent is transferred or destroyed within 90 days; that the agent is properly secured against theft, loss, or release; and that the CDC or USDA is notified within 90 days.

Sources for Additional and Updated Information

Information and guidelines on bioterrorism continue to change and may be updated if a bioterrorism event is detected. Microbiologists are encouraged to remain current with local, state, and federal guidelines and should contact the local health department regarding emergencies and to report suspected or confirmed exposures to biological agents. In addition, laboratory personnel and clinicians can use the CDC Emergency Response Hotline (phone: 770-488-7100) at all hours to receive emergency consultation from subject matter experts in bioterrorism, chemical exposure, and natural disasters. Check http://www.bt.cdc.gov/emcontact/ for updated contact information.

Other current information on terrorism preparedness can be obtained from a variety of Internet sites, including the following.

1. Federal agencies and preparedness

CDC—http://www.cdc.gov
CDC Bioterrorism Preparedness and Response Program—http://www.bt.cdc.gov
Department of Health and Human Services—http://www.hhs.gov
Department of Homeland Security—http://www.dhs.gov
Federal Emergency Management Agency—http://www.fema.gov
National Response Plan—http://www.dhs.gov/interweb/assetlibrary/ NRP_FullText.pdf
National Incident Management System—http://www.fema.gov/nims/
Interim National Preparedness Goal—http://www.ojp.usdoj.gov/odp/docs/InterimNationalPreparednessGoal_03-31-05_1.pdf
Interim Public Health and Healthcare Supplement to the National Preparedness Goal—http://www.hhs.gov/ophep/npgs.html

2. General information

ASM—http://www.asm.org
Center for Biosecurity of the University of Pittsburgh Medical Center (UPMC)—http://www.upmc-biosecurity.org/
Center for Infectious Disease Research and Preparedness—http://www.cidrap.umn.edu/index.html

3. Sentinel laboratory testing protocols

ASM—http://www.asm.org/Policy/index.asp?bid=6342
CDC—http://www.bt.cdc.gov

4. Bioterrorism readiness plans and templates for health care facilities

Association of Professionals in Infection Control—http://www.apic.org/Content/NavigationMenu/PracticeGuidance/Topics/Bioterrorism/Bioterrorism.htm
CDC—http://www.bt.cdc.gov/planning/#healthcare
ASM—http://www.asm.org/Policy/index.asp?bid=520
DHS—National Disaster Medical System (NDMS)—http://www.oep-ndms.dhhs.gov

5. Packaging and shipping regulations

Department of Transportation Hazardous Materials Regulations (49 CFR parts 171 to 180, shipment of biological and clinical specimens)—http://hazmat.dot.gov/
IATA—http://www.iata.org
World Health Organization—http://www.who.org
International Civil Aviation Organization—http://www.icao.org
TDGR—http://www.cftcanada.com/c203547.2.html

6. Select agent regulations

CDC—http://www.cdc.gov/od/sap/
USDA-APHIS—http://www.aphis.usda.gov/programs/ag_selectagent/

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Infection Control Epidemiology and Clinical Microbiology

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10

In 2000, the Institute of Medicine issued a landmark report on medical errors, estimating that between 44,000 and 98,000 deaths per year in U.S. hospitals are a result of injuries or complications sustained during the delivery of health care (44). Health care-associated (or nosocomial) infections represent one of the most common complications of care, affecting approximately 2 million persons admitted to acute-care hospitals each year (8). For this reason, every health care facility should have an infection control program charged with monitoring, preventing, and controlling the spread of infections in the health care environment. Because infection control requires the ability to detect infections when they occur, the clinical microbiology laboratory is inextricably linked to any comprehensive infection control program. In this chapter we will discuss the impact of nosocomial infections, outline the organization of the hospital infection control program, and describe the important role of the clinical microbiology laboratory in the prevention and control of health care-associated infections.

NOSOCOMIAL INFECTION

Definition

A nosocomial infection is one that is acquired in a hospital or health care facility (i.e., the infection was not present or incubating at the time of admission). For most bacterial infections, an onset of symptoms more than 48 h after admission is evidence of nosocomial acquisition. To determine whether some infections such as legionellosis are hospital acquired, one must consider the usual incubation periods and determine whether the patient was hospitalized during that time period. Because hospital stays are getting shorter and more patients are treated in the outpatient setting, many health care-associated infections are not recognized during hospitalization. Infection control programs must therefore devise strategies for effective outpatient surveillance in order to accurately monitor nosocomial infection rates (38).

Infection Rates and Predominant Pathogens

At least 5% of patients may acquire an infection during hospitalization (6, 34). The urinary tract is the most commonly involved site, with 30 to 40% of all nosocomial infections occurring at this site. Surgical wound and lower respiratory tract infections are the next most frequent, with each accounting for about 15 to 20% of nosocomial infections, followed by bloodstream infections (5 to 15%). The vast majority of nosocomial infections are related to devices (e.g., urinary tract catheters, endotracheal tubes in ventilated patients, and central venous catheters). For this reason, and as a way to adjust for risk when comparing rates over time or between similar units in different facilities, the Centers for Disease Control and Prevention (CDC) recommends calculating nosocomial infection rates in the intensive care unit (ICU) by using device days as the denominator (Table 1 gives definitions of common epidemiology terms).

Table 2 lists the five most common bacterial pathogens isolated from various sites of nosocomial infection in U.S. hospital ICUs (17, 80). Over the past 3 decades, the spectrum of nosocomial pathogens has shifted from gram-negative to gram-positive organisms and *Candida* spp. have emerged as a major problem (55). The incidence of nosocomial infections caused by staphylococci and enterococci has increased, in part because these organisms are becoming increasingly resistant to antimicrobial agents (21).

Morbidity, Mortality, and Cost

Nosocomial infections cause or contribute to thousands of deaths annually (6, 76). Because patients with the most severe underlying illnesses are also those most vulnerable to nosocomial infections, it is very difficult to estimate the proportion of crude or overall mortality that is directly attributable to nosocomial infections. Studies that attempt to address this question by carefully controlling for many potentially confounding variables are called attributable mortality studies. Estimates of the attributable mortality associated with nosocomial bloodstream infections have ranged from 14% for infections caused by coagulase-negative staphylococci (49), 31 and 37% for infections caused by vancomycin-susceptible and vancomycin-resistant enterococci (26, 46), respectively, and 38 to 49% for infections caused by *Candida* spp. (32, 78) (Table 3).

Nosocomial infections also increase hospital costs and lengths of stay (LOS), thereby costing the health care system billions of dollars annually. At the University of Iowa, the median excess LOS for nosocomial bloodstream infections caused by coagulase-negative staphylococci and *Candida* spp. were 8 and 30 days, respectively (49, 78). Nosocomial bloodstream infections in the ICU were associated with an excess LOS of 24 days and excess hospital costs of $40,000

TABLE 1 Commonly used terms in health care epidemiology[a]

Term	Definition or Summary
Epidemiology	Study of the occurrence, distribution, and determinants of health and disease in a population. Hospital or health care epidemiology is the study of disease occurrence and distribution in the hospital or health care system.
Nosocomial infection	An infection acquired in a hospital or other health care facility.
Endemic infections	Infections occurring as part of the background or usual rate of infection in a specified population.
Epidemic infections	Infections occurring as part of an outbreak (or epidemic) of infection—defined as a significant increase in the usual rate of that infection in the specified population.
Incidence rate	Ratio of the number of new cases of infection in a specified population at risk during a defined time period to the overall number of people in the population at risk (the denominator).
Device-associated incidence rate	Ratio of the number of new cases of device-related infection in a specified population at risk during a defined time period to the number of days of device utilization in the population at risk.
Prevalence rate	Total number of cases of infection in the defined population at risk at one point in time (point prevalence) or in a given time period (period prevalence).
Observational or descriptive study	Study of the natural course of events, without an intervention in the process.
Case control study	Study frequently done as part of an outbreak investigation: a group of patients with the outcome of interest (e.g., cases of nosocomial infection) is compared to a control group of patients without the outcome. A comparison of specific factors between groups (e.g., exposures of interest) may suggest why infection occurred.
Crude or overall mortality rate	Ratio of the number of patients who die to the overall number of patients in a specified population.
Attributable mortality	Ratio of the number of patients who die as a direct result of the disease of interest to the overall population with the disease.

[a] Adapted from reference 50.

per survivor (60). Kirkland et al. found that surgical site infections increased LOS by more than 6 days and increased hospital costs by more than $3,000 per infection (42).

The premise upon which infection control programs operate is that many of these life-threatening and costly nosocomial infections are preventable. The Study of the Efficacy of Nosocomial Infection Control indicated that the presence of an active surveillance and infection control program was associated with a 32% decrease in nosocomial infection rates and that the absence of such a program was associated with an 18% increase in nosocomial infection rates (35). More recently, the CDC National Nosocomial Infection Surveillance (NNIS) system of hospitals reported a reduction in risk-adjusted infection rates in ICUs for all monitored infection sites (urinary tract, bloodstream, and lung) during the 1990s. The elements that were critical for reducing rates included targeted surveillance of high-risk populations (using standard definitions); adequate numbers of trained infection control professionals (ICPs), who inform health care providers of infection rates; and prevention efforts designed to address issues identified during evaluation of infection rates (18). Clearly, an effective infection control program improves patient care, saves lives, and decreases health care costs.

TABLE 2 Distribution of the five most common nosocomial pathogens isolated from major infection sites in the ICU

Pathogen	% of total at each infection site
Bloodstream infection[a]	
CoNS[b]	35.9
S. aureus	16.8
Candida spp.	10.1
Enterococcus spp.	9.8
Pseudomonas aeruginosa	4.7
Pneumonia[c]	
S. aureus	18.1
Pseudomonas aeruginosa	17.0
Enterobacter spp.	11.2
Klebsiella pneumoniae	7.2
Haemophilus influenzae	4.3
Urinary tract infection[c]	
Escherichia coli	17.5
Candida albicans	15.8
Enterococcus spp.	13.8
Pseudomonas aeruginosa	11.0
Klebsiella pneumoniae	6.2

[a] Data from SCOPE (80); collected between March 1995 and September 2002.
[b] CoNS, coagulase-negative *Staphylococcus* spp.
[c] Data from NNIS (17); collected between January 1990 and May 1999.

TABLE 3 Attributable mortality of nosocomial bloodstream infection due to selected pathogens[a]

Organism	Mortality among cases (%)	Mortality among matched controls (%)	Attributable mortality (%)	Reference(s)
CoNS[b]	31	17	14	49
Enterococcus spp.	43	12	31	46
VRE	67	30	37	26
Candida spp.	57–61	12–19	38–49	32, 78

[a] Adapted from reference 23, with permission.
[b] CoNS, coagulase-negative *Staphylococcus* spp.

THE HOSPITAL INFECTION CONTROL PROGRAM

The hospital infection control program should include surveillance and prevention of nosocomial infections, continuing education of medical staff, control of infectious disease outbreaks, protection of employees from infection, and advice on new products and procedures. The program is generally directed by a physician-epidemiologist and enforced by the infection control committee. Every hospital must also have a working infection control staff, comprising one or more ICPs. The ICPs should collect data on nosocomial infections and provide the data to the infection control committee.

Infection Control Committee

The infection control committee is responsible for reporting and evaluating nosocomial infection data and for drafting and implementing policies, procedures, and guidelines pertinent to the practice of infection control. Of note, in some hospitals the infection control program staff performs the functions just described and the infection control committee approves the reports, policies, procedures, and guidelines. The committee should be multidisciplinary, with representatives from all departments, including clinical microbiology, and should meet every 1 to 3 months to review hospital-specific nosocomial infection data and to formulate policy. The members bring the needs and perspectives of their departments to the committee and, in turn, take back important information about infection control initiatives and policies, etc. Other responsibilities of the committee include reviewing technical information about new products, devices, or procedures pertinent to infection control and instituting all necessary control measures in the event of an outbreak or other infection control emergency.

A clinical microbiologist must be on the infection control committee in order to provide expertise in the interpretation of culture results, advice about the appropriateness and feasibility of microbiological approaches to an infection control problem, and input regarding the laboratory resources necessary to accomplish the goals of the committee. One of the most important contributions of the clinical microbiologist is to inform the infection control committee of the strengths and limitations of methods employed to detect and characterize nosocomial pathogens. He or she should describe how changes in the methods used for detection, identification, and susceptibility testing of nosocomial pathogens would affect the infection control program. For example, if the laboratory introduces a urinary antigen detection test for diagnosis of legionellosis, the clinical microbiologist must inform the committee that the test is sensitive and specific only for detection of *Legionella pneumophila* serogroup 1 and that culture is required to evaluate for nosocomial legionellosis due to other species or serogroups. The committee should also be made aware of the budgetary and personnel constraints under which the laboratory operates to ensure that they do not expend valuable laboratory resources unless there is a clear epidemiologic indication to do so.

Nosocomial Infection Surveillance

Active nosocomial infection surveillance programs are associated with a reduction in infection rates and their consequent morbidity and mortality (22, 35), and national and state accrediting agencies require hospitals to do surveillance for nosocomial infections. Thus, systematic surveillance of nosocomial infections is the infection control program's most important activity. Surveillance is also the infection control program's most costly and time-consuming activity. Surveillance allows the infection control program staff to monitor the frequency and types of nosocomial infections, detect outbreaks, evaluate compliance with infection control guidelines, provide data for policy development, and monitor the effect of infection control interventions on nosocomial infection rates. To accomplish the overall goal of decreasing infection rates, the infection control program personnel must give the surveillance data and suggestions for improvement, including reminders to staff of existing infection control practices, back to the clinicians as soon as possible. Infection control programs that follow the CDC's advice about using device days as the denominator for calculating rates of nosocomial infection in ICUs can compare their rates with national benchmarks compiled and reported by the CDC NNIS system (17, 18). Figure 1 is a sample format for comparing infection rates in an ICU of a tertiary-care center with national benchmark data. The infection control program should provide infection rates, recommendations for improving rates, and assistance in

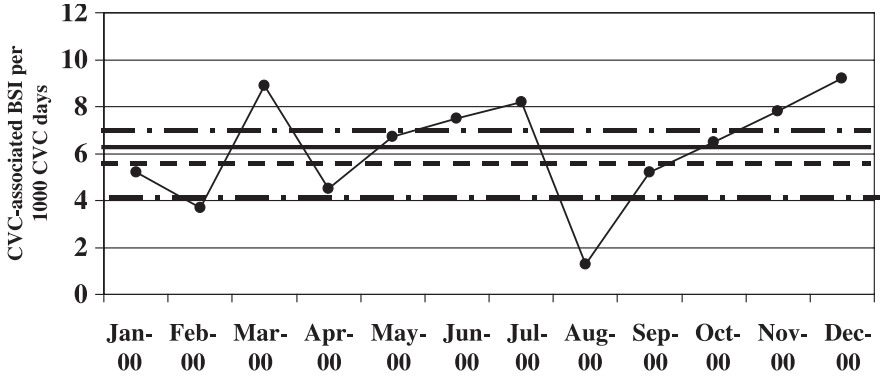


FIGURE 1 Medical ICU catheter-associated bloodstream infection rates. •———•, central venous catheter (CVC)-associated bloodstream infection rate per 1,000 catheter-days; ———, mean CVC-associated bloodstream infection rate in Hospital A medical ICU; – – – –, pooled mean CVC-associated bloodstream infection rate for the CDC NNIS hospital medical ICUs (n = 131); — ▪ —, 25th and 75th percentiles for CVC-associated bloodstream infection rate in NNIS hospitals.

implementing interventions to unit personnel such as medical directors, nurse managers, and clinicians.

The infection control program staff should design a surveillance system that is appropriate for the specific needs of the hospital and feasible based on the hospital's budget. For example, program personnel wishing to use device days as a denominator must develop a system for counting or accurately estimating device utilization in their ICUs. Because surveillance consumes more resources than any other infection control activity (27), infection control programs must devise the most efficient surveillance system possible. The most complete and accurate surveillance program might require an ICP to review charts of all hospitalized patients daily, but this approach obviously is not practical in any but the smallest of hospitals. Infection control programs should focus their limited resources on the highest-risk areas (e.g., intensive care, hematology-oncology, burn, and organ transplant wards) and use various screening techniques to increase surveillance efficiency. ICPs can use microbiology reports, nursing care plans, antibiotic orders, radiology reports, vital signs, and discharge diagnoses to determine which charts should be further reviewed.

Review of microbiology reports is probably the most common method for case finding, and it compares favorably in some circumstances with more comprehensive ward-based surveillance (31, 77). For example, Yokoe and colleagues reported that review of microbiology data alone was both more resource efficient and as effective as applying the CDC's definition of nosocomial bloodstream infection (85). Such laboratory-based surveillance allows the ICP to efficiently review a large amount of data. Moreover, medical information systems can enhance laboratory-based surveillance further by linking laboratory data with data from many sources (71), including pharmacies (e.g., antimicrobial use), radiology departments, billing departments (e.g., diagnostic codes), and nursing notes (e.g., vital signs and care plans).

Although reviewing microbiology reports is an essential part of surveillance, these data alone may not detect all nosocomial infections or all outbreaks. The sensitivity and specificity of laboratory-based surveillance depend upon both the frequency at which clinicians obtain cultures and the quality of the culture specimens received by the laboratory. In addition, while laboratory-based surveillance may quickly detect outbreaks due to unusual pathogens, or infections at unusual sites, outbreaks or clusters due to common pathogens at common sites (e.g., *Escherichia coli* urinary tract infection) may go undetected for longer periods of time. An optimal surveillance program will include data from more than one source (e.g., nursing care plans and microbiology reports) to help ICPs determine which charts deserve further review. The University of Iowa, Iowa City, previously validated a surveillance strategy using primarily microbiology reports and nursing care plans and found the sensitivity and specificity to be 81 and 98%, respectively (5). More recently, we introduced a computer-based screening algorithm that provides a list for each ICP each day of all patients in their units that had positive cultures or that had a *Clostridium difficile* toxin test or a respiratory syncytial virus antigen test ordered or one of the following combinations of tests ordered within a 24-h period: chest radiograph and culture of respiratory secretions or cultures from two or more body sites. After reviewing this information, the ICPs review the medical records of a small percentage of patients, thereby reducing the amount of time required for surveillance.

The frequency of surveillance of specific hospital units should be determined by the infection control committee based on the types of patients hospitalized, the procedures and treatments done in the facility, the resources available for surveillance, prevailing infection rates, and other factors. In addition, the infection control committee, in consultation with clinical microbiology personnel, should decide the mechanism by which the laboratory provides specific information needed for surveillance (e.g., all positive results or selected or sorted reports, etc.).

The recent movement toward public disclosure of nosocomial infection rates (16, 75, 81) has important implications for surveillance. If interhospital comparisons of nosocomial infection rates are to have any meaning, all hospitals must use the same methods for surveillance and risk adjustment (16, 75). Unfortunately, at the time this chapter was written, methods for nosocomial infection surveillance varied widely among hospitals and validated methods for risk adjustment rates were not available (40). Thus, although public disclosure of nosocomial infection rates is a laudable goal and the policy is being introduced by law in many states, much work needs to be done to ensure that such reporting improves, rather than hinders, efforts to prevent nosocomial infections.

Process Surveillance

Several recent studies clearly demonstrate that implementing evidence-based infection control practices, such as good hand hygiene (61) and guidelines for the placement and care of central venous catheters (4, 10), can dramatically reduce nosocomial infection rates (4, 61, 86). The obvious implication of these studies is that health care workers do not routinely adhere to the safest processes of care. For this reason, ICPs must now perform surveillance not only for important outcomes (infections) but also for important processes (e.g., rates of hand hygiene performance, use of maximal sterile barriers during central venous catheter placement, and elevation of the heads of beds to 30° for ventilated patients, etc.). Process measures can help infection control personnel understand some of the variation in nosocomial infection rates. In addition, reporting compliance rates to personnel may improve practices and reduce nosocomial infection rates.

ROLE OF THE MICROBIOLOGY LABORATORY IN INFECTION CONTROL

With this overview of the structure and activities of the hospital infection control program in mind, we will now focus on the most important specific roles played by the microbiology laboratory in the day-to-day practice of infection control.

Specimen Collection

Many nosocomial pathogens (e.g., coagulase-negative staphylococci) also commonly colonize patients' skin or mucous membranes and can easily contaminate cultures if specimens are not collected or handled properly. If contaminants are mistakenly considered to be infecting organisms, ICPs may inadvertently count these as representatives of nosocomial infections, thereby inflating the infection rates (2). Consequently, microbiologists must monitor specimen quality carefully and also set and enforce strict criteria for acceptable clinical specimens (see chapter 5).

Accurate Identification and Susceptibility Testing of Nosocomial Pathogens

Commercial identification and susceptibility testing systems allow most laboratories to identify microorganisms to the species level and perform antimicrobial susceptibility testing (AST). However, the expanding spectrum of organisms that colonize and infect seriously ill patients challenges the ability of the clinical microbiology laboratory to identify and characterize nosocomial pathogens accurately (56). For example, while many nosocomial pathogens (e.g., *Staphylococcus* spp. and the *Enterobacteriaceae*) are easily detected and identified with commonly used automated systems, many nonfermentative gram-negative organisms that cause nosocomial infections can be much more difficult to identify. Laboratories that identify nosocomial pathogens to the species level may find outbreaks that would otherwise have been undetected because clusters of unusual organisms or unusual clusters of common organisms may be clues to outbreaks. Thus, laboratories should establish a system for sending unusual nosocomial pathogens to a reference laboratory for definitive identification. In addition, viral, fungal, and mycobacterial pathogens can cause nosocomial infections and also can be difficult to identify to a level appropriate for infection control needs.

New antimicrobial resistances continue to emerge, and existing resistances are increasing in frequency. To guard against significant AST errors for some organism-antimicrobial combinations, laboratories must supplement automated systems with additional methods. AST errors are most likely for organisms that display heteroresistance or inducible resistance mechanisms or for newly emerging resistances. For example, some systems previously underestimated oxacillin resistance among *Staphylococcus* species (82) and did not adequately detect extended-spectrum beta-lactamase (ESBL) production by certain *Enterobacteriaceae* (3, 29, 70). Some automated systems have not detected all enterococci with certain vancomycin resistance phenotypes (68). More recently, investigators have found that automated systems were not adequate to detect vancomycin-resistant *Staphylococcus aureus* (VRSA), and therefore, laboratories must use new screening methods to detect this organism (67). If the laboratory uses methods that do not accurately identify organisms or particular resistance patterns, the infection control program may not identify serious problems or even outbreaks. Conversely, infection control personnel may investigate spurious problems, thereby diverting and wasting precious resources.

Laboratories that recognize such problems should bring them to the manufacturers' attention so that manufacturers can improve the instrumentation, panels, or software programs and, thereby, improve accuracy. This process of ongoing independent evaluation of automated systems and feedback to responsive industry representatives is extremely important. Unfortunately, in the era of managed care and shrinking laboratory resources, fewer laboratories have the ability to perform rigorous internal evaluations of new technology. The most important resistances emerging in nosocomial pathogens include ESBL production among *Enterobacteriaceae* (58, 64), glycopeptide resistance among enterococci (21) and staphylococci (9, 11, 12, 14, 19, 51, 65), and methicillin resistance among *S. aureus* strains (25). Moreover, four patients are known to have been infected with VRSA (11, 12, 14, 51), which is particularly concerning because *S. aureus* is a virulent organism and options for bactericidal therapy for infections caused by multiply resistant methicillin-resistant *S. aureus* (MRSA) are limited. The infection control program must implement control measures to prevent the spread of these important antimicrobial-resistant pathogens. However, the success of the program depends upon the ability of the laboratory to detect these organisms. Laboratory directors must read current literature regarding automated systems' ability to detect emerging resistances and implement, if necessary, additional methods to detect or confirm particular resistance patterns. The CDC website provides fact sheets summarizing current recommendations for detecting these resistances (http://www.cdc.gov/ncidod/hip/lab/lab.htm). Antimicrobial resistance detection is also reviewed in chapters 17, 74, and 78 of this Manual.

Laboratory Information Systems

An information system that can do prospective data mining and interface with other parts of the computerized patient record could help ICPs do surveillance, monitor patient-to-patient spread of pathogens, and detect outbreaks early (54, 59). Thus, persons choosing a laboratory information system must consult with both laboratory and infection control personnel before purchasing the best system for the hospital. Chapters 4 and 17 include more complete discussions of laboratory information systems and expert systems for data analysis.

Rapid Diagnostic Testing

During the past decade, numerous rapid diagnostic tests have been developed that use molecular or immunologic methods. For example, a variety of methods are now available for rapid detection of respiratory syncytial virus (41), *Clostridium difficile* (30), *Mycobacterium tuberculosis* (36, 39), and *Legionella pneumophila* serogroup 1 (62). Rapid methods for detecting important antimicrobial resistances are also being developed. Latex agglutination testing for the altered penicillin binding protein 2a (84)—or real-time molecular detection of the *mecA* gene coding for this protein (7, 73, 87)—detects MRSA more rapidly than traditional methods. Some hospitals, including that at the University of Iowa, are using methods that detect the *vanA* and *vanB* genes in real time to identify patients who carry vancomycin-resistant *Enterococcus* spp. (VRE) (24). A positive result from any of these tests will allow clinicians to implement appropriate isolation precautions quickly in order to prevent the spread of the organisms. Thus, such tests can help infection control programs control the spread of important pathogens. Of course, if clinicians order the tests indiscriminately or the laboratory has poor quality control, rapid diagnostic tests can lead to errors, including falsely positive tests that lead to inappropriate treatment and isolation of patients. Erroneous results may also cause the infection control program staff to waste time investigating a pseudo-outbreak (48). The clinical microbiologist must also assess the negative predictive value of any rapid tests provided by the laboratory. If the negative predictive value is not high enough, decisions to discontinue isolation precautions should not be based on the results of the rapid test.

Reporting of Laboratory Data

Culture and AST results are an important data source for infection control and are usually reviewed daily by ICPs. Thus, routine microbiology laboratory results should be readily accessible to ICPs. In most cases, results are stored in a computer database, facilitating retrieval and analysis. The laboratory should store the following information: specimen

type, date of collection, patient name, hospital number, hospital service, ward location, organisms identified, AST results, and the results of any specialized testing performed (e.g., typing). Both clinicians and ICPs benefit from periodic summaries of selected microbiology results such as an antibiogram specifically for nosocomial pathogens. These results can be presented in a table that includes the antibiograms of the most common nosocomial pathogens organized by anatomical site and hospital service and that also includes cost information for the most commonly used antimicrobials. This information will help clinicians choose empiric antimicrobial therapy for patients with nosocomial infections. The Clinical and Laboratory Standards Institute has developed guidelines for antibiogram preparation, which are discussed in chapter 4 (20).

Laboratory personnel should call the ICP directly to report some culture results to ensure that appropriate control measures are implemented. Examples include sterile-site cultures positive for *Neisseria meningitidis* and *Legionella pneumophila* and smears or cultures positive for acid-fast bacilli, enteric pathogens such as *Salmonella* and *Shigella* spp., and certain antimicrobial-resistant pathogens such as MRSA, VRSA, and VRE. In addition, new or unusual pathogens and potential agents of bioterrorism (e.g., *Bacillus anthracis*, *Yersinia pestis*, and orthopox viruses) should be reported promptly to the ICP.

In addition to providing printed and verbal reports, laboratory staff should meet regularly with infection control staff to ensure that their communication is direct and clear. They can discuss areas of mutual concern, such as the status of epidemiological and microbiological investigations of clusters or outbreaks. Together they can determine whether supplementary studies such as molecular typing or environmental cultures will be necessary. If these studies are necessary, they can determine exactly what needs to be done, who will perform these procedures, and when they will be carried out.

Outbreak Recognition and Investigation: Epidemic versus Endemic Infections

Most nosocomial infections are not associated with outbreaks, that is, they are endemic rather than epidemic infections. If rates of nosocomial infections are consistently defined by prospective surveillance, infection control personnel may occasionally identify outbreaks of nosocomial infections—an increase in infection rate beyond that expected during a defined time period—by reviewing these rates. However, more often infection control personnel learn about potential outbreaks while interacting with personnel in the ward, in clinics, or in the laboratory.

When the infection control team detects a cluster or outbreak of nosocomial infections, they must act promptly to identify the etiologic agent if it is not known, define the extent of the outbreak, learn the mode of transmission of the pathogen, and institute appropriate control measures. The clinical microbiology laboratory must provide appropriate laboratory support during this time. Table 4 outlines recommended steps in an outbreak investigation and points out the important role of the clinical microbiology laboratory at each step.

TABLE 4 Steps in nosocomial outbreak investigation, and the role of the laboratory at each step[a]

Investigative step	Role of the clinical microbiology laboratory
Recognize problem	Maintain surveillance and early-warning system—ideally part of the laboratory information system; notify infection control personnel of clusters of infections, unusual resistance patterns, and possible patient-to-patient transmission
Establish case definition	Assist and advise regarding inclusion of laboratory diagnosis in case definition
Confirm cases	Perform laboratory confirmation of diagnosis
Complete case finding	Characterize isolates with accuracy; store all sterile-site isolates and epidemiologically important isolates; search laboratory database for new cases
Establish background rate of disease, compare to attack rate during suspected outbreak	Provide data for use in ongoing surveillance, which provide baseline rates for selected units and infection sites; search laboratory database for all prior cases of the entity if baseline rate is not prospectively monitored
Characterize outbreak (descriptive epidemiology)	Perform typing of involved strains and compare to previously isolated endemic strains to determine if the outbreak involves a single strain (see chapter 11); this can be done only if selected pathogens are routinely stored (see above)
Generate hypotheses about causation: Identify potential reservoirs Identify potential modes of spread Identify potential vectors Perform case control study or cohort study	Perform supplementary studies or cultures as needed, but only if justified by epidemiologic link to transmission: personnel, patients, environment
Institute control measures	Adjust laboratory procedures as necessary
Perform ongoing surveillance to document efficacy of control measures	Maintain surveillance and early-warning function of the laboratory

[a] Adapted from reference 50.

Because the demands on the laboratory may be great during outbreaks, the laboratory staff should prepare in advance. Laboratory personnel periodically should ask ICPs what types of outbreaks have occurred in the past or may be anticipated in the future and what laboratory resources would be required should similar outbreaks occur. Laboratory staff should also anticipate the extra costs associated with outbreak investigations so that they can work with hospital administrators to include funds for these efforts in annual budgets. Costs should not be borne by the laboratory or charged to individual patients involved in the outbreak.

Some problems and potential pitfalls of outbreak investigation are pertinent to the clinical microbiology laboratory and bear specific mention. Foremost among these is the problem of determining when to proceed with an outbreak investigation in the first place. The number of cases necessary to constitute an outbreak depends upon the organism, the patient population, and the institution involved. For example, while numerous cases of *Escherichia coli* urinary tract infection in a long-term-care facility may not constitute an outbreak, even a single nosocomial case of group A streptococcal surgical wound infection or VRSA infection merits an outbreak investigation. Laboratories should consider instituting a computerized program that recognizes clusters of pathogens within the hospital. Organisms that appear to be part of a cluster could be further characterized to evaluate whether they are genetically related (see chapter 11), which would suggest patient-to-patient spread or exposure to a contaminated common source. Investigators at Northwestern University hospital implemented such a system and noted that their rates of nosocomial infections decreased in temporal association with this intervention (33).

A second important problem is that of a pseudo-outbreak. A pseudo-outbreak has occurred when an apparent outbreak turns out not to be an outbreak after all. The usual cause of a pseudo-outbreak is either misdiagnosis (e.g., infection has not actually occurred) or misinterpretation of epidemiologic data (e.g., infections have occurred, but clustering or epidemic transmission has not). The microbiology laboratory can be the source of pseudo-outbreaks (1, 28, 37, 45, 47, 66, 72, 83). Problems in the laboratory that lead to pseudo-outbreaks include contamination of reagents for stains (37), false AST results (28), and contamination of culture specimens (often from construction or renovation projects [47] or cross-contamination during specimen processing [83]). Careful attention to quality control, use of sterile techniques for specimen processing, and preventive measures during construction and renovation projects can decrease the likelihood of pseudo-outbreaks that originate in the laboratory.

Molecular Typing To Support Infection Control Activities

Outbreaks of nosocomial infection often result when a number of hospitalized patients are exposed to a contaminated common source or a reservoir of a pathogenic agent (e.g., water from a hot water tank colonized with *Legionella* spp.). The organisms causing such outbreaks would all derive from a single strain (i.e., they are clonally related). The infection control program team may, therefore, request that the microbiology laboratory characterize isolates that may be associated with outbreaks to determine whether they are genetically related. In the appropriate clinical setting, species-level identification and AST results (antibiogram) may provide strong evidence for an epidemiologic link. However, more sensitive methods of strain delineation are often necessary. In this setting, genotypic or DNA-based typing methods have essentially replaced phenotypic typing methods (e.g., AST, biochemical profiles, and bacteriophage susceptibility patterns), which discriminate poorly among isolates (52, 63, 74).

Genotypic typing methods provide meaningful data and are cost-effective only when they are used for well-defined epidemiologic objectives. These objectives include (i) determining the source and extent of an outbreak, (ii) determining the mode of transmission of a nosocomial pathogen, (iii) evaluating the efficacy of preventative measures, and (iv) monitoring transmission of pathogens in high-risk areas (e.g., ICUs), where cross-infection is a recognized hazard.

The ideal genotypic typing system should be standardized, reproducible, stable, sensitive, broadly applicable, readily available, and inexpensive. The typing method should also have proven value in previous epidemiologic investigations. Further discussion of the relative advantages and disadvantages of the many available typing systems is beyond the scope of this chapter and has been summarized in several reviews (52, 63, 69, 74) and in chapter 11 of this text.

Organism Storage

Of course, the laboratory cannot provide the infection control program with supplemental testing such as molecular typing if the appropriate isolates have not been saved. The laboratory should plan ahead and be sure to save all epidemiologically important isolates (see chapter 6). Laboratory and infection control personnel should decide which isolates should be banked and how long they should be stored based upon their epidemiological importance and the available resources. We recommend that all isolates from normally sterile sites (e.g., blood and cerebrospinal fluid), important antibiotic-resistant organisms (MRSA, VRE, and ESBL-producing *Enterobacteriaceae*) from any site, and other epidemiologically important pathogens (e.g., *Mycobacterium tuberculosis*) be saved for a period of 3 to 5 years.

Cultures of Specimens from Hospital Personnel and the Environment

The laboratory should perform cultures of specimens from hospital personnel and the environment (surfaces, air, and water) rarely and only when the epidemiologic evidence suggests that personnel or the environment was associated with transmission of a nosocomial pathogen. Various potential sources and appropriate culture methods are outlined in Table 5. Although infection control staff frequently consider obtaining such cultures, these cultures are labor-intensive, nonstandardized, and difficult to interpret and rarely provide useful information.

Because health care workers' hands can transmit nosocomial pathogens from patient to patient, hand cultures are sometimes useful in confirming the mechanism of cross-infection during an outbreak investigation (79). Similarly, because the anterior nares represent the usual reservoir for *S. aureus* (including MRSA) colonization in humans (43), nares cultures from patients and health care personnel are sometimes appropriate during an *S. aureus* infection outbreak.

Laboratory and infection control personnel should weigh two important factors before deciding to culture specimens from hospital personnel during an outbreak investigation: (i) finding the outbreak strain on the hands or in the nares of a health care worker does not establish the direction of transmission or definitively implicate a health care worker as the source or reservoir for the outbreak, and (ii) culturing specimens from hospital personnel indiscriminately can lead

TABLE 5 Cultures of personnel or environmental sources of infection in the hospital[a,b]

Source	Culture method	Comment
Blood products	Broth culture incubated aerobically and anaerobically at 30–32°C for 10 days	Following transfusion reaction; obtain simultaneous blood cultures by venipuncture
Environmental surfaces	Swab-rinse or impression plate	No evidence that any particular level of contamination correlates with nosocomial infection
Disinfectants and antiseptics	Plating of serial dilutions of the product with and without specific neutralizers	Organisms usually nonfermenting gram-negative aerobic bacilli
Air	Mechanical air sampler (preferred); settling plates (poor)	No uniform agreement on acceptable levels of contamination; lack of correlation with infection
Water (for *Legionella* spp.)	Membrane filter for water samples, swab of faucets and showerheads	Number of sites positive for *Legionella* spp. may correlate with risk for nosocomial cases; culture after confirmed case of nosocomial legionellosis; no consensus on performance of routine water cultures for *Legionella* (see text)
Hands of personnel	Broth-bag: 10–20 ml nutrient broth in sterile plastic bag; wash hands in broth and plate semiquantitatively	May confirm the mechanism of cross-infection; impress the importance of hand washing
Anterior nares of personnel	Swab culture	Carriage of outbreak strain may be eradicated by application of topical agent (e.g., mupirocin for *S. aureus*); recolonization with the same strain is frequent

[a] Cultures to be performed only if clearly indicated by epidemiologic data.
[b] Adapted from references 23 and 57, with permission.

to confusing results and can generate ill will toward the infection control program. In general, only specimens from health care workers epidemiologically linked to cases should be cultured. We recommend that infection control programs obtain cultures of specimens from hospital personnel only after consulting with a hospital epidemiologist experienced in outbreak investigation.

As a general rule, routine cultures of specimens from hospital personnel and the environment should not be performed. Exceptions include routine monitoring of sterilized items, infant formula, products prepared in the hospital, blood components (e.g., platelets) prepared in an open system, and hemodialysis fluid. The CDC and other experts have not reached a consensus about the utility of routine water cultures for the presence of *Legionella* spp. While the CDC suggests that such culturing may be an important aspect of preventing nosocomial legionellosis among very high risk patients (e.g., transplant recipients), the CDC has not made a recommendation about obtaining routine water cultures in health care settings other than transplant units if cases of nosocomial legionellosis have not been identified (13, 15). A complete discussion of this issue is beyond the scope of this chapter but can be found in the CDC guidelines (13, 15) and in a recent review by O'Neill and Humphreys (53).

Routine surveillance cultures should not be obtained from the following persons or items because the cost is high and the cultures rarely provide useful clinical or epidemiologic information: patients and hospital personnel, commercial patient care items, antiseptics and disinfectants that are in use, random blood units, respiratory therapy equipment, peritoneal dialysate, and air. Routine cultures from these sources are a burden to the laboratory and seldom, if ever, provide useful information or lead to specific interventions (13).

CONCLUSION

The clinical microbiology laboratory is an essential component of any effective infection control program. The development and application of new technologies in the clinical laboratory can greatly enhance infection control efforts. A good working relationship between clinical laboratory and infection control personnel will greatly facilitate the investigation and control of health care-associated infections.

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Laboratory Procedures for the Epidemiological Analysis of Microorganisms

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11

In dealing with an infection, one often is faced with the need for species identification in order to prescribe effective treatment. In some clinical cases, however, one must pursue the identity of the infecting organism to the subspecies level. The typing techniques that have evolved for such discrimination at both of these levels of relatedness must be as rapid as possible. These techniques, therefore, usually focus on phenotypic characteristics and rarely provide the resolution necessary to obtain measurements of genetic relatedness among isolates of the same species or the same subspecies. Such resolution is essential for a number of epidemiological questions. In order to accurately identify the origin of a nosocomial infection, track the transmission of a disease, the emergence of a new hypervirulent or drug-resistant strain, or the microevolution of a commensal or infecting strain, and examine the general population structure of a pathogen, one must move from species typing to strain and substrain typing. Methods must be selected that provide information on the level of genetic relatedness. Methods have evolved that indeed provide such information, but researchers rarely validate the method they select for characterizing relatedness (135) or ask if the method they have selected has the resolving power for the question posed. Their results may, therefore, suggest stability when in fact the infecting organism is undergoing rapid microevolution. Alternatively, researchers may ask a question related to strain grouping that requires the genesis of deep-rooted dendrograms and then apply a method that relies mainly on hypervariable changes. Again, their results may not provide them with a valid answer, in this case because hypervariable changes will affect clustering due to homoplasy, which is the presence of identical characteristics in distinct phylogenetic lineages that are acquired not through descent, but rather through convergence, parallelism, or reversion. It is, therefore, imperative that one accurately formulate the question to be answered, define the level of genetic relatedness that must be assessed, and select a genetic fingerprinting method with the resolution necessary to answer that question (135).

BIOTYPING AND GENETIC FINGERPRINTING TECHNIQUES

Prior to the development of DNA-based techniques for assessing genetic relatedness, scientists relied on biotyping techniques, which measure phenotypic differences. The logic behind this approach was that phenotype reflects genotype, and so if one employs a number of phenotypic parameters, one can obtain a measure of genetic relatedness. In discriminating between genuses and species in both bacteria and fungi, biotyping still provides us with fast and reliable diagnostic methods. Kits measuring assimilation patterns (biochemical profiles) are still a mainstay for discriminating among a variety of bacterial and fungal species (25, 76, 78, 82, 106, 139, 163), antibody-based tests (serotyping) continue to be used to discriminate among groups within bacterial species and fungi like *Cryptococcus neoformans* (7, 132, 138, 174), and phage typing continues to be used to discriminate among groups within bacterial species (9, 70). However, these types of methods in general do not provide the kind of genetic discrimination usually necessary for addressing epidemiological questions. First, most of these tests do not provide enough unrelated parameters to obtain a good reflection of genotype. Usually they discriminate among only a limited number of groups, as in the case of serotyping. Second, and more important, the expression of many genes is affected by environmental changes and by developmental programs or reversible phenotypic switching (11, 54, 134, 143). In addition, phage and plasmids can be transmitted horizontally (23, 173). Most biotyping methods, therefore, fall short as genetic fingerprinting techniques. There is, however, one major exception, multilocus enzyme electrophoresis (MLEE) (105, 115). MLEE represents a robust genetic fingerprinting method that exhibits performance parity with many of the most effective DNA fingerprinting methods (111, 156).

DNA fingerprinting techniques, by virtue of the fact that they assess differences in genetic material, have been assumed to be the most accurate methods for genetic fingerprinting. This is not necessarily the case (135). As noted, some are as effective as MLEE and others fall short. Some are poor indicators of microevolution, and others are good indicators of microevolution but measure DNA sequences that are too hypervariable for deep-rooted cluster analyses (111). Some techniques are effective for analyzing bacteria but less effective for analyzing eukaryotes, and vice versa, because of inherent genomic differences. Finally, while some DNA fingerprinting techniques are excellent for cluster analyses of large collections of isolates, they are not favored by evolutionary biologists because they do not provide

codominant markers (150), although this requirement has been challenged quite effectively (154, 155).

What, then, are the general methods of DNA fingerprinting? The methods that will be reviewed in this chapter have, for the most part, been used for bacteria, fungi, and parasites. One of the earliest methods applied to bacteria and fungi took advantage of the fact that in the divergence of strains within a species, restriction sites identified by restriction enzymes, or endonucleases, change, leading to changes in the lengths of DNA sequences between sites. These changes accumulate as strains diverge during evolution, and the sum of the changes provides an indicator of evolutionary distance. The pattern of restriction fragments is referred to as the restriction fragment length polymorphism (RFLP) pattern. The comparison of the RFLP patterns between different isolates is referred to as RFLP analysis. This method has proved to be effective in DNA fingerprinting of bacteria, especially with the use of infrequent cutters in combination with pulsed-field gel electrophoresis (PFGE) (151), but less effective in fingerprinting of eukaryotic pathogens like the infectious fungi (10, 123). To obtain a more limited and more specific pattern, Southern blots can be hybridized with a DNA probe. Although this method is also referred to as RFLP analysis in the literature, we will distinguish it by calling it RFLP with a probe. The probes in this case must distinguish more than one fragment and, therefore, usually contain a repetitive sequence (124, 141, 145) or a combination of unique and repetitive sequences (41, 72, 91). The former are referred to as repetitive-element probes, and the latter are referred to as complex probes (135). A second approach to DNA fingerprinting takes advantage of PCR to amplify a variety of sequence fragments from the genome by utilizing sequences identified by the primers used in the PCR. The use of arbitrary primers for amplification has been referred to as random amplification of polymorphic DNA (RAPD) analysis (165, 167). This method has been used for DNA fingerprinting of a number of prokaryotic and eukaryotic pathogens (35, 77, 109, 156, 165). In recent years, PCR-based methods have been developed that employ primers that recognize identified sequences and in some cases these methods have been used in combination with RFLP analysis. Some of these methods are preferred by evolutionary biologists since the data they generate represent identified alleles (150). A third DNA fingerprinting method that has evolved in the last decade is based on PFGE methods for separating chromosome-sized DNA fragments (26, 126). This method has been successfully used to karyotype many fungi (14, 36, 79) and other eukaryotic microorganisms (69, 133) and, in the case of prokaryotes, to separate large fragments generated by endonucleases that identify infrequent restriction sites (3, 15, 35). Finally, sequencing of portions of one or several genes is a basic tool for answering evolutionary questions and a rapidly emerging tool for assessing epidemiological questions (42, 75, 95). Multilocus sequence typing (MLST) (95) has evolved rapidly in the past 5 years for DNA fingerprinting of both bacteria and lower eukaryotes (20, 37, 46, 149, 157). Finally, microarray typing based on probing oligonucleotides representing alleles of loci with PCR products provides a very new and potentially powerful method of discrimination (51, 56, 86, 140). Since MLEE and the variety of DNA fingerprinting methods outlined above are all tools for epidemiological studies of the genetic relatedness of isolates, we will refer to them as genetic fingerprinting methods.

GENERAL REQUIREMENTS OF AN EFFECTIVE GENETIC FINGERPRINTING METHOD

Before considering in detail each DNA fingerprinting method, a consideration of the general requirements of fingerprinting methods is in order. Although a list of requirements will be formulated, it should be realized that each epidemiological question posed will require different levels of resolution or stringency.

A Method Should Provide Data That Reflect Genetic Distance at the Level Necessary for Answering the Question Posed

An effective genetic fingerprinting system should do more than demonstrate that two isolates are nonidentical or that a collection of isolates differ. In many cases, a researcher must know how unrelated (or related) two or more isolates are. If one does not know the resolving power of the genetic fingerprinting method applied, one may conclude that because there are differences in the fingerprinting data, two isolates are unrelated or that because the fingerprinting patterns are identical, the isolates represent the same strain. However, if in the former case the method identifies hypervariable changes in the genome that can occur as frequently as 1 in every 200 cell divisions, the isolates may in fact be highly related, and in the latter case, if the method measures only rare changes, the isolates may in fact be quite unrelated. With this in mind, what are the levels of relatedness that we must consider? Because few methods provide a true measure of genetic relatedness, with some functioning better at discriminating one level than another, and since evolutionary time can only be estimated, we will categorize the levels rather than consider them as a continuum. The categories, diagrammed in Fig. 1, are "identical," "highly related but nonidentical," "moderately related," and "unrelated." The utility of using these categories will become evident in considering the effectiveness of the separate methods.

A Genetic Fingerprinting Method Should Be Resistant to Environmental Perturbations and High-Frequency Genomic Reorganization

As in the case of MLEE, the targeted sequences for a DNA fingerprinting method must be carefully selected. For instance, plasmid DNA and minichromosomal DNA may in

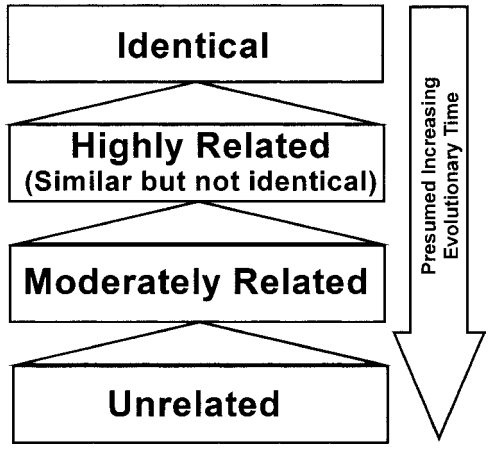


FIGURE 1 Categories of genetic relatedness for isolates within a species.

some cases be bad choices since growth rate and cellular phenotype may affect the maintenance of the genetic markers and the rate of reorganization (22, 27). Sequences involved in phase transitions in such bacteria as *Salmonella enterica* serovar Typhimurium and *Escherichia coli* undergo reversible transitions (61), as is also the case for the expressed mating type locus in *Saccharomyces cerevisiae* (65). In addition, some genomic reorganization events are repressed by silencing genes (58) and derepressed in mutants (63) and the frequencies of other reorganizational events are affected by the expressed phase in phenotypic switching systems (113).

In Most Cases, a Genetic Fingerprinting Method Should Be Fast, Feasible, Affordable, and Amenable to Computer-Assisted Analysis

In many instances, a genetic fingerprinting study involves few isolates and limited goals. If a study, however, involves large numbers of isolates requiring complex measurements of relatedness, and if retrospective use of the data in the future is entertained, one must select a method amenable to automatic computer-assisted analysis and storage. One must also select a method that is within one's technical abilities, that is affordable, that can be accomplished in a reasonable amount of time, and that will answer the question posed.

COMMON GENETIC FINGERPRINTING METHODS

There are several basic genetic fingerprinting methods that have been used repeatedly for prokaryotic and eukaryotic pathogens to answer a variety of epidemiological questions. In the sections that follow, the most commonly used methods are described and evaluated.

Multilocus Enzyme Electrophoresis

It is immediately obvious that a method based on enzyme electrophoresis is not DNA based. However, MLEE fulfills the requirements set forth for DNA-based fingerprinting systems (135) and has been demonstrated to attain resolution at all of the levels categorized in Fig. 1 (105, 111, 115, 156). The MLEE method relies on phenotypic polymorphisms, but these polymorphisms are rooted in protein structures that are reflections of the sequences of the structural genes that encode them. The MLEE method resolves polymorphisms through differences in the electrophoretic mobilities of the gene products of different alleles of the same gene. Electrophoretic mobility depends primarily on the net charge of the protein, which is a consequence of the primary protein structure but which is also influenced by secondary, tertiary, and quaternary structures. The latter levels of structure can conceal or reveal charged amino acid residues that contribute to the total charge of a protein and therefore to its exact electrophoretic mobility. In addition, MLEE detects polymorphisms only within the coding region of a gene. Even in coding regions, many mutations do not cause a polymorphism. It is, therefore, difficult to estimate what proportion of base changes leading to amino acid substitutions or other mutations in a protein is detected by the MLEE method, but it is generally accepted that only approximately 15% of the amino acid changes in an average protein can be resolved by MLEE (115). This level of sensitivity is sufficient for assessing the various levels of genetic relationships outlined in Fig. 1.

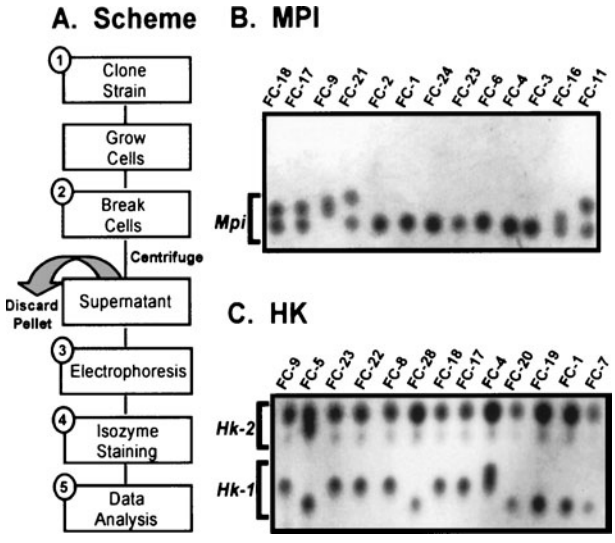


FIGURE 2 MLEE. (A) Generic version of the steps in the MLEE method. (B and C) Examples of enzyme phenotypes using starch gel electrophoresis for mannose-6-phosphate isomerase (MPI) (B) and hexokinase (HK) (C) in 13 *C. albicans* isolates (111). Note that there are two different HK genes, *Hk-1* and *Hk-2*.

In Fig. 2A, a generic version of the MLEE method is presented.

Preparation of Cells

One must begin with a clone. Primary isolates must be cloned, and each clone must be individually grown for protein extraction. For most infectious microorganisms, homogeneous cell populations can be grown in culture to the levels necessary for standard assays. Precaution must be taken, however, in the growth of the different isolates in a test set. Growth conditions for isolates must be uniform, which is not as stringent a requirement for DNA-based methods. If some isolates grow at different rates, reach stationary phase at different times, or express different general phenotypes under the same culture conditions, there may be effects on the isozyme patterns that would not represent changes in DNA sequence.

Preparation of Protein Extracts

Cells are harvested (e.g., pelleted by centrifugation) and washed (e.g., with distilled water). The cell sample (e.g., 200 μl of wet cells) is mixed with glass beads (e.g., 200 μl) and distilled water (e.g., 200 μl) in a 1.5-ml microcentrifuge tube and vortexed. The extraction solution must be appropriate for the maintenance of enzyme activity. Distilled water is in many cases appropriate for large, concentrated biological samples. In some cases, proteinase inhibitors must be employed. Bead friction during vortexing will produce heat that can denature proteins. To avoid this, cooling intervals (e.g., placing the tube in ice water) are interspersed with vortexing pulses. A number of different lysis methods are available, each with different attributes. Immediately after disruption, samples are centrifuged to remove insoluble materials. The supernatant containing the soluble enzymes of interest can then be divided into small aliquots (e.g., 50-μl aliquots) and immediately used or stored at ⩽20°C. Enzymes must be selected that retain activity through the extraction procedure.

Electrophoresis

Electrophoretic methods employing different supporting gels have been used in MLEE. These include methods utilizing cellulose acetate, agarose, starch, and polyacrylamide. In the case of cellulose acetate, migration of proteins occurs on the surfaces of gels, in the film created by the buffer. Proteins migrate as a function of their electrophoretic mobilities alone. In the cases of both starch and agarose, the supporting gels are made up of a network of constantly sized pores, large enough for the migration of most proteins without retardation due to friction. In both cases, proteins migrate as a function of their electrophoretic mobilities alone. In the case of polyacrylamide gels, it is possible to regulate pore size by changing the acrylamide/bisacrylamide ratio. Therefore, polyacrylamide gels can be used to separate proteins based both on electrophoretic mobility and on size or conformation. In specific cases, one of these four methods may be more effective in separating a particular set of isozymes than another. Urea and sodium dodecyl sulfate (SDS), used in denaturation gels, can never be included in the electrophoresis procedure. The electrophoretic temperature usually must be maintained at close to 4°C in order to avoid thermal denaturation of the protein. The composition of the electrophoresis buffer must be optimized empirically by the enzymes selected for analysis. Buffer characteristics that affect both activity and separation include pH, ionic strength, and the specific concentrations of cations and anions.

Staining of Enzymes for Visualization of Specific Activities

Once enzymes are separated, enzyme positions must be visualized. This is achieved by using the specific activities of the isozymes. This basic approach requires that the mutations that affect mobility do not affect enzyme activity. In visualizing enzyme activity, one of the enzyme products is usually stained by a second reaction. Examples of stained isozymes are presented in Fig. 2B and C.

Application and Analysis

If the patterns of a single set of isozymes for a particular gene are used to assess genetic relatedness, the information level is too meager. For a haploid organism, one band is obtained per sample, and for diploids, one or two bands are usually obtained. Electrophoretic patterns may be more complex if the enzyme is active in multimeric forms, but this does not increase the information that can be used to assess genetic relatedness. In the patterns generated by MLEE (Fig. 2B and C), the alleles are codominant, which means that for heterozygous loci in diploid organisms, both alleles are expressed. Electrophoretic conditions should, therefore, be developed that provide the best separation of the different isozymes of each gene. For monomeric enzymes (composed of a single polypeptide), a homozygote will exhibit one band and a heterozygote will exhibit two bands. For a dimeric enzyme (composed of two polypeptides) in diploid organisms, heterozygotes could show three bands. Similarly, a heterozygote for a tetrameric enzyme should show up to five bands. Such patterns are sometimes difficult to resolve. On rare occasions, enzymes are composed of two or more polypeptides encoded by independent genes, and even greater complications then arise. To generate complex enough data to assess relatedness at all levels (Fig. 1), several genes must be analyzed. On average, 10 to 20 genes should make up the data set for each microorganism (see, e.g., Table 1). The selected genes must exhibit variability between independent isolates. Therefore, for each organism, a set of enzymes must be established empirically. For well-studied organisms, this job usually has already been performed, so scrutiny of the literature should provide one with a list of enzymes and references to the chemical reactions for visualization. The general approach for selecting enzymes was recently described by Pujol et al. (110, 111) in developing an MLEE method for *Candida albicans*. In two independent studies, 21 enzyme loci were tested with collections of 55 and 29 isolates of *C. albicans*. In both studies, 13 loci (62%) exhibited variability among the test strains and their products were selected as the set of analytical enzymes. In MLEE studies of bacterial collections, 9 polymorphic enzymes were used to analyze *Campylobacter jejuni* isolates (103) and 16 were used to analyze the genetic diversity of *Helicobacter pylori* (66). In MLEE studies of fungal collections, 7 polymorphic enzymes were used to analyze *Aspergillus fumigatus* (17) and 12 were used to analyze *Cryptococcus neoformans* (16). In MLEE studies of parasite collections, 22 polymorphic enzyme loci were used to analyze *Trypanosoma cruzi* isolates (12) and 16 were used to analyze *Leishmania* spp. (8). Examples of sets of enzymes used effectively in an MLEE study of a bacterium (13), a fungus (111), and a parasite (2) are presented in Table 1.

The result of an MLEE analysis involving, for example, 10 analyzed genes is a phenotype composed of 10 sets of values for each isolate. Each analyzed test isolate must then be compared with every other isolate at every locus in order to obtain a summed similarity coefficient. The method for doing this will be dealt with later in this chapter.

Restriction Fragment Length Polymorphism without Hybridization

As previously noted, one of the earliest methods for DNA fingerprinting of infectious microorganisms was restriction enzyme analysis, more commonly referred to as RFLP analysis. RFLP has been useful in answering limited epidemiological questions posed for a number of lower eukaryotic pathogens, but because of the composition of eukaryotic genomes, RFLP as presently applied presents limitations as a DNA fingerprinting method. For this reason, the application of RFLP to eukaryotic microorganisms will first be described and discussed and a discussion of its application to bacteria, which has been far more effective, will follow separately. The basic method of RFLP has also been incorporated into a number of additional, more complex fingerprinting methods, usually involving repetitive-hybridization probes.

Use of RFLP To Analyze Lower Eukaryotic Pathogens

The general method of RFLP analysis is relatively straightforward (Fig. 3A). Total cellular DNA is extracted from cells, digested usually with one endonuclease, separated by agarose gel electrophoresis, and stained usually with ethidium bromide. The final banding pattern represents differences in the sizes of the digestion fragments. Differences between patterns are assumed to represent differences in genetic relatedness. Fragment polymorphisms are determined by the positions of restriction sites identified by the particular endonuclease(s) employed. Differences in the banding patterns of two isolates of the same species are due to differences in fragment sizes that can occur as a result of changes in restriction site sequences, secondary modifications of restriction sites, deletion or insertion of restriction sites, or deletion or insertion of sequences between

TABLE 1 Examples of enzyme sets used to perform MLEE analyses of an infectious bacterium, an infectious fungus, and a protozoan parasite

Enzyme sets used for analysis of:		
Neisseria meningitidis[a,d]	*Candida albicans*[b,d]	*Plasmodium falciparum*[c,d]
ADH (EC 1.1.1.1)	ADH (EC 1.1.1.1)	LDH (EC 1.1.1.27)
ME (EC 1.1.1.40)	SDH (EC 1.1.1.14)	IDH (EC 1.1.1.42)
IDH (EC 1.1.1.42)	MDH (EC 1.1.1.37)	6PGD (EC 1.1.1.44)
G6PD (EC 1.1.1.49)	IDH (EC 1.1.1.42)	NAD-GDH (EC 1.4.1.2)
UDH (EC 1.1.1)	G6PD (EC 1.1.1.49)	GSR (EC 1.6.4.2)
NAD-GDH (EC 1.4.1.2)	SOD (EC 1.15.1.1)	HK (EC 2.7.1.1)
NADP-GDH (EC 1.4.1.4)	AAT (EC 2.6.1.1)	NH-i (EC 3.2.2)
IPO (EC 1.9.3.1)	HK (EC 2.7.1.1)	LAP (EC 3.4.11.1)
AK (EC 2.7.4.3)	PK (EC 2.7.1.40)	PEP1 (EC 3.4.11; Leu-Leu-Leu)
AKP (EC 3.1.3.1)	EST (EC 3.1.1.1)	PEP2 (EC 3.4.11; Leu-Ala)
PEP (EC 3.4)	LAP (EC 3.4.11.1)	ADA (EC 3.5.4.4)
ACO (EC 4.2.1.3)	PEP1 (EC 3.4.13.18; Val-Leu)	GPI (5.3.1.9)
FUM (EC 4.2.1.2)	PEP2 (EC 3.4.11.4; Leu-Gly-Gly)	
	PEP3 (EC 3.4.13.9; Phe-Pro)	
	MPI (EC 3.5.1.8)	
	ALD (EC 4.1.2.13)	
	FUM (4.2.1.2)	
	GPI (EC 5.3.1.9)	
	PGM (EC 5.4.2.2)	

[a] Data from Bart et al. (13).
[b] Data from Pujol et al. (111).
[c] Data from Abderrazak et al. (2).
[d] Enzyme activities are indicated (with Enzyme Commission numbers). ADH, alcohol dehydrogenase; ME, malic enzyme; IDH, isocitrate dehydrogenase; G6PD, glucose-6-phosphate dehydrogenase; UDH, unidentified dehydrogenase; NAD-GDH, glutamate dehydrogenase (NAD^+ dependent); NADP-GDH, glutamate dehydrogenase ($NADP^+$ dependent); IPO, indophenol oxydase; AK, adenylate kinase; AKP, alkaline phosphatase; PEP, peptidase (the substrates are indicated for the different peptidases used with *C. albicans* and *P. falciparum*); ACO, aconitase; FUM, fumarase; SDH, sorbitol dehydrogenase; MDH, malate dehydrogenase; SOD, superoxide dismutase; AAT, aspartate aminotransferase; HK, hexokinase; PK, pyruvate kinase; EST, esterase; LAP, leucine aminopeptidase; MPI, mannose-6-phosphate isomerase; ALD, aldolase; GPI, glucose-6-phosphate isomerase; PGM, phosphoglucomutase; LDH, lactate dehydrogenase; 6PGD, 6-phosphogluconate dehydrogenase; GSR, glutathione reductase; NH-i, nucleoside hydrolase (substrate inosine); ADA, adenosine deaminase.

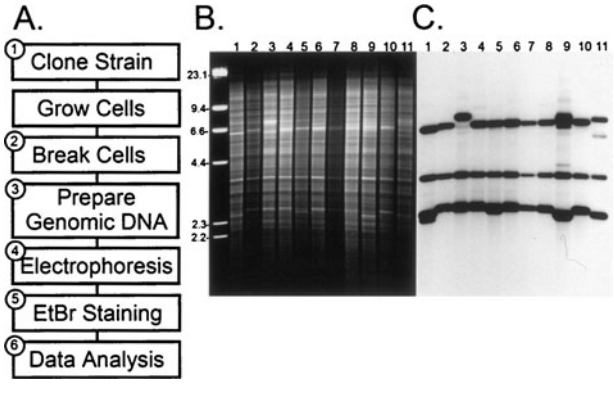


FIGURE 3 RFLP. (A) Generic version of the steps in the RFLP method as it is commonly applied to lower eukaryotic pathogens. EtBr, ethidium bromide. (B) Example of an ethidium bromide-stained gel of EcoRI-digested DNA from 10 test isolates of *C. albicans* (lanes 2 to 11) and a control isolate (lane 1). The first lane, not numbered, contains molecular mass standards. Molecular sizes (in kilobases) are indicated to the left. (C) The gel in panel B Southern blotted and hybridized with a ribosomal probe that identifies 28, 17, and 5S rRNA genes.

restriction sites. Therefore, selection of the most effective endonuclease for generating a pattern must be empirical. A good example of an RFLP pattern for isolates of the yeast pathogen *C. albicans* is presented in Fig. 3B. One should immediately recognize first that not all bands are easily resolvable. Because of the complexity of the eukaryotic genome, the number of digestion fragments is greater than that in prokaryotes. This reduces resolution between bands in an agarose gel and leads to the normally smeared nature of an average RFLP profile. The dominant bands are resolvable, but unfortunately, these bands represent primarily the repetitive ribosomal cistrons and to a lesser extent mitochondrial DNA sequences. Unlike those of prokaryotes, the ribosomal cistrons of eukaryotes are clustered on one or two chromosomes (62, 128). The cistrons are in tandem with interspersed spacer regions and are structurally quite homogeneous. Therefore, endonuclease digestion that results in a complex RFLP pattern results in a relatively simple rRNA gene pattern. To demonstrate this point, the ethidium bromide-stained gel with *C. albicans* whole-cell DNA in Fig. 3B was destained, Southern blotted, and hybridized with an rRNA gene probe containing the high-molecular-mass (28S), low-molecular-mass (17S), and 5S rRNA gene sequences (Fig. 3C). Only three intense bands and a few minor bands were resolved with a reasonable degree of resolution in each pattern. The number and variability of the major RFLP bands

among independent *C. albicans* isolates, therefore, do not provide enough complexity for resolving differences between moderately or highly related isolates and in some cases may not even be able to distinguish between unrelated isolates. In contrast, the complexity of the pattern of unique-sequence DNA, which should be great enough for assessing genetic distance, is blurred by the congestion of bands in a normal gel. Even so, the use of RFLP patterns for distinguishing between unrelated isolates or identifying different isolates of the same strains of eukaryotic pathogens continues and can sometimes be reasonably effective, if one does not demand resolution at the levels of moderate relatedness (Fig. 1).

In Fig. 3A, a generic version of the RFLP method (121) as applied to eukaryotic pathogens is presented. The major steps are dealt with individually below.

Preparation of Cells

As in the case of the MLEE method, one must begin with a clone. Each clone must be individually grown for DNA extraction. In contrast to MLEE, however, RFLP analysis does not require one to worry about uniform growth conditions since genotypes should be stable under different conditions.

Preparation of DNA

Many lower eukaryotic pathogens are encased in cell walls, and the walls must be removed prior to cell lysis. Here, a general protocol is described for infectious yeast. Cells from agar cultures are grown to stationary phase in a rich liquid growth medium. Cells are pelleted, washed, and resuspended in medium that removes cell walls and osmotically maintains the integrity of the resulting spheroplasts (cells that have had their walls removed). For generation of spheroplasts, cells are first suspended in the medium SPP, containing 1 M sorbitol and 50 mM potassium phosphate, pH 7.4. To remove the cell wall, a variety of enzymes are used, depending on the nature of the wall. To remove yeast cell walls, the snail enzyme Zymolyase can be used. For *C. albicans*, which has a very tough wall, 15 μl of a solution containing 100 mg of Zymolyase 20 T (Seikagaku America, Ijamsville, Md.) in 800 μl of 50 mM sodium phosphate (pH 6.5)–50% glycerol is added to 0.7 ml of SPP containing 10^8 cells. Cells are then incubated in suspension for 30 to 90 min at 37°C. The time for wall removal varies, even between strains of the same species. A preparation is assessed microscopically for spheroplast formation and is usually terminated when the proportion of spheroplasts in the population is >80%. The percentage of spheroplasts can be assessed by adding 1 μl of 10% KOH to a droplet of spheroplasts on a microscope slide and counting the proportion of lysed cells. At the time of 80% lysis, the spheroplasts are pelleted at 2,500 × *g* for 10 min at room temperature, washed in SPP, and finally resuspended in 500 μl of a lysis buffer containing 50 mM Tris-HCl, pH 7.4, and 20 mM EDTA. Fifty microliters of 10% SDS is added to the preparation, which is then incubated for 30 min at 65°C. Two hundred microliters of 5 M potassium acetate is then added, and the preparation is incubated in an ice bath for 1 h. The lysate is centrifuged at 10,000 × *g* for 10 min at 4°C, and the supernatant, containing DNA, is extracted with an equal volume of a 1:1 solution of phenol-chloroform. The DNA in the supernatant is precipitated with an equal volume of cold isopropanol. The DNA is then washed twice with 750 μl of 75% ethanol, dried, and resuspended in 100 μl of a solution containing 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA. Contaminating RNA can be removed by adding 2 μl of a 10-mg/ml solution of RNase A and incubating for 1 h at 37°C. This solution is again extracted with phenol-chloroform and precipitated, as in earlier steps. The final precipitate is then resuspended in 100 μl of the Tris-HCl–EDTA solution and may be stored at 4°C. This solution should not be frozen.

Endonuclease Digestion

Three micrograms of DNA extracted from each test isolate is incubated in 25 μl of reaction buffer containing one or more selected endonucleases according to the manufacturer's instructions. The units of endonuclease should be threefold over the recommended value to ensure complete digestion, a necessary prerequisite for obtaining reproducible, well-separated RFLP patterns. Endonucleases, or restriction enzymes, identify precise sequences throughout the genome. For example, EcoRI identifies and cleaves (cuts) at sites with the sequence 5′GAATTC3′, BamHI cleaves at 5′GGATCC3′, and HinfI cleaves at 5′GANTC3′, where N is any nucleotide. Restriction sites vary in abundance. An infrequent cutter is an enzyme that identifies infrequent sites in a particular genome and is the basis for the RFLP method combined with PFGE that is frequently applied to prokaryotes. Two examples of infrequent cutters used in fungal studies are NotI, which cleaves sites with the sequence 5′GCGGCCGC3′, and SfiI, which cleaves sites with the sequence 5′GGCCNNNNNGGCC3′, where N is any nucleotide. In bacteria, SmaI is an infrequent cutter which cleaves sites with the sequence 5′CCCGGG3′. An infrequent cutter will generate a limited number of genomic fragments. These fragments may be too large to be separated by standard gel electrophoresis. On the other hand, some restriction sites are too common, resulting in patterns containing too many bands for good pattern resolution. It should be clear from this brief discussion that one must carefully choose the endonuclease or combination of endonucleases to be used in an RFLP analysis.

Electrophoresis

After digestion is complete and prior to electrophoresis, 3 μl of DNA loading dye (e.g., 40% Ficoll and 0.01% bromophenol blue) is added to each sample. The low-molecular-weight dye will migrate close to the front of the sample and, therefore, provide a measure of migration progress. Electrophoresis, in this case, is usually performed with agarose gels cast in and run with 1× TBE buffer, containing 89 mM Tris-HCl (pH 8.1), 89 mM H_3BO_3, and 2 mM EDTA. In a standard experiment, 3 μg of digested DNA is loaded into a well of a 13- by 24-cm gel containing 12 to 15 wells. A set of standards, DNA fragments of known sizes, should also be loaded onto each gel. The percent agarose used will depend upon the range of fragment sizes, which will in turn be a function of genome size and the selected restriction enzyme(s). For example, for the gel in Fig. 3B, digestion of *C. albicans* DNA with the restriction enzyme EcoRI generated a complex pattern separated in a 0.8% agarose gel. For these gel specifications, the dye front should migrate 16 cm from the loading well.

Staining

To visualize the banding patterns of RFLPs, the gel is soaked for 1 h in a solution of 0.2 μg of ethidium bromide/ml. The pattern is then viewed and photographed with a UV light source. It is suggested that a ruler be placed next to the gel when the gel is photographed so that migration distances can be assigned.

Analysis

The standard RFLP method for eukaryotic pathogens generates patterns that are sometimes difficult to compare. Because of band crowding, automatic computer-assisted analyses are difficult and too much information is lost due to the lack of discrimination. The positions of the major bands in RFLP patterns can be manually digitized into a database, but as noted above, the major bands represent primarily rRNA gene fragments. However, when RFLP studies involve small numbers of isolates, and all that is demanded from the data is an assessment of identity and nonidentity, qualitative interpretations can be sufficient. The problems of RFLP analyses of eukaryotic pathogens are resolved to some extent by applying hybridization probes, the subject of a later section of this chapter.

Use of RFLP To Analyze Prokaryotes

The bacterial genome is usually composed of a single DNA molecule (i.e., a single chromosome). These genomes can vary significantly in size and complexity, but in the majority of cases, bacterial genomes are less complex than lower eukaryotic genomes. One, therefore, may expect greater resolution in an RFLP pattern generated with a prokaryotic genome, but that is really not the case. When a frequent cutter is used, the ethidium bromide-stained pattern is still crowded. Standard RFLP analysis without probes has, therefore, not been a popular epidemiological tool for bacteria. Instead, bacterial epidemiologists have used infrequent cutters to generate a limited number of DNA fragments, which are then separated by an electrophoretic method customized for large fragments (33). In Fig. 4A, a generic version of this method is presented. A clone is grown to stationary phase in medium recommended for the particular species. In many cases, this represents an overnight growth culture. Cells are usually pelleted by centrifugation and resuspended in a medium customized for the species under analysis. For instance, in a procedure applied to *Staphylococcus aureus* (68), cells are washed in a solution containing 0.15 M NaCl and 10 mM EDTA, pH 8.0, and resuspended in a solution containing 1 M NaCl and 10 mM EDTA, pH 8.0. This cell suspension is then mixed with an equal volume of 1.2% low-melting-temperature agarose, and the mixture is allowed to solidify in 100-μl molds. The blocks are then incubated in lysis solution containing 1 M NaCl, 10 mM EDTA, 10 mM Tris-HCl (pH 8.0), and a lysis cocktail that includes 0.5% (wt/vol) Brij 58, 0.2% (wt/vol) deoxycholate, 0.5% (wt/vol) Sarkosyl, 1 mg of lysozyme/ml, and 4 mg of acromopeptidase/ml. The blocks are next incubated in a solution containing 0.25 M EDTA, 1% (wt/vol) Sarkosyl, and 0.1 mg of proteinase K/ml at 50°C, followed by a solution containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. Sections of blocks are then incubated with restriction enzyme and electrophoresed in a 0.9% agarose gel by using contour-clamped homogeneous electric field electrophoresis (29), the PFGE system of choice for this form of fingerprinting. Staining and data analysis are as described for RFLP analysis of lower eukaryotes. An example of the application of RFLP-PFGE to *Staphylococcus aureus* is presented in Fig. 4B. A standardized protocol for the refined preparation of DNA for PFGE analysis has been described by Chang and Chui (28) and should serve as a good starting point for individuals interested in an RFLP method for fingerprinting of bacteria. Chung et al. (30) give a protocol for PFGE analysis of *Staphylococcus aureus* with specific details on troubleshooting.

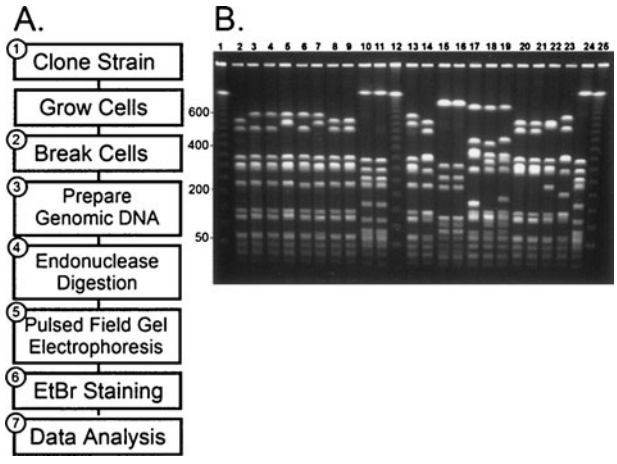


FIGURE 4 RFLP as it is commonly applied to prokaryotic pathogens, using PFGE to separate large DNA fragments. (A) Generic version of the steps in the RFLP-PFGE method. EtBr, ethidium bromide. (B) Example of RFLP-PFGE as it was applied to 22 *Staphylococcus aureus* isolates (provided by M. Pfaller, University of Iowa). Standards were run in lanes 1, 12, and 25. Molecular sizes are given in kilobases.

Restriction Fragment End Labeling

Restriction fragment end labeling was developed as a fingerprinting method for bacteria because of the compact size of the bacterial genome. In this method, total genomic DNA is digested with a restriction endonuclease and all of the fragments are end labeled using the Klenow fragment of DNA polymerase I. Following labeling, the fragments are separated by electrophoresis through a polyacrylamide-urea gel, such as what would be used for DNA sequencing. The number of bands will depend upon the species and isolates of the bacteria that will be analyzed and on the characteristics of the gel. van Steenbergen et al. (161) found that of the 11 species of bacteria that they analyzed, they could distinguish between 30 and 50 bands per isolate in the 100- to 400-bp size range. This method has been successfully applied to the molecular epidemiology of several bacterial species, including *Streptococcus pneumoniae* (19) and *Helicobacter pylori* (159).

Application of Hybridization Probes to RFLPs

Because hybridization probes allow visualization of a subset of fragments in an RFLP pattern, the resolution of the pattern increases over that with RFLP without a probe. The increase in resolution is evident in the comparison between the RFLP pattern (Fig. 3B) and the Southern blot pattern of the same gel hybridized with an rRNA gene probe (Fig. 3C). The success of this fingerprinting strategy has been mixed and depends upon the selected hybridization probe. Therefore, after a description of the general method, the caveats of the method will be reviewed. In Fig. 5A, a generic version of the RFLP method with a hybridization probe is presented.

Steps i to vi

Steps i through vi are identical to those already described for the RFLP method without a probe, and a description of them will not be repeated.

Steps vii and viii

To hybridize with a probe, the ethidium bromide-stained DNA is transferred onto a nitrocellulose or nylon membrane,

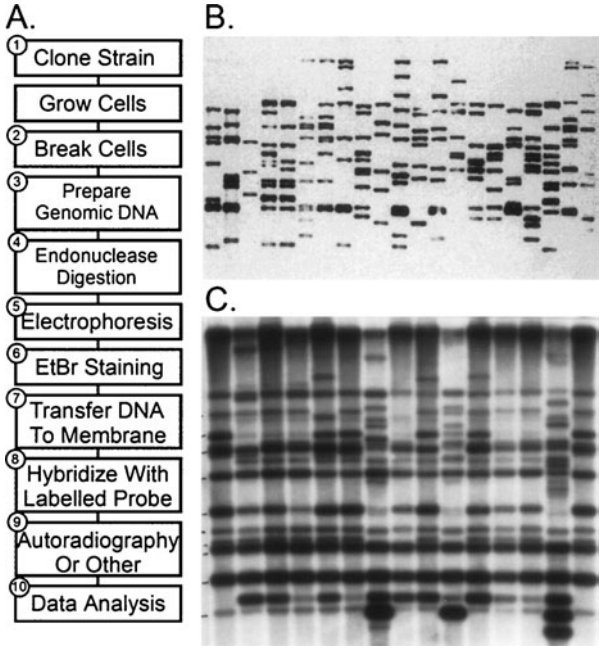


FIGURE 5 RFLP with probe. (A) Generic version of the steps in the RFLP-with-probe method. EtBr, ethidium bromide. (B) Example of RFLP with probe applied to a bacterium, in this case an IS probe applied to PvuII-digested *Mycobacterium tuberculosis* DNA (160). Molecular sizes are presented in kilobases to the left of the gel. (C) Example of RFLP with probe applied to a yeast, in this case the complex probe Ca3 applied to EcoRI-digested *C. albicans* DNA (S. Joly and D. R. Soll, unpublished results).

a process referred to as Southern blotting. The method for this process can be found in *Current Protocols in Molecular Biology*, volume 1 (6), or in *Molecular Cloning: a Laboratory Manual* (121). In brief, the gel is first treated with 0.25 M HCl. This step results in partial depurination and strand cleavage of DNA. The gel is rinsed in water and soaked in a solution containing 0.5 M NaOH and 1.5 M NaCl. This treatment denatures the DNA, a necessary step prior to hybridization. If a nitrocellulose membrane is used, the gel must then be neutralized to a pH below 9.0. The transfer to a membrane can then be achieved by a number of protocols, the most common ones based on upward or downward capillary transfer (60, 121). The Southern blot is then ready for the hybridization step. The probe must be labeled. The most common method is random priming with [α-^{32}P]dCTP or [α-^{32}P]dATP. A nonradioactive method employs a digoxigenin label (67), which can generate sharper banding patterns than radioactive probes but is usually less sensitive. For both methods, the gel must first be treated with hybridization buffer containing 150 μg of sheared, denatured salmon sperm, calf thymus, or other DNA per ml for approximately 4 h at 65°C to block nonspecific binding of the probe. Hybridization is then performed with the same prehybridization buffer containing the labeled probe. One standard hybridization buffer contains 50 mM NaH_2PO_4 (pH 7.5), 50 mM EDTA, 0.9 M NaCl, 5% dextran sulfate, and 0.3% SDS. The hybridization reaction is performed for 16 to 24 h at 65°C. Hybridization is terminated by washing the membrane at 45°C with a solution containing 0.3 M NaCl, 0.03 M sodium citrate (pH 7.0), and 2% SDS.

Step ix

The hybridization pattern generated by a radioactive probe is visualized by exposing the blot to X-ray film with the aid of intensifying screens. Examples of Southern blot hybridization patterns for bacteria and yeast are presented in Fig. 5B and C, respectively. To visualize digoxigenin-labeled patterns, one can treat the gel with anti-digoxigenin antibody conjugated to horseradish peroxidase (39). The antibody binds to digoxygenin. Next, one adds the substrate luminol under alkaline conditions. Horseradish peroxide and hydrogen peroxide catalyze luminol oxidation. Oxidized luminol decays, releasing light, which can be visualized with X-ray film.

Step x

Because hybridization is a result of ionic rather than covalent bonding, one can strip a Southern blot of a probe and rehybridize with additional probes.

Selection of a Hybridization Probe

Selection of a probe for a study should be based on a firm understanding of the exact information a particular probe will provide. Some of the first probes used for DNA fingerprinting of the infectious fungi included single gene sequences, rRNA genes, and mitochrondrial DNA. It should be evident that a single-gene probe will usually hybridize to only one band in a haploid organism and one or two bands in a diploid organism. Although differences in the sizes of the fragments carrying the gene may exist between isolates, the data are never sufficient for epidemiological studies that require measurements of moderate relatedness.

The use of rRNA genes as a fingerprinting probe for both prokaryotic and eukaryotic pathogens was predicated on the idea that single-copy DNA probes would not provide adequate data complexity. In bacteria, rRNA gene probes, which are the basis of the most popular automatic typing systems such as the Riboprinter microbial characterization system (Qualicon, Wilmington, Del.), are effective because rRNA cistrons are dispersed throughout the single chromosome (104). Variability in banding patterns results from changes in bordering sequences (34, 43, 97, 107, 169).

In contrast, the rRNA cistrons of eukaryotes are clustered, usually in only one chromosome (62, 128). These cistrons are separated by spacers. Endonuclease digestion usually results in a very limited number of bands, generating unexpectedly simple patterns (Fig. 3C) that are not much more complex than those generated by single-copy probes. Therefore, other repetitive sequences were sought that provided more complex patterns. In particular, repetitive sequences were sought that were dispersed throughout the genome. The expectation was that changes in the flanking sequences would generate differences in the patterns of different isolates that could be interpreted in terms of genetic distance. In some bacteria, transposable elements have been used as DNA fingerprinting probes. For instance, in *Mycobacterium tuberculosis*, insertion sequences such as IS*6110* (also known as IS*986*) have been used as probes (83). The copy number of IS*6110* per strain varies between 1 and 19, but the majority of strains carry between 8 and 15 copies dispersed throughout the chromosome (114). Therefore, patterns generated with the IS*6110* probe and PvuII-digested *Mycobacterium tuberculosis* DNA are relatively complex and vary between unrelated isolates (Fig. 5B).

For the fungi, a variety of moderately repetitive sequences have been used. For instance, poly(GT) and several oligonucleotides, such as $(GGAT)_4$, $(GTG)_5$, $(GATA)_4$, and

$(GACA)_4$, have been used for DNA fingerprinting of *Candida* species (142). These sequences, which identify microsatellite regions, generate complex patterns that may prove to be effective fingerprinting probes, but none have been fully verified. In *C. albicans*, the repeat sequence CARE2 (84), which is represented 10 to 14 times in the genome, generates a relatively complex Southern blot hybridization pattern. However, CARE2 and other repetitive sequences that reorganize at a rapid evolutionary rate are sometimes ineffective in identifying clusters of moderately related isolates of a species, a problem to which we will return.

For fungi, complex probes have been cloned that have proven effective at all levels of relatedness. Complex probes are genomic fragments between 10 and 20 kb in length that contain and therefore identify highly variable repetitive sequences, moderately variable sequences, and monomorphic sequences. These probes hybridize in a species-specific fashion and generate patterns that are complex but distinct enough for automatic computer-assisted methods of analysis. They have been cloned from every fungal species so far tested, including *C. albicans* (4, 89, 119), *C. tropicalis* (71), *C. glabrata* (91), *C. dubliniensis* (72), *C. parapsilosis* (41), *C. lusitaniae* (S. Lockhart and D. R. Soll, unpublished results), and *Aspergillus fumigatus* (60). The methods for cloning and characterizing complex probes have been recently reviewed by Lockhart et al. (88). An example of patterns obtained with the Ca3 probe for *C. albicans* is presented in Fig. 5C.

Random Amplification of Polymorphic DNA

RAPD is one of the most frequently used methods for DNA fingerprinting of eukaryotic organisms (24, 165, 167). As we shall see in a following section dealing with verification, this method, when developed correctly, is highly effective for assessing relatedness at all levels of resolution. However, in contrast to RFLP, RFLP-PFGE, and RFLP with a hybridization probe, the RAPD method is compromised by problems of reproducibility among laboratories. The method, presented in a generic form in Fig. 6A and B, is straightforward. With the use of random primers of approximately 10 bases in length, amplicons throughout the genome are amplified by PCR as described in Fig. 6A. The amplification products are separated on an agarose gel and visualized by ethidium bromide staining. Polymorphisms arise when the distances between primer hybridization sites change or when primer sites appear, disappear, or change location due to insertion, deletion, or recombination. If a primer hybridizes to a large number of sites on opposing Crick and Watson strands (i.e., identifies a significant number of amplicons), that primer will generate a complex pattern. Usually, however, each 10-bp primer will generate one or a few major bands and a few minor bands (Fig. 7). Because some minor bands may not be highly reproducible in repeat experiments in the same laboratory, only the major bands are usually used for analysis. This irreproducibility is probably the result of low annealing temperatures and short primer sequences, which result in mispairing. Several primers must be used and the data must be pooled to obtain the necessary level of complexity for epidemiological studies. As a general guideline, it is recommended that one use approximately eight primers, each generating at least two reproducible, strong bands, resulting in at least 16 polymorphic bands. Too low a degree of polymorphism will bias a study. Based on our experience with the RAPD method, we recommend the selection of polymorphic bands present in between 10 and 90% of isolates (41, 91, 111). To

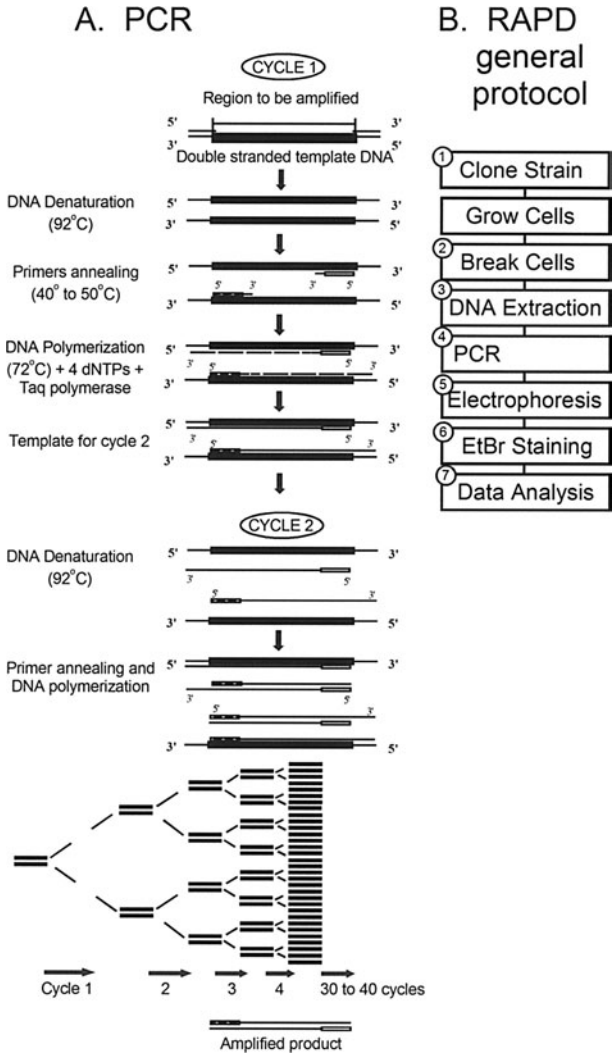


FIGURE 6 RAPD. (A) Description of PCR. (B) Generic version of the steps in the RAPD method. EtBr, ethidium bromide.

obtain an effective collection of primers, one should begin with 30 or 40 primers and test each with a representative collection of isolates if one is setting up one's own RAPD protocol. Primer collections can be readily obtained commercially. The test sequences can usually be obtained from collections used for studies of related organisms. In an analysis of *C. glabrata*, Lockhart et al. (91) selected the following nine primers: GGACTGCAGA, GTGACATGCC, CAGGCCCTTA, TGCCGAGCTG, AATCGGGCTG, GTGATCGCAG, TCGGCGATAG, AGCCAGCGAA, and AGGTGACCGT. In an analysis of *C. albicans*, Pujol et al. (111) selected the following eight primers: CCAGATGCAC, GTGACATGCC, TTATCGCCCC, GGACTGCAGA, ACGGCGTATG, AACGGTGACC, GGAAGCTTGG, and ACGGTACCAG. In an analysis of *Salmonella enterica* serovar Typhimurium isolates, Malorny et al. (96) selected 13 primers, and in an analysis of *Burkholderia pseudomallei* (85), 30 primers were tested for their efficacy in RAPD analysis. In contrast, in several studies, isolates have been grouped using a single primer (31). In some cases, a single primer can in fact provide enough data to generate a dendrogram (see, e.g., reference 112).

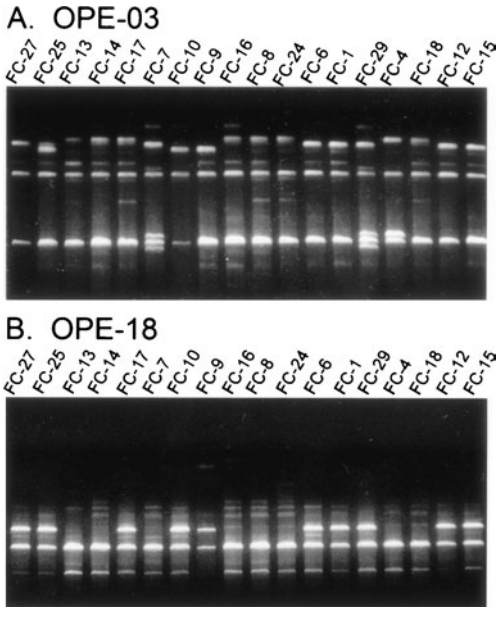


FIGURE 7 Examples of RAPD patterns with the primers OPE-03 (A) and OPE-18 (B) applied to 18 isolates of the yeast *C. albicans*. Reprinted from reference 111 with permission.

Once a primer or primers are selected, the method of RAPD analysis is relatively straightforward. In Fig. 6B, a generic version of the RAPD method is presented.

Preparation of Cells

As in the case of the MLEE, RFLP, and RFLP-with-probe methods, one must begin with a clone. In contrast to the MLEE method, but similar to the RFLP and RFLP-with-probe methods, the RAPD method does not require one to be too concerned about uniform growth conditions.

Preparation of DNA

The preparation of DNA is similar to that for RFLP, with some modifications. First, since the DNA has to be pure to facilitate the PCR, several chloroform extractions are necessary. To prevent DNA degradation, EDTA can be added during extraction, but EDTA should be absent from the final DNA suspension solution since 0.1 μM EDTA can affect the efficiency of *Taq* polymerase in the PCR. The following protocol is relatively simple, has been successfully used for the analysis of several fungal species, and can be adapted for other organisms. A 500-μl suspension of cells (5×10^9 cells per ml) in a solution of 10 mM Tris-HCl (pH 8.0)–1 mM EDTA is mixed with an equal volume of glass beads (0.45 mm in diameter) in a 1.5-ml microcentrifuge tube and disrupted in a bead beater. SDS is then added to a final concentration of 2% (wt/vol), and the suspension is mixed by repeatedly inverting the tube. DNA is then purified by two rounds of phenol extraction followed by three rounds of chloroform extraction. DNA is precipitated in 0.3 M sodium acetate (pH 4.5) and 2 volumes of 100% ethanol. The final DNA precipitate is resuspended in distilled water.

PCR Amplification

The following is a simple version of PCR amplification. To a 0.5-ml microcentrifuge tube is added 25 μl of a reaction mixture containing 5 ng of genomic DNA, 2.5 μl of 10× buffer provided by the manufacturer with purchased *Taq* DNA polymerase, 1.5 mM $MgCl_2$, 0.5 to 1.5 U of *Taq* DNA polymerase, 250 μM (each) dATP, dCTP, dGTP, and dTTP, and 0.2 mM selected 10-bp primers. Amplification is performed in a thermal cycler programmed for 45 cycles of 1-min duration at 94°C, 2 min at 36°C, and 2 min at 73°C. Amplification products are separated by electrophoresis in a 1.5% agarose gel run for 4 h at 110 V so that the bromophenol blue marker dye migrates approximately 10 cm. For the separation of low-molecular-mass (<500 bp) amplification products, acrylamide gels can be used.

Visualization of Bands

The gel is stained with ethidium bromide as described for RFLP gels and viewed with a UV light box.

Problems Inherent in the RAPD Method and Controls That Must Be Applied

Because there is a problem of reproducibility not only between laboratories but also within the same laboratory over time, one must be cognizant of the pitfalls in the procedure. Most aspects of PCR affect reproducibility, including small differences in the primer-to-template ratios, the concentrations of magnesium, and temperatures (40, 94, 99). Second, variation can occur as a result of the source of *Taq* enzyme (94, 99).

The following steps should, therefore, be taken to obtain reproducible banding patterns. First, DNA from different strains should be tested at a variety of dilutions to assess the impact of dilution on pattern stability. Second, the DNA of a single strain should be extracted several times to test whether minor variations in the preparation of DNA affect patterns. Finally, the amplification reaction should be performed in parallel and in sequence for a single DNA preparation that will be used as a control to test intralaboratory reproducibility.

Other PCR-Based Methods for DNA Fingerprinting

A variety of additional DNA fingerprinting methods have been developed that are PCR based. One modification to RAPD is the amplified fragment length polymorphism method, in which restriction fragments are selected for amplification (130, 158, 164). In this method, DNA is first digested with a restriction enzyme and random restriction fragments are singled out by using a specific base sequence at the 3′ ends of the primers. While the amplified fragment length polymorphism method has been developed to target restriction sites, PCR methods have also been developed to target other known sequences distributed throughout the genome, such as microsatellite sequences and spacer regions between tRNAs or rRNAs. In interrepeat PCR, the variable-length segments found between consecutive repeat elements, rather than the repeat elements themselves, are amplified. Oligonucleotide primers are designed such that they hybridize to the 5′ and/or 3′ ends of repetitive elements, but instead of amplifying the elements, they face outward from the elements and amplify the internal spacer sequences. PCR fragments are separated by agarose gel electrophoresis following amplification and are viewed by staining with ethidium bromide. The number of bands produced is dependent upon the number of repetitive elements within the genome as well as the number that are adjacent and close enough for *Taq* DNA polymerase to amplify the sequences between them (usually around 5,000 bp). Single-primer interrepeat PCR using the M13 core sequence or the microsatellite primers

$(GTG)_5$ and $(GACA)_4$ has been successfully used for a number of fungal species, including *Candida* spp. (101) and *Cryptococcus neoformans* (100), and the parasite *Leishmania* spp. (125). In bacteria, there are at least two commonly used inverted-repeat elements, BOX and the enterobacterial repetitive intergenic consensus (ERIC) sequence. These elements have been successfully used for typing of *Streptococcus pneumoniae* with the single BOXA primer (80) and *Pseudomonas aeruginosa* with both BOXA and primers ERIC1 and ERIC2 (146). A specific protocol for fingerprinting of *Mycobacterium tuberculosis* strains called double repetitive element PCR utilizes two sets of primers, two of which amplify out from the repetitive element IS*6110* and two of which amplify out from the polymorphic GC-rich repetitive sequence PGRS element, in order to generate patterns for isolates with small numbers of IS*6110* repetitive elements (53). Another typing method that has been applied to *Mycobacterium* is spoligotyping. This method uses interrepeat PCR to amplify the regions between a repetitive element known as DR. Rather than separating the interrepeat regions on a gel, one of the primers is end labeled and the products are hybridized to an array of oligonucleotides corresponding to 43 known inter-DR regions (38, 73, 81).

Variable-number-of-tandem-repeat elements (VNTRs) or microsatellites are very short tandem repetitive elements found within the genomes of both prokaryotes and eukaryotes. The VNTR fingerprinting method is PCR based and relies on the amplification of specific VNTRs by using primers specific to the outside flanking regions of the elements. The bands that are generated are distinguished by size following gel electrophoresis. In an article by Lott et al. (93), VNTR profiles were generated for one dimorphic and three polymorphic loci from 114 isolates of *C. albicans* and the methodology was verified by comparing the deduced phylogeny to that which was found using some of the same strains in a previously verified fingerprinting system (111). Mazars et al. (98) identified 12 VNTR regions within the *Mycobacterium tuberculosis* genome that had from two to eight alleles each in the 72 isolates that were tested. This corresponds to a potential for more than 16 million combinations of alleles. VNTR typing has also been successfully applied to the parasite *Plasmodium falciparum*. Anderson et al. (5) described 12 variable-length microsatellite loci and were able to do a population structure analysis of isolates from 465 cases of infection.

Because some evolutionary biologists believe that patterns must account for identified alleles (i.e., a change in a band size in a pattern must be attributed to a change in the size or another aspect of a known sequence), PCR-based methods have been developed that employ genetic markers, either identified or anonymous. Karl and Avise (74) have devised a method that has been applied to a number of lower eukaryotic pathogens, in which arbitrary primers are used to identify monomorphic amplicons, which are then partially sequenced. Customized pairs of primers are then developed for specific bands. Changes in these identifiable bands are then assessed through either RFLP or single-strand conformational polymorphism (SSCP) analysis, which involves the identification of single base pair changes in a sequence through nonhomologous renaturation of DNA and separation on a sequencing gel. For the use of these markers in fungi, see the review by Taylor et al. (150). For *C. albicans*, both PCR-SSCP (52, 64) and PCR-RFLP (171, 172) have been used with equally good results. PCR and the use of specific allelic probes have also been applied to *C. albicans* (32). Similar approaches have also been developed that target the noncoding regions of loci. Development of primer pairs is based on published sequences, and polymorphisms can again be identified by SSCP or RFLP. In these types of PCR-based methods, several pairs of primers must be employed and the pairs must be selected not only because they identify a known sequence but also because they produce the necessary levels of variation between strains. Although these methods have been argued to be superior to standard RFLP because one knows the identity of bands in different strains, analyses of the efficacy of RAPDs in band identification have demonstrated levels of identity between patterns that far exceed those required for effective analysis. Reiseberg (116) tested the homology of 220 pairs of comigrating RAPD fragments from three closely related species (not strains!) of sunflower and found that 91% exhibited homology. Thormann and coworkers (152) demonstrated an extremely high degree of similarity of hybridization patterns of RAPD bands within several plant species. Both these interspecies and intraspecies analyses support the conclusion that RAPD fragment size within a plant species is a strong indicator of homology, and by inference, this should be true for microbial pathogens. More important, verification of the RAPD method through comparison of its clustering abilities with those of unrelated methods has demonstrated its efficacy, as will be discussed in a later section of this chapter. The bottom line is that it is not necessary to identify the RAPD bands being analyzed to use the RAPD method in genetic fingerprinting, as has been amply argued by Tibayrenc (154–156).

Electrophoretic Karyotyping

Since lower eukaryotes possess multiple chromosomes within the size separation range of PFGE technology, this method (26, 126) has been used as a way of fingerprinting eukaryotic pathogens (Fig. 8). If the cell possesses a wall, the wall must be removed. The resulting spheroplasts are embedded in an agarose plug. Detergent and proteinase are added, which removes membrane, digests protein, and releases nucleic acid. The agarose matrix reduces shear forces and thus protects the large chromosomal DNAs from fragmenting. The agarose plug is placed in a well of an agarose slab gel, and electrophoresis is

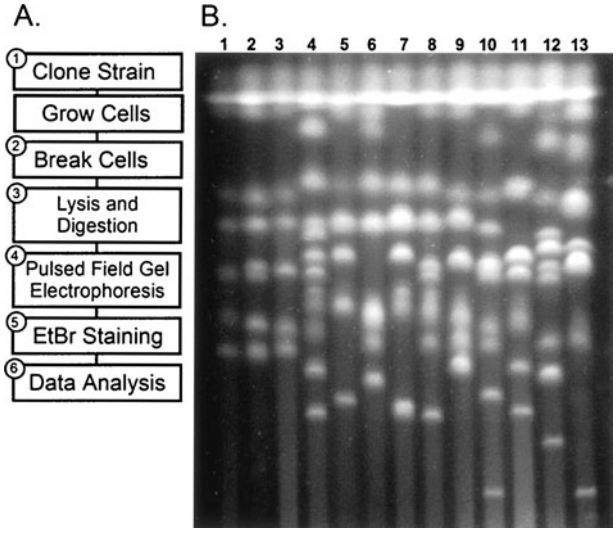


FIGURE 8 PFGE. (A) Generic version of the steps in the PFGE method. EtBr, ethidium bromide. (B) Example of ethidium bromide-stained chromosomes of isolates of the yeasts *C. albicans* (lanes 1 to 3) and *C. dubliniensis* (lanes 4 to 13).

carried out according to the protocol for the specific system used (e.g., orthogonal-field-alternation gel electrophoresis [127], contour-clamped homogeneous electric field electrophoresis [29], or transverse alternating-field electrophoresis [57]). After electrophoresis, chromosomal DNAs are visualized by ethidium bromide staining. Chromosomes can then be identified by Southern blot hybridization with chromosome-specific probes. For infectious yeast, Sangeorzan et al. (122) demonstrated that patterns were reproducible among experiments and relatively insensitive to the method of preparation. However, there have been other studies that demonstrate pattern variability due to reagents, sample preparation, and eletrophoretic conditions. Thrash-Bingham and Gorman (153) demonstrated that in spite of karyotypic variability between strains, the general organization of the genome was maintained, although some chromosomal translocations contributed to karyotypic variability between strains of *C. albicans*. In addition, it has been demonstrated that the frequency of changes in electrophoretic karyotypes can be dramatically influenced by high-frequency phenotypic switching (113). In a sequence of high-frequency switching, the electrophoretic karyotype of a strain diverged from and then reverted to the original pattern, a process that would invalidate the use of this method for assessing moderate levels of relatedness (113). There are reasons to believe that the majority of changes in the chromosome harboring rRNA cistrons in *C. albicans* were due to a release from silencing (113), a process involving *SIR* genes in *Saccharomyces cerevisiae* (58, 63). Whatever the mechanism proves to be, the problem that arises from these observations is that the rate of change identified by PFGE methods may not always reflect genetic distance.

Multilocus Sequence Typing

Although nucleotide sequencing provides the most exact data for assessing polymorphisms within a species, until recently it has been used primarily to analyze mutations associated with phenotypic change and to generate single-locus phylogenetic trees at the interspecies level. At the intraspecies level, multiple loci must be sequenced and the combined data must be analyzed. Recent advances in automated sequencing and the complete sequence data of genomes of select microorganisms have led to the use of sequence data to type isolates and perform population genetic studies of bacteria and fungi. MLST (95) has evolved rapidly over the past several years for DNA fingerprinting of bacteria and fungi (20, 21, 37, 45, 49, 157). It replaces MLEE, providing more alleles per locus and resulting data that are highly reproducible between laboratories. In addition, sequence data can be easily stored and shared via the Internet for comparison and use in large-scale epidemiology studies (1).

A general protocol for MLST has recently been described by Lockhart et al. (90). For template DNA extraction, the protocol proposed for the RAPD method is appropriate. Alternatively, commercial DNA extraction kits can also be used. PCR fragments should be around 500 bp in length to be easily sequenced in both directions. The PCR products must be purified by using any commonly available PCR cleanup kit before sequencing.

Choice of Loci

A selection of six to seven genes is commonly used. These are usually selected among core metabolic genes (housekeeping genes) that are believed to be unaffected by selection. Their use, therefore, precludes microevolutionary analysis. In a few studies, microevolutionary changes have been assessed by using hypervariable genes or genes that are known to be affected by selection. Morelli et al. (102) have analyzed the microevolution of a clonal lineage of *Neisseria meningitidis* by sequencing multiple DNA fragments, including genes encoding cell surface antigens and intergenic regions. Similarly, Feavers et al. (44) used antigen sequences to analyze an outbreak of *Neisseria meningitidis* infection. More recently, Robinson and Enright (117) have used surface-associated genes to supplement their MLST data in order to document the emergence of methicillin-resistant *Staphylococcus aureus* isolates. While MLST schemes have been used to fingerprint fungi (21, 37, 55, 149), the MLST approach was originally designed for bacterial analyses. The use of coding sequences is appropriate for the majority of bacteria because many bacterial species present a high number of polymorphisms. If noncoding sequences were to be used, homoplasy may render interpretations difficult. However, for some microorganisms, and fungi in particular, this approach may not be optimum due to lower genetic variability than that in bacteria. For instance, in the yeast *C. parapsilosis*, three distinct genetic groups can be distinguished (41, 87, 147). Group I strains are the most pervasive and account for between 60 and 90% of isolates (41, 87). Groups II and III are less frequently found and have recently been considered distinct species (147). Tavanti et al. (147) have sequenced a total of 7.5 kb over 11 genes for 21 *C. parapsilosis* group I isolates. They found limited genetic variability, with only two polymorphic nucleotide positions in their entire collection.

MLST and Heterozygosity

The MLST method has been used primarily for fingerprinting of species with haploid genomes. MLST has been successfully used for at least one diploid species, the yeast *C. albicans* (20, 21, 148). While direct sequencing of PCR products identifies single alleles in haploid species, in diploid species, the sequences obtained reflect the combination of two homologous alleles. Heterozygosities are revealed by double peaks in the sequencing traces. Thus, where heterozygosities exist at multiple nucleotide sites in any particular locus, assignment of individual alleles is not possible. This issue may be overcome by cloning the PCR product into a suitable plasmid vector and sequencing one cloned allele. The remaining allele may then be inferred. In diploid genomes, loci containing insertion or deletion polymorphisms need to be avoided. Where such polymorphisms are present, the sequences of the two alleles will be out of phase and the sequencing traces will be unreadable.

Microarray Typing

The latest development in typing techniques has evolved from advances in genome sequencing and DNA microarray technology. Microarray typing methods are rapidly emerging as powerful high-throughput typing strategies. The general method is based on the use of oligonucleotides of around 30 bp in length. These oligonucleotides are selected to target variable single nucleotide polymorphism sequences based on the presence of known polymorphisms. A set of specific oligonuclotides representing each allele of a given locus is used to detect polymorphisms in PCR products of the respective loci. It is a multilocus-based method with which several loci can be analyzed simultaneously. This approach has been used to fingerprint several bacteria (56, 129, 144, 168), viruses (59, 86), parasites (140), and fungi (51). The method has been shown to be effective in detecting heterozygosities in diploid organisms (51).

VERIFYING THE EFFICACY OF A GENETIC FINGERPRINTING METHOD

As noted earlier, one must be sure that a fingerprinting method provides the correct level of genetic resolution for the question posed. Therefore, one must select a method that has already been verified or perform tests to verify the method. The most straightforward test is comparison with an unrelated method by using a set of test isolates with known or implied relatedness. The levels to be tested are (i) identical, (ii) highly similar but nonidentical, (iii) moderately related, and (iv) unrelated (Fig. 1). The test collection is fingerprinted by the individual methods, and similarity coefficients are computed by the same analytical method for every pair of isolates. Dendrograms based on the independent sets of data are then generated and compared. If the unrelated methods both identify known identical isolates as identical, presumed highly related isolates as similar but nonidentical, and presumed unrelated isolates as dissimilar and if the independent methods group isolates into the same clusters, they in essence cross-verify each other's efficacy at every level of relatedness. If they identify as identical presumably identical isolates and identify as similar but nonidentical highly related isolates but do not cluster in a similar fashion moderately related to unrelated isolates, then one or both methods may be ineffective in assessing lower levels of relatedness. This general method of cross-verification has been used to assess the efficiency of a variety of fingerprinting methods for bacterial, fungal, and parasitic pathogens (see, e.g., references 81, 111, and 156). It should be emphasized that without cross-verification, a genetic fingerprinting method is always suspect for the different levels of relatedness (Fig. 1), no matter how valid the system seems to be. One must realize, however, that such cross-verification is possible only for microorganisms with clonal population structures (111, 154–156).

DATA ANALYSIS

Computing Similarity Coefficients

Genetic fingerprinting data come in different forms. MLEE provides multiple spot patterns, RAPD provides single or multiple banding patterns, RFLP, RFLP with a probe, and PFGE provide single or multiple banding patterns, and MLST provides sequence data. Regardless of the data form, the goal of a genetic fingerprinting method is to compute similarity coefficients (S_{AB}s) for every pair of isolates in order to generate a dendrogram, or tree. The formula for computing S_{AB}s and the formula for generating a tree from the S_{AB}s must be carefully selected, and excellent reviews of these computations are available (47, 131, 166). Here, we will review only the most common of these. For a detailed description of these methods, the reader is referred specifically to Sneath and Sokal's *Numerical Taxonomy: the Principles and Practice of Numerical Classification* (131). The challenges of MLST data analysis will be dealt with in a separate section.

Band Positions Alone

For methods that produce patterns of bands, the presence or absence of a band is described by the binary value 1 or 0, respectively, n_{AB} is the number of common bands in the two patterns A and B (coded 1, 1), a is the number of bands in A with no counterpart in B (coded 1, 0), b is the number of bands in B with no counterpart in A (coded 0, 1), and c is the number of bands absent in A and B (coded 0, 0). The sample size x represents the total number of bands, described by the following formula: $x = n_{AB} + a + b + c$. The number of matches (m) is, therefore, $m = n_{AB} + c$, and the number of mismatches (u) is $u = a + b$. Using this basic logic, a number of formulas for S_{AB}s based on band position alone have been developed, most notably those for the coefficient of Jaccard (S_j):

$$S_j = \frac{n_{AB}}{n_{AB} + a + b}$$

the coefficient of Dice (S_D):

$$S_D = \frac{2n_{AB}}{2n_{AB} + a + b}$$

the sample-matching coefficient (S_m):

$$S_m = \frac{n_{AB} + c}{n_{AB} + a + b + c}$$

the Pearson coefficient (S_Ψ):

$$S_\Psi = \frac{(n_{AB}c - ab)}{\sqrt{[(n_{AB} + a)(n_{AB} + b)(b + c)(a + c)]}}$$

and others such as mean square difference/total bands. It should also be noted that data such as those obtained by MLEE or RAPDs, in which a series of primers provide binomial data, can be converted into binary values to generate a matrix similar to that obtained with banding data that can then be used to compute S_{AB}s for every pair of isolates. The same formulas for S_{AB}s can then be considered.

Band Position and Intensity

In many cases, the information garnered from band intensity is added to that of band position. In these formulas, X_{iA} and X_{iB} represent the intensities of bands in patterns A and B, respectively, $\bar{X}_{iA}$ and $\bar{X}_{iB}$ represent the respective means of all intensities, and n represents the total number of bands. A number of formulas for S_{AB}s based on band position and intensity have been developed, most notably those for Pearson's product-moment correlation coefficient (S_r):

$$S_r = \frac{\sum_{i=1}^{n}[(X_{iA} - \bar{X}_{iA})(X_{iB} - \bar{X}_{iB})]}{\sum_{i=1}^{n}(X_{iA} - \bar{X}_{iA})^2 \sum_{i=1}^{n}(X_{iB} - \bar{X}_{iB})^2}$$

absolute difference/total area (S_{AB}):

$$S_{AB} = 1.0 - \frac{\sum_{i=1}^{n}|X_{iA} - X_{iB}|}{\sum_{i=1}^{n}|X_{iA} + X_{iB}|}$$

and others such as absolute difference/maximum intensity similarity coefficient, mean square difference/intensity similarity coefficient, and mean square difference/maximum intensity similarity coefficient.

In selecting a method for computing S_{AB}s, several points should be kept in mind. First, if the reproducibility of band intensities is not good, then an S_{AB} based on band positions

alone is obviously more accurate. Second, if a method involves the presence or absence of bands as if they were alleles, S_{AB}s based on band position alone are used. One must realize, however, that in most of the DNA fingerprinting methods employed, allelism is not always verified. Third, it must be realized that in methods based on repeat sequences, band intensities may provide as much or more information on relatedness as position, and a method based on both may, therefore, be preferable.

The different similarity coefficients described above do not require the identification of alleles at given loci. For this reason, they are favored when cross-verifying methods are applied since one or more of the methods may rely on dominant markers. Genetic similarity can also be assessed by using genetic distances based on the frequencies of codominant markers (see references 47, 131, and 166).

Generating Dendrograms

The computation of S_{AB}s leads to a matrix of values generated for every pair of isolates in an epidemiological study. These values are the basis for generation of a dendrogram. The most commonly used method for connecting isolates into a tree is the unweighted-pair group method using arithmetic averages (UPGMA) first employed by Rohlf (118). This method generates a rooted tree. The Fitch-Margoliash method with evolutionary clock (50) also generates a rooted dendrogram, with the additional feature that it tests alternative topologies for the tree by rearranging nodes to minimize the sum of squares computed between genetic distances and branch lengths, making it more time-consuming to perform. There are also methods that do not assume a common molecular clock for all strains and, therefore, generate unrooted trees. These methods, which include the neighbor-joining method (120) and the Fitch-Margoliash method without evolutionary clock (50), generate unrooted trees that in fact should have more exact topographies. Because the neighbor-joining method and more particularly the UPGMA are faster than the other methods, they may be preferable for large experimental samples. There are, therefore, tradeoffs among methods. It should be realized that these methods were developed for generating trees at the species level rather than subspecies levels of relatedness, but they still result in interpretable trees at the subspecies and strain levels. If the data are robust, similar dendrograms should be generated no matter which method is applied. Dendrograms generated for intraspecies analysis should not be considered a purely phylogenetic representation of lineages but rather a practical tool that provides a visual description of genetic similarities and divergence between strains and that may be useful in identifying groups, or clades. The derived dendrograms represent true phylogenies only when each strain is derived through a purely clonal lineage (i.e., with no genetic exchange) and homoplasy is negligible. These conditions are rarely verified for the markers used in epidemiological studies.

Let us consider how the UPGMA is performed. The S_{AB} matrix is scanned for the most similar isolates. If more than one group (two or more) are identified, the first is arbitrarily taken as group one. The isolates are joined at the appropriate position along the S_{AB} axis. The matrix is scanned once again for the next most similar isolate or group of isolates, which is then connected along the S_{AB} matrix to the first group, and this function is repeated over and over again until all isolates are incorporated into the tree. A sample dendrogram that includes 67 *C. albicans* isolates is presented in Fig. 9. In this dendrogram, thresholds and

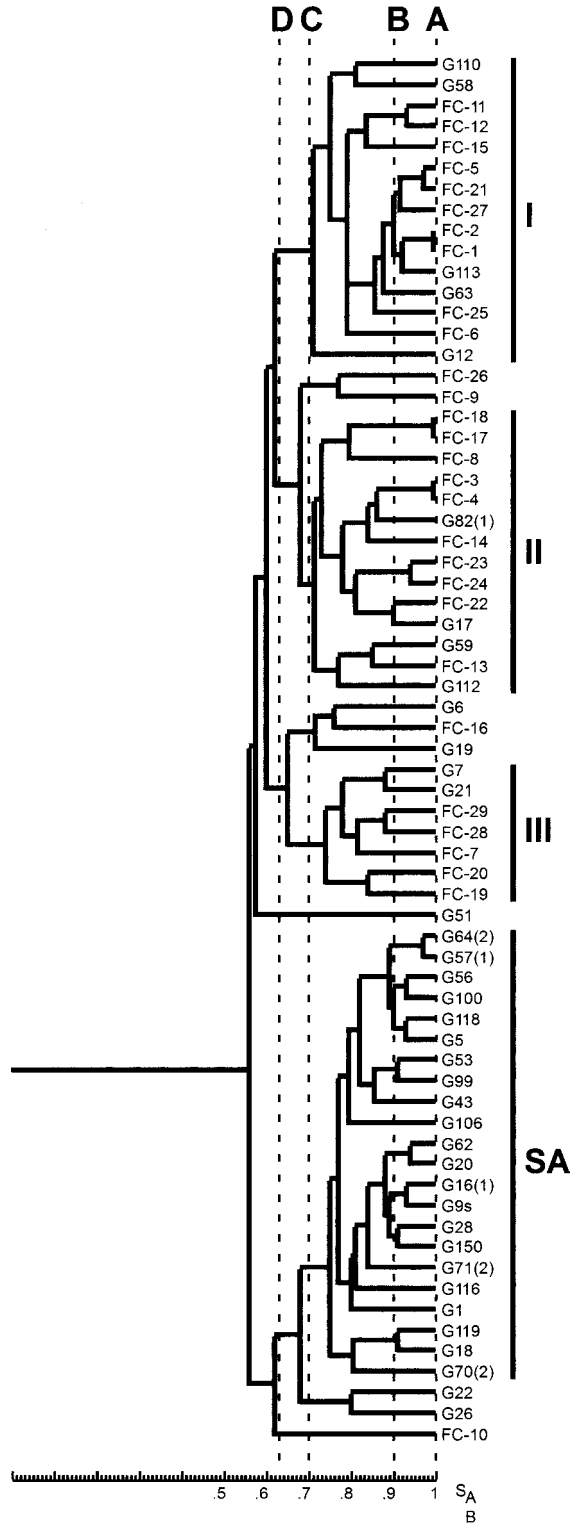


FIGURE 9 Example of a dendrogram. The similarity coefficients (S_{AB}s) were generated from the patterns of 67 isolates of *C. albicans* fingerprinted with the complex Ca3 probe. The value A represents identicalness, at an S_{AB} of 1.00; threshold B demarcates highly related isolates at an S_{AB} of 0.90; threshold C demarcates clades at an S_{AB} of 0.70; and the value D represents the average S_{AB} at 0.63. In this example, a South African-specific clade of *C. albicans* (SA) was identified in addition to the three clades (I, II, and III) present in U.S. collections (18).

landmarks are noted, one (A) which represents identity, at an S_{AB} of 1.00; one (B) which demarcates highly related isolates, at an S_{AB} of 0.90; one (C) which demarcates moderately related groups, in this example at an S_{AB} of 0.70; and one (D) which denotes the average S_{AB} for all strains in the dendrogram, in this example at an S_{AB} of 0.63. The C threshold is the one which best demarcates clades. The selection of thresholds and landmarks in a dendrogram is sometimes somewhat arbitrary (135) but extremely important in interpreting a tree. Confidence in these values comes with increased familiarity with the origins of isolates and assumptions of relatedness based on those origins.

Testing the Integrity of Clusters and Statistical Analyses

Although the selection of a threshold for defining clusters may be somewhat arbitrary, one can demonstrate that the content of a cluster is stable and that the identity of the cluster is in fact valid. First, the order in which isolates are chosen in the generation of a dendrogram can be randomized (2), an important control when the UPGMA is used since this method is prone to mistakes in higher-order clusters. For instance, one can apply 10 random starts to assess whether a cluster remains intact. Second, "noise" can be introduced by adding or subtracting a set percentage of the S_{AB}s in every pairwise comparison (108). Noise can affect and, therefore, be used to assess the stability of second-tier groupings. Third, one can use the comparison of clustering patterns generated by two independent genetic fingerprinting methods to assess the stability of a cluster (81, 111, 156).

The most common method used by evolutionary biologists to analyze the integrity of clusters is bootstrapping (48). In this process, one generates deletions and duplications by random sampling. This process is normally performed 1,000 times. A consensus tree of all dendrograms is then generated, and a "majority rule consensus" algorithm is used to compute the percentage of occurrence of each node. A percentage above 80% suggests a stable node. In a second common method called jackknifing (170), subsets of the collection are randomly selected approximately 100 times, a consensus dendrogram is generated, and the percentage of occurrence at each node is computed. These methods, however, were developed for DNA fingerprinting methods in which the data represent alleles of identified loci, not for patterns like those generated by RFLP or RAPD. They are, therefore, not commonly used in general epidemiological studies. They can, however, be used for RAPD data if each primer is considered an independent gene.

For comparing the relatedness of different populations of isolates, it is often the case that the mean S_{AB} of an entire collection is not informative, although comparisons of mean S_{AB}s of different collections may be informative. The Student *t* test can assess whether mean S_{AB}s are significantly different. In applying the Student *t* test, a probability value of 0.05 (i.e., less than 5%) usually represents significance.

Challenges of MLST Data Analysis

MLST data have also been typically represented by dendrogram-based methods. In addition to that approach, an increasingly popular method, called eBURST (for "based upon related sequence types"), has been developed to accommodate the specific needs associated with the analysis of large MLST data sets (46, 137). The eBURST approach was developed to provide a simple representation of relationships between closely related isolates. As such, eBURST does not try to represent relationships among all analyzed isolates. The development of eBURST was warranted by the fact that dendrograms based on the bifurcating of lineages cannot correctly represent patterns of evolutionary descent within closely related strains from a clonal group. In addition, dendrograms of thousands of strains are hard to display and to analyze. The solution proposed by Feil et al. (46) and Spratt et al. (137) is based on a simple evolutionary model in which clonal lineages are assumed to be founded by a parental clone that, as it increases in frequency, will diversify by accumulation of mutations or by recombination. The eBURST algorithm identifies clonal lineages, predicts their founding genotypes, and displays the most parsimonious pattern of descent for the isolates of each clonal group from the putative founding genotype.

The eBURST algorithm first divides the collection analyzed into groups of closely related strains. The level of relatedness used is user defined. As a general rule, members assigned to the same group share identical alleles at a minimum of one locus with at least one other member of the group. This is the most stringent definition for clonal groups. A threshold of two (or fewer) loci can also be used, but the outcome may be harder to interpret. This step results in a set of nonoverlapping groups of related strains. Then, based on the number of single-locus variants, strains that differ at a single locus, observed for each strain, the algorithm predicts which genotype is the founder for each group. The strain with the highest number of single-locus variants will be defined as having the founder genotype. This process can be supplemented by bootstrap analysis in troublesome cases. The descent from the founder genotype to all of the other genotypes of a given group is then inferred and represented as a radial diagram. The eBURST approach can be used to analyze large samples of thousands of isolates (46, 137).

While the eBURST method is becoming very popular in bacterial MLST studies due to its attractive and convenient representation of evolutionary descent in clonal groups, users should be aware of a few limitations. The eBURST analysis will perform better on large samples. In small samples, clonal groups may not be correctly inferred due to the absence of intermediary genotypes that may have connected groups otherwise deemed independent. Even in larger samples, however, this problem may persist if the missing link has become extinct. Because the founder genotype is inferred from the genotypes present in the sample, predictions of the founder genotype may be affected by sampling biases. Again, this problem should be more limited in large samples. The eBURST method, therefore, offers a useful representation that complements the more commonly used dendrogram-based methods.

ROLE OF COMPUTERS IN GENETIC FINGERPRINTING

For epidemiological studies in which only a few isolates are compared or in which qualitative comparisons are sufficient to obtain an answer, it is simple enough to interpret results, including the computation of similarity coefficients, without the use of computers. However, when dealing with bigger collections, one must recruit a customized computer program. Computer programs are available that can assist in virtually every step in a cluster analysis. For fingerprinting methods that create complex patterns, the systems will automatically acquire the original image and with the assistance of the user will remove distortion, identify lanes, normalize to a universal standard, identify bands, measure the intensity of bands, compute similarity coefficients, generate dendrograms, identify clusters, and perform statistical tests (135). Computer systems will also store data at any or all levels of analysis for

retrospective studies and future comparisons with new collections. An automatic genetic fingerprint analysis system can perform searches based on any aspect of the fingerprinting data (e.g., a particular band length), any characteristic of a patient (e.g., human immunodeficiency virus positivity), or any characteristic of the infecting organism (e.g., drug susceptibility). An outline of the steps for computer-assisted analysis of generic complex RFLP, RFLP-with-probe, and select PCR-based patterns follows. The generic software will be referred to simply as the Program.

Digitizing the Pattern

The pattern of an autoradiogram or its equivalent is digitized into the computer's hard drive (Fig. 10A) by using a scanner with software that supports grayscale scanning that saves the image in the correct format (e.g., bitmap or tiff). The scanner should be sensitive to a reasonable range of grayscales (e.g., around 256 grayscales).

Local Standard

In order to straighten a pattern for automatic analysis and compare patterns obtained on different gels, landmark bands must exist in a set of standards either run in lanes alongside test samples, added to the test samples, or incorporated as components of the pattern (e.g., monomorphic bands). In all three cases, standards must include bands that span the entire molecular-weight range of bands in test patterns.

Global Standard

For studies in which the patterns of multiple gels are compared, each test pattern must be normalized to a global standard in the database. The global standard must share bands in the local standard.

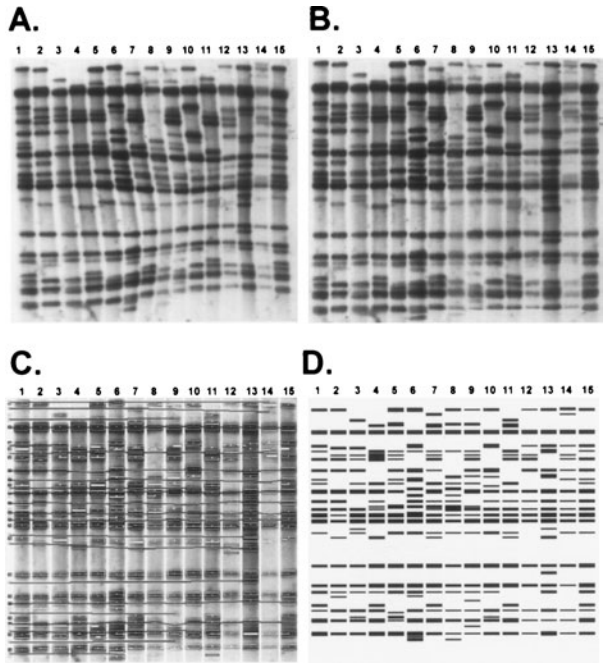


FIGURE 10 Computer-assisted processing and analysis of DNA fingerprint patterns generated with the complex Ca3 probe of 15 isolates of *C. albicans*. (A) Original digitized image with inherent distortions; (B) straightened patterns; (C) correlations of bands with universal standards; (D) model generated from data.

Initial Image Processing

The need for processing stems from artifacts that distort the pattern. First, because of uneven polymerization or electrophoresis, the entire gel pattern may contain "smiles," "frowns," skews, and other linear or nonlinear distortions (Fig. 10A). Unwarping is the process of pattern straightening. Local standards are essential for unwarping. Horizontal lines are drawn between common bands for horizontal distortions, and vertical lines are drawn through spaces for vertical distortions. Once lines are drawn, the Program reconstructs the processed pattern (Fig. 10B). Second, because of unequal loading, the pattern in a single lane can be intensified or deintensified.

Automatic Lane and Band Detection

The Program scans and automatically detects lanes and separates them by spaces to facilitate band identification. At this point, the Program should allow editing for final band alignment that includes lane sliding, stretching, or compressing. This may be necessary if lanes were loaded unequally, which can result in slightly different migration rates for bands of the same molecular weight. Next, the Program should perform a densitometric analysis of grayscales along the lane, subtracting background intensity. Integration at peaks or an intensity threshold can be used for band identification, and band intensity can be categorized (e.g., from 0 to 5), directly measured, or converted according to a normalization curve. Models of the gel can then be generated from band position and intensity (Fig. 10D).

Calibrating to the Global Standard and Linking

The local standard is calibrated to the global standard (Fig. 10C), providing molecular weights of the standard bands. The Program then automatically links bands in test lanes to bands in the local standard. The user must specify a degree of tolerance for considering bands to be the same size. Bands with no correlate in the standard can be linked, and the option should exist for adding new bands to the global standard, if desired.

Creating the Basic Data File

The preceding data provide a text map in which the band number, molecular size, or pixel distance of each band is listed in descending order along the vertical axis for each labeled isolate. At each position, either the binary value 0 or 1 for position alone or the band intensity is listed. If one has collected binary data by using MLEE, RAPD, MLST, or other multilocus methods, one can manually generate the data file at this point in the computer program, or the software can combine the data automatically.

Computing Similarity Coefficients and Generating Dendrograms

The Program must provide the user with a choice of formulas for computing S_{AB}s. Once a particular formula is selected, the Program generates a matrix of S_{AB}s for all pairs of isolates. The Program must then provide the user with a choice of algorithms for generating a dendrogram from the S_{AB} matrix and software for separating groups by thresholding, testing of the stability of clusters, and statistical analyses (Fig. 9).

Other Useful Functions

The Program should provide accessible storage and comparative capabilities so that every new set of genetically fingerprinted isolates can be compared with previously

fingerprinted isolates in the database. It must also allow searches based on other elements such as patient and isolate characteristics. The former include disease state, treatment, geographical location, anatomical origin of the isolate, age, sex, weight, and predisposing conditions. The latter include growth characteristics, sugar assimilation patterns, antigenicity, drug susceptibility, switching repertoire, and other phenotypic traits, most notably those involved in virulence and pathogenicity. One must also be able to mine the genetic fingerprint database for particular characteristics, such as the presence of a particular MLEE allele or RFLP fragment.

THE COLLECTION

Because genetic fingerprinting is time-consuming, one must carefully consider the original collection. Yet, this first step is often the most poorly conceived. This problem stems no doubt in part from the assumption that colonizing populations of pathogens are genetically homogenous. It has become increasingly apparent that in many cases, infecting populations, especially in immunocompromised patients, may consist of multiple species, multiple strains of the same species, or diverging substrains. Species heterogeneity has been demonstrated in recurrent infections and commensals (92, 162). In each case in which heterogeneity was demonstrated, care was taken in obtaining the original collection.

In most standard methods of collection, the primary samples are aspirates, tissue, blood, other fluids, or swabs. These materials may be transported to a microbiology laboratory for anaerobic or aerobic culturing. In some cases, the microorganism must be released from the material. Blood cells may be lysed, tissue may be minced, and fluids may be filtered or centrifuged. The problem is clonality. If the sampled colonizing population is homogeneous (i.e., consists of one genetically homogenous strain), there is no problem. However, if the population is heterogeneous (consists of multiple species, strains, or substrains), several problematic scenarios can arise. First, if the culture is genetically heterogeneous at the time of genetic fingerprinting, a mixed fingerprint will arise. Second, if the primary culture contains mixed strains or substrains and is mass cultured, the strain or substrain that grows fastest under the culture conditions employed will enrich. Finally, a problem arises when a primary culture containing mixed strains or substrains is cultured and only one clone is picked for analysis. The single clone may not represent the majority genotype of the infecting population. The solutions to these problems are straightforward. First, the primary sample should be clonally plated, which may entail assessment of concentration, serial dilution, or knowledge of approximate density. Second, indicator agars can be used for initial screening of species. Third, more than one colony can be picked for genetic fingerprinting. The number picked and analyzed will depend on the size of the collection and the objectives of the study. These considerations apply mainly to bacteria and fungi. The problems may be harder to resolve for many parasites that cannot be grown as isolated colonies on agar media.

In addition to sample homogeneity, a second problem arises in some cases that is related to space and time. There is growing evidence of body location specificity in commensalism, geographical specificity of strains, and rapid microevolution (88, 89, 108, 135). It is prudent to consider what body location is sampled, since a pathogen like *C. albicans* can colonize multiple sites (136). It is also important to realize that if one is testing strain specificity for a particular disease, geographical differences may outweigh disease specialization. For instance, within a geographical region, one might find that isolates from a particular disease state are genetically similar, but when isolates from that disease state are collected from different geographical regions and compared, they may prove to be genetically dissimilar (108). One must, therefore, consider restricting the geographical boundaries of the collection or choosing distinct multiple populations. Finally, one must consider the speed of microevolution and the timing of sampling. If one is analyzing substrain specialization, the linear rate of microevolution may outweigh substrain differences related to different disease states or antibiotic resistance. One must, therefore, consider restricting the time window of collection.

CONCLUDING REMARKS

The preceding survey of methods used to fingerprint microbial pathogens should demonstrate that there is no dominant method that has emerged for all of the pathogen categories (bacteria, fungi, and parasites) or even for all species within a category. For instance, in bacteria, although RFLP-PFGE is the most commonly used method for *Streptococcus pneumoniae* and *Staphylococcus aureus*, RFLP with a probe is the most commonly used method for *Mycobacterium tuberculosis* (Table 2). The same variability holds true between fungal and parasite species (Table 2). The reason is twofold. First, differences between the genomes of different species warrant in some cases different methods. Second, different methods are effective for measuring different levels of relatedness. Third, and maybe

TABLE 2 Rough estimates of the use of the most common genetic fingerprinting methods[a]

Pathogen category	Genus or species	Most common genetic fingerprinting methods
Bacteria	*Streptococcus pneumoniae*	42% RFLP-PFGE; 17% RepE1 PCR; 10% restriction fragment end labeling
	Mycobacterium tuberculosis	54% RFLP + probe: 23% spoligotyping
	Staphylococcus aureus	56% RFLP-PFGE; 13% PCR-RFLP; 11% RAPD
Fungi	*Candida*	30% RAPD; 29% karyotyping; 22% RFLP + probe; 12% RFLP
	Aspergillus	71% RAPD; 18% MLEE; 11% RFLP + probe
	Cryptococcus	53% RAPD; 21% karyotyping; 18% RFLP + probe
Parasites	*Trypanosoma*	47% MLEE; 30% RAPD
	Leishmania	69% MLEE; 16% RAPD
	Plasmodium	81% PCR-RFLP; 11% RAPD

[a] Estimates were made from a literature search beginning in 1996.

not as scientific, a particular method may take hold for a particular species due to a consensus of the scientists working in that area and the history of the methods first applied. Fourth, selections may be based on expediency. Whatever the reason, the take-home message of this chapter should be clear. Select a fingerprinting method that will answer the question posed. Make sure that the method has been verified for efficacy at the level of genetic relatedness necessary. Use computer-assisted systems, and save data in a format accessible for retrospective analyses and comparisons with new data. Finally, carefully consider the methods used for obtaining the collection.

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Investigation of Foodborne and Waterborne Disease Outbreaks*

TIMOTHY F. JONES

12

Each year approximately 4 billion cases of diarrhea occur worldwide (26). In 2002, foodborne and waterborne diarrheal diseases were estimated to have killed 1.8 million people, most of whom were under 5 years of age. In the United States alone, foodborne diseases are estimated to cause 76 million illnesses, 325,000 hospitalizations, and 5,000 deaths each year (21). Food and water safety issues are complex, and foodborne and waterborne diseases can have a myriad of potential causes. Because outbreaks can affect large numbers of persons, prompt and thorough investigations of potential outbreaks can prevent substantial morbidity. In addition, outbreak investigations provide information that increases our understanding of the epidemiology of these diseases and allows us to develop preventive measures.

Foodborne and waterborne disease outbreaks are generally defined as clusters of two or more persons with a similar illness resulting from ingestion of a common food (6) or epidemiologically linked to recreational or drinking water (10). Foodborne and waterborne disease outbreaks may be caused by bacteria, viruses, protozoa, fungi, helminths, prions, and biologic or environmental toxins. Viral agents cause two-thirds of foodborne disease outbreaks in the United States, bacteria cause approximately one-third, and parasites are relatively uncommon etiologic agents. Of viruses, noroviruses are by far the most common cause of foodborne disease outbreaks. The most common bacterial causes are *Salmonella* and *Campylobacter* (21).

Many foodborne and waterborne diseases are self-limited and characterized by gastrointestinal symptoms such as vomiting and diarrhea. Others, however, may manifest as systemic or neurological disease and result in substantial morbidity and mortality. Examples include infection with *Escherichia coli* O157:H7, which can be associated with hemolytic-uremic syndrome; *Helicobacter pylori,* which has been linked to gastric cancers; *Listeria,* which can cause meningitis and miscarriage; *Salmonella,* which can cause reactive arthritis; and prions, which cause invariably fatal new-variant Creutzfeldt-Jakob disease.

Improved basic sanitation, medical care, and diagnostic capabilities have helped substantially reduce both the risks for foodborne and waterborne diseases and the frequency of complications. However, other factors have allowed old scourges to persist and new threats to emerge. Noroviruses, rotaviruses, astroviruses, *Cryptosporidium parvum, Campylobacter* spp., *E. coli* O157:H7, hepatitis E, and the prion associated with bovine-spongiform encephalopathy are among foodborne and waterborne pathogens identified since 1972. The population of vulnerable persons is growing, and new populations are being exposed to potential pathogens because more people are living longer, are living with immunosuppressive conditions, or are traveling internationally. Moreover, the food supply has been globalized so that people are eating at home foods that travel medicine physicians tell them not to eat when they are traveling abroad. Outbreaks of cryptosporidiosis in Milwaukee, Wis., in 1993, which sickened 400,000 persons and led to 100 deaths, primarily in human immunodeficiency virus-infected persons (20), and *E. coli* O157:H7 in Japan, which caused over 6,000 illnesses among persons who ate radish sprouts from a single supplier (24), are examples demonstrating the large and dramatic sequelae of such social changes.

It is important to investigate foodborne outbreaks so that the etiology, vehicle, and mechanisms associated with disease can be identified and similar outbreaks can be prevented in the future. This chapter provides a framework for understanding the general principles and strategies of outbreak investigations. Details on the clinical and epidemiologic characteristics of specific pathogens can be found in other chapters of this Manual. Additional information on outbreak investigation and testing of environmental and food specimens is available from other published sources (http://www.cdc.gov/foodborneoutbreaks/, http://www.cdc.gov/ncidod/dpd/healthywater/professional.htm, and http://www.cdc.gov/healthyswimming/outbreak.htm) (5, 12–14, 16–18).

EPIDEMIOLOGY OF FOODBORNE AND WATERBORNE DISEASE OUTBREAKS

Beginning in 1938, the U.S. Public Health Service has maintained and reported data on foodborne and waterborne disease outbreaks (6). Between 2001 and 2003, the Centers for Disease Control and Prevention (CDC) received an annual mean of 1,214 reports of outbreaks from federal, state, and local epidemiologists throughout the United States who conducted outbreak investigations (http://www.cdc.gov/foodborneoutbreaks/report_pub.htm) (11). These outbreaks involved approximately 24,000 persons. Of reported

*This chapter contains information presented in chapter 13 by John Besser, James Beebe, and Bala Swaminathan in the eighth edition of this Manual.

outbreaks during this period, approximately one-fifth were due to bacterial pathogens, about one-eighth were due to viruses, and less than one-twentieth were due to chemicals and parasitic agents. It is noteworthy that the etiology of two-thirds of the outbreaks was not identified. The pathogens most commonly identified in outbreaks reported in 2003 are shown in Fig. 1.

In the United States over one-half of the outbreaks of foodborne and waterborne disease are associated with restaurants. In contrast, nearly 90% of the global burden of diarrheal disease is attributable to unsafe water supplies (25). In 2001–2002, 96 waterborne disease outbreaks in the United States were reported to the CDC, of which 31 were associated with drinking water and 65 were associated with recreational water (10). Figure 2 illustrates the etiologic agents causing outbreaks associated with drinking water, and Fig. 3 describes the clinical syndromes seen in outbreaks related to recreational water.

The response to preventive measures and the treatment of clinical infections vary markedly depending on the etiologic agent involved. It is therefore of concern that the etiologic agents were not identified for nearly two-thirds of foodborne disease outbreaks reported to the CDC in 2001–2003 and for one-fourth of drinking-water-associated outbreaks reported in 2001–2002. This deficit is due in part to the fact that stool specimens are rarely tested in such outbreaks. In fact, stool specimens were tested for only one of seven waterborne outbreaks of acute gastrointestinal illness for which the etiology was unknown (10) and stool specimens were not collected for laboratory testing in two-thirds of foodborne disease outbreaks of unknown etiology occurring at seven sites in 1998 and 1999 (19). While many factors can impede investigators as they work up outbreaks, laboratory testing is usually necessary to confirm an etiology.

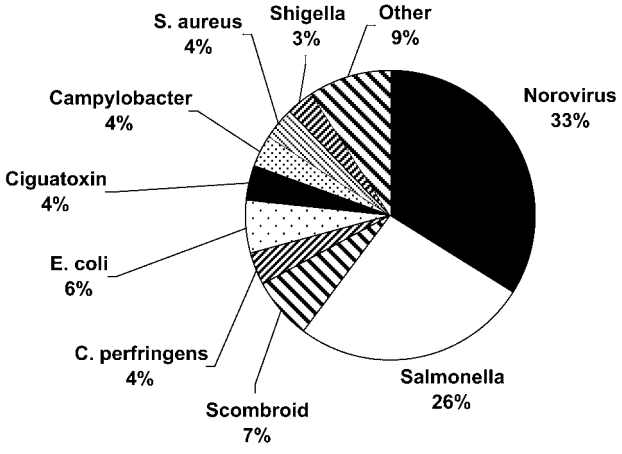


FIGURE 1 Causes of foodborne disease outbreaks with a confirmed etiology, among those reported to the Centers for Disease Control and Prevention, 2003.

GENERAL APPROACH TO AN OUTBREAK INVESTIGATION

Standard steps in an outbreak investigation are outlined in Table 1. Potential foodborne and waterborne disease outbreaks may come to the attention of health authorities through a number of mechanisms. Laboratorians, clinicians, or public health agencies may recognize unusual numbers or types of illness and report them, or patients may report illnesses to public health agencies directly. Foodborne and waterborne disease outbreaks must be reported to public health departments in most jurisdictions, and public health personnel frequently coordinate community-wide investigations, although clinicians, laboratorians, environmentalists, and institutional representatives all may play important roles in outbreak investigations. Local and state public health authorities generally have legal responsibility for outbreak investigations and may involve the CDC or other local, state, and federal agencies as appropriate. The roles of various government agencies in outbreak investigations are outlined in Table 2.

One of the first steps in responding to a possible foodborne or waterborne disease outbreak is confirming whether an "outbreak" has actually occurred. The CDC defines a foodborne disease outbreak as "the occurrence of two or

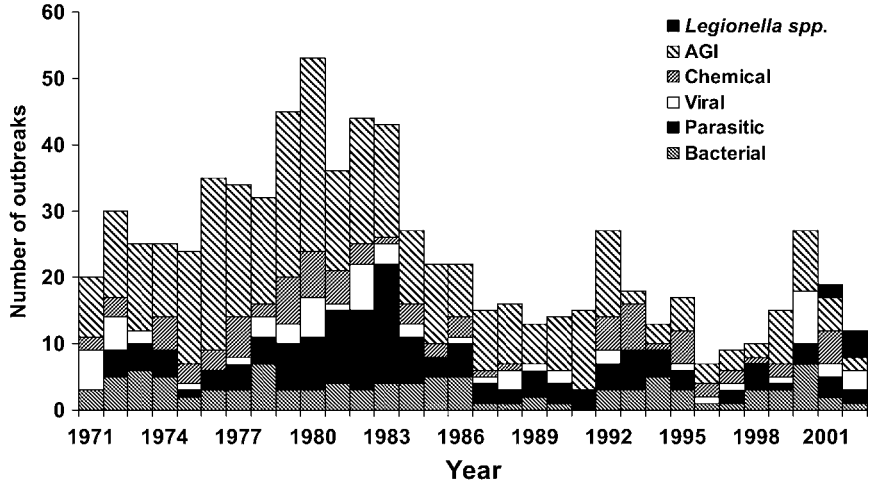


FIGURE 2 Drinking-water-associated outbreaks, by year and etiologic agent—United States, 1971–2001 (n = 764). AGI, acute gastrointestinal illness of unknown etiology. For *Legionella* spp., Legionnaires' disease was added to the surveillance system (and *Legionella* spp. were classified separately) beginning with 2001. Adapted from reference 10.

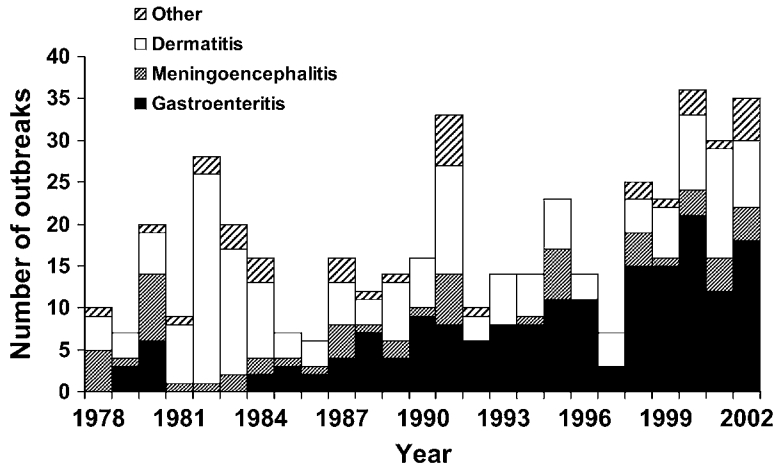


FIGURE 3 Waterborne-disease outbreaks ($n = 65$) associated with recreational water, by etiologic agent—United States, 1978–2002. "Other" includes keratitis, conjunctivitis, otitis, bronchitis, meningitis, hepatitis, leptospirosis, Pontiac fever, and acute respiratory illness. "Meningoencephalitis" also includes data from report of ameba infections (28). Adapted from reference 10.

TABLE 1 Steps of an outbreak investigation[a]

1. Establish the existence of an outbreak.
2. Verify the diagnosis.
3. Define and count cases.
4. Determine the population at risk.
5. Describe epidemiology.
6. Develop hypotheses.
7. Evaluate hypotheses.
8. Perform additional epidemiologic, environmental, and laboratory studies, as necessary.
9. Implement control and prevention measures.
10. Communicate findings.

[a]Adapted from references 5 and 14, with permission.

more cases of a similar illness resulting from ingestion of a common food" (6). The CDC defines a recreational water-associated outbreak as ≥2 persons experiencing a similar illness after exposure to water or air encountered in a recreational water setting (this criterion is waived for single cases of laboratory-confirmed primary amebic meningoencephalitis, wound infections, or chemical poisoning if water quality data indicate contamination by the chemical), with epidemiologic evidence implicating recreational water or a recreational water setting as the probable source of the illness (10). Drinking-water outbreaks are defined similarly, with the implication of drinking water rather than recreational water. Information on local and federal water quality standards is available from a number of government agencies (http://www.cdc.gov/nceh/ehhe/water/data.htm). Outbreaks caused by contamination of water or ice at the point of use (e.g., a contaminated faucet or serving container) are not classified as drinking-water-associated outbreaks (10). As these definitions suggest, it is important to quickly ascertain whether apparent clusters of patients with similar symptoms or laboratory-confirmed disease are linked epidemiologically by exposure to a common food or water source. In practical terms, many outbreaks are recognized through routine surveillance for diseases that must be reported (Table 3). If public health officials identify unusual numbers of cases clustered by time or place, they often investigate further and identify a previously unrecognized epidemiologic link between cases. To recognize unusual clusters of illness, public health officials obviously must have knowledge of what "normal" or baseline rates of a disease are in the affected community. This important information may be available from routine disease surveillance performed by public health authorities and institutional infection control personnel. To decide whether a potential cluster of disease warrants further investigation, one needs basic epidemiologic data about the potential cluster and one must consider other factors such as the resources available for an investigation, the severity of the disease, the community affected, and the likelihood that useful data can be obtained from an investigation.

To recognize and characterize a potential outbreak, one must identify the persons affected by the outbreak ("cases") and organize data about these cases by time, place, exposure history, and other characteristics. Investigators should develop a "case definition," or criteria by which a person will be judged to be part of an outbreak (or not), early in their inquiry. While this definition may change as additional information is collected, it is critical to collect sufficient data on all possible cases to determine whether each is part of the group of interest. Typically, cases are defined by criteria including symptoms, time of onset, and the time and place of potential exposure. Examples include "a case is defined as any person reporting vomiting or diarrhea within 5 days of consuming food from Restaurant A between March 2 and 5" or "a case is defined as any person seeking medical care for a rash illness, with or without fever, within 2 weeks of swimming at Lake X."

The incubation period (time from exposure to an etiologic agent until onset of symptoms) provides an important clue to the possible etiology of an outbreak. Textbooks and references frequently categorize infectious agents by incubation period. It is important to remember, however, that a definitive incubation period can rarely be determined based on a single patient's history. Typical signs and symptoms and the mean incubation period and duration of illness are most useful when evaluated in aggregate, for a number of ill patients. In addition, patients often assume that recent meals or particular events caused their illnesses, when

TABLE 2 Roles of selected government agencies in responding to possible foodborne or waterborne disease outbreaks in the United States[a]

Agency	Responsibilities	Product or situation of interest
Local and state health departments	Generally authorized under state laws to be responsible for surveillance for notifiable diseases and investigation of most foodborne and waterborne outbreaks in their jurisdiction. Often responsible to inspect and regulate restaurants within jurisdiction. State health laboratories support outbreak investigations.	Any legally notifiable disease or outbreak of public health importance.
Local and state water regulatory agencies	Generally authorized under state laws to have oversight of surface water, municipal water systems, wells, and other water supplies. Responsibility may be under the jurisdiction of different agencies, including health, agriculture, or environmental safety departments.	Regulation of recreational and drinking water supplies including surface water, wells, source water protection, and public water systems.
State department of agriculture	Enforce state food safety laws and perform investigations associated with facilities or products they regulate.	Farms, food production facilities and warehouses, milk production facilities, water bottling facilities, grocery stores, and many retail food establishments within the state.
Federal Centers for Disease Control and Prevention (CDC)	Assists state and local authorities in outbreak investigations, by invitation. May provide extensive laboratory and epidemiology support. Frequently participates in multistate and international outbreaks.	Any disease or outbreak of public health importance.
U.S. Food and Drug Administration (FDA)	May assist local authorities in investigations associated with products they regulate, perform interstate or international product tracebacks, and provide laboratory and regulatory support.	Manufacturers, processors, and distributors of human and animal foods (except meat, poultry, and processed egg products); potable water, bottled water, and dietary supplements shipped in interstate commerce.
U.S. Department of Agriculture, Food Safety and Inspection Service (FSIS)	May assist local authorities in investigations associated with products they regulate, perform interstate or international product tracebacks, and provide laboratory and regulatory support.	Domestic and imported meat, poultry, and egg products, including soups, stews, pizzas, and frozen foods which contain meat or poultry.
Environmental Protection Agency (EPA)	Works with other agencies in responding to outbreaks associated with contaminated water or environmental contaminants.	Drinking water, toxic substances, and wastes to prevent their entry into the environment and food chain.
Law enforcement	Federal Bureau of Investigation (FBI) has the authority to lead the investigation of any outbreak suspected of being associated with terrorism. Works with other health and regulatory agencies on the investigation.	Outbreaks involving criminal actions, including acts of terrorism.

[a]This table provides a general outline of typical responsibilities for different agencies involved in investigation of food- and waterborne outbreaks. All states have unique food and water safety laws, policies, and organizational structures that will affect investigations, and many other agencies and organizations may play important roles in certain situations. Additional information is available at the Gateway to Government Food Safety Information website (http://www.foodsafety.gov/) and the EPA website (http://www.epa.gov/ebtpages/water.html).

further epidemiologic investigation implicates earlier exposures. Moreover, many foodborne and waterborne infections present with clinical signs and symptoms that are indistinguishable from those of other common diseases, and few have pathognomonic clinical features. Thus, clinicians evaluating individual patients may have difficulty identifying the source of the infection, and persons investigating an outbreak should not jump to conclusions regarding the source of infection. In any epidemiologic study of a potential outbreak, the investigators must systematically and completely assess the population of affected persons and systematically identify the involved cohort or well controls, which may be challenging. Laboratories can assist the investigators by reporting all laboratory-confirmed cases of the disease of

TABLE 3 Foodborne and waterborne diseases and conditions designated as notifiable at the national level in the United States, 2003[a]

Bacterial diseases and conditions
Anthrax
Botulism
Brucellosis
Cholera
Enterohemorrhagic *E. coli*
Hemolytic-uremic syndrome, postdiarrheal
Listeriosis
Salmonellosis
Shigellosis
Typhoid fever (*S. enterica* serovars Typhi and Paratyphi infections)
Viral diseases
Hepatitis A
Parasitic diseases
Cryptosporidiosis
Cyclosporiasis
Giardiasis
Trichinellosis

[a]Adapted from reference 9. Additional diseases and conditions may be reportable at the state level, and outbreaks of public health importance are reportable regardless of etiology.

interest within a specific geographic area or time period. Public health authorities may augment routine surveillance for notifiable diseases by contacting medical providers, hospitals, and laboratories directly or by pursuing other means of active surveillance. Media reports can encourage ill persons to report their illnesses directly to public health authorities, and known cases may be able to identify other ill persons or well persons with similar exposures who could be controls for epidemiologic studies.

To identify the entire exposed group, investigators may need to collect information on all persons who attended a large function, residents of an institution, patrons of a restaurant, or other potentially exposed groups. Public health authorities have substantial legal authority to gather data from individuals, groups, and institutions when investigating outbreaks that threaten the public health. For example, they may at times review credit card receipts to identify patrons of a restaurant, use records generated by grocery stores' frequent-shopper discount cards, or make public announcements to identify enough cases or controls for an investigation. However, epidemiologists must assess potential biases introduced into a study by the mechanisms through which they identify the cases or controls.

EPIDEMIOLOGIC INTERVIEWS

After confirming the existence of an outbreak, investigators must interview affected persons to identify demographic characteristics of cases, the nature and timing of symptoms, and potential causative exposures. As noted previously, interviews can also help investigators identify other ill persons or well persons with similar characteristics who may be enrolled in the epidemiologic studies. The initial broad-based and open-ended interviews provide data that allow investigators to generate hypotheses about the suspected etiologic agents and exposures (e.g., particular foods, restaurants, water sources, etc.) in order to efficiently focus subsequent investigations.

Standardized written questionnaires can help investigators collect data consistently and completely from all study participants. The CDC and others maintain sample templates of questionnaires, which can be modified, for preliminary outbreak investigations (http://www.cdc.gov/foodborneoutbreaks/standard_questionnaire.htm). While standard templates are a useful starting point, a "one-size-fits-all" approach is rarely optimal, and questionnaires likely will need to be modified depending on the suspected etiology, affected population, and particular details surrounding a specific outbreak. Moreover, investigators inevitably want to be comprehensive, but this desire must be balanced by practical considerations including the ease with which support staff can use the questionnaire and its acceptability to study participants. Complete answers to reasonably limited questions are generally preferred over incomplete answers to an unreasonable number. The design of a questionnaire should be tailored for the population to whom it is directed, the mode of administration, and the sophistication of those collecting the data. Collection of data via telephone, face-to-face interview, written survey, self-administered questionnaire, and electronic data capture all have associated advantages and disadvantages that must be considered in the planning stages of a study. Before the study begins, all persons who will collect data must be taught how to introduce the study to participants and answer questions. Data collectors must also be given guidelines for obtaining data and standard definitions so that the data are elicited consistently and completely, thereby minimizing bias and erroneous information.

Data on cases can be organized in various formats for different purposes. Basic information about cases is frequently entered on a "line-listing" or spreadsheet. The distribution of cases over time (frequently categorized by date or time of symptom onset) can be represented graphically as an "epidemic curve" (Fig. 4), and the geographic distribution of cases can be plotted on a map. The line-list, the epidemic curve, and the map can give the investigator clues about the source of an outbreak. For example, if these representations suggest that the outbreak is acute and associated with an exposure at a single time and place ("point source outbreak"), such as all patrons of a single restaurant on a single day, the outbreak may have been caused by a contamination event that is relatively easy to identify and correct. In contrast, if the line-list, epidemic curve, and map indicate that the outbreak has occurred over a large area or over a prolonged time period, the investigators may need to search for contaminated food or water products distributed over a wide area, a source of intermittent or low-level contamination, a means by which products are recurrently contaminated during production, storage, or serving, or a component of person-to-person spread (i.e., secondary cases) following an initial food or water-related source, and a potential ongoing threat to the public health.

The intensity of an investigation may vary widely, depending on the nature of an outbreak, the population involved, the etiologic agent, and available resources. In some cases, basic laboratory testing and interviews of a limited number of ill persons may provide investigators with sufficient information to identify the source of the outbreak and institute adequate interventions and preventive measures. In many cases, more extensive epidemiologic investigation is required. If the population affected by the outbreak is well defined, such as attendees of a church picnic or patrons of a single restaurant within a 3-day period, a retrospective cohort study can be performed. In this situation, all members or a representative sample of the affected group (the "cohort")

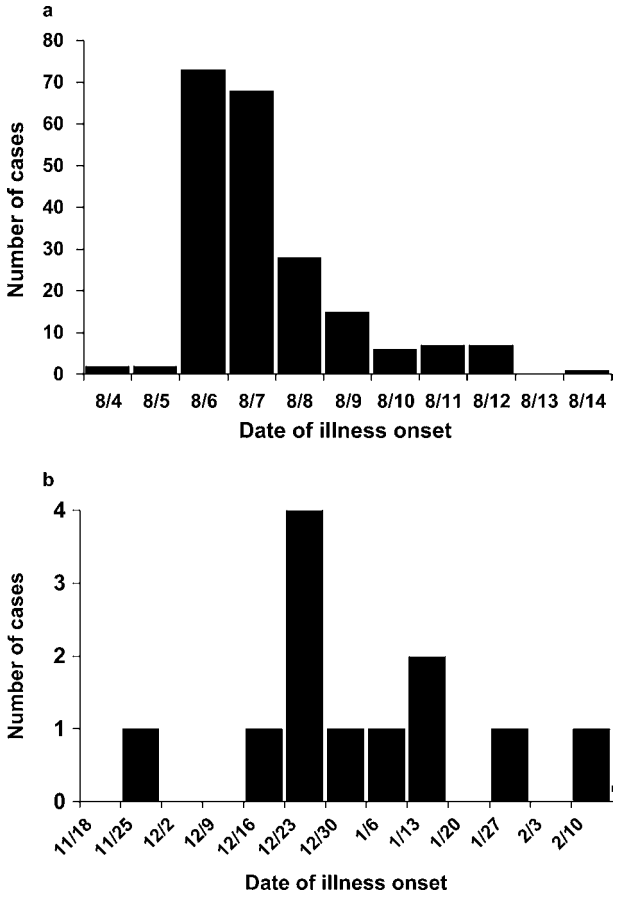


FIGURE 4 (a) Epidemic curve, point-source outbreak of *Salmonella* associated with a restaurant in Tennessee. All ill patrons ate at the establishment on the same day, and the distribution of illness onset times reflects natural variation in incubation period. (b) Epidemic curve, continuing common-source outbreak of *Yersinia* associated with chitterlings in Tennessee. The distribution of dates of illness onset suggests that the source of infection was a contaminated product handled over several weeks. Under different conditions, a similar epidemic curve might reflect a propagated outbreak, in which earlier cases were the source of infection for subsequent cases.

are interviewed to assess whether they were ill and what foods or water they consumed. Rates of illness among persons who did or did not consume particular foods (or among persons who were or were not exposed to other factors of interest) are compared by appropriate statistical methods to help investigators identify likely causes of an outbreak (5, 14). If the affected population is not well defined or a cohort study is not practical, a case-control study may be performed. In such outbreaks, persons with the illness of interest (cases) are compared to those without it (controls).

Statistical analyses can help investigators summarize their findings from the epidemiologic investigation and help them draw conclusions from data that may be complicated. Basic descriptive statistics should include the number of ill persons, a summary of the characteristics of cases and the population at risk, the proportion of ill persons experiencing particular symptoms, mean and/or median incubation period (time between exposure and onset of symptoms), and mean and/or median duration of illness. Data from cohort studies may be used to calculate the attack rate (proportion of persons exposed to an etiologic agent who subsequently developed disease). Data from cohort and case-control studies can be used to estimate the risk (using "risk ratios" and "odds ratios," respectively) of developing disease after exposure to particular foods, water sources, or other risk factors. Basic two-by-two tables or comparisons of relative risks of illness among those exposed to and those not exposed to particular foods frequently suffice to support the most imperative decisions during an outbreak investigation. More advanced statistical techniques, such as stratified analyses, regression modeling, and sensitivity analyses, may also be appropriate. Detailed information on doing and interpreting statistical analyses of epidemiologic data is available from multiple sources (5, 14, 22, 23). While statistical analytic techniques can be powerful tools, investigators must remember that all statistics must be interpreted cautiously and with a component of judgment. The obvious cause of a foodborne or waterborne disease outbreak should not be discounted simply because its associated "*P* value" (a commonly cited measure of statistical significance) is greater than 0.05, and likewise measures of statistical significance (particularly in larger studies) do not necessarily prove that a result is biologically plausible or meaningful in a given situation.

LABORATORY INVESTIGATION

Laboratory testing is usually needed to confirm the etiology of an outbreak. In most foodborne or waterborne outbreaks, the etiologic agent should be isolated from the stool specimens of two or more ill persons or from the epidemiologically implicated food (Table 4). In a few situations, such as mushroom poisoning, ciguatera fish poisoning, or other chemical intoxications, it is sufficient to document the clinical syndrome among affected persons. *Staphylococcus* can also be problematic because the organism may not be viable in stool or food samples and most laboratories cannot test for enterotoxin. Thus, investigators must collect sufficient numbers of specimens (potentially including stool and other clinical specimens and also food, water, or environmental specimens) and handle them appropriately to ensure that laboratory testing identifies the etiologic agent.

Epidemiologists, environmentalists, or others participating in outbreak investigations should communicate early and openly with laboratorians to ensure that the specimens collected can be tested for all suspected etiologic agents and that appropriate specimens are collected. Many organisms (including most bacterial causes of foodborne and waterborne diseases) can be isolated from both clinical and environmental specimens. In such cases, molecular methods (such as pulsed-field gel electrophoresis [PFGE]) can be used to demonstrate similarities between isolates from affected persons and the putative source, thereby confirming the epidemiological studies. Other common agents, such as norovirus, are not recovered easily from food or water, whereas many toxins are more readily identified in food than in clinical specimens. Moreover, if the laboratory needs to identify or quantify pathogens in food, the epidemiologic investigators must tell the laboratory which pathogens are suspected so that resources are not wasted.

Most clinical laboratories currently cannot test specimens for norovirus, which is the most common cause of foodborne disease in the United States. Most state health department laboratories offer reverse transcriptase (RT)-PCR testing for norovirus and can help coordinate appropriate testing of

TABLE 4 Typical characteristics of foodborne and waterborne disease outbreaks and guidelines for confirmation[a]

Etiologic agent	Incubation period	Clinical syndrome	Typical vehicle	Diagnostic testing	Confirmation of outbreak etiology
Bacterial					
Bacillus cereus					
Preformed toxin	1–6 h	Vomiting; some patients with diarrhea; fever uncommon	Improperly stored cooked or fried rice, meats	Clinical diagnosis; clinical laboratories do not routinely identify; stool and food specimens may be tested at a reference laboratory for culture and toxin identification	Isolation of organism from stool of two or more ill persons and not from stool of control patients OR Isolation of 10^5 organisms/g from epidemiologically implicated food, provided specimen is properly handled
Diarrheal toxin	10–16 h	Diarrhea, abdominal cramps, and vomiting in some patients; fever uncommon	Cereal products, soups, custards and sauces, meatloaf, sausage, cooked vegetables, reconstituted dried potatoes, refried beans	Stool and food specimens may be tested at a reference laboratory for culture and toxin identification	Isolation of organism from stool of two or more ill persons and not from stool of control patients OR Isolation of 10^5 organisms/g from epidemiologically implicated food, provided specimen is properly handled
Brucella	7–21 days	Weakness, fever, headache, sweats, chills, arthralgia, weight loss, splenomegaly, bloody stools	Raw milk, goat cheese from unpasteurized milk, contaminated meats	Blood cultures and serology	Two or more ill persons and isolation of organism in culture of blood or bone marrow OR Greater-than-fourfold increase in standard agglutination titer (SAT) over several weeks OR Single SAT of 1:160 in person who has compatible clinical symptoms and history of exposure
Campylobacter jejuni/coli	2–10 days; usually 2–5 days	Diarrhea (often bloody), abdominal pain, fever, vomiting	Unpasteurized milk, raw and undercooked poultry, contaminated water	Routine stool culture; requires special media and incubation at 42°C	Isolation of organism from clinical specimens from two or more ill persons OR Isolation of organism from epidemiologically implicated food
Clostridium botulinum (toxin)	12–72 h	Vomiting, diarrhea, blurred vision, diplopia, dysphagia, descending muscle weakness	Canned low-acid foods, smoked fish, cooked potatoes in foil, garlic in oil, fish, marine mammals	Stool, serum, and food tested for toxin; stool and food cultured for organism; testing available at state health department or CDC laboratories	Detection of botulinum toxin in serum, stool, gastric contents, or implicated food OR

					Isolation of organism from stool or intestine
Clostridium perfringens (toxin)	8–16 h	Watery diarrhea, nausea, abdominal cramps; vomiting and fever uncommon	Meats, poultry, gravy, dried or precooked foods, time- and/or temperature-abused foods	Stools tested for enterotoxin and cultured; because C. *perfringens* can normally be found in stool, quantitative cultures must be done	Isolation of 10^6 organisms/g from stool of two or more ill persons, provided specimen is properly handled OR Demonstration of enterotoxin in the stool of two or more ill persons OR Isolation of 10^5 organisms/g from epidemiologically implicated food, provided specimen is properly handled
Escherichia coli					
Enterohemorrhagic *E. coli* (EHEC) including *E. coli* O157:H7 and other Shiga toxin-producing *E. coli* (STEC)	1–8 days	Diarrhea (often bloody), abdominal pain, vomiting; fever is rare	Undercooked beef (especially hamburger), unpasteurized milk and juice, raw fruits and vegetables (e.g., sprouts), contaminated water	Stool culture; Shiga toxin may be detected using commercial kits; positive isolates should be forwarded to public health laboratories for confirmation and serotyping	Isolation of *E. coli* O157:H7 or other Shiga-like toxin-producing *E. coli* strains from clinical specimen from two or more ill persons or from epidemiologically implicated food
Enterotoxigenic *E. coli* (ETEC)	1–3 days	Watery diarrhea, abdominal cramps; vomiting and fever less common	Water or foods contaminated with human feces	Stool culture; special laboratory techniques are required to identify ETEC; if suspected, request specific testing	Isolation of organism of same serotype that produces heat-stable and/or heat-labile enterotoxin from stool of two or more ill persons
Enteropathogenic *E. coli* (EPEC)	Variable	Diarrhea, fever, abdominal cramps	Water, fecal-oral contamination	Stool culture; special laboratory techniques are required to identify EPEC; if suspected, request specific testing	Isolation of organism of same enteropathogenic serotype from stool of two or more ill persons
Enteroinvasive *E. coli* (EIEC)	Variable	Diarrhea (might be bloody), fever, abdominal cramps	Salads and other foods not subsequently heated, water	Stool culture; special laboratory techniques are required to identify EIEC; if suspected, request specific testing	Isolation of same enteroinvasive serotype from stool of two or more ill persons
Listeria monocytogenes					
Invasive disease	2–6 weeks	Meningitis, neonatal sepsis, fever	Coleslaw, milk, soft cheese, pâté, turkey franks, processed meats	Blood or cerebrospinal fluid cultures; asymptomatic fecal carriage occurs; antibody to listerolysin O	Isolation of organism from normally sterile site
Diarrheal disease	Unknown	Diarrhea, abdominal cramps, fever	Corn salad, chocolate milk	Stool culture	Isolation of organism of same serotype from stool of two or more ill persons exposed to food that is epidemiologically implicated or from which organism of same serotype has been isolated

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TABLE 4 Typical characteristics of foodborne and waterborne disease outbreaks and guidelines for confirmation[a] *(Continued)*

Etiologic agent	Incubation period	Clinical syndrome	Typical vehicle	Diagnostic testing	Confirmation of outbreak etiology
Salmonella Non-Typhi	1–3 days	Diarrhea, often with fever and abdominal cramps	Poultry, eggs, meat products, raw milk or juice, cheese, contaminated raw fruits and vegetables (sprouts, melons)	Stool culture	Isolation of organism of same serotype from clinical specimens from two or more ill persons OR Isolation of organism from epidemiologically implicated food
Serovar Typhi	3–60 days; usually 7–14 days	Fever, anorexia, malaise, headache, and myalgia; sometimes diarrhea or constipation	Shellfish, any food contaminated by infected person, raw milk, meat contaminated after processing, cheese, watercress, water	Stool culture, blood culture.	Isolation of organism from clinical specimens from two or more ill persons OR Isolation of organism from epidemiologically implicated food
Shigella spp.	24–48 h	Diarrhea (often bloody), often accompanied by fever and abdominal cramps	Any food contaminated by infected person; frequently salads, poi, water	Stool culture	Isolation of organism of same serotype from clinical specimens from two or more ill persons OR Isolation of organism from epidemiologically implicated food
Staphylococcus aureus (preformed toxin)	1–6 h	Sudden onset of severe nausea and vomiting, diarrhea; fever may be present	Unrefrigerated or improperly stored meats, potato and egg salads, cream pastries	Normally a clinical diagnosis; stool, vomitus, and food can be tested for toxin	Isolation of organism of same phage type from stool or vomitus of two or more ill persons OR Detection of enterotoxin in epidemiologically implicated food OR Isolation of 10^5 organisms/g from epidemiologically implicated food, provided specimen is properly handled
Streptococcus, group A	1–4 days	Fever, pharyngitis, scarlet fever, upper respiratory infection	Raw milk, egg-containing salads	Throat culture, culture of food	Isolation of organism of same M- or T-type from throats of two or more ill persons OR Isolation of organism of same M- or T-type from epidemiologically implicated food
Vibrio cholerae O1 or O139	24–72 h	Profuse watery diarrhea and vomiting; dehydration and death can occur within hours	Raw fish, shellfish, crustaceans, contaminated water	Stool culture; special media required to isolate the organism; request specific testing if suspected	Isolation of toxigenic organism from stool or vomitus of two or more ill persons OR Significant rise in vibriocidal, bacterial agglutinating, or antitoxin antibodies in acute- and early

					convalescent-phase sera among persons not recently immunized OR Isolation of toxigenic organism from epidemiologically implicated food
Non-O1 and non-O139	1–5 days	Watery diarrhea	Shellfish, fish	Stool culture; special media required to isolate the organism; request specific testing if suspected	Isolation of organism of same serotype from stool of two or more ill persons
Vibrio parahaemolyticus	2–48 h	Watery diarrhea, cramps, nausea, vomiting	Undercooked or raw seafood or cooked seafood contaminated with seawater or by utensils used on raw seafood	Stool culture; special media required to isolate the organism; request specific testing if suspected	Isolation of Kanagawa-positive organism from stool of two or more ill persons OR Isolation of 10^5 Kanagawa-positive organisms/g from epidemiologically implicated food, provided specimen is properly handled
Yersinia enterocolitica and *Y. pseudotuberculosis*	24–48 h	Diarrhea, abdominal pain (often severe), appendicitis-like symptoms	Undercooked pork, unpasteurized milk, tofu, contaminated water, chitterlings	Stool, vomitus or blood culture; special media required to isolate the organism; request specific testing if suspected; serology available in reference laboratories	Isolation of organism from clinical specimen from two or more ill persons OR Isolation of pathogenic strain of organism from epidemiologically implicated food
Chemical					
Marine toxins					
Ciguatera toxin	1–48 h; usually 2–8 h	Usually gastrointestinal symptoms followed by neurologic symptoms (including paresthesia of lips, tongue, throat, or extremities) and reversal of hot and cold sensation	Numerous varieties of tropical fish, e.g., barracuda, grouper, red snapper, amberjack, goatfish, skipjack, parrotfish	Radioassay for toxin in fish or a consistent history	Demonstration of ciguatoxin in epidemiologically implicated fish OR Clinical syndrome among persons who have eaten a type of fish previously associated with ciguatera fish poisoning (e.g., snapper, grouper, or barracuda)
Scombroid toxin (histamine)	1 min–3 h; usually <1 h	Flushing, dizziness, burning of mouth and throat, headache, gastrointestinal symptoms, urticaria, and generalized pruritis	Fish: tuna, mackerel, Pacific dolphin (mahi mahi), bluefin, marlin, escolar	Detect histamine in food or clinical diagnosis	Demonstration of histamine in epidemiologically implicated fish OR Clinical syndrome among persons who have eaten a type of fish previously associated with histamine fish poisoning (e.g., mahimahi or fish of order Scombroidei)

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TABLE 4 Typical characteristics of foodborne and waterborne disease outbreaks and guidelines for confirmation[a] *(Continued)*

Etiologic agent	Incubation period	Clinical syndrome	Typical vehicle	Diagnostic testing	Confirmation of outbreak etiology
Paralytic or neurotoxic shellfish poison	30 min–3 h	Paresthesia of lips, mouth, or face and extremities; intestinal symptoms or weakness, including respiratory difficulty	Mussels, clams, scallops	High-pressure liquid chromatography to detect toxin in food or water where fish are located	Detection of toxin in epidemiologically implicated food OR Detection of large numbers of a dinoflagellate species associated with shellfish poisoning in water from which epidemiologically implicated mollusks are gathered
Puffer fish, tetrodotoxin	10 min–3 h; usually 10–45 min	Paresthesia of lips, tongue, face, or extremities, often following numbness; loss of proprioception or floating sensations	Puffer-type fish	Toxin testing of fish	Demonstration of tetrodotoxin in epidemiologically implicated fish OR Clinical syndrome among persons who have eaten puffer fish
Heavy metals: antimony, cadmium, copper, iron, tin, zinc	5 min–8 h; usually <1 h	Vomiting, often metallic taste	High-acid foods and beverages, metal-colored cake decorations	Testing foods	Demonstration of high concentration of metal in epidemiologically implicated food
Monosodium glutamate (MSG)	3 min–2 h; usually <1 h	Burning sensation in chest, neck, abdomen, or extremities; sensation of lightness and pressure over face or heavy feeling in chest	Foods seasoned with MSG	Clinical diagnosis; testing food	Clinical syndrome among persons who have eaten food containing MSG (e.g., usually 1.5 g of MSG)
Mushroom toxins					
Shorter-acting toxins	2 h	Vomiting and diarrhea; other symptoms differ with toxin	Many species of wild mushrooms	Typical syndrome, identify mushroom, detect toxin	Clinical syndrome among persons who have eaten mushroom identified as toxic type OR Demonstration of toxin in epidemiologically implicated mushroom or food containing mushroom
Muscimol		Confusion, visual disturbance			
Muscarine		Salivation, diaphoresis			
Psilocybin		Hallucinations			
Coprinus artrementaris		Disulfiram-like reaction			
Ibotenic acid		Confusion, visual disturbance			

Longer-acting toxins (e.g., *Amanita* spp.)	6–24 h	Diarrhea and abdominal cramps for 24 h followed by hepatic and renal failure	Wild mushrooms	Typical syndrome, identify mushroom, detect toxin	Clinical syndrome among persons who have eaten mushroom identified as toxic type OR Demonstration of toxin in epidemiologically implicated mushroom or food containing mushrooms
Parasitic					
Cryptosporidium parvum	2–28 days; median, 7 days	Diarrhea, nausea, vomiting, fever	Water, any uncooked food or food contaminated after cooking	Request specific examination of stool, food, or water for *Cryptosporidium*	Demonstration of organism or antigen in stool or in small-bowel biopsy specimens of two or more ill persons OR Demonstration of organism in epidemiologically implicated food
Cyclospora cayetanensis	1–11 days; median, 7 days	Fatigue, protracted diarrhea, often relapsing	Produce including raspberries, lettuce, basil; water	Request specific examination of stool, food, or water for *Cyclospora*	Demonstration of organism in stool of two or more ill persons
Giardia lamblia	3–25 days; median, 7 days	Diarrhea, gas, cramps, nausea, fatigue	Any uncooked food or food contaminated by ill food-handler, water	Stool examination for ova and parasites; may require at least 3 specimens	Two or more ill persons and detection of antigen in stool or demonstration of organism in stool, duodenal contents, or small-bowel biopsy specimen
Trichinella spp.	1–2 days for intestinal phase; 2–4 weeks for systemic phase	Fever, myalgia, periorbital edema, high eosinophil count	Pork, bear meat, walrus flesh, cross-contaminated ground beef, lamb	Positive serology, demonstration of larvae in muscle biopsy specimen, increase in eosinophils	Two or more ill persons and positive serologic test or demonstration of larvae in muscle biopsy OR Demonstration of larvae in epidemiologically implicated meat
Viral					
Hepatitis A	15–50 days; median, 28 days	Jaundice, dark urine, fatigue, anorexia, nausea	Water, raw shellfish, any food contaminated by infected person	Increase in ALT, bilirubin; positive IgM and anti-hepatitis A antibodies	Detection of immunoglobulin M anti-hepatitis A virus in serum from two or more persons who consumed epidemiologically implicated food
Noroviruses (and other caliciviruses)	12–48 h	Vomiting, cramps, diarrhea, headache, fever	Shellfish, any food contaminated by infected person	Routine RT-PCR and electron microscopy on fresh unpreserved stool samples; negative bacterial cultures; stool negative for white blood cells	Detection of viral RNA in stool or vomitus by RT-PCR OR Visualization of small, round-structured viruses that react with patient's convalescent-phase sera but not

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TABLE 4 Typical characteristics of foodborne and waterborne disease outbreaks and guidelines for confirmation[a] *(Continued)*

Etiologic agent	Incubation period	Clinical syndrome	Typical vehicle	Diagnostic testing	Confirmation of outbreak etiology
					acute-phase sera by immune-electron microscopy OR More than fourfold rise in antibody titer to Norwalk virus or Norwalk-like virus in acute and convalescent sera in most serum pairs
Astrovirus	15–77 h; usually 24–48 h	Vomiting, cramps, diarrhea, headache	Ready-to-eat foods contaminated by infected food-handler	Identification of virus in early acute-phase stool specimen, serology; commercial enzyme-linked immunosorbent assay kits available	Detection of virus antigen by enzyme immunoassay OR Detection of viral RNA in stool or vomitus by RT-PCR OR Visualization of viruses with characteristic surface morphology by electron microscopy

[a] Adapted from references 6 and 18.

specimens if this agent is suspected as the cause of an outbreak. Likewise, many laboratories do not serotype *Salmonella,* and few can definitively identify enterotoxigenic *E. coli,* non-O157:H7 enterohemorrhagic *E. coli* strains, or staphylococcal enterotoxin. Consequently, investigators should notify the appropriate health departments early in the course of an outbreak investigation if such laboratory services are necessary.

Guidelines for collecting appropriate specimens during an outbreak investigation are listed in Table 5. It can be difficult to collect adequate specimens for laboratory testing. If the outbreak is not reported promptly or the investigation is delayed, affected persons may have recovered and appropriate clinical specimens may not be available for testing. Investigators may need to expend substantial effort convincing patients to provide stool specimens and may need to provide transportation for the patients or the specimens, convenient collection materials, and free testing so that sufficient specimens are obtained. Investigators should promptly contact laboratories that may have received previous specimens from outbreak-associated patients to ask that the original specimens be held for possible additional testing. Suspected "vehicles," such as water or food samples, should be collected as soon as possible after an outbreak is recognized. If possible, investigators should collect specimens from the batches or lots of food or water that cases actually ate or drank. If this is not possible (as is often the case in outbreaks caused by pathogens with long incubation periods), the investigators should collect food or water from stored products, leftovers, suppliers, or other sources that represent as closely as possible what was consumed by the cases. If investigators suspect that the source of an outbreak is contaminated packaged food, they should collect unopened packages from the implicated lot. In such cases, investigators should work with the appropriate regulatory authorities to ensure that specimens are handled and tested properly because the specimens might provide important evidence for product tracebacks or other regulatory interventions. If specimens, such as leftovers from an implicated meal, have been handled and stored by ill persons, investigators must interpret the results cautiously, because the case may have contaminated the products after becoming infected.

Molecular microbiology technology has markedly changed the nature of many acute-disease epidemiology investigations. For example, PFGE can provide DNA fingerprints of bacterial isolates from clinical and food specimens. If the PFGE patterns are the same, the investigators have evidence that the suspected food item is implicated in the event. PFGE can also help investigators include related cases and exclude concurrent cases that are epidemiologically unrelated to the outbreak. PulseNet, a CDC-sponsored program that allows PFGE patterns to be shared nationally, has greatly helped public health officials recognize multistate outbreaks affecting small numbers of persons scattered over time or large geographic areas. In the absence of this technology, the epidemiologic link between cases is often missed.

Bender et al. evaluated the PFGE patterns of *E. coli* O157:H7 isolates obtained during a 2-year period in Minnesota (2). These investigators identified 10 outbreaks, of which 40% were identified solely based on subtype-specific surveillance. In addition, 8 of 11 (73%) "apparent outbreaks" (i.e., increases in numbers of reported cases) were not associated with clonal outbreaks, and 2 of the remaining 3 outbreaks were actually multiple clonal outbreaks occurring within the same time period. The same group studied the molecular epidemiology of *Salmonella enterica* serovar Typhimurium and identified 16 outbreaks over a 5-year period, including 10

TABLE 5 Guidelines for collection and handling of stool specimens during a foodborne or waterborne disease outbreak investigation[a]

Procedure	Instructions regarding specimens to be tested for:			
	Bacteria	Parasites[c]	Viruses[d]	Chemicals
Collection[b]				
When to collect	During period of active diarrhea (preferably as soon as possible after onset of illness).	Anytime after onset of illness (preferably as soon as possible).	Within 48–72 h after onset of illness.	Soon after onset of illness (preferably within 48 h of exposure to contaminant).
How much to collect	Two rectal swabs or swabs of fresh stool from 10 ill persons; whole stool is preferred if testing for nonbacterial pathogens is considered.	A fresh stool sample from 10 ill persons; to enhance detection, 3 stool specimens per patient can be collected >48 h apart.	As much stool sample as possible from 10 ill persons (a minimum of 10 ml of stool from each).	A fresh urine sample (50 ml) from 10 ill persons; samples from 10 controls also can be submitted; collect vomitus, if vomiting occurs within 12 h of exposure; collect 5–10 ml of whole blood if a toxin/poison that is not excreted in urine is suspected.
Method of collection	For rectal swabs, moisten 2 swabs in an appropriate transport medium (e.g., Cary-Blair, Stuart, Amies; buffered glycerol-saline is suitable for *E. coli, Salmonella, Shigella,* and *Y. enterocolitica* but not for *Campylobacter* and *Vibrio*); insert swabs 1–1.5 inches into rectum and gently rotate; place both swabs into the same tube deep enough that medium covers the cotton tips; break off top portion of sticks and discard; alternatively, swab whole stools and put the swabs into Cary-Blair medium.	Collect bulk stool specimen, unmixed with urine, in a clean container; place a portion of each stool sample into 10% formalin and polyvinyl alcohol preservative at a ratio of one part stool to three parts preservative; mix well; save portion of the unpreserved stool placed into a leakproof container for antigen or PCR testing.	Place fresh stool specimens (liquid preferable), unmixed with urine, in clean, dry containers, e.g., urine specimen cups.	Collect urine, blood, or vomitus in prescreened containers[e]; if prescreened containers are not available, submit field blanks with samples[f]; most analyses from blood require separation of serum from red cells; cyanide, lead, and mercury analyses require whole blood collected in prescreened EDTA tubes; volatile organic compounds require whole blood collected in a specially prepared gray-top tube.
Storage	Refrigerate swabs in transport media at 4°C; when possible, test within 48 h after collection; otherwise, freeze samples at −70°C; refrigerate whole stool, process it within 2 h after collection; store portion of each stool specimen frozen at less than −15°C for antigen or PCR testing.	Store specimen in fixative at room temperature or refrigerate unpreserved specimen at 4°C; a portion of unpreserved stool specimen may be frozen at less than −15°C for antigen or PCR testing.	Immediately refrigerate at 4°C; store portion of each stool specimen frozen at less than −15°C for antigen or PCR testing.	Immediately refrigerate at 4°C and if possible freeze urine, serum, and vomitus specimens at less than −15°C; refrigerate whole blood for volatile organic compounds and metals at 4°C.

(Continued on next page)

TABLE 5 Guidelines for collection and handling of stool specimens during a foodborne or waterborne disease outbreak investigation[a] *(Continued)*

Procedure	Instructions regarding specimens to be tested for:			
	Bacteria	Parasites[c]	Viruses[d]	Chemicals
Transportation	For refrigeration, follow instructions for viral samples. For frozen samples, place bagged and sealed samples on dry ice. Mail in insulated box by overnight mail.	For refrigeration, follow instructions for viral samples. For room-temperature samples, mail in waterproof container.	Keep refrigerated; place bagged and sealed specimens on ice or with frozen refrigerant packs in an insulated box; send by overnight mail. Send frozen specimens on dry ice for antigen or PCR testing.	Place double-bagged and sealed urine, serum, and vomitus specimens on dry ice; mail in an insulated box by overnight mail. Ship whole blood in an insulated container with prefrozen ice packs. Avoid placing specimens directly on ice packs.

[a] Adapted from reference 7.
[b] Put the samples in sealed, waterproof containers (i.e., plastic bags). Label each specimen container in a waterproof manner. Batch the collection and send by overnight mail to arrive at the testing laboratory on a weekday during business hours unless other arrangements have been made in advance with the testing laboratory. Contact the testing laboratory before shipping, and give the testing laboratory as much advance notice as possible so that testing can begin as soon as samples arrive. When etiology is unclear and syndrome is nonspecific, consider collecting all four types of specimens.
[c] For more detailed instructions on how to collect specimens for specific parasites, please see the CDC DPDx website (http://www.dpd.cdc.gov/dpdx/).
[d] For more detailed instructions on how to collect specimens for viral testing, please see http://www.cdc.gov/mmwr/PDF/RR/RR5009.pdf.
[e] The containers must be tested for the presence of the chemical of interest prior to use.
[f] Unused specimen collection containers that have been brought to the field and subjected to the same field conditions as the used containers. These containers are then tested for trace amounts of the chemical of interest.

FIGURE 5 Patterns of PFGE of isolates of *Salmonella enterica* serovar Typhimurium collected in Minnesota during 4 months in 1995. Molecular subtyping allowed investigators to recognize three concurrent outbreaks caused by different strains and associated with different restaurants and to associate particular strains with particular outbreaks or document that particular strains are not associated with particular outbreaks. Adapted from reference 3, with permission.

institutional outbreaks with small numbers of cases. Four of six larger community outbreaks would not have been recognized without molecular subtyping (Fig. 5) (3).

Genetic sequencing technology has become more readily available and has been useful for assessing the relatedness of various pathogens involved in food- and waterborne outbreaks. For example, sequencing of hepatitis A viruses collected during three large outbreaks associated with green onions demonstrated that similar virus strains caused all three outbreaks and that this strain was related to hepatitis A strains commonly isolated from patients living in the region of Mexico where the green onions were grown (8). Sequencing of noroviruses is also becoming increasingly useful in identifying relatedness among potential outbreak-associated viruses.

CONTROL AND PREVENTION

The most immediate priorities for anyone investigating outbreaks of foodborne or waterborne disease are to implement appropriate measures to control the outbreak, mitigate associated morbidity, and prevent recurrences. While the steps of an outbreak investigation (Table 1) are listed sequentially, in practice many of those steps will be addressed concurrently. Not infrequently, public health officials must institute substantial and occasionally controversial control measures before the investigation is complete and before all desired data are available because there appears to be considerable potential for continued transmission of infection and thus continued morbidity or mortality. Control measures may include confiscating food products from markets, excluding foodhandlers or ill cases from work, closing retail food establishments or implicated venues, and publicly notifying persons who may have been exposed. Because such interventions can have important medical, emotional, and economic implications for all parties involved, investigators, laboratorians, regulatory partners, and other involved groups must assimilate available data rapidly and communicate effectively with each other and the public. Such collaboration is imperative to ensure that

ongoing transmission is eliminated while minimizing the adverse consequences associated with control measures.

The causes of foodborne and waterborne disease outbreaks can be multifactorial and complex. A single incident or event, such as an ill foodhandler with poor hygiene who prepared cake frosting barehanded or chlorination that was not adequate to disinfect well water contaminated by agricultural runoff, may be identified as the proximate cause of an outbreak. Frequently, however, seemingly simple explanations may belie complex underlying factors that contributed to the final outcome. For example, foodhandlers may be poorly educated regarding proper techniques for preparing food, may not understand English, or may lose their jobs if they miss work because they are ill. In addition, food may be prepared at temperatures inadequate to kill organisms, food may be stored at temperatures that encourage microbial growth, or equipment for preparing or storing food may fail to reach the appropriate temperature. These and other such factors can contribute to the immediate cause of an outbreak. The CDC attempts to capture some data on "contributing factors" on the standard reports it uses for foodborne disease outbreaks (http://www.cdc.gov/foodborneoutbreaks/report_f.htm). To ensure that similar incidents do not recur after an outbreak, investigators should identify modifiable contributing factors and recommend measures to correct them.

INTENTIONAL OUTBREAKS

Most foodborne and waterborne disease outbreaks have resulted from unintentional contamination of food or water sources at any of innumerable points in the long food production and service chain. In recent years public health officials and the public have become concerned that the food supply system is a potential target of intentional acts of contamination, sabotage, or terrorism. In fact, the food and water supply is considered a high-risk target for potential terrorist activity because it is difficult to protect the myriad stages from "farm-to-fork," tremendous volumes of product are rapidly distributed worldwide, and techniques for detecting intentional events are inadequate. Virtually all governmental agencies associated with food safety are increasing efforts to improve security at all stages of the food supply chain (http://www.foodsafety.gov).

Given the existence of active terrorist groups, persons investigating outbreaks of foodborne or waterborne disease must consider intentional contamination as a possible cause. Several outbreaks related to intentional contamination of food have been identified in the United States (1). In 1984, a religious group deliberately contaminated food at salad bars in Oregon with *S. enterica* serovar Typhimurium in an attempt to influence a local election. In total, 751 persons became ill. In 1996, a disgruntled laboratory employee in Texas deliberately contaminated doughnuts with a laboratory strain of *Shigella*, leading to illness in 12 coworkers. In 2003, a Michigan supermarket employee intentionally contaminated 200 pounds of ground beef with a nicotine-containing pesticide, causing illness in 92 persons (http://www.fda.gov/ora/training/orau/FoodSecurity/). In another incident that year, a parishioner put arsenic in coffee served at a church in Maine, causing 12 persons to become ill; one person died (15). While these have been isolated incidents, they demonstrate the range of agents and modes by which persons could deliberately cause an outbreak.

In some instances of intentional contamination, the perpetrators may claim responsibility for the subsequent outbreak. In general, however, these outbreaks are likely to be identified and reported in the same way that unintentional foodborne and waterborne disease outbreaks are reported. Thus, continued surveillance is necessary and standard epidemiologic methods remain essential to the investigations. Potential clues that might suggest bioterrorism or intentionally caused outbreaks are described in Table 6. If purposeful contamination is suspected as a cause, investigators must include law enforcement officials in the investigation. In any instance involving food- or water-related terrorism, the Federal Bureau of Investigation will lead the investigation and will coordinate the response.

TABLE 6 Potential clues that might increase suspicion that intentional contamination is the cause of a foodborne or waterborne disease outbreak

Occurrence of a rare or novel disease
Outbreak due to a disease occurring outside its normal range of endemicity
Disease occurring during unusual season
Unusual drug resistance
Unusual epidemiologic characteristics
Unusual demographic population affected
Unusual clinical presentation of disease
Widespread contamination without apparent explanation
Claims or social context suggesting intentional contamination

REPORTING AND SUMMARIZING OUTBREAKS

Under the law in most states, all suspected foodborne and waterborne disease outbreaks must be reported to the public health department. Public health authorities will work with other involved parties to determine whether further investigation is warranted and who will perform those investigations. Whether public health authorities or other agencies or institutions perform the investigation, health departments have the authority and obligation to ensure that the response to a potential outbreak protects the public's health. By mutual agreement, state or local departments of health collect basic descriptive data on all foodborne and waterborne disease outbreaks in their jurisdictions and share reports with the CDC, which periodically summarizes those data. Examples of the forms, the electronic database used for foodborne disease outbreaks, and recent data are accessible online (http://www.cdc.gov/foodborneoutbreaks/info_healthprofessional.htm). Organizations other than the CDC regularly monitor outbreaks reported via public health agencies and other venues (4).

Public health and regulatory agencies and most other institutions involved in outbreak investigations adhere to strict guidelines protecting the confidentiality of data collected during those investigations. Although confidentiality laws may vary by jurisdiction and agency, personally identifiable medical information regarding involved case patients is strictly protected in all cases. In the absence of a court subpoena or permission from the patient, personally identifiable information cannot be released to other parties. Such assurances of confidentiality are necessary to ensure that persons involved in the outbreak trust the investigators and provide critical information during investigations. In many cases, proprietary information and the names of institutions involved in outbreaks are also protected from public dissemination. Occasionally, such information may be disseminated publicly if it is necessary to protect people from infection.

TABLE 7 Recommended components of an outbreak investigation summary report

List of participants in the investigation and contact information for lead investigator
Dates of outbreak and investigation
Description of process by which outbreak was recognized and reported
Description of the epidemiologic steps followed in the investigation
Definition of cases included in the outbreak
Summary of total number of affected persons and description of the population exposed
Description of clinical syndrome, including proportion of persons experiencing common symptoms, summary of incubation period, duration of illness, and indicators of morbidity such as proportion seeking medical care or mortality rates
Description of results from epidemiologic studies, including numbers of cases or controls enrolled in cohort or case-control studies, and results of analyses assessing possible sources
Summary of supporting laboratory data obtained by testing clinical, food, or environmental specimens
Summary of findings of various groups contributing to the investigation, including epidemiologists, environmentalists/sanitarians, regulatory agencies, institutional representatives, medical providers, or others not primarily writing report
Conclusions about the etiology, vehicle, cause, and underlying contributing factors leading to the outbreak
Lessons learned during the investigation, which may benefit others in similar situations in the future
Limitations of the investigation
Specific recommendations made and control measures implemented (including dates and how and when communicated)
Results of intervention, if known
Recommendations for preventing similar incidents in the future

For example, media announcements may be used to notify restaurant patrons potentially exposed to hepatitis A from a foodhandler that they should get prophylactic immune globulin. In such cases, public health authorities and regulators work closely with other involved parties to ensure that appropriate measures are taken to avoid an inappropriate breach of confidentiality.

Investigators frequently summarize the results of outbreak investigations in narrative reports, which are maintained locally and disseminated based on institutional policy. These reports are an important medical, legal, and scientific resource for documenting the process of an investigation, nature of an outbreak, conclusions about its causes and control, and preventive measures instituted. Sufficient detail is required in those reports to ensure that the audience understands the outbreak and investigation, while maintaining confidentiality of protected information. An example of the components that should be in an outbreak investigation report is shown in Table 7. Summary reports should be shared with appropriate persons and agencies, and outbreak descriptions should be published in peer-reviewed literature when the information can contribute to improving future investigations or the general understanding of diseases, epidemiology, laboratory methods, or other aspects of outbreak response.

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DIAGNOSTIC TECHNOLOGIES IN CLINICAL MICROBIOLOGY

II

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Actinomadura madurae (P. Conville, NIH).

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Microscopy

DANNY L. WIEDBRAUK

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The history of microscopy is closely linked to the beginning of microbiology; Hooke, Divini, Kircher, and van Leeuwenhoek were among the first individuals to describe microscopic life-forms (7). Hooke, in his 1665 treatise, "*Micrographica,*" included illustrations of mold forms and the anatomy of the flea (7). Antonie van Leeuwenhoek provided detailed descriptions of protozoa and bacteria in his letters to the Royal Society in London. His descriptions of "very small animalcules" included drawings of basic organism shapes and movement (7). Today, light microscopy is used not only in microbiology and cell biology but also in metallurgy, computer chip design, and microsurgical applications. This chapter attempts to describe the basic concepts of light microscopy as they are practiced in the microbiology laboratory.

TECHNICAL BACKGROUND AND DEFINITION OF TERMS

Aberration

Aberrations are unwanted artifacts in the microscopic image that are caused by elements in the optical path. Aberration can be caused by physical objects such as dust or oils on the optical surfaces, by alterations in the light path caused by improper alignment or aperture settings, and by imperfections in the lens systems. Two main types of optical aberrations can occur when white light passes through a convex lens: spherical aberration and chromatic aberration. Spherical aberration is hallmarked by images that appear to be in focus in the center of the field and out of focus at the periphery (5). Chromatic aberration occurs because shorter light wavelengths are refracted to a greater extent than longer wavelengths (5). This wavelength separation (also called dispersion) produces color fringes within the image field. Chromatic aberration is reduced or eliminated in optical systems by combining two lenses with different color dispersion characteristics (5).

Contrast

Contrast is a measure of the differences in image luminance that provides gray-scale or color information. Contrast is expressed as the ratio of the difference in luminance between two points divided by the average luminance in the field (1). Under optimum conditions, the human eye can detect the presence of 2% contrast (1).

Depth of Field and Depth of Focus

Depth of field is a subjective measure of the vertical distance between the nearest and farthest objects in the specimen that appear to be in sharp focus. Depth of field decreases as the numerical aperture (NA) of the lens increases (3). Depth of focus is the area around the image plane where the image appears to be sharply focused. The image plane is formed within the microscope tube at or near the level of the ocular lenses. Microscopes with greater depth of focus allow the user to employ ocular lenses with different working distances, magnification factors, and visual compensation systems without losing image sharpness. Like depth of field, depth of focus depends on the NA of the objective. However, depth of focus increases as the NA increases (3).

Immersion Fluid (Immersion Oil)

Immersion fluid is a term used to describe any liquid that occupies the space between the object and microscope objective lens. Immersion fluids are usually required for objectives that have working distances of 3 mm or less (5). Many microscopy applications employ immersion fluids that possess the same refractive index as the glass slide (refractive index = 1.515) (3, 5). This procedure produces a homogeneous optical path which minimizes light refraction and maximizes the effective NA of the objective lens. Immersion fluids are also used between the condenser and the microscope slide in transmitted light fluorescence microscopy and in dark-field microscopy to minimize refraction, increase the NA of the objective, and improve optical resolution (3, 5).

Köhler Illumination

Köhler illumination was first introduced in 1893 by August Köhler of the Carl Zeiss corporation as a method of providing the optimum specimen illumination (5). In this procedure, the collector lens projects an enlarged and focused image of the lamp filament onto the plane of the aperture diaphragm. Because the light source is not focused at the specimen, the specimen is bathed in a uniformly bright, glare-free light that is not seriously affected by dust or imperfections on the glass surfaces of the condenser. Köhler illumination is required to produce the maximum optical resolution and high-quality photomicrographs (4, 5).

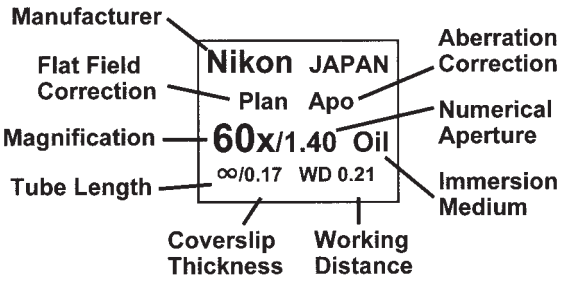


FIGURE 1 Objective lens labeling. Objective lenses are labeled with information on the manufacturer, correction factors, NA, tube length, coverslip thickness, working distance, and expected immersion medium. Objectives without a listed aberration correction are considered achromats. Objectives without a listed immersion medium (Oil, Oel, W, Gly) are considered dry objectives and are meant to operate with air between the lens and the specimen.

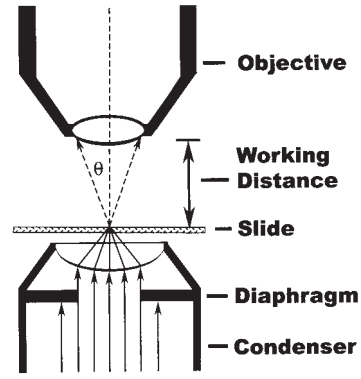


FIGURE 2 Typical configuration for bright-field microscopy. The column of light generated by the field lens and the field diaphragm enters the bottom of the condenser and is focused on the slide by the condenser lens. The condenser diaphragm controls the angle of the light, the NA of the condenser, and the amount of contrast in the image. The working distance is the vertical distance from the top of the specimen to the leading edge of the objective lens. The semiangle of the objective aperture (θ) is used to calculate the NA. Modified from reference 7.

Mechanical Tube Length

Mechanical tube length describes the light path distance within the microscope body tube. Tube length is measured from the objective opening in the nosepiece to the top edge of the observation tube. Tube length is usually inscribed on the barrel of the objective as the length in millimeters (e.g., 160, 170, 210, etc.) for fixed lengths, or the infinity symbol (∞) for infinity-corrected tube lengths (Fig. 1). Many of the newer objectives are infinity corrected, whereas older objectives are corrected for 160-mm (Nikon, Olympus, Zeiss) or 170-mm (Leica) tube lengths (5).

Numerical Aperture

NA is a measure of the light-gathering capability of a lens or condenser. Higher NA objectives have better resolving power and brighter images than lower NA objectives. Higher NA objectives also have shallower depth of field. The equation for determining NA is as follows: $NA = n \cdot \sin(\theta)$, where n is the refractive index of the imaging medium between the objective and the specimen and θ is one-half the angular aperture of the objective (Fig. 2) (3, 5, 7, 12).

Refractive Index (Index of Refraction)

Index of refraction is the ratio of the velocity of light in a vacuum to its velocity in a transparent or translucent medium (3, 5, 7, 11, 12). As the refractive index of a material increases, light beams entering or leaving a material are deflected (refracted) to a greater extent. The refractive index of a medium depends upon the wavelength of light passing through it. Light beams containing multiple wavelengths (e.g., white light) are dispersed when they move into a different medium because each wavelength in the beam is refracted to a slightly different degree. Light dispersion causes chromatic aberration in microscope objectives (5). Refractive index is also an important variable in calculating NA (see "Numerical Aperture" above). Moving from a high-dry microscope objective that uses air as the imaging medium (refractive index of air = 1.003) to an oil immersion objective of the same power (refractive index of immersion oil = 1.515) increases the maximum theoretical NA of a given lens from 1.0 to 1.5, producing a 50% increase in light-gathering capability (3, 5).

Resolution (Resolving Power)

The resolution of an optical microscope is defined as the shortest distance between two points that can be distinguished by the observer or camera system as separate entities (3, 7, 12). The resolving power of a microscope is the most important feature of the optical system because it defines our ability to distinguish fine details in a specimen. The theoretical limit of resolution for a given lens is defined mathematically as $r = \lambda/(2NA)$, where r is the resolution, λ is the imaging wavelength, and NA is the numerical aperture of the lens (3, 7, 12). From this equation it is obvious that only the light wavelength and NA directly affect the resolving power. Thus, a 40× oil objective with an NA of 1.30 can have the same resolving power as a 100× oil objective (Table 1). In the same manner, the resolving power of a 100× oil objective is higher when using UV wavelengths than it is when using visible light (Table 1).

TABLE 1 Resolving power of selected lenses with different NAs

Lens system	NA	Light color	Avg wavelength (nm)	Medium	Resolution (μm)
Eye		White	550	Air	700
Hand magnifier	0.03	White	550	Air	10
10× objective	0.30	White	550	Air	0.92
40× objective	0.75	White	550	Air	0.37
40× objective (oil)	1.30	White	550	Oil	0.21
100× objective	1.30	White	550	Oil	0.21
100× objective	1.30	UV	400	Oil	0.15

Working Distance

Working distance is the distance between the objective front lens and the top of the cover glass when the specimen is in focus (Fig. 2). The working distance of an objective generally decreases as magnification increases (3). The working distance of an objective may not be inscribed on the barrel of older objectives, but newer objectives often contain the working distance in millimeters (Fig. 1). Longer working distance objectives are important when examining the inside surface of glass tubes (tube cultures) and cell culture flasks.

SIMPLE MICROSCOPE

Common objects such as jewelers' loupes, photographic slide viewers, and simple magnifying or reading glasses are all examples of simple microscopes in routine use today. A simple microscope is composed of a single biconvex magnifying lens which is thicker in the center than at the periphery. In contrast with compound microscopes, simple microscopes produce a magnified image that is in the same orientation as the original object. Because of their low NA, simple microscopes have limited resolution and magnifying power. Most commercial magnifiers are able to produce a magnification of ×2 to ×30, and the better lenses have a resolution of about 10 μm. Simple magnifiers are useful for dissection, examination of bacterial colonies, and interpretation of agglutination reactions.

COMPOUND MICROSCOPE

The first compound microscopes were constructed around 1590 by Dutch spectacle makers Zaccharias Janssen and Hans Janssen. The Janssen microscope consisted of an object lens (objective) that was placed close to the specimen and an eye or ocular lens that was placed close to the eye. The lenses were separated by a body tube. In this microscope, the objective lens projected a magnified image into the body tube and the eyepiece magnified the projected image, thereby producing a two-stage magnification. Modern compound microscopes still use this general design and have two separate lens systems mounted at opposite ends of a body tube.

The stereoscopic microscope combines two compound microscopes which produce separate images for each eye. Stereoscopic microscopes may have one or two separate objectives, and many have a zoom magnification function. These microscopes are used for reflected or transmitted illumination, but the absence of a substage condenser limits their NA and resolution. Stereomicroscopes are useful in examining colonial morphology of bacteria, fungi, and cell cultures (12).

The modern light microscope is composed of optical and mechanical components that, together with the mounted specimen, make up the optical train. The optical train of a typical bright-field microscope consists of an illuminator (light source and collector lens), a substage condenser, a specimen, an objective, an eyepiece, and a detector. The detector can be a camera or the observer's eye.

Specimen illumination is one of the most critical elements in optical microscopy. Inadequate or improper sample illumination can reduce contrast in the specimen and significantly decrease the resolving power of any microscope. There are numerous commercially available illuminators for microscopes, but 50- or 100-W tungsten halogen lamp systems are frequently used due to their low cost and long life. Light generated by the light source is passed through a collector and a field lens (Fig. 3) before being directed into the substage condenser and onto the specimen. Image-forming light rays are captured by the microscope objective and passed into the eyepieces or a camera port. Alignment of the optical components of a microscope is critical to produce a good image.

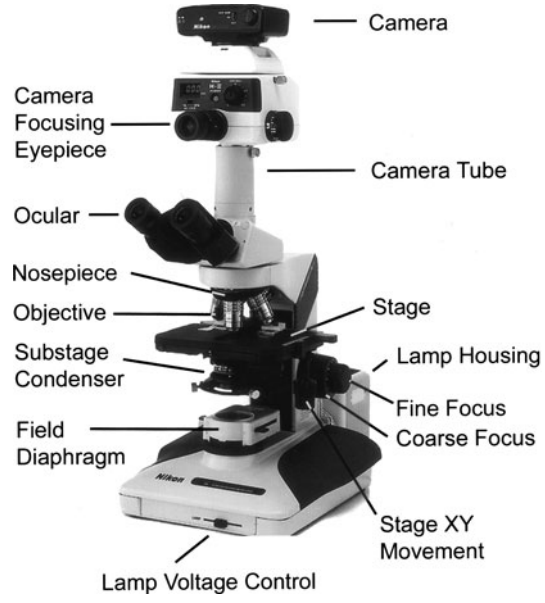


FIGURE 3 Anatomy of a typical clinical microscope with an integral camera.

Field Diaphragm

The field diaphragm is located in the light path between the light source and the substage condenser (Fig. 3). This iris-like mechanism controls the width of the light beam that enters the substage condenser. The field diaphragm does not affect the optical resolution, NA, or the intensity of illumination. However, the field diaphragm should be centered in the optical path and opened far enough that it just overfills the field of view. This adjustment is important for preventing glare and loss of contrast in the observed image. When the field diaphragm is opened too far, scattered light and reflections can degrade image quality.

Substage Condenser

The substage condenser is typically mounted beneath the microscope stage in a bracket that can be raised or lowered independently of the stage (Fig. 3). The substage condenser gathers light from the field diaphragm and concentrates it into a cone of light that illuminates the specimen with uniform intensity over the entire field of view. Adjustment of the substage condenser is probably the most critical element for achieving proper illumination, and it is the main source of image degradation and poor-quality photomicrography. The condenser light cone must be properly adjusted to optimize the intensity and angle of light entering the objective. Because each objective has different light-gathering capabilities (NA), the substage condenser should be adjusted to provide a light cone that matches the NA of the new objective. This is done by adjusting the aperture (or condenser) diaphragm control. Substage condensers on newer microscopes have a scale embossed on the condenser and an index mark on the aperture control that allows the user to quickly switch from one NA range to another. Many manufacturers

are now synchronizing the NA gradations to correspond with the approximate NA of the objectives.

In clinical laboratory practice, the condenser aperture is often made smaller to improve the contrast of wet mounts of some stained preparations (7). This practice, while effective for some applications, results in decreased resolution (1, 7). It should be noted that the intensity of illumination should not be adjusted by opening and closing the condenser aperture diaphragm or by moving the condenser laterally in the light path. Illumination intensity should be controlled through the use of neutral density filters placed into the light path or by reducing voltage to the lamp. Reducing the voltage, however, also alters the color of the incoming light. For this reason, lamp voltage changes are not recommended for photomicroscopy (4).

Objectives

The objective lens is the most important single determinant of the quality of the image produced by a particular microscope (12). When choosing a microscope, the purchaser must select the magnification factor, NA, and the level of correction for each objective. Lenses with higher NA values have higher resolution and produce a brighter field of view. The level of optical correction in the objective depends on the ultimate use of the microscope. Achromatic (achromat) objectives are the least expensive objectives found on laboratory microscopes. Achromat objectives are corrected for axial chromatic aberration in two wavelengths (red and blue), and they are corrected for spherical aberration in one color (green) (5). The limited correction of achromatic objectives can cause a number of optical artifacts when specimens are examined and photographed in color (e.g., green images often have a reddish magenta halo) (5). Achromat objectives produce the best results with light passed through a green filter and when performing black and white photomicroscopy. Flatness of field is also a problem when using straight achromat objectives because the center of the field is in focus while the edges are out of focus (5). In the past few years, most manufacturers have begun providing flat-field corrections for achromat objectives. These objectives are called plan-achromats.

The next higher level of correction and cost is found in objectives called fluorites or semiapochromats. Fluorite objectives are produced from advanced glass formulations that allow for greatly improved correction of optical aberration. Like the achromat objectives, the fluorite objectives are also corrected chromatically for red and blue light (5). Fluorites are also corrected spherically for two or three colors instead of a single color, like achromats (5). The superior correction of fluorite objectives compared to achromats enables these objectives to be made with a higher NA. Fluorite lenses therefore produce brighter images than achromats. Fluorite objectives also have better resolving power than achromats and provide a higher degree of contrast, making them better suited for color photomicrography in white light (3, 5).

Apochromats

Apochromats are the most highly corrected microscope lenses and the most costly. Apochromats are corrected chromatically for three colors (red, green, and blue), almost eliminating chromatic aberration, and are corrected spherically for either two or three wavelengths (5). Apochromat objectives are the best choice for color photomicrography in white light. Because of their high level of correction, apochromat objectives usually have, for a given magnification, higher numerical apertures than do achromats or fluorites (3, 5).

Fluorescence Objectives

Fluorescence objectives are designed with quartz and other special glasses that have high transmission rates of UV, visible, and infrared light. These objectives are extremely low in autofluorescence and use specialized optical cements and antireflection coatings that protect the lens and allow it to operate with a wide variety of excitation wavelengths. The correction for optical aberration and NA values in UV fluor objectives usually approaches that of apochromats, which contributes to image brightness and enhanced image resolution (2, 5). The primary drawback of high-performance fluorescence objectives is that many are not corrected for field curvature and produce images that do not have uniform focus throughout the entire field of view. This is not a large problem when performing direct or indirect fluorescent-antibody testing, but it can be troublesome if you have to use the same objectives to do bright-field and phase-contrast microscopy.

Microscope objectives that use air as the medium between the coverslip and the objective lens are considered dry objectives. The maximum working NA of a dry-objective system is limited to 0.95, and greater values can be achieved only by using optics designed for immersion media. Immersion media have the same refractive index and dispersion values as glass (refractive index = 1.51). The use of immersion media produces a homogeneous light path from the coverslip to the lens so that light is not refracted away from the objective. The use of immersion fluids and lenses significantly increases the NA and the optical resolution of the system. In addition to "oil" lenses, specially corrected objective lenses designed for glycerin and water immersion are available commercially. The proper immersion fluid type is always stamped on the side of the objective. The advantages of oil immersion objectives are severely compromised if the wrong immersion fluid is utilized. Microscope manufacturers produce immersion objectives with tight refractive index and dispersion tolerances (5). It is therefore advisable to use only the immersion fluid recommended by the objective manufacturer. Mixing of immersion fluids from different manufacturers should be avoided because mixing can produce unexpected crystallization artifacts or phase separations that compromise image quality.

Many high-power (NA, $\geq$0.8) dry objectives are engineered to operate through 0.17-mm coverslips (designated as number 1½). In practice, however, the total thickness of the specimen-coverslip sandwich can be greater or less than 0.17 mm due to variations in coverslip and/or mounting fluid thickness (3, 5). Under these conditions, there will be noticeable spherical aberration in the microscopic image (3, 5). A 0.2-mm deviation in coverslip thickness produces an 8% decrease in image intensity when using a 0.79 NA objective and 57% with a 0.85 NA high-dry objective (5). Therefore, some of the more advanced dry objectives are engineered with a coverslip correction collar that adjusts the objective lens elements to compensate for coverslip thickness variations. Objectives with a coverslip correction collar are labeled Corr, w/Corr, or CR. However, this labeling is usually unnecessary because the objective has a distinctive knurled ring and graduated scale on the side. The expected coverslip thickness for an objective is etched on the barrel of the objective (Fig. 1).

The eyepiece or ocular objective contains the final lens system in the optical train. The purpose of the ocular objective is to magnify and focus the projected image onto the eye of the viewer. Ocular lenses generally have a magnification factor of $\times$10 to $\times$20 and the total magnification of the microscope is the product of the objective magnification and the ocular magnification. (5, 7, 11, 12). Thus, a microscope

with a 40× objective and a 10× ocular lens would have a magnification value of ×400. Many eyepieces have a shelf at the level of the fixed eyepiece diaphragm that allows for the insertion of ocular micrometers, pointers, or crosshairs. This shelf is located at the focal plane of the image projected by the objective lens so that the inserted element is in focus when the specimen image is in focus.

DARK-FIELD MICROSCOPY

Dark-field microscopy is a specialized illumination technique that is used in the clinical laboratory to detect thin organisms such as spirochetes and *Leptospira* (refer to chapters 61 and 63). High-resolution dark-field microscopy utilizes a specialized high-NA cardioid dark-field condenser that blocks the central light path light and produces a hollow cone of illumination that is directed away from the objective lens at an oblique angle (Fig. 4). Bacteria on the slide have a refractive index slightly different from that of the surrounding medium, and light rays passing through the organism are refracted into the objective lens. Light rays that do not pass through an organism do not enter the lens. This type of illumination produces bright organism profiles against a dark background. Dark-field microscopy requires careful alignment of the condenser and the placement of immersion oil between the slide and the substage condenser. Dark-field microscopy, when done correctly, increases the resolution of the microscope to 0.1 μm or less (1, 7). The resolution of bright-field microscopy is 0.2 μm (7, 12).

PHASE-CONTRAST MICROSCOPY

Many unstained biological specimens are virtually transparent when observed under bright-field illumination. To improve visibility in wet mounts and cell cultures, microscopists often reduce the opening size of the substage condenser iris diaphragm (7), but this maneuver is accompanied by a serious loss of resolution and the introduction of diffraction artifacts (1, 5). Phase-contrast microscopy significantly improves the contrast in these specimens without significant loss in resolution (1).

In phase-contrast microscopy, a ring annulus is placed directly under the lower lens of the condenser to produce a hollow cylinder of light. This light is essentially unchanged as it passes into the objective, and it arrives at the rear focal plane of the objective in the shape of a ring. Light that goes through the specimen is refracted and slowed slightly so that it is out of phase with the unchanged light by about one-quater wavelength. This light is spread over the entire focal plane. Light passing through the rear focal plane of the objective interacts with a ring-shaped phase plate that alters the direct light path by one-quarter wavelength (1). When the direct light and the refracted light arrive at the image plane, they are out of phase by one-half wavelength. This out-of-phase light interacts destructively so that specimen details appear as dark areas against a lighter background (1). Because the phase-shifting calculations are based upon on a one-quarter wavelength of green light, the phase image has the best resolution when a green filter is placed in the light path (1). Green filters also allow the microscopist to use less expensive achromat lenses that are spherically corrected for green light. Phase microscopy is an important tool for examining living and/or unstained material in wet mounts and cell cultures. However, phase-contrast microscopy has lower resolution than bright-field microscopy of stained specimens (1). In addition, viewed objects are often surrounded by halos that can obscure boundary details. Phase-contrast microscopy does not work well with thick specimens because the phase shift may be greater than the expected one-quarter wavelength.

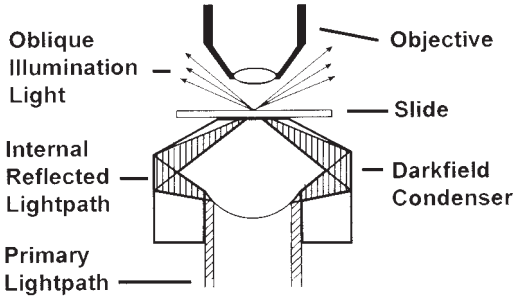


FIGURE 4 Dark-field illumination. The central light path interacts with the silvered dome located at the bottom of the condenser and is reflected away from the specimen. Peripheral light is reflected into the condenser and is reflected again by the internal condenser surfaces to produce a cone of light that is directed obliquely away from the objective.

FLUORESCENCE MICROSCOPY

The fluorescence microscope was developed in the early 1900s, and many of the initial microscopic studies involved identification and localization of compounds that autofluoresced when irradiated with UV light. In the 1930s a number of investigators began using fluorescent compounds to identify specific tissue components and infectious agents that did not autofluoresce (2). These stains are not organism specific, but rather, they bind to and stain specific structures within the organism. Examples of this type of staining include acridine orange (intercalcates into DNA and RNA), auramine-rhodamine (mycolic acids), calcofluor white (fungal cell wall polysaccharides), Evans blue (cytoplasm of fixed cells), and Hoechst 33258 (minor groove of AT-rich double-stranded DNA).

The use of fluorochrome-antibody conjugates (immunofluorescence) was first described in the 1940s when Coons et al. (8, 9) used fluorescein-labeled antibodies to detect pneumococcal polysaccharide antigens in tissue sections of infected mice. Fluorescent antibody staining expanded significantly with the development of fluorescein isocyanate in 1950 (10) and the more stable fluorescein isothiocyanate (FITC) derivative in 1958 (13, 15, 16, 22, 24). Today fluorescence microscopy is also used in conjunction with nucleic acid hybridization to visualize the location of fluorescent in situ hybridization (FISH) and multicolor FISH probes (23, 26).

Fluorescence microscopy is dependent on the ability of fluorescent substances to absorb near-UV light energy and reemit that energy (light) at a lower wavelength (2). To work properly, the fluorescence microscope must irradiate the specimen with UV excitation light and separate the much weaker emitted light from the brighter excitation light so that only the emitted light reaches the eye. The resulting image consists of brightly shining areas against a dark background (2). Older fluorescent microscopes are configured for dark-field illumination. In these instruments, UV excitation light enters a dark-field condenser and the light is directed onto the specimen at an oblique angle (Fig. 4). Fluorescent compounds in the specimen absorb the excitation light, and the emitted light is collected by the objective lens. The emitted light then passes through a barrier filter to remove any

excitation light that may enter the objective. These microscopes are difficult to use for routine diagnostics because the condenser and the objective must be carefully oiled. The dark-field condenser also reduces the effective NA of the objectives, thereby producing dim images that lack resolution (2). Most modern fluorescence microscopes use reflected light (epifluorescence). In these instruments, the excitation light is directed downward through the objective and onto the specimen. The emitted light and the reflected excitation light are collected by the objective, and they pass through a dichroic mirror which removes the excitation light and allows the longer-wavelength emitted light to form an image. With epifluorescence, the objective acts as a condenser and the alignment and oiling issues associated with a dark-field condenser are eliminated (2). The visual field is brighter with epifluorescence, the resolution is higher, and fluorescence quenching occurs only in the field of view (2).

Fluorescence microscopy requires high levels of illumination because the quantum yield of most fluorochromes is low. The most common lamps are mercury vapor (HBO) lamps, ranging in wattage from 50 to 200 W, or xenon vapor (XBO) lamps, which range from 75 to 200 W. It should be noted that lamp wattage is not necessarily a measure of usable brightness in a fluorescence lamp. The HBO 100-W lamp is 4 times brighter than the 200-W HBO lamp and 11 times brighter than the XBO 150-W lamp (2). When purchasing a fluorescence microscope, it is also important to determine whether the emission spectrum of the lamp is compatible with the fluorochromes used in the laboratory. HBO and XBO lamps are under high pressure, and care must be taken to prevent the lamps from exploding. One should never touch these lamps with bare hands because oils on the fingers can etch or discolor the glass.

Fluorochromes must be excited by specific light wavelengths in order to generate the maximum amount of emitted light. Therefore, specific exciter and barrier filter combinations are used to maximize the quantum yield of the fluorophore. Exciter filters are used to select the required light wavelengths from the spectrum of light generated by the lamp (2). Excitation filters are provided in narrow, medium, and wide bandpass configurations that pass a narrower and wider range of light frequencies, respectively. Barrier filters block shorter light wavelengths and allow longer wavelengths to pass through the filter. Barrier filters are important because they remove the high-intensity excitation light that could overwhelm the low-intensity emitted light. Barrier filters also prevent UV light from entering the eye, where it can cause cataracts and retinal damage. Wide bandpass barrier filters generally produce brighter images, but care must be taken to prevent the introduction of background light that could overwhelm the emitted light. Epifluorescence microscopes also have a dichromatic mirror (beam splitter) that reflects the incoming excitation light to the objective and allows the emitted light to pass to the barrier filter and on to the objectives. In most modern epifluorescence microscopes, the barrier filter, the excitation mirror, and the beam splitter are housed in removable optical blocks and several of these blocks can be installed in the microscope at one time. This configuration allows the user to quickly change the excitation and barrier filters to accommodate different fluorochromes. Care must be exercised when selecting optical blocks. The excitation filter should match the excitation wavelength of the fluorophore (Table 2), and the emission barrier should allow the emitted light to pass through. For example, direct fluorescent-antibody testing for viral antigens in cell smears typically employs FITC-labeled antibodies and an Evans blue counterstain. Choosing an optical block with a 450- to 490-nm excitation filter and a 515-nm-wide bandpass barrier filter produces a bright field of view, and the counterstained cells will appear orange-red. By selecting a more restricted bandpass barrier filter (520 to 560 nm), the field of view will be darker and the red emitted light from the Evans blue counterstain will not be visible. The images produced by this optical block have more contrast because the background is darker. Both filter combinations are appropriate for this task, but the final choice depends on user preference.

One of the major problems in the use and examination of fluorescent microscopic images is the tendency of fluorophores to lose fluorescence when exposed to excitation light for several minutes. This loss of fluorescence is caused by two mechanisms, photobleaching and quenching. Photobleaching (fading) is a permanent loss of fluorescence that is caused by chemical damage to the fluorophore (2). Quenching is caused by the presence of free radicals, salts of heavy metals, or halogen compounds (2). Quenching can also be caused by transfer of emission light energy to other fluorescent molecules in close proximity to the fluorophore in a process called fluorescent resonance energy transfer. To lessen the effect of quenching, slides should be stored in the dark at 2 to 8°C. In addition, the user should block the excitation light path when not viewing or photographing the specimen. Most epifluorescence microscopes have a shutter in the light path for this purpose. Quenching can be a significant problem when photographing fluorescent images because the shutter may be open for 1 min or more. Quenching can be reduced somewhat

TABLE 2 Excitation and emission wavelengths of commonly used fluorochromes[a]

Fluorescent compound	Excitation wavelength (nm)	Emission wavelength (nm)
Acridine orange (single-stranded nucleic acid)	500	526
Acridine orange (double-stranded nucleic acid)	460	640
Auramine O	460	550
Calcofluor white	440	500–520
Ethidium bromide	545	605
Evans blue	550	610
FITC	490	525
Hoechst 33258	352	461
Rhodamine B	540	625
TRITC[b]	555	580

[a] Excitation and emission wavelengths can vary depending on the solvent and the pH of the solution.
[b] TRITC, tetramethylrhodamine isothiocyanate.

by adding free-radical scavengers such as *p*-phenylenediamine (16), 1,4-diazabicyclo(2,2,2)-octane (DABCO) (17), or *n*-propylgallate (14) to the mounting fluid. *P*-Phenylenediamine and *n*-propylgallate can be used to reduce quenching in FITC and rhodamine. DABCO is slightly less effective than *p*-phenylenediamine for FITC fluorescence, but unlike *p*-phenylenediamine, DABCO does not darken when exposed to light and it is safer to use. Quench-resistant mounting media are also available from Vector Laboratories (Burlingame, Calif.), Molecular Probes Inc. (Carslbad, Calif.), and Bio-Rad Laboratories (Hercules, Calif.).

LINEAR MEASUREMENTS (MICROMETRY)

The first reported micrometric procedures were credited to Antonie van Leeuwenhoek, who used fine grains of sand as a gauge to determine the size of human erythrocytes. Since then, a variety of methods have been used to determine the dimensions of microscopic organisms. The crudest method for determining size in the clinical laboratory involves comparing the object size to the measured or calculated view field size. Other micrometric techniques include the addition of polystyrene beads of known size into the specimen. Comparative measurements are then performed utilizing a photomicrograph or digital image. The accuracy of this method is variable and depends on the homogeneity of the comparison objects. Direct measurements of microorganisms can be done by placing them on calibrated microscope slides or counting chambers. The accuracy of this method depends on the separation distance between ruled lines but averages between 10 and 50 μm. The most common procedure used in the clinical laboratory utilizes a graduated scale (reticle) located within one of the eyepieces (21). Reticles must be calibrated against a stage micrometer for each objective (21). The accuracy of this type of measurement is approximately 2 to 10 μm (3 to 5%), depending on magnification and the resolution of the stage micrometer (21).

PHOTOMICROSCOPY

Microscopists began capturing microscopic images on film shortly after the photographic process was invented (4). Micrographic images have long been used for investigations of morphology, in scientific publications and lectures, and in teaching. Modern film technologies have high resolution and clarity, but the use of photomicrographs in day-to-day microscopy has been hampered by long turnaround times associated with film development and printing. Reacquiring fluorescence images is a particular concern because the fluorescence can fade (2). The availability of high-quality digital cameras has significantly changed how photomicrographs are used in the microbiology laboratory. Today it is not unusual for digital photomicrographs to be shared with experts via the Internet. This process significantly extends the capabilities of the on-site microbiologist and can enhance patient care. Microscope-based digital cameras and video systems are also used to perform "plate rounds" in remote hospitals and clinics within a multihospital system. Newer Internet technologies involving robotic microscopes and high-resolution video systems now allow microbiologists to change the focus and the slide positioning of a microscope located anywhere in the world and view the resulting images on a monitor in their office. The availability of digital photomicroscopy has significantly enhanced the microbial identification process and has helped to standardize microbe identification.

A wide variety of microscopes that have integrated camera systems and sophisticated light metering and exposure controls are currently available. Accessory cameras are also available from a large number of aftermarket manufacturers. The performance and optical characteristics of these systems are too diverse to discuss in a single chapter. Photographs are a stern judge of microscopic quality (4), and the purchase of an expensive camera system does not automatically confer the ability to produce high-quality images. High-quality photomicrographs require proper specimen illumination and optical train alignment to achieve its ultimate potential (4). Color photography can be very demanding because specimens may appear yellow or blue under tungsten halogen (3200K color temperature) light depending on whether the lamp voltage is above or below the recommended 9-V setting. Photographs will also appear blue if the daylight blue filter is not removed from the light path. Photographs can also appear yellow when tungsten halogen (3200K) illumination is used in conjunction with daylight (5500K) film or digital cameras designed for daylight photography. Under these conditions, a Kodak 80A (3200K to 5500K) color conversion filter should be placed in the light path to achieve the proper 5500K color temperature (4).

Not all microbiologists can afford a microscope with an integrated camera system. Simple eyepiece cameras can also be used to capture bright-field images. The simplest configuration for eyepiece photography involves the use of a point-and-shoot digital camera. Some improvisation may be necessary with this method because few adapters are available for coupling a fixed-lens camera to the microscope eyepiece. Instead, the microscopist can use a camera tripod or some other support bracket to hold the camera in its proper position. Entry of stray light can be minimized by using a piece of black polyvinyl chloride pipe with an appropriate diameter and a black camera cloth. During photography, the camera lens system should be set to infinity focus (the default in fixed-lens cameras) and the lens should be positioned so that it is at the eyepoint (focal point) of the eyepiece. Location of the eyepoint can be determined by holding a piece of white paper just above the objective with the microscope turned on and focused (4). A bright circle of light will be projected onto the paper. The eyepoint is the position where the light circle is smallest (4). Photographs produced under these conditions are often acceptable, but they may be dark. Cameras with adjustable aperture settings should be set to the largest aperture value (smallest f-stop number) to maximize the amount of light entering the camera. This method also produces some chromatic aberration (due to different lens correction factors) and vignetting (pipe view effect).

Another method for photomicroscopy is to use the camera port on microscopes fitted with a trinocular head. Olympus and Nikon have introduced adapters that allow their digital cameras to attach to the camera tube of their microscopes. In addition, camera tube and eyepiece adapters for a number of digital cameras are available from Microscope Depot (Tracy, Calif.). Photography under these conditions is best done using a camera with through-the-lens exposure metering. These devices work well if the exposure is not longer than several seconds or shorter than one-third of a second (4). Many of these cameras have built-in flashes that should be turned off during photomicroscopy. These cameras may have problems with fluorescence microscopy due to the extreme contrast of fluorescent images and the tendency of metering systems to average exposure values over the entire field (4).

CARE AND USE OF THE MICROSCOPE

Proper care and maintenance of the microscope will prolong the usable life of the instrument and allow for more accurate interpretation of microbiological images. The microscope should be kept in a low-vibration, low-dust environment to facilitate viewing and decrease damage to the optical systems. The optical elements should be kept completely free of dust, dirt, oil, solvents, and any other contaminants (21). Ideally, the microscope should be covered and the lamp should be turned off when the microscope is not in use. Do not touch the optical surfaces with your fingers (21). Keep the lenses clean and be sure to remove oil or mounting fluid from the objectives, condenser, and mechanical stage after each session. Avoid dragging the high-dry objective through oil or fluorescence mounting fluid. One way to avoid accidental contact with these fluids is to place the high-dry objective and the oil immersion objective in the nosepiece on opposite sides of the low-power objective (4). Lenses should be dusted with residue-free compressed air and cleaned with lens paper and a commercial lens cleaner that is approved by the microscope manufacturer. Organic solvents such as alcohols and acetone should not be used on the lenses because the solvent may dissolve the optical mounting cement (7). Unused spaces in the nosepiece (Fig. 3) should be plugged, and the eyepieces should remain installed at all times to prevent introduction of dust into the body tube. The stage should be cleaned regularly and any spilled immersion oil must be removed or else slides will stick when they are moved across the stage. Spilled immersion oil also collects dust and grit that can damage the optical and mechanical parts. Microscopists should not attempt to remove or disassemble the objectives, as this increases the potential for damage (7, 21). This is a job that is best left to professionals (21). The gears and rackwork should be cleaned and treated with new grease at intervals specified by the manufacturer. Do not use light oil on the gears or bearing surfaces because this may cause the condenser and stage to sink from their own weight (21). Periodic cleaning and adjustment by a professional microscope repair person also help to extend the usable life of the microscope.

ERGONOMICS

Peering into a microscope eyepiece for long periods is not an activity for which the body is well adapted. Microscope work requires the head and arms to be locked in a forward position and inclined toward the microscope with rounded shoulders. This unusual positioning is further exaggerated when the feet are placed on the ring-style footrests that are common to many laboratory stools. Poor posture and awkward positioning during microscopy can cause pain or injury to the neck, wrists, back, shoulders, and arms. In one regional survey of cytotechnologists, Kalavar and Hunting (18) found that 70.5% of respondents reported neck, shoulder, or upper back pain during microscopy and 56% had an increased prevalence of hand and wrist symptoms. Eyestrain and leg and foot discomfort have also been documented with long-term microscope use (6). When using older microscopes, users often have their heads inclined up to 45° from vertical and their upper backs may be inclined by as much as 30°. Even 30° inclinations of the head can produce significant muscle contractions, fatigue, and pain (6). For this reason, microscopists should be taught to sit upright and hold their head in a neutral position (25).

During microscopy, the laboratorian should sit erect and maintain the natural curve of the spine (25). The lower back and shoulder blades should be supported by the chair and a lumbar support cushion should be used if necessary. The legs and feet should rest firmly on the floor or a footrest. The chair should have a pneumatic height adjustment (21), and the seat should have a sloping front edge to prevent undue pressure on the thighs. The backrest should be adjustable for both height and angle, and the chair should have a five-pointed star base with caster wheels. Knee spaces, which are often used for laboratory storage, should be free from obstructions, and there should be a minimum of 2 in. of clearance between the thigh and the bottom of the desk or counter (18). Obstructions that prevent the microscopists from holding their shoulders perpendicular to the ocular axis of the microscope should be removed (27). The upper arms should be perpendicular to the floor with the elbows close to the body. The forearms should be parallel with the floor, and the wrists should be straight. The head should be upright and the neck should bend as little as possible, preferably no more than 10 to 15°. The eyepieces should be just below the eyes, and the eyes should look downward at a 30 to 45° angle. The use of tilting microscope heads can significantly improve the comfort of the microscopist (18, 19, 27). Repetitive motions of the hands and the contact stress of arms resting on (the edge of) a hard surface can cause pain and nerve injury, leading to repetitive stress injuries and/or carpal tunnel syndrome. The use of padded arm rests can moderate some of these problems. In addition, microscopes should not be placed under an air vent in order to prevent stiffening of the muscles during microscopy.

Most laboratory microscopes are used by multiple individuals, and it is often a challenge to find conditions or microscope configurations that satisfy everyone. Some laboratories place microscopes on books or heavy blocks of wood to accommodate taller microscopists (21). This configuration creates a number of problems. If the microscope is raised to a sufficient height to prevent neck flexion, users may be forced to bend their wrists into an unnatural position. If the microscope is lowered to allow the forearms to remain parallel to the floor, the neck is forced to bend. Lowering the chair to its lowest position causes leg discomfort. Vertically challenged individuals may have to raise the chair to a level where their feet no longer touch the floor. Foot rests can ameliorate this problem, but some individuals may have insufficient space under the bench top to accommodate their legs. In practice, most laboratories will elect to use a suboptimum but workable microscope configuration that all users can employ. Under these conditions, microscopists can reduce stress and fatigue by taking 1-min "microbreaks" every 10 to 15 min during which they can stand, stretch, and allow the eyes to focus at a distance.

Eye fatigue can be a major problem for microscope users, especially if they have poor vision. The diopter adjustment provided on most microscope eyepieces can be adjusted to compensate for minor near- and far-sightedness, thereby allowing the users to remove their glasses during microscope use. The diopter adjustments do not adjust for astigmatism, and users with moderate to severe astigmatism should wear glasses when using the microscope. Most microscope manufacturers now produce high-eyepoint eyepieces that move the visual observation point further from the eyepiece, thereby facilitating the use of glasses during microscopy. Ensuring that the microscope images are as bright, sharp, and crisp as possible will also help to reduce eye fatigue and associated headaches. The importance of proper alignment of the microscope and optical components cannot be overstressed. Proper optical alignment and the use of newer objectives with higher NA values will produce brighter

images and better resolution, which eases the strain of searching for tiny specimen details. The use of a neutral blue (daylight) filter during bright-field microscopy can also help to lessen eyestrain when examining microbiological specimens. In the future, many new microscopes will display the specimen image on a computer monitor. This innovation could alleviate many of the eyestrain problems that develop during extended microscope use (27).

Microscopes are as different as the people who use them, and the previous comments should not be construed as a prescription for alleviating strain or repetitive motion injuries in every situation. When purchasing a microscope, every effort should be made to allow microscopists to evaluate the new microscope under their normal working conditions. Some microscopes will be comfortable for some users and uncomfortable for others. In the long run, the feel and fit of the microscope are just as important as the optical characteristics.

CONCLUSION

Advances in the design, resolution, and ergonomics of modern microscopes have greatly enhanced our ability to study and identify microorganisms. Microscopy still has a central role in the detection of infectious agents despite highly publicized advances in DNA and RNA detection systems. Microscopic examination of clinical specimens provides a rapid and inexpensive "first pass" in the detection and identification of infectious agents. Thus, clinical microscopy will continue to be a core competency in clinical microbiology laboratories for the foreseeable future.

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Principles of Stains and Media*

KIMBERLE C. CHAPIN

14

DIRECT EXAMINATION OF SPECIMENS

The first step in the processing of most clinical material is the microscopic examination of the specimen. Direct examination is a rapid and cost-effective diagnostic aid developed to reveal and enumerate microorganisms and eukaryotic cells. Visible microorganisms or the lack thereof may denote the presumptive etiologic agent, guiding the laboratory in the selection of the appropriate isolation media and the physician in the selection of the appropriate empirical antibiotic therapy. The quality of the specimen and the measure of the inflammatory response can also be evaluated. In addition, the direct smear serves as a quality control indicator for attempts to cultivate observed organisms (18). The first part of this chapter discusses the principles of staining methods used in the direct examination of specimens. Tables 1 to 3 give a brief overview of all commonly performed staining methods noted in this Manual. Procedures for performance of the stains are available in the chapters on reagents, stains, and media for bacteriology (chapter 21), virology (chapter 81), mycology (chapter 117), and parasitology (chapter 134). Technical aspects of microscopy are described in chapter 13. An excellent website with exquisite detail containing all aspects of microscopy is available at http://www.zeiss.com.

CHEMICAL BASIS OF STAINING

Simple wet mounts, consisting of clinical material in a drop of saline, allow determination of cellular composition, morphology, and motility. However, cellular material and organisms are usually transparent and best distinguished by the use of dyes or biological stains. Antonie van Leeuwenhoek was the first to attempt the differentiation of bacteria with the use of natural colored agents such as beet juice in 1719 (16). Staining procedures and the understanding of the chemical basis of staining developed extensively in the area of histology, where cellular constituents were desired to be more clearly demarcated. The specific chemical basis of many stains used in clinical microbiology is described in detail in histology texts (10, 12, 13, 21). Additionally, websites pertaining to the basic properties of stains for histology are helpful, including the following site that lists stains alphabetically, http://focosi.immunesig.org/histochemistry.html.

Metachromasia is a characteristic color change which natural dyes (aniline dyes or products of such natural products) exhibit when bound to certain substances either in tissue or in aqueous solution. With the exception of hematoxylin, natural dyes have for the most part been replaced by artificial dyes. Artificial dyes are products of chemical derivatives from substances in coal tar, especially benzene. Two other important chemical groups, the chromophore and auxochrome, complete the dye compound (12, 13).

A chromophoric group is the group of atoms within a dye molecule responsible for its color. Benzene, an aromatic organic compound, undergoes substitution reactions with radicals to form these new compounds, which constitute the dye resonance system. Some of the molecular changes result in a colored product. The most important chromophoric groups are C=C, C=O, C=S, C=N, N=N, N=O, and NO_2. The number of chromophores in a compound determines the intensity of the compound color. Benzene plus a chromophore group is a chromogen. Although the chromogen is colored and typically ionic, it does not have great affinity for bacteria or tissues, and washing or mechanical processes will readily remove the compound. Thus, this group does not in itself constitute a dye. The molecule must also possess an ionizing group called an auxochrome, which allows the dye molecule as a unit to have affinity (cationic or anionic) for a compound and to function as a dye. The auxochrome group gives the compound the property of electrostatic dissociation, or the ability to form salt linkages with the ionizable radicals on proteins, glycoproteins, and lipoproteins on tissue or organism cellular components. This process can occur either directly or through a chelating action of a mordant (13). Commonly occurring auxochromes are amino groups ($-NH_2^+$), hydroxyl groups (–OH), sulfates ($-SO_3^-$), and carboxyl groups (–COOH). With the exception of the amino groups, which ionize to produce a positive charge and are considered basic (cationic), all of these auxochromes ionize to produce negative charges and are acidic (anionic). Some dyes have more than one auxochrome. Even in combinations with basic and acid auxochromes, the negative charge typically predominates (10, 12, 21).

Dyes are usually sold as salts; thus, it is the auxochrome group that usually determines whether a dye is classified as cationic (basic) or anionic (acidic). Most dyes will retain their cationic or anionic properties throughout the pH range of

*This chapter contains information presented in chapter 18 by Kimberle C. Chapin and Patrick R. Murray in the eighth edition of this Manual.

staining (pH 3 to 9) and thus reliably stain those structures that are oppositely charged. For example, DNA phosphate groups and mucopolysaccharides, which are negatively charged and acidic, will be stained with a basic dye. Basic (positively charged) components in cytoplasm will stain with an acid dye. Crystal violet, methylene blue, and safranin are typical cationic (basic) dyes, and eosin, acid fuchsin, and picric acid are typical anionic (acid) dyes.

WET MOUNTS AND SINGLE-STAIN METHODS

Wet-mount preparations are used to determine the cellular composition of a specimen as well as the morphology of organisms, their gross structure, and their biological activity, including motility (Table 1). The specimens can be examined by bright-field, phase-contrast, or dark-field microscopy. True wet mounts do not involve fixation of the clinical material and are viewed immediately upon preparation. Single-stain methods, such as methylene blue or iodine, enhance visualization of organisms by increasing the contrast of structures and can be performed either as a wet mount or with fixed material (e.g., M'Faydean stain) (3, 4). All organisms and cellular components stain shades of a similar color.

DIFFERENTIAL STAINING

While direct visualization of specimens in various wet mounts is useful, differentially stained specimens are the most helpful for presumptive grouping of the majority of pathogens. The

TABLE 1 Wet mounts and single-stain methods

Direct-examination method	Applications	Principle	Time required (min)	Advantages	Disadvantages
Wet mount	Direct clinical examination of stool, vaginal discharge, urine sediment, aspirates	Used to detect organism motility and morphology of parasitic forms and fungi	1	Rapid	Limited contrast and resolution; Brownian movement may be confused with motility; experienced microscopist required
10% KOH	Direct examinations of specimens for fungi, e.g., skin scrapings, fluid aspirates	Proteinaceous host cell components are partially digested by alkali; fungal cell walls stay intact	5–10	Rapid detection of fungi	Background material may cause confusion; experienced microscopist required
10% KOH with lactophenol cotton blue	Direct examination of specimens for fungi, e.g., skin scrapings, fluid aspirates	Adds contrast for detection of fungi	5–10	Dye enhances detection of fungi	Background material may cause confusion; experienced microscopist required
Colloidal carbon (India ink, nigrosin)	Direct examination of CSF[a] and other body fluids for *Cryptococcus neoformans*	Polysaccharide capsule excludes ink particles producing a halo appearance	1	Rapid; diagnostic in CSF when present	Not as sensitive as cryptococcal antigen; cells and artifacts may cause confusion; experienced microscopist required
Lugol's iodine	Direct examination of stool	Nonspecific contrast dye to help differentiate parasitic cysts from leukocytes; cysts retain dye and appear light brown	1	Rapid; enhances differentiation	Background material may cause confusion; experienced microscopist required
Methylene blue staining	Direct examination of stool for leukocytes; detection of bacteria, particularly poorly staining gram-negative organisms, spirochetes, and *Corynebacterium diphtheriae*	Leukocytes and bacteria stain blue	1; up to 10 min if *C. diphtheriae*	Rapid; enhances differentiation	Leukocytes may disintegrate if stool is not examined promptly; overstaining may mask granules
M'Fadyean staining	Fixed examination of clinical specimens from patients suspected to have anthrax	Methylene blue dye stains *Bacillus anthracis* deep blue and demarcated pink capsule zone	4	Rapid; enhances differentiation of capsule	Stain rarely performed

[a] CSF, cerebrospinal fluid.

Gram stain and acid-fast stain are examples of differential stains. In addition, fluorescent stains (see below) may aid in the identification of organisms when specific attachment of fluorochromes occurs with organism components: auramine, calcofluor white, and fluorescein isothiocyanate (FITC) bound to monoclonal antibodies or synthetic protein nucleic acids (PNAs) (19).

In fixed differential smear preparations, four components are typically used in a progressive manner: the primary stain, a mordant, a decolorizing agent(s), and a secondary stain or a counterstain. The primary stain usually stains all cellular components and organisms in the specimen the same color, as seen in simple procedures with a single stain (e.g., methylene blue). The mordant aids in the attachment of a dye to cellular components. Heat, phenol, and iodine are examples of mordants. Decolorizing agents are typically acids and alcohols, such as the acetone and alcohol mixture used in the Gram stain and sulfuric acid used in the modified Kinyoun (modified acid-fast) stain. Removal of the primary stain with the decolorizing agent allows a secondary stain (counterstain) to be taken up by any decolorized organisms and background material. The secondary stain differentiates between those cells that retain the primary stain and those that do not. Examples include the purple (primary stain) and pink (counterstain) organisms seen with the Gram staining procedure or the organism and the background, as seen with a pink acid-fast organism (primary stain) in a blue-counterstained background.

Gram Stains

The Gram stain is the most commonly performed differential fixed stain in microbiology. The Gram reaction, morphology, and arrangement of the organisms give the physician clues to the preliminary identification and significance of the organisms. Gram-positive organisms are thought to retain the crystal violet dye because of the increased number of cross-linked teichoic acids and the decreased permeabilities of their cell walls to organic solvents because they contain little lipid. While the gram-positive organisms take up the counterstain, their color is not altered. The cell walls of gram-negative organisms, because of the higher lipid content associated with the cell wall, show increased permeability to decolorizer, and these organisms lose the crystal violet dye and take up the counterstain dye, safranin (6).

Some enhancement techniques are basic modifications of more standard differential or single-stain methods. These include the use of tartrazine and light green (enhanced Gram stain [Remel]) in place of safranin in the Gram stain and the addition of basic fuchsin to methylene blue (Wayson stain). Many other combinations and manipulations exist (Table 2). The main purpose of these stains is to make organisms that are normally difficult to detect by the Gram staining method stand out more prominently. Typically, these enhancement techniques are used for the better visualization of gram-negative organisms. This is done by two methods. One method is to simply use a stain that will make the organism a darker color so that the normally weakly staining gram-negative organisms are visible (4, 9, 17). The second method is to make the background inflammatory cells and mucus, which often masks gram-negative organisms, a different color than the usual red-pink. This method is used with the tartrazine-light green stain, which makes the background gray or green and the organism easily visible. In this staining method the Gram reaction of the organisms is preserved (J. Kee, A. Hill, and K. Chapin, *Abstr. 94th Gen. Meet. Am Soc. Microbiol. 1994*, abstr. C-242, 1994).

Acid-Fast Stains

The cells of certain organisms contain long-chain (34- to 90-carbon) fatty acids (mycolic acids) that give them a coat impervious to crystal violet and other basic dyes. Heat or detergent must be used to allow penetration of the primary dye fuchsin into the bacterium. Once the dye has been forced into the cell, it forms a stable complex and the usual acid-alcohol solvent cannot decolorize the organism. The acid-fast stain is useful for identification of a specific group of bacteria (e.g., *Mycobacterium, Nocardia, Rhodococcus, Tsukamurella, Gordonia, and Legionella micdadei*) and the oocysts of *Cryptosporidium, Isopora, Sarcocystis*, and *Cyclospora*. A number of modifications have been developed from the original acid-fast stains described by Ziehl in 1882 and Neelsen in 1883. The modifications most commonly used are the Kinyoun and modified Kinyoun (modified acid-fast). Age of the organisms or slight differences in the fatty acids between species can also alter the stain choice (1, 14; J. Brown, CDC, personal communication).

FLUORESCENT MICROSCOPY

See Table 3 and chapter 13 for technical aspects of fluorescent microscopy.

ANTIBODY STAINING METHODS

See chapter 18 on immunoassays.

HISTOLOGICAL TISSUE SPECIMEN INTERPRETATION

The clinical microbiologist is often asked to consult on smear interpretation of blood and body fluid specimens and histologically stained sections of tissue. The differences between organisms stained directly from blood and those stained in fixed tissues need to be noted. In general, it should be remembered that most fungi, parasites, viral inclusions, and bacteria in histological preparations cannot be identified definitively. For instance, the stain typically used in the hematology laboratory for blood and body fluids is the Wright-Giemsa preparation, which uniformly stains all bacteria blue. Thus, one must not call a blue coccus a gram-positive coccus until Gram staining can be done to help in differentiation. In addition, in stained tissue preparations, pathogens may be significantly different in appearance owing to the staining and fixative practices used in the histology laboratory. For instance, vacuolated areas may give the appearance of a capsule in a nonencapsulated organism. However, some organisms, such as *Borrelia, Anaplasma*, and *Ehrlichia,* are best visualized in direct blood smears of buffy coats with Wright-Giemsa.

Histological preparations often stain organisms similarly to the stains used in microbiology but have been modified for tissue. The Brown-Brenn (Gram) stain and Fite-Faraco (acid-fast) stain are examples of tissue stains with which the organisms retain the color appearance seen with their microbiology counterpart (2, 15). Other common tissue stains often viewed by microbiologists include silver stains, such as Warthin-Starry or Dieterle, and the Wright-Giemsa stain. The Wright-Giemsa stain does not stain bacteria or fungi reliably in tissue, with the important exception of *Histoplasma capsulatum*, which will stain by Wright staining of bone marrow and peripheral blood. These histological preparations, as well as others, can also be used to stain infected cell culture monolayers and aid in the visualization of viral and

TABLE 2 Differential fixed staining methods

Differential fixed staining method	Application	Principle	Time required	Advantages	Disadvantages
Gram staining (conventional or Atkins' modification)	Differential bacterial and yeast stains; used to assess suitability of specimen for bacterial culture	Gram-positive organisms retain crystal violet and stain blue; gram-negative organisms do not retain crystal violet and stain pink due to the counterstain safranin	3 min	Rapid; commonly performed; can aid in choice of antibiotic therapy; used to assess specimen for culture and compare smear result to culture result	Organisms with damaged cell walls will stain unpredictably; *Nocardia* and fungi may not take up crystal violet completely; background and cellular elements stain pink, often masking gram-negative organisms
Anaerobic Gram staining variation	Differential stain used to detect anaerobic organisms (especially gram-negative bacteria) not easily seen with regular Gram stain	0.1–0.5% basic carbol fuchsin used as counterstain instead of safranin; enhances detection of gram-negative organisms	3 min	Darker staining of gram-negative anaerobes such as *Fusobacterium* as well as other slender gram-negative organisms, such as *Helicobacter* and *Campylobacter*	
Tartrazine-fast green Gram staining variation	Differential bacterial stain that enhances detection of organisms because cellular background is green	Use of fast green and tartrazine before safranin counterstain allows significant suppression of red-pink color of the background material; organisms still stain purple (gram positive) or pink (gram negative)	3 min	Allows excellent enhancement of small gram-negative organisms and detection of mixed cultures	Slight change in color appearance of organisms compared with regular Gram stain may be confusing
Spore staining (Wirtz-Conklin)	Differential stain for detection of bacterial spores	Spores take up the malachite green stain and the cellular debris, and bacteria appear pink from the safranin counterstain.	Slides can be stained for 45 min or gently heated to steaming for 3–6 min.	Facilitates detection of bacterial spores which may otherwise be difficult to observe. While a heating step is more cumbersome, it enhances uptake of the stain into the spore.	Gentle heating of slide may be difficult to control
Wayson staining	Direct examination of CSF[a] and other specimens for bacteria and amebae	A mixture of the dyes basic fuchsin and methylene blue that results in contrast staining between bacteria that stain deep blue and other material that stains light blue or purple	3 min	Rapid; enhances differentiation	Staining reagents unstable; slides cannot be restained with Gram stain
Acid-fast staining (Ziehl-Neelsen, Kinyoun, modified Kinyoun)	Detection of acid-fast and weakly acid-fast organisms (e.g., *Mycobacterium, Nocardia, L. micdadei, Rhodococcus, Tsukamurella, Gordonia, Cryptosporidium, Isospora, Cyclospora,* and *Sarcocystis*)	Presence of long-chain fatty acids (mycolic acids) in cell wall or cystic forms makes organisms resistant to decolorization; organisms retain the carbol fuchsin dye and appear pink	2 h	Used to detect acid-fast and partially acid-fast organisms; presence generally significant from direct specimens	Low organism number makes slide examination tedious; tissue homogenates often mask presence of organisms because of deeply staining background

(Continued on next page)

TABLE 2 Differential fixed staining methods *(Continued)*

Differential fixed staining method	Application	Principle	Time required	Advantages	Disadvantages
Periodic acid-Schiff staining	Detection of fungi, specifically yeast cells and hyphae, in tissue specimens. *Tropheryma whipplei* bacilli appear as inclusions in macrophages.	Combination of acid hydrolysis and staining of cell wall carbohydrates; fungi stain pink-magenta or purple, and background appears orange or green if picric acid or light green, respectively, is used as the counterstain. Diastase-resistant, magenta-stained inclusions within macrophages are pathognomic of Whipple's disease.	1 h	Most fungi stain	Time-consuming; respiratory specimens must be digested or mucin will also stain pink-magenta. Inclusion staining pattern seen in diseases other than Whipple's disease.
Toluidine blue O staining	Rapid examinations of lung biopsy imprints and respiratory specimens for *P. jiroveci*	Background material removed by sulfation reagent and appears light blue; *P. jiroveci* cysts stain reddish blue or dark purple against the lighter background	20 min	Rapid method for detection of *P. jiroveci* cysts from appropriate specimen, such as bronchoalveolar lavage specimen	Differentiation of *P. jiroveci* from yeast may be difficult; cysts often appear crescent shaped; trophozoites are not discernible
Wright-Giemsa staining	Detection of parasites from blood smears, viral and chlamydial inclusions, toxoplasmosis, *Pneumocystis carinii* trophozoites, *Histoplasma* yeast forms in tissue, *Yersinia, Helicobacter, Ehrlichia,* and *Rickettsia*	Differential staining of basophilic and acidophilic material. Combination of stains allows uptake by multiple structures.	10 min–1 h	Detection of multiple organisms and cellular inclusions	Not specific for inclusions (*Chlamydia* is the exception); cannot determine bacterial Gram reaction
Gimenez staining	Intracellular organisms, especially *Coxiella, Rickettsia* from cell cultures, and *Legionella pneumophila*	Gram-negative organisms take up the carbol fuchsin and appear red against a green background with the counterstain that contains malachite and fast green.	3 min	Provides enhanced contrast of the gram-negative cell wall not able to be achieved with the Gram stain	Stain needs to be heated to 37°C 48 h prior to use and then filtered. Not commonly available in microbiology laboratories. Not specific.
Trichrome staining, Wheatley	Detection of intestinal protozoan cysts and trophozoites	Provides contrast between parasite cytoplasm (blue-green tinged) and internal structures (red or purplish red) and background debris (green to blue-green) by using chromotrope 2R and light green combination	1 h	Permits detection of diagnostic internal structures of protozoa	Helminth eggs are generally stained very dark; human cells, yeast, and artifacts may also stain.

(Continued on next page)

TABLE 2 *(Continued)*

Differential fixed staining method	Application	Principle	Time required	Advantages	Disadvantages
Trichrome staining, modified (Weber-Green) (Ryan-Blue)	Detection of microsporidial spores	Increase in chromotrope 2R stain concentration and staining time permits detection of pink microsporidial spores.	2 h	Permits detection of pink microsporidial spores against a green (Weber) or blue (Ryan) background	Time-consuming stain
Iron hematoxylin staining (Delafield's)	Detection of microfilariae	Provides contrast so internal filarial structures can be visualized. Hematein, an amphoteric dye, when combined with iron that acts as a mordant, creates an ionically charged dye lake that has affinity for cellular components.	45 min	Permits greater detection of nuclei and sheath of microfilaria compared to Giemsa or Wright's stains	Stain not available commercially and involves extensive aging process
Iron hematoxylin staining	Detection of intestinal protozoan cysts, inclusions, nuclei, and trophozoites	Provides contrast between parasites and background debris. Cytoplasm will have a blue-gray color, sometimes with a tinge of black; cysts tend to be slightly darker; nuclei and inclusions have a dark gray-blue color. Background is pale gray or blue.	60 min	Permits detection of diagnostic structures of protozoa	Helminth eggs are generally stained too dark to discern specific structural differences.

Chlamydia inclusion bodies, *Coxiella, Ehrlichia,* and *Toxoplasma* in tissue. Silver stains provide a differential stain that is best for detection of fungi and *Pneumocystis jiroveci* cyst walls in tissue (20). However, the stain also can detect bacteria and parasites. Differentiation of various yeast forms of *P. jiroveci* as well as other yeasts such as *Cryptococcus* is difficult, and interpretation should be done with caution and in conjunction with the use of other specific stains, such as Fontana-Mason (melanin) and mucicarmine (mucopolysaccharide). Thus, for the best outcome in the histological diagnosis of infectious etiologies, good communication among the surgical pathologist, hematologist, microbiologist, and primary physician will result in the securing of tissue for staining as well as for culture.

PRINCIPLES OF MEDIA

The purposes and descriptions of media for bacteriology, virology, mycology, and parasitology are provided in chapters 21, 81, 117, and 134, respectively. This chapter focuses on the principles of media used mainly for the isolation of bacteria.

General Considerations

Many components optimize the growth of microorganisms on media. The basic requirements for a medium include a nutrient source, a solidifying agent (for solid media), a specific pH, and any number of specific additives. The nutritional requirements of most microorganisms are complex. Most utilize an array of nutrient sources including nitrogen, carbon, inorganic salts, minerals, and other diverse substances. While some organisms can utilize a very simple medium such as nitrate or ammonia, most require protein hydrolysates or peptones. Peptones are the most common nutrient additives in media and are water-soluble materials prepared by enzymatic or acid hydrolysis of animal tissues or products and vegetable substances. Meat infusions were the initial growth-supporting components in media, but because they are cumbersome to prepare and lack batch-to-batch consistency, they are not truly defined. However, meat infusions are still used in some media. Agar serves as the solidifying agent and is derived from red seaweed. The acidity or alkalinity (pH) of a medium is important because microorganisms have strict pH requirements, with most growing in the range of pH neutrality. Components may be added to a medium for purposes of evaluating the pH. These include dyes that change color at a specific pH secondary to the production of acid or alkaline by-products by the organism and buffers that allow determination of the hydrogen ion concentration. Other selective agents, such as antibiotics, dyes, and other nutrients, can be incorporated

TABLE 3 Fluorescent staining methods

Fluorescent stain	Application	Principle	Time required	Advantages	Disadvantages
Acridine orange stain (8, 11)	Detection of bacteria in blood cultures, CSF,[a] buffy coats, and corneal scrapings; detection of fungi	Fluorochrome intercalates nucleic acid in both native and denatured states; bacterial and fungal DNA fluoresces orange and mammalian DNA fluoresces green with UV excitation	3 min	Sensitive method of detection in blood, CSF, and tissues; detects organisms difficult to see with Gram stain and low numbers of organisms such as *Bartonella* and *Helicobacter;* thick or bloody smears can be used; can Gram stain same slide to confirm result	Cellular specimens with an abundance of DNA may be difficult to interpret; interobserver variability seen with some specimens (e.g., buffy coat smears)
Auramine-rhodamine stain	Detection of mycobacteria and other acid-fast organisms	Nonspecific fluorochromes that bind to mycolic acids and resist decolorization by acid-alcohol (identical to acid-fast stains); organisms fluoresce orange-yellow with UV excitation and use of the secondary potassium permanganate stain	30 min	Allows rapid screening of specimens at lower magnification; is more sensitive than other acid-fast stains; can use other acid-fast stains on the same slide to confirm suspicious organisms	Low numbers of organisms may be difficult to confirm with routine acid-fast procedures
Calcofluor white stain (7)	Detection of fungi, *P. jiroveci* cysts, and parasites, such as microsporidia and cysts of free-living amebae in clinical specimens	Nonspecific fluorochrome that binds to cellulose and chitin. Organisms fluoresce blue-white or green if a barrier filter is used. Optimal fluorescence occurs with UV and blue-violet excitation. For eye protection, barrier filters of 510–530 nm are recommended.	KOH clearing if necessary, and then immediate viewing after adding stain	Can be mixed with KOH to clear specimens such as hair, skin, and nails for dermatophytes; rapid, sensitive, and inexpensive screening test. Smears can be restained with conventional stains such as Gram stain.	Difficulty in interpretation with cellular specimens that contain fluorescing collagen and elastic fibers. Variable fluorescence is seen with darkly pigmented fungi. Use with Evans blue helps to suppress green background fluorescence and imparts a red color when blue-violet excitation is used.
Fluorescein-conjugated antibodies and PNA probes	Identification of specific organisms in clinical material; used to confirm specific organism identification from cultures (CMV,[b] *Erhlichia, Francisella*) and growth from blood culture broth (yeast and bacteria)	Monoclonal antibodies or probes bound to the fluorochrome FITC to detect antigens or nucleic acids for specific pathogens in clinical specimens; pathogens fluoresce apple green or red depending on barrier filters used	1–3 h	Rapid and specific organism identification; especially useful for commonly occurring blood culture isolates, specifically staphylococci, *Candida albicans,* enterococci, *Bordetella, Legionella, Pneumocystis*, and viruses.	Adequate clinical specimen must be submitted if done with direct specimen

[a] CSF, cerebrospinal fluid.
[b] CMV, cytomegalovirus.

into media for the isolation of a particular organism. Other considerations that allow optimal microorganism growth include the incubation temperature and the gas in the growth environment. Most clinically significant organisms are mesophiles, which means that they will grow optimally at temperatures of between 25 and 40°C. In addition, most species grow in ambient air, but others require CO_2 or the total removal of O_2. Liquid media require all of the ingredients and conditions described above but lack a solidifying agent (e.g., agar).

Medium Types

Transport Media and Preservatives

Transport media are used in the collection and transport of specimens and were devised initially because fastidious organisms would not survive transport from the bedside to inoculation in the laboratory. Now transport media are even more crucial in providing an appropriate environment for specimens as more and more specimens are transported from distant sites and for long periods.

Generally, bacterial transport media come packaged in a plastic tube sleeve or in tubes with a small amount of liquid medium. A single or a double swab attached to a cap is used for collection of the specimen, which is then placed into the tube and secured. The cap allows the swab(s) to be easily removed from the transport medium for inoculation. The swab component and shaft material, such as Dacron versus rayon and aluminum versus calcium alginate, respectively, may be significant depending on the organism targeted for isolation or the assay system used. Generally, transport media provide a nonnutrient source that sustains the viability of both aerobic and anaerobic organisms without allowing significant growth. Most transport media have specific ingredients that accomplish these goals. These include a small amount of agar or sponge to allow a solid base to which the organisms can attach and to reduce desiccation, an indicator oxidation-reduction agent which shows when oxidation has occurred, and reagents that maintain the pH. Other additives allow the survival of specific organisms, such as sodium thioglycolate for anaerobes or charcoal, which reduce the effects of toxic metabolic products and which subsequently enhance the growth of the pathogens. Other ingredients are added for specific purposes and are noted below. Transport media generally allow stability of specimens for 6 to 12 h at ambient temperatures and should not be refrigerated because some organisms do not survive at colder temperatures. When the specimen arrives in the laboratory, it should be plated as soon as possible. The material in the swab is extracted and placed onto the medium of choice. Care should be taken to inoculate the material from the swab itself when it is extracted from the tube and not the material that may have been in the swab system, such as the gel.

Viruses and chlamydiae have different transport requirements. Viral and *Chlamydia* transport media are designed to provide an isotonic solution containing protein, antibiotics to control bacteria, and a buffer to control pH. The media come in polypropylene centrifuge tubes that contain approximately 1 to 3 ml of medium, 1-dram freezer vials with up to 2 ml of medium, and a tube and swab form with a gel base. While separate transport media for viral pathogens and *Chlamydia* exist, more often laboratories use systems that can accomplish the culture of both of these types of pathogens as well as the *Ureaplasma* and *Mycoplasma* groups. The antibiotics used in these media are not inhibitory to the bacterial pathogens desired or viruses.

Parasitology transport media are actually preservatives meant to maintain the integrity of the parasite and not to maintain viability.

General-Purpose, Enriched, Selective, Differential, and Specialized Media

General purpose, enriched, selective, differential, and specialized are the general categories of media that are used for growth and cultivation of microorganisms. Each type of medium is not exclusive; e.g., many selective media are also differential media. An example is MacConkey agar, which is selective for gram-negative organisms but which is also differential in that it is used to identify lactose-fermenting organisms. Descriptions of each type of medium follow.

General Purpose

General-purpose media are those media capable of detecting most aerobic and facultatively anaerobic organisms. An example of a medium in this category is sheep blood agar, which is commonly used for the general isolation of organisms directly from primary specimens inoculated onto the agar.

Enriched

Enriched media are media that allow fastidious organisms to grow. These organisms may not grow well on general media. An example in this category is the growth of *Francisella* on chocolate agar because the agar is supplemented with cysteine.

Selective

Selective media are media that contain additives that enhance the detection of the desired organism by inhibiting other organisms. Most commonly, selection is attained with a dye or with the addition of an antibiotic. Examples include MacConkey agar that contains crystal violet, which inhibits most gram-positive organisms, and colistin-nalidixic acid agar, which contains antibiotics that inhibit most gram-negative organisms. The effectiveness of selective media varies and is not always complete. Thus, small colonies of partially inhibited organisms may be present on the media. In addition, the ingredients that make media highly selective may actually inhibit the desired pathogen, e.g., a vancomycin-supplemented medium that is selective for *Neisseria gonorrhoeae* (gonococci [GC]) may inhibit some strains of GC.

Differential

Differential media aid in the presumptive identification of organisms based on the organisms' appearance on the media. This can be demonstrated by colony color or a precipitate that forms on or around the colony. Examples include the agars used for the isolation of enteric pathogens (e.g., MacConkey, Hektoen enteric, and xylose-lactose-desoxycholate agars). In the case of MacConkey agar, lactose fermentation by the organism and exhibition of a bright pink magenta color by the colony mean that the organism is utilizing lactose. Further enhancements of differential media exist, such as specific chromogenic media that are also very selective, and allow groups of organisms within a genus to be more clearly recognized than with use of the usual primary media alone. See chromogenic media in chapter 21 for specific descriptions.

Specialized

Specialized media are those media developed with additives for the purpose of isolating a specific pathogen. Such media include buffered charcoal yeast extract medium (BCYE), which is designed for the purpose of isolating *Legionella* species. Specialized media typically include nutrients that the specific pathogen requires but that are not found in general-purpose or enriched media. In the case of BCYE, cysteine and ferric pyrophosphate are provided. Other examples include virology culture media with essential amino acids that are required for the maintenance of cell lines and growth of viruses and anaerobic media that typically include vitamin K, hemin, and reducing agents.

Susceptibility Media

Susceptibility testing media have well-defined formulations designed to support the growth of the most common bacterial

and fungal isolates. Hydrolysates of casein and beef extract with low concentrations of thymidine and thymine are used because excess amounts of thymine and thymidine can make organisms appear to be more susceptible to sulfonamides and trimethoprim. Adjustment of small ion concentrations may be necessary for correct susceptibility reporting. Calcium and magnesium ion concentrations are adjusted to allow correct interpretations of *Pseudomonas* susceptibility results with the aminoglycosides, colistin, and tetracycline.

Mycobacteriology Media

Most of the nonselective media used for the isolation and cultivation of mycobacteria are enriched media that are egg based or agar based and that contain additives with fatty acids essential for growth of the organism. Common additives include albumin, which protects the tubercle bacilli from toxic agents; inorganic salts essential for growth; glycerol as a carbon and an energy source; and malachite green, which partially inhibits contaminating bacteria other than mycobacteria and acts as a pH indicator. Because malachite green is a photosensitive dye, a medium with this ingredient should be stored in the dark. Mycobacteria prefer moisture, and tubes of media should be tightly sealed before inoculation. Liquid media are used for the optimum recovery of mycobacteria and for decreasing the time to detection of the organisms. The broths are also used to subculture stock strains and for other tests, such as susceptibility testing and tests with DNA probes.

Anaerobic Media

All general-purpose nonselective anaerobic blood agar media have similar formulations and include peptones, yeast extract, vitamin K (which is required for some *Porphyromonas* spp.), hemin (which enhances the growth of some *Bacteroides* spp.), 5% sheep blood (which allows for the detection of hemolysis), and reducing agents. All allow the isolation and cultivation of both strictly anaerobic and fastidiously anaerobic organisms. The difference in each of the media is the small variation in the peptones used and the inclusion of dextrose in some media as an energy source. These differences may make some of the media better for gram-negative or gram-positive organisms with slight variations in colonial characteristics. Additives used with some of these media allow the media to have both selective and differential properties. Enrichment broths are available in a number of formulations but are increasingly less commonly used for the routine isolation of anaerobes. It should be noted that media are available that are prepared and stored in a prereduced atmosphere specifically for optimization of anaerobic isolation (prereduced anaerobically sterile [PRAS] media).

Preparation of Media

When preparing media from dehydrated materials, the manufacturers' instructions should be followed closely. Chemically cleaned glassware and distilled and/or demineralized water should always be used unless specified otherwise. Care in terms of accuracy should be taken when measuring liquid and dry ingredients. Mixing and solubilization of ingredients are typically done on hot plates, with magnetic stir bars placed in the bottom of the flask or beaker. Excessive heating should be avoided. Autoclaving or filtration sterilizes the media. Autoclaving of volumes of up to 500 ml at 121°C for 15 min is adequate. Larger volumes may require up to 20 to 30 min. The stir bars should be removed before sterilization. For quality control of autoclaving, specialized tape or paper is placed on the medium flask at the time of autoclaving. Enrichments such as blood and other labile additives such as filter-sterilized antibiotics should be added aseptically after the base medium has cooled.

Quality Control

The Clinical and Laboratory Standards Institute has specific requirements for quality assurance of commercially prepared media, which have been updated and documented in standard M22-A3 (3). However, these recommendations do not apply to all media. In addition, any medium that is prepared by the user requires its own specific quality control. Storage of media should be in the dark at 2 to 8°C. Storage in the dark is preferred because additives, such as dyes, will deteriorate faster in the light. This is especially true for chromogenic media that rely heavily on color differentiation between organisms in the same genus. The date that the medium was received in the laboratory and the medium expiration date should be marked and easily visible when stored. Media should be in use only up to the expiration date. Prolonged or incorrect storage of media, including transport media, can lead to desiccation of the medium and to changes in the composition of nutrients and selective agents, and it can compromise organism isolation.

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Manual and Automated Systems for Detection and Identification of Microorganisms*

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The last two decades have seen a trend away from the use of conventional, tube-based methods for the detection and identification of microorganisms and toward the use of instrument-based methods. Automation in microbiology first occurred in the early 1970s with the introduction of the first semiautomated blood culture instruments, followed by the early instrumented systems for identification and susceptibility testing of bacteria. In the 1980s, an instrumented screening device for the detection of bacteriuria was introduced. The trend toward automation has accelerated with the introduction and development of automated continuous-monitoring blood culture systems (CMBCSs) and more rapid systems for antimicrobial identification and susceptibility testing. Improvements to these instruments have included expansion of databases, implementation of combinations of technologies to decrease time to detection, and significant improvements in expert computer systems that provide laboratories with the ability to interpret, store, and manipulate data. Commercially available platforms have made improvements in their databases to incorporate or improve identification of category A and B potential agents of bioterrorism. Since the last edition of this manual, few publications have appeared evaluating these conventional methods, with the exception of the literature on a new automated identification and susceptibility testing instrument (Phoenix; BD Diagnostics, Sparks, Md.) (16, 50, 66). More has been published on susceptibility testing performance of some of these instruments (reviewed in chapter 17).

Progress has been made in the development of molecular platforms that provide real-time and simultaneous detection of multiple pathogens, most of which target viruses and agents of sexually transmitted diseases. However, it is unlikely that molecular technologies will completely replace the less expensive phenotypic methods in the short term. Regardless of whether a laboratory is using manual, automated, or molecular methods, the fundamental principles that provide the scientific bases for both detection and identification of microorganisms remain important.

This chapter reviews the systems used for the detection of microorganisms in clinical specimens, with primary emphasis on the underlying principles and systems for blood cultures, systems for microorganism identification, and criteria for assessing and selecting a system. For an expanded review and discussion of the blood culture issues, the reader is referred to more detailed reviews (5, 48, 63, 67). Discussions relevant to systems for antimicrobial susceptibility testing (chapter 17), immunoassays (chapter 18), molecular diagnostics (chapter 16), and rapid detection of mycobacteria (chapter 36) are found elsewhere in this Manual.

BLOOD CULTURE DETECTION SYSTEMS

Development and introduction of automated CMBCSs during the 1990s accelerated the trend away from conventional manual methods. However, the fundamental principles that provide the scientific basis for modern blood culture methods remain important, even with the new, automated technologies. The key variables will be reviewed briefly. For expanded review and discussion of these issues, the reader is referred to more detailed treatises (5, 55, 74).

Technical Variables That Affect Blood Cultures

Volume of Blood Cultured

The volume of blood obtained for culture is one of the most important variables in the detection of bloodstream infections (BSIs) (12, 56, 87). It has been well documented that BSIs in adults may be characterized by fewer than a single microorganism per 10 ml of blood. Studies have shown a direct relationship between the diagnostic yield of blood cultures and the volume of blood obtained for culture (12, 23, 30, 52, 69). Consensus guidelines recommend obtaining 20 to 30 ml per culture from adults (5). This cannot be accomplished with the use of a single blood culture set, and laboratories that accept single sets should discourage this practice (see discussion below). The importance of volume for detecting BSIs in infants and small children has become evident in recent years as well. Isaacman et al. (31) showed that the detection rate with 6 ml of blood was double that with 2 ml of blood from the same blood sample. Kellogg et al. (32) documented that low-level bacteremia occurs in children and recommended that

* This chapter contains information presented in chapter 14 by Caroline Mohr O'Hara, Melvin P. Weinstein, and J. Michael Miller in the eighth edition of this Manual.

4 to 4.5% of a child's blood volume be obtained for optimal detection of BSIs in this patient population. With tiny, premature infants, however, it may be impossible to obtain the volumes recommended by Kellogg et al. (32).

Culture Medium

No one medium or commercial product is capable of optimally detecting all microorganisms. Decisions by microbiologists as to medium formulations should be based on data from well-controlled field trials in which large numbers of cultures were assessed. The most widely used medium for blood cultures is soybean casein digest broth; brain heart infusion (BHI) broth may be equivalent or even superior for the recovery of yeasts and some bacteria (88).

Ratio of Blood to Broth

A number of substances in human blood are capable of inhibiting microbial growth, including leukocytes, complement, and lysozyme. Moreover, nearly one-third of patients from whom blood samples were obtained in a recent study (84) already were receiving antimicrobials at the time the blood samples were obtained. Dilution of blood in broth by a ratio of at least 1:5 has been shown to enhance detection (3, 58), probably by reducing the concentrations of the natural inhibitory substances and antimicrobial agents to subinhibitory levels. Some commercial media, notably those containing resins, may have blood-to-broth ratios of less than 1:5; however, these media have been shown to have sufficiently improved recovery rates such that the suboptimal blood-to-broth ratios are overcome.

Some manufacturers have marketed "pediatric" blood culture bottles with decreased volumes of broth medium designed to maintain a blood-to-broth ratio of 1:5 to 1:10 when only small volumes of blood can be obtained from young children. The broth media in these bottles are supplemented with X and V factors to enhance the yield of *Haemophilus influenzae* and have reduced concentrations of sodium polyanetholsulfonate (SPS) for improved detection of *Neisseria* species. Although these bottles have become popular, there are few objective data to indicate that they provide higher yields or detect microorganisms earlier than conventional blood culture bottles. Moreover, with the availability of the *H. influenzae* type b vaccine, *H. influenzae* bacteremia in children is now rare. Thus, whether the use of pediatric blood culture bottles is truly necessary remains an unanswered question for clinical microbiology laboratories.

Anticoagulants

The yield from blood cultures may be reduced if the blood clots. Therefore, all broth-based blood culture medium formulations contain anticoagulants, the most common being SPS in concentrations of 0.025 to 0.050%. In addition to inhibiting clotting, SPS inhibits lysozyme, inactivates aminoglycoside antibiotics, and inhibits parts of the complement cascade and phagocytosis. SPS has some negative attributes, albeit fewer than some other anticoagulants used in blood culture media over the years. SPS has been shown to inhibit the growth of *Neisseria gonorrhoeae, Neisseria meningitidis, Gardnerella vaginalis, Streptobacillus moniliformis, Peptostreptococcus anaerobius, Francisella tularensis,* and *Moraxella catarrhalis* (19, 54, 55, 65). In general, higher concentrations of SPS have enhanced the growth of gram-positive cocci but inhibited the growth of gram-negative bacteria. Although SPS has its limitations, no other anticoagulant has been shown to be superior.

Neutralization and Inactivation of Antimicrobials

Because many patients are already being treated with antimicrobials before blood samples are obtained (84), potentially reducing test sensitivity, some manufacturers market media designed to bind, absorb, or inactivate these agents. Some medium formulations include additives designed to bind or absorb antimicrobial agents, thereby enhancing the yield of microorganisms. The BACTEC blood culture system (BD Diagnostics) utilizes antibiotic-binding resins on tiny glass beads, whereas the BacT/Alert blood culture system (bioMerieux Inc., Durham, N.C.) uses activated charcoal. In both systems, culture media containing these additives have been shown to have improved abilities to detect microorganisms overall, especially staphylococci and yeasts, and improved yields for patients receiving theoretically effective antimicrobial therapy (41) compared to medium formulations without the additives (15, 63, 77, 88). More coagulase-negative staphylococcal contaminants may be detected in the media containing the resins and activated charcoal than in the media without these additives (77, 88).

Atmosphere of Incubation

Traditional blood cultures have consisted of two blood culture bottles, one designed to support the growth of aerobes and facultative anaerobic bacteria and the other designed to support obligate anaerobes as well as facultative microorganisms. Aerobic blood culture bottles usually contain an ambient atmosphere in the bottle headspace to which various amounts of carbon dioxide have been added to support the growth of certain microorganisms. Anaerobic blood culture bottles usually contain carbon dioxide and nitrogen but no oxygen in the bottle headspace. With the decrease in the proportion of bacteremias caused by obligate anaerobes in recent decades (5, 17, 38, 48), some investigators have concluded that the routine use of anaerobic blood culture bottles in a culture set is not necessary (46, 48, 61, 91). Rather, use of a second aerobic bottle is recommended to enhance the detection of the more common aerobic and facultative organisms and yeasts and to ensure that at least 20 ml of blood from adults will be cultured. An anaerobic bottle would be used only selectively for patients deemed at high risk for anaerobic bacteremia. However, a recent study using media with activated charcoal that compared two aerobic bottles with an aerobic and anaerobic pair of bottles found improved overall detection of microorganisms with the aerobic-anaerobic pair (57). A potential limitation of the study was the presence of few fungemias in the study population. Whether to use only aerobic bottles or to use a more traditional aerobic and anaerobic pair of bottles remains controversial (5, 74).

Bottle Agitation

Several studies have assessed the value of bottle agitation, documenting an enhanced yield and improved speed of detection of positive blood cultures from aerobic bottles (27, 53, 75). All of the commercially available CMBCSs agitate aerobic bottles, and most agitate anaerobic bottles as well.

Subcultures

The processing of conventional manual blood cultures includes Gram staining and blind subculturing of the aerobic culture bottles, usually after the first overnight incubation and, if the cultures remain negative, at the end of the

incubation period. Blind subcultures of the anaerobic culture bottles in manual systems and of all bottles in instrumented systems are unnecessary (5).

Length of Incubation

In routine situations, manual blood cultures need not be incubated for more than 7 days. Studies of the instrumented blood culture systems have shown that 5 days of incubation is sufficient for the detection of most pathogens (12, 20, 25, 39, 86). Some investigators have suggested that incubation periods of 4 days (14) and even 3 days (8, 24) may be sufficient for certain systems and media. However, the current standard remains a 5-day duration of incubation. Although it has been common to extend the incubation period when infective endocarditis is suspected, Washington (71) noted that this practice rarely increases the ability to detect the etiologic agent. A recent study from the Mayo Clinic, using a CMBCS, demonstrated that 99.5% of nonendocarditis BSIs and 100% of endocarditis episodes were detected within the standard 5-day incubation period (12). Similarly, in the best medium formulations of the modern CMBCSs, extended incubation periods appear not to be necessary for the detection of the most common *Candida* species. However, published data for *Candida glabrata* and *Cryptococcus neoformans* are lacking.

Clinical Practices That Affect Blood Cultures

Skin Antisepsis and Prevention of Contamination

The probability that a positive blood culture represents infection rather than contamination is a function of the effectiveness of skin antisepsis at the time of the venipuncture or, when blood is obtained from an indwelling device, a function of the effectiveness of antisepsis of that device. Growth of blood culture contaminants, especially coagulase-negative staphylococci, which are the most common etiologic agents of catheter-associated bacteremia as well as the most common blood culture contaminants, not only may be confusing to clinicians but also is associated with substantial expense (6). Thus, reducing contamination is a key issue for both the microbiology laboratory and the health care system in general. For many years, contamination rates (number of contaminated blood culture sets/total number of blood culture sets obtained) of $<3\%$ were considered the benchmark for good blood culture practices. A 1998 report from the College of American Pathologists of 640 institutions determined that the median contamination rate was 2.5% (60). In that study, the contamination rate for laboratories in the 10th percentile was 5.4% and that for laboratories in the 90th percentile was 0.9%.

The traditional recommendation for skin preparation has been the application of 70% alcohol followed by either povidone-iodine or 2% iodine tincture. Povidone-iodine preparations require 1.5 to 2 min of contact time for maximum antiseptic effect (74), whereas iodine tincture requires 0.5 min (34). Two studies have documented lower contamination rates with the use of iodine tincture rather than with an iodophor (37, 68). Recently, chlorhexidine has been recommended for use prior to venipuncture. One study demonstrated lower contamination rates with this preparation than with an iodophor (43). Another report compared chlorhexidine tincture with iodine tincture and found equivalent contamination rates (4).

Regardless of the type of skin preparation used, meticulous care and an aseptic technique are required to reduce contamination. Studies have demonstrated that a dedicated blood culture team and/or phlebotomists are less likely than other health care workers to contaminate blood cultures (73; R. B. Sivadas, B. Vazirani, S. Mirrett, and M. P. Weinstein, *Abstr. 101st Gen. Meet. Am. Soc. Microbiol.,* abstr. C10, 2001). Lastly, blood samples for culture obtained by peripheral venipuncture are less likely than those obtained from indwelling catheters to grow contaminating microorganisms (11, 90; Sivadas et al., *Abstr. 101st Gen. Meet. Am. Soc. Microbiol.*).

Number of Blood Samples Cultured

Studies with manual blood culture systems into which 20 ml of blood was inoculated for culture provided good evidence that culture of two or three blood samples will detect virtually all ($\geq$99%) BSIs in adults (70, 83). A recent study using the same volume of blood obtained from adults and inoculated into one of the CMBCSs reported a nonendocarditis BSI detection rate of 96% with culture of three blood samples (12).

Culture of a single blood sample should be discouraged if not forbidden altogether (2). A single sample will provide insufficient blood volume for detection of some infections. Moreover, the growth of a coagulase-negative staphylococcus, a viridans group streptococcus, or a diphtheroid in a single blood culture most often represents contamination but may represent a clinically important infection (84). Interpretation of the positive result under these circumstances is very difficult.

Timing of Blood Cultures

Few studies have systematically addressed the timing of collection of blood for culture. Although bacteremia is associated with rigors (7), this physiologic event usually precedes fever, and it is the latter that most often triggers the request for a blood culture. Some authorities have recommended that blood for culture be drawn at arbitrary intervals (67). However, in a retrospective study, Li et al. (35) showed no difference in yields whether blood samples obtained during a 24-h period were drawn simultaneously or at spaced intervals. The clinician and microbiologist should be guided by the patient's clinical status and suspected diagnosis. With a septic, unstable patient, blood samples should be cultured promptly so that therapy can be instituted. Conversely, if subacute infective endocarditis is suspected in an otherwise stable patient, several blood samples can be obtained at spaced intervals.

BLOOD CULTURE SYSTEMS

Manual Systems

Only three manual blood culture systems are currently marketed in the United States: Septi-Chek (BD Diagnostics), Signal (Remel Inc., Lenexa, Kans.), and Isolator (Wampole Laboratories, Cranbury, N.J.). The Septi-Chek system originally was developed as a labor-saving alternative to conventional blood cultures, which had to be subcultured manually. It consists of a conventional aerobic broth blood culture bottle to which is attached an agar-coated paddle in a clear plastic cylinder, creating a biphasic system similar to that of the classic Castaneda bottle. After blood is inoculated into the bottle, the paddle is attached and the blood-broth mixture is inverted to flood the agar, inoculating onto the agar any microorganisms that may be present. A companion anaerobic bottle that does not use the paddle

attachment, which is permeable to oxygen, can be used as well and processed manually. The bottles are incubated with or without agitation and inspected macroscopically for evidence of microbial growth once or twice daily. The agar paddle can be removed from its cylinder for better inspection. Following each examination of the agar paddle, the bottle is inverted, in effect repeating the subculture. There are several Septi-Chek broth medium formulations. The paddles contain three agars: chocolate, MacConkey, and malt. The Septi-Chek system performed well in published clinical trials (10, 51, 80–82).

The Signal is a one-bottle manual blood culture system that also was developed as a labor-saving alternative to conventional manual blood cultures. After blood is inoculated into the bottle in a conventional fashion, a clear plastic signal device is attached to the top of the bottle. An outer plastic sleeve that slides over the neck of the bottle anchors this signal device. Within the device is a long needle that extends beneath the level of the blood-broth mixture. If microbial growth occurs in the bottle, gases are produced in the bottle headspace. This creates increased atmospheric pressure, which forces some of the blood-broth mixture through the needle and into the clear plastic signal cylinder, where it can be detected visually by the microbiologist who inspects the bottles daily. Only one medium formulation has been marketed. In published controlled clinical trials done in the United States, the Signal system performed less well than its competitors (47, 75, 76, 78).

The Isolator blood culture system is unique as the only commercial system that does not utilize a broth culture medium. Rather, it is based on the principle of lysis-centrifugation. Blood is inoculated into an Isolator tube that contains a lysing solution consisting of saponin, the anticoagulant EDTA, and a fluorocarbon that acts as a cushion during the centrifugation step of blood processing. After the blood is lysed and centrifuged, the tube is placed into the Isostat system, which applies a disposable cap that penetrates the rubber stopper, permitting access to the contents of the tube. The disposable supernatant pipette is used to remove the supernatant, and the concentrate pipette is used to transfer the sediment from the tube directly to culture media that will support the growth of the pathogens of which detection is desired. The Isolator can be used for detection of routine bacterial pathogens; however, it has been reported to have a reduced ability to detect anaerobes, *Haemophilus* species, and pneumococci if specimens are not processed within 8 h (28, 29, 33, 72). The Isolator is an excellent system for detecting yeasts and dimorphic fungi, mycobacteria, and *Bartonella* species (9). The system is labor-intensive compared to the newer automated CMBCSs, especially during the initial processing of specimens in the laboratory.

Instrumented Systems

All of the commercially available CMBCSs have a number of characteristics in common. Some of the relevant information pertaining to these systems is shown in Table 1.

The CMBCSs have been adapted or modified so that they can be used to detect the growth of mycobacteria; additional information is provided in chapter 36 of this Manual. The CMBCSs have also been used, as have manual and earlier automated systems, to detect the growth of microorganisms from other normally sterile body fluids, for example, peritoneal fluid (22, 62).

The BacT/Alert system (bioMerieux Inc.) was the first CMBCS and was marketed in 1990; the system was updated in 1999 as the BacT/Alert 3D, which has a smaller instrument footprint than the original and a computer touch screen to ease technologist manipulations. Each incubator module has a capacity of 240 culture bottles. At the base of each bottle is a colorimetric CO_2 sensor that is separated from the blood-broth mixture by a CO_2-semipermeable membrane that monitors the amount of CO_2 in the bottle. At the base of each bottle's holding cell in the incubator unit are light-emitting and light-sensing diodes. With microbial

TABLE 1 Commercially available continuous-monitoring blood culture systems[a]

System (manufacturer)	Method for detecting growth	Capacity per module (no. of bottles)	Maximum no. of modules (no. of bottles) per system	Test cycle (min)	Agitation type/speed (no. of back-and-forth strokes/min)	Dimensions (cm)
BacT/Alert 240 (bioMerieux Inc.)	CO_2, colorimetric	240	6 (1,440)	10	Rocking/34	175 by 87 by 66
BacT/Alert 120	CO_2, colorimetric	120	6 (720)	10	Rocking/34	87 by 87 by 55
BacT/Alert 3D	CO_2, colorimetric	240	12 (2,880)	10	Rocking/34	90 by 49 by 61
BACTEC 9240 (BD Diagnostics)	CO_2, O_2,[b] fluorescence	240	5 (1,200)/20 (4,800)/ 50 (12,000)[c]	10	Rocking/30	93 by 128 by 55
BACTEC 9120	CO_2, O_2,[b] fluorescence	120	5 (600)/20 (2,400)/ 50 (6,000)[c]	10	Rocking/30	61 by 129 by 56
BACTEC 9050	CO_2, O_2,[b] fluorescence	50	1 (50)	10	Continuous rotation	61 by 72 by 65
VersaTREK (TREK Diagnostic Systems)	Manometric	96–240	6 (1,440)	12 (aerobic), 24 (anaerobic)	Vortexing, aerobic only	40 by 52 by 36
VersaTREK	Manometric	528	6 (3,168)	12 (aerobic), 24 (anaerobic)	Vortexing, aerobic only	76 by 52 by 31

[a] Adapted from Reimer et al. (55) and Wilson and Weinstein (87) and modified by Weinstein and Reller (79).
[b] O_2 detection is for Myco/F-Lytic medium only.
[c] Maximum number of bottles accommodated depends on data management system selected (core, Vision, or Epicenter).

growth and production of CO_2, the bottle's sensor changes color, altering the amount of light reflected. The change in reflectance is measured by the instrument, and the information is transmitted to the instrument's computer. The computer has several algorithms to report the detection of a positive culture that is noted when (i) the reflectance exceeds an arbitrary threshold, (ii) the instrument recognizes a linear increase in the CO_2 level, or (iii) there is a change in the rate of CO_2 production. Several medium formulations are available: (i) standard aerobic (SA) and anaerobic (SN) media that contain 40 ml of supplemented tryptic soy broth (TSB) and accept up to 10 ml of blood; (ii) aerobic FAN (FA) and anaerobic FAN (FN) media that contain 30 and 40 ml, respectively, of peptone-enriched TSB, supplemented with BHI solids, and activated charcoal designed to inactivate or bind antimicrobial agents and other inhibitory substances in the blood; and (iii) a FAN medium with a lower volume (20 ml) of peptone-enriched TSB, supplemented with BHI solids, and activated charcoal marketed for use with pediatric patients and those elderly patients from whom it is difficult to obtain larger volumes of blood. A detailed review of published comparative clinical trials is beyond the scope of this chapter, and more comprehensive reviews can be found elsewhere (57, 79, 89). Overall, the system is equivalent to the other commercially available CMBCSs with regard to the yield and speed of detection of microorganisms (79, 89). BacT/Alert media now are available in clear, shatter-resistant, plastic bottles that have performance characteristics equivalent to those of glass bottles.

The BACTEC 9000 (BD Diagnostics) CMBCS offers three instrument formats. The incubator module for the 9240 instrument holds 240 bottles, versus 120 bottles per module in the 9120 instrument. For small laboratories, the company markets the bench-top 9050 instrument that holds 50 bottles. Similar to the BacT/Alert system, the BACTEC system features a CO_2 sensor at the base of each culture bottle, but unlike BacT/Alert, the BACTEC instrument uses a fluorescence-sensing mechanism to detect the growth of microorganisms. When the amount of CO_2 increases, the concomitant increase in fluorescence is detected by the instrument; the principal detection criteria are a linear increase in fluorescence and an increase in the rate of fluorescence. The BACTEC system has multiple medium formulations: (i) standard aerobic and anaerobic media that contain 40 ml of soybean casein digest broth, (ii) aerobic and anaerobic Plus media that contain 25 ml of soybean casein digest broth plus antibiotic-binding resins on glass beads, (iii) an anaerobic lytic medium that contains 40 ml of soybean casein digest broth plus a lysing agent, (iv) a resin medium formulated for pediatric patients, and (v) a medium designated Myco/F-Lytic designed for improved detection of fungi and mycobacteria but which also supports the growth of bacterial pathogens. All BACTEC bottles accept up to 10 ml of blood, except the pediatric bottle, which accepts up to 5 ml. Published comparative clinical evaluations of the BACTEC 9000 system versus other CMBCSs have demonstrated that the system performs in a fashion relatively equivalent to those of its competitors in terms of both sensitivity and speed of detection of positive cultures (79).

The VersaTREK (TREK Diagnostic Systems) blood culture system uses the same technology as its commercial predecessor, the ESP system, and differs from BacT/Alert and BACTEC 9000 in several ways. In this system, bottles are placed into the instrument and monitored with a transducer for pressure changes within the bottle headspaces as gases (oxygen, hydrogen, nitrogen, and carbon dioxide) are either produced or consumed by metabolizing microorganisms. Aerobic (REDOX 1) bottles are monitored every 12 min, and anaerobic (REDOX 2) bottles are monitored every 24 min. Pressure is plotted against time to yield growth curves, and positive cultures are signaled according to the instrument's proprietary algorithms. Aerobic bottles are agitated by vortexing of the blood-broth mixture with a small, stainless-steel stir bar within each bottle, whereas agitation is accomplished by gentle rocking in the other two systems. Anaerobic bottles are not agitated, whereas anaerobic bottles in the other two systems are agitated in the same manner as their aerobic counterparts. In the VersaTREK system, the basal culture medium is supplemented soy casein-peptone broth in the aerobic bottle and modified proteose-peptone broth in the anaerobic bottle. As of this writing, there are no published comparative clinical evaluations of the VersaTREK system versus either the BacT/Alert or BACTEC 9000 system. The ESP blood culture system, which was marketed by the same manufacturer and which preceded VersaTREK, was shown to compare favorably with BacT/Alert and earlier BACTEC instrument systems in studies where the latter two systems utilized their standard medium formulations (45, 79, 92). However, fewer staphylococci and enteric gram-negative rods were detected by using ESP aerobic bottles than by using BacT/Alert aerobic FAN bottles (14, 45, 92).

Interpretation of Positive Blood Cultures

In most general hospitals, 8 to 14% of blood samples obtained will be positive by culture. Of the isolates in these positive blood cultures, one-half to two-thirds will be isolates that are the causes of bacteremia or fungemia and the remainder will be contaminants or isolates of unknown clinical significance. Thus, interpretation of the clinical significance of positive blood cultures is sometimes a vexing clinical problem. Misinterpretation of positive results can be expensive for both the patient and the institution (6). Several useful criteria may assist in interpretation. These include the identity of the microorganism itself, the presence of more than a single blood culture positive for the same microorganism, and growth of the same microorganism as that found in the blood from another normally sterile site.

Microorganisms that almost always represent true infection when isolated from blood include *Staphylococcus aureus*, *Escherichia coli*, and other members of the family *Enterobacteriaceae, Pseudomonas aeruginosa, Streptococcus pneumoniae,* and *Candida albicans* (84). Isolates from blood that rarely represent true bacteremia include *Corynebacterium* species, *Bacillus* species, and *Propionibacterium* species (84). Coagulase-negative staphylococci are perhaps the most problematic group with regard to interpreting clinical significance, in part because of their ubiquity and also because 12 to 15% of blood isolates are pathogens rather than contaminants (84). The number of positive culture bottles in a blood culture set is not a reliable criterion for decisions regarding the clinical significance of coagulase-negative staphylococci (44; S. J. Peacock, I. C. J. W. Bowler, and D. W. M. Crook, Letter, *Lancet* **346:**191–192, 1995).

A useful interpretive factor is the number of culture sets that are positive relative to the number of sets obtained. If most or all sets are positive for the same microorganism(s),

clinical significance is virtually assured (83). Although, ultimately, it is the physician who must make the final judgment, the microbiologist may provide important guidance regarding the clinical significance of blood isolates.

ORGANISM IDENTIFICATION SYSTEMS

Overview of Methods and Mechanisms of Identification

From the early years of diagnostic methods in microbiology until the 1960s, when advances in microbial identification began to emerge, skill in interpretive judgment and the use of tubed and plated media were the bases of microbial identification. Organisms were identified by what we now refer to as "conventional procedures," which include reactions in tubed media and observation of physical characteristics, such as colony morphology and odor, coupled with the results of Gram staining, agglutination tests, and antimicrobial susceptibility profiles. These conventional procedures eventually defined the genera and species of bacteria and yeasts and became the reference methods by which we confirm the identities of isolates.

The next step in the evolution of identification methods simply miniaturized commonly used biochemical reactions into a more convenient format (26). A system-dependent approach became the industry standard, and it remains the approach upon which most currently used substrate profile systems rely. In a system-dependent methodology, a set of substrates that will allow positive and negative reaction patterns to emerge is carefully selected. These patterns create a metabolic profile that can be compared with an established database profile. In many systems, it is necessary to use different sets of substrates to identify rapidly growing members of the family *Enterobacteriaceae,* slower-growing gram-negative non-*Enterobacteriaceae,* gram-positive cocci, gram-negative cocci, and anaerobes. Yeasts require yet another profile set.

Biochemical profiles are determined by the reactions of individual organisms with each of the substrates in the system. The accuracy of the reactions is dependent upon the users' following the directions of the manufacturer regarding inoculum preparation, inoculum density, incubation conditions, and test interpretation. Most systems rely upon one or a combination of several indicators. These include (i) pH changes resulting from utilization of the substrate, (ii) enzymatic reactions that allow the release of a chromogenic or fluorogenic compound, (iii) tetrazolium-based indicators of metabolic activity in the presence of a variety of carbon sources, (iv) detection of volatile or nonvolatile acids, and (v) recognition of visible growth (Table 2). Additional tests for microbial identification that use other means of detecting a positive response for a given substrate may also be included.

Although no formal definition of "rapid" exists for describing the time required for results to be generated, most microbiologists expect rapid systems to provide usable results within 2 to 4 h of incubation. Clearly, the generation times of microbes (usually 30 min or longer) will not allow growth-dependent methods to generate detectable biochemical responses within this time. To overcome the problem of generation times, manufacturers of rapid systems use novel substrates with which preformed enzymes, produced by the organisms to be tested, may react to elicit responses detectable within 2 to 4 h.

Most recently, molecular methods that amplify particular gene targets novel enough to distinguish among genera and species and automated sequencing technology have

TABLE 2 Basis of identification system reactivity

System reactivity	Need for growth	Analyte	Indicator of positive result	Examples of system
pH-based reactions (mostly 15–24 h)	Yes	Carbohydrate utilization	Color change due to pH indicator; carbohydrate utilization = acid pH; protein utilization or release of nitrogen-containing products = alkaline pH	API panels, Crystal panels, Vitek cards, MicroScan conventional panels, Phoenix panels, Sensititre panels
Enzyme profile (mostly 2–4 h)	No	Preformed enzymes	Color change due to chromogen or fluorogen release when colorless complex is hydrolyzed by an appropriate enzyme	MicroScan rapid panels, IDS panels, Crystal panels, Vitek cards, Phoenix panels, Sensititre panels
Carbon source utilization	Yes	Organic products	Color change as a result of metabolic activity transferring electrons to colorless tetrazolium-labeled carbon sources and converting the dye to purple	Biolog
Volatile or nonvolatile acid detection	Yes	Cellular fatty acids	Chromatographic tracing based on detection of end products, which are then compared to a library of known patterns	MIDI
Visual detection of growth	Yes	Various substrates	Turbidity due to growth of organism in the presence of a substrate	API 20C AUX panels

supplanted phenotypic methods for microbial identification for difficult-to-identify microorganisms. These methods have expanded our knowledge of pathogenesis and have expanded and resolved erroneous taxonomic classifications in some cases. Where appropriate throughout this text, more detailed descriptions of molecular methods for detection and identification of pathogens are provided in discussions of particular organism groups. The future will likely see more widespread implementation of these platforms as costs decrease and technologies improve.

System Construction

Microbial identification systems are either manual or automated. Manual methods offer the advantage of using the analytical skills of the technologists for reading and interpreting the tests, whereas automated systems offer a hands-off approach, allowing more technologist time for other duties. For all systems, the backbone of accuracy is the strength and utility of the database. Databases are constructed by using known, clinically relevant strains and include the type strains of most taxa. In some cases, before an organism is added to the database, it is evaluated to confirm its relationship to other strains in the same taxon by using the likelihood fraction. This compares the biochemical characteristics of the new strain to those of a typical culture of the same species.

The number of species included in a database may vary from just a few for some manual assays to over 1,900 for some of the automated systems, particularly if the system is to be used not only in clinical laboratory settings but also in environmental and research settings. For most commercial systems, database maintenance is a continuous process and software upgrades incorporating major taxonomic changes are provided by the manufacturer at intervals of up to every 4 years. Some systems may allow users to make minor changes at the local workstation.

System identifications are supported by algorithm-based decision making that is generally available through a computer. Occasionally, these identifications are compiled into a preprinted index, which is used to manually convert the organism's biochemical profile number into an identification. Bayes's theorem, or modifications of it, is often the basis of algorithm construction from data matrices.

Bayes's theorem is one of the statistical methods that manufacturers use to arrive at an identification of a certain taxon based on the reaction profile produced by the unknown clinical isolate (85). Bayes's theorem considers two important issues in order to arrive at an accurate conclusion: (i) $P(t_i/R)$ is the probability that an organism exhibiting test pattern R belongs to taxon t_i, and (ii) $P(R/t_i)$ is the probability that members of taxon t_i will exhibit test pattern R. Before testing, we make the assumption that an unknown isolate has an equal chance of being any taxon and that each test used to identify the isolate is independent of all other tests. In this case, Bayes's theorem can be written as

$$P\left(t_i / R\right) = \frac{P\left(R / t_i\right)}{\sum_i P\left(R / t_i\right)}$$

By observing reference identification charts derived by conventional biochemical tests, we know the expected pattern of the population of taxon t_i (e.g., *Escherichia coli* is indole positive and citrate negative). R in the formula is the test pattern composed of $R_1, R_2, \ldots R_n$, where R_1 is the result for test 1 and R_2 is the result for test 2, etc., for a given taxon. We can then incorporate the percentages (likelihoods that t_i will exhibit R_1, etc.) into Bayes's theorem to arrive at an accurate taxon.

Clinical microbiologists must not, however, become dependent upon these likelihoods and percentages when interpretive judgment would suggest an alternative taxonomic conclusion. Bacteria often tend to stretch the rules of nomenclature when isolated from clinical specimens, and they may not react as expected in a commercial system, even though a legitimate result is produced (e.g., lactose-positive *Salmonella* spp. or H_2S-positive *Escherichia coli*). The result from the most reliable system can be misleading. In these cases, an alternative method of identification must be used. D'Amato et al. (13) have described how the systems use the database profiles and probability matrices to arrive at an identification of an unknown taxon.

The manufacturers of commercial identification systems rely heavily on input from their customers. Laboratories are encouraged to communicate with the product manufacturer about problems such as unusual organism identifications that develop when a method or system is being used. Manufacturers depend on customer satisfaction, and most are willing to assist in problem solving or in projects that could add strength to their systems. These companies, like their users, are clearly interested in the highest quality of cost-effective patient care. Tables 3 to 8 provide a detailed summary of the available identification systems and compare the features offered by the automated and nonautomated organism identification methods.

CRITERIA FOR SELECTING INSTRUMENTED SYSTEMS

Whether selecting a blood culture system or a method for identification and susceptibility testing, the laboratorian must consider several important issues. Criteria for determining the need for an instrument-based system and its selection are provided in detail in the chapter on laboratory management (chapter 2). Supervisors and managers in the laboratory should make such major decisions carefully and with expert consultation. The process begins by answering key questions about the needs for a new system in the context of laboratory versus patient benefits.

Once these questions are answered, the next step is to begin the search for the right instrument or system to meet the needs of the laboratory and the medical staff. As a general rule, it is best not to be the first to purchase a new system without having seen in the peer-reviewed literature the results of evaluations performed by reputable clinical laboratories. If microbiology journals are unavailable, the manufacturer's representative can be asked to supply peer-reviewed articles about the ability of the system to correctly identify the range of isolates usually seen in your laboratory in the case of identification and susceptibility testing instruments or the results of well-designed, controlled comparative clinical evaluations with large numbers of observations (e.g., more than 5,000 comparisons and more than 500 positive cultures) in the case of blood culture systems. This phase requires demonstrations and conversations regarding space requirements, technical applications, manufacturer issues such as interface capabilities and service contracts, and personnel-related concerns such as sample preparation and throughput.

It is often helpful to visit other laboratories similar to one's own that are using the system under consideration to

TABLE 3 Summary of identification systems available in 2005

System	Manufacturer	Organisms targeted	Storage temp (°C)	No. of tests	Incubation	Automated
Systems for anaerobe identification						
AN Microplate	Biolog	Anaerobes	2–8	95	20–24 h	Yes
ANI Card	bioMerieux	Anaerobes	2–8	28	4 h; aerobic	Fill only[a]
API 20A	bioMerieux	Anaerobes	2–8	21	24–48 h; anaerobic	No
BBL Crystal Anaerobe	BD[b]	Anaerobes	2–8	29	4 h; aerobic	No
RapID ANA II	Remel	Anaerobes	2–8	18	4–6 h; aerobic	No
Rapid Anaerobe	Dade MicroScan	Anaerobes	2–8	24	4 h; aerobic	Yes
Rapid ID 32A	bioMerieux	Anaerobes	2–8	29	4 h; aerobic	No
Systems for *Enterobacteriaceae* and other gram-negative bacilli						
API 20E	bioMerieux	*Enterobacteriaceae* and nonfermenting gram-negative bacteria	2–8	21	18–24 h; 48 h; aerobic	No
API Rapid 20E	bioMerieux	*Enterobacteriaceae*	2–8	20	4 h	No
BBL Crystal E/NF	BD	*Enterobacteriaceae*, and some gram-negative nonfermenters	2–25	30	18–20 h	Reader only
Enterotube II	BD	*Enterobacteriaceae*	2–8	15	18–24 h	No
EPS (Enteric Pathogen Screen)	bioMerieux	*Edwardsiella, Salmonella, Shigella,* and *Yersinia* spp.	2–8	10	4–6 h	Yes
GN2 Microplate	Biolog	Aerobic gram-negative bacteria	2–8	95	4–24 h	Yes
Microbact 12A[c], 12E	Oxoid	*Enterobacteriaceae* and miscellaneous gram-negative bacilli	2–8	12	18–48 h	No
Microbact 12B[c]	Oxoid	Gram-negative bacilli not detected by Microbact 12A	2–8	12	24–48 h	No
Microbact 24E[c]	Oxoid	*Enterobacteriaceae* and other gram-negative bacilli	2–8	24	24–48 h	No
Micro-ID	Remel	*Enterobacteriaceae*	2–8	15	4 h	No
NEG ID Type 2	Dade MicroScan	*Enterobacteriaceae* and other fermenting and nonfermenting bacteria	2–25	32	16-18 h	Yes
PASCO Gram-NEG MIC/ID	BD	*Enterobacteriaceae* and other gram-negative bacilli	–70 – –20	27	16–20 h	Reader only
PASCO Tri-Panel	BD	Gram-negative and gram-positive bacteria	–70 – –20	27	16–20 h	Reader only
Phoenix NID[d]	BD	*Enterobacteriaceae* and other gram-negative bacilli	RT[e]	45	2–12 h	Yes
Rapid NEG ID Type 3	Dade MicroScan	*Enterobacteriaceae* and other fermenting and nonfermenting bacteria	2–8	36	2.5 h	Yes
RapID ONE	Remel	*Enterobacteriaceae* and other oxidase-negative bacteria	2–8	19	4 h	No
RapID SS/u	Remel	Common urinary tract pathogens and *Enterobacteriaceae*	2–8	12	2 h	No
r/b Enteric Differential System	Remel	*Enterobacteriaceae*	2–8	15	18–24 h	No
Sensititre GNID	TREK Diagnostic Systems, Inc.	*Enterobacteriaceae* and nonfermenting gram-negative bacteria	RT	32	5 h (Aris); 18–24 h off-line	Yes
UID-1/UID-3[f]	bioMerieux	Urinary tract pathogens directly from urine	2–8	9	1–13 h	Yes
ID-GNB Vitek 2	bioMerieux	Gram-negative fermenting and nonfermenting bacilli	2–8	41	3 h	Yes
Vitek GNI+	bioMerieux	*Enterobacteriaceae*, vibrios, and nonfermenting bacteria	2–8	28	2–12 h	Yes
Vitek 2 GN	bioMerieux	Fermenting and nonfermenting gram-negative bacilli	2–8	47	≤10 h (3–10 h)	Yes
Systems for identification of gram-negative non-*Enterobacteriaceae*						
API 20 NE	bioMerieux	Gram-negative non-*Enterobacteriaceae*	2–8	20	24–48 h; aerobic	No
Oxi-Ferm II	BD	Gram-negative, oxidase-positive glucose fermenters and nonfermenters	2–8	14	48 h	No
RapID NF Plus	Remel	Nonfermenting and selected fermenting gram-negative bacteria	2–8	17	4 h	No
Uni-N/F Tek plate[g]	Remel	Gram-negative nonfermenting bacteria	2–8	13	18–48 h	No

(Continued on next page)

TABLE 3 Summary of identification systems available in 2005 *(Continued)*

System	Manufacturer	Organisms targeted	Storage temp (°C)	No. of tests	Incubation	Automated
Systems for identification of fastidious gram-negative organisms						
API NH	bioMerieux	*Neisseria* and *Haemophilus* spp. and *Moraxella catarrhalis*	2–8	12	2 h; aerobic	No
BBL Crystal *Neisseria/ Haemophilus*	BD	*Neisseria*, *Haemophilus*, *Moraxella*, and *Gardnerella* spp. and other fastidious pathogens	2–8	29	4 h	No
HNID	Dade MicroScan	*Neisseria* and *Haemophilus* spp., *Moraxella catarrhalis*, and *Gardnerella vaginalis*	2–8	18	4 h	Yes
Neisseria Enzyme Test	Remel	Three *Neisseria* species and *Moraxella catarrhalis*	2–8	3	30 min	No
NHI	bioMerieux	*Neisseria* and *Haemophilus* spp. and other fastidious bacteria	2–8	15	4 h	No
RapID NH	Remel	*Neisseriaceae*, *Haemophilus* spp., and other gram-negative bacteria	2–8	13	4 h; 1 h for gonococci	No
Systems for identification of gram-positive cocci						
API 20 Strep	bioMerieux	Streptococci and enterococci	2–8	20	4–24 h; aerobic	No
API Staph	bioMerieux	Staphylococci and micrococci	2–8	20	18–24 h	No
BBL Crystal Gram-Positive	BD	Gram-positive cocci and bacilli	2–8	29	18–24 h	Reader only
BBL Crystal Rapid Gram-Positive	BD	Gram-positive cocci and bacilli	2–8	29	4 h	No
GP2 Microplate	Biolog	Aerobic gram-positive bacteria	2–8	95	4–24 h	Yes
GPI	bioMerieux	Gram-positive cocci and bacilli	2–8	29	2–15 h	Yes
ID-GPC Vitek 2	bioMerieux	Gram-positive cocci	2–8	46	2–6 h	Yes
Microbact Staph 12S	Oxoid	Staphylococci	2–8	12	24 h	No
PASCO Gram-Pos MIC/ID	BD	Gram-positive bacteria	–70 – –20	27	16–20 h	Reader only
Phoenix PID[d]	BD	Gram-positive bacteria	RT	45	2–16 h	Yes
Pos ID 2	Dade MicroScan	Gram-positive cocci and *Listeria* spp.	2–25	27	16–18 h	Yes
RAPIDEC STAPH	bioMerieux	Staphylococci	2–8	4	2 h	No
Rapid POS ID	Dade MicroScan	Gram-positive cocci and *Listeria* spp.	2–8	34	2.5 h	Yes
RapID STR	Remel	Streptococci and related organisms	2–8	14	4 h	No
Sensititre GPID	TREK Diagnostic Systems, Inc.	Gram-positive bacteria	RT	32	24 h	Yes
Vitek 2 GP	bioMerieux	Gram-positive cocci and bacilli	2–8	43	≤8 h(3–8 h)	Yes
Systems for identification of gram-positive bacilli						
API Coryne	bioMerieux	*Corynebacteria* and corynebacterium-like organisms	2–8	20	24 h; aerobic	No
Microbact Listeria 12L	Oxoid	*Listeria* spp.	2–8	12	4 h; 18–24 h	No
Micro-ID Listeria	Remel	*Listeria* spp.	2–8	15	24 h	No
RapID CB Plus	Remel	Coryneform bacilli	2–8	18	4 h	No
Systems for identification of yeasts and fungi						
API 20C AUX	bioMerieux	Yeasts	2–8	19	48–72 h	No
FF Microplate	Biolog	Filamentous fungi and selected yeasts	2–8	95	1–4 h; 7 days	Yes
ID-YST Vitek 2	bioMerieux	Yeasts	2–8	46	15 h	Yes
Rapid Yeast ID	Dade MicroScan	Yeasts	2–8	27	4 h	Yes
RapID Yeast Plus	Remel	Yeasts and yeast-like organisms	2–8	18	4 h	No
Uni-Yeast Tek[h]	Remel	Yeasts	2–8	11	24 h–7 days	No
YBC	bioMerieux	Yeasts	2–8	26	24–48 h[i]	Yes
YT Microplate	Biolog	Yeasts	2–8	94	24–72 h	Yes
Vitek 2 Yeast	bioMerieux	Yeasts and yeast-like organisms	2–8	46	18 h	Yes

[a] Cards are filled automatically but read visually.
[b] BD, BD Diagnostics.
[c] Microbact 12A and 12B are in a strip format; the 12E and 24E have microplate formats.
[d] Also available are combination identification and susceptibility panels: NMIC/ID and PMIC/ID for gram-negative and gram-positive organisms, respectively.
[e] RT, room temperature.
[f] For one sample and three samples, respectively.
[g] Part of the N/F system which also includes the N/F Screen 42P and the N/F GNF screen which are tubed media that are used for the identification of pigmented strains of *Pseudomonas aeruginosa* and the *Pseudomonas fluorescens-Pseudomonas putida* group.
[h] Part of the yeast system which also contains the C/N screen for *Cryptococcus neoformans*; GBE tube for presumptive identification of *Candida albicans* and *C. stellatoidea*, and the SAM tube for differentiation of *Candida albicans* and *Candida stellatoidea.*
[i] Off-line incubation required.

TABLE 4 Comparison of features of automated identification systems[a]

Feature	Values for automated identification systems							
	Vitek Legacy[b]	Vitek 2[b,c]	autoSCAN-4[d]	WalkAway SI[d]	Phoenix[e]	Sensititre Aris 2X[f]	Biolog[g]	MIDI[h]
Capacity of system	60/240/480	60/120	Unlimited	40/96	100	64	50	200+[i]
No. of species in database[j] (no. of substrates)								
Gram-negative organisms	116 (30)	130 (47)	142 (24)	142 (24),[k] 149 (44)[l]	167 (45)	128 (32)	524 (95)	482 (NA)[m]
Gram-positive organisms	52 (30)	54 (49)	49 (27)	49 (27),[k] (42)[l]	140 (45)	70 (32)[n]	351 (95)	451 (NA)
Anaerobes	85 (29)[o]	No	54 (24)	54 (24)	NA	No	361 (95)	732 (NA)
Fastidious organisms	9 (30)[o]	Expected release in 2006	21 (18)	20 (18)	Included in tests for gram-negative and gram-positive organisms	No	Included in tests for gram-negative and gram-positive organisms	50 (NA)
Environmental organisms	No	No	No	No	No	No	Included in tests for gram-negative and gram-positive organisms	Included in tests for gram-negative and gram-positive organisms
Yeasts	36 (30)[o]	54 (46)	44 (27)	44 (27)	No	No	267 (95)	196 (NA)
Mycobacteria	No	No	No	No	No	No	No	31 (NA)
Inoculation	Automated	Automated	Manual	Manual	Manual	Automated	Manual	Automated
Type of incubation, incubation time	On-line	On-line	Off-line	On-line	On-line	On-line	On-line	On-line
Gram-negative organisms	2–18 h	3 h	16–18 h	2.5 or 16–18 h	2–16 h	5 h	4–6 or 16–24 h	9–30 min
Gram-positive organisms	2–15 h	2–6 h	16–18 h	2.5 or 16–18 h	2–16 h	24 h	4–6 or 16–24 h	9–30 min
Anaerobes	4 h	NA	4 h	4 h	NA	NA	20–24 h	30 min
Fastidious organisms	4 h	NA	4 h	4 h	NA	NA	4–6 or 16–24 h	9–30 min
Environmental organisms	NA	NA	NA	NA	NA	NA	NA	9–30 min
Yeasts	24–48 h	15 h	4 h	4 h	NA	NA	24, 48, 72 h	30 min
Mycobacteria	NA	NA	NA	NA	NA	NA	NA	30 min
Manual reagent addition	No	No	Yes	No	No	No	No	No
Additional tests required before incubation	Yes	No	Yes	Yes	No	No	Yes	No
Storage temp	2–8°C[p]	2–8°C	RT[q]	RT, 4°C	RT	RT	4°C	RT
Other features								
Susceptibility testing	Yes	Yes	Yes	Yes	Yes	Yes	No	No
Urine screen or identification	Yes	No	No	No	No	No	No	No
DMS[r]	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

(Continued on next page)

TABLE 4 Comparison of features of automated identification systems[a] (*Continued*)

Feature	Values for automated identification systems							
	Vitek Legacy[b]	Vitek 2[b,c]	autoSCAN-4[d]	WalkAway SI[d]	Phoenix[e]	Sensititre Aris 2X[f]	Biolog[g]	MIDI[h]
Computer interface	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes

[a] Modified from O'Hara (50) and Stager and Davis (64) and updated in 2005.
[b] Manufacturer: bioMerieux Inc., 100 Rodolphe St., Durham, NC 27712. Phone: (800) 682-2666. http://www.bioMerieux.com.
[c] A compact model of this instrument (capacity 30/60) was released in 2005. It uses the same cards as Vitek 2.
[d] Manufacturer: Dade Behring, MicroScan, Inc., 1717 Deerfield Rd., Deerfield, IL 60015. Phone: (847) 267-5300. http://www.dadebehring.com.
[e] Manufacturer: BD Diagnostics, Inc., 1 Becton Drive, Franklin Lakes, NJ 07417. Phone: (201) 847-6800. http://www.bd-com/us/.
[f] Manufacturer: TREK Diagnostic Systems, Inc., 982 Keynote Circle, Suite 6, Cleveland, OH 44131. Phone: (800) 871-8909. http://www.trek.com.
[g] Manufacturer: Biolog, Inc., 21124 Cabot Blvd., Hayward, CA 94545. Phone: (510) 785-2564. E-mail: info@biolog.com.
[h] Manufacturer: MIDI, 125 Sandy Dr., Newark, DE 19713. Phone: (800) 276-8068.
[i] Based upon whether the rapid or standard method is used.
[j] Species in database indicates the groups, genera, or species identified.
[k] Conventional identification panel.
[l] Fluorogenic identification panel.
[m] NA, not applicable.
[n] U.S. clinical database (veterinary database not included).
[o] Although these panels have off-line incubation, they use the Vitek filling module for inoculation and the data management system for generation of identification.
[p] RT, room temperature.
[q] All rapid identification panels.
[r] DMS, data management system.

ask if they like the system, whether they would buy it again, how much downtime they have experienced, whether the service from the manufacturer has been acceptable, and whether the system has been mechanically reliable.

The laboratory should select a system that has been fully evaluated and whose accuracy exceeds 90% in its overall ability to identify common and uncommon bacteria normally seen in your hospital or laboratory. The system should be able to identify commonly isolated organisms with at least 95% accuracy compared with conventional methods.

The accuracy of antimicrobial susceptibility testing for combination panels is as important as the accuracy of identification, perhaps more so. Chapter 17 of this Manual discusses the issues involved in instrument susceptibility test methods.

EVALUATING AN INSTRUMENT OR SYSTEM

Several references provide useful information on the approach to evaluation, verification, and validation of kits, assays, and instruments in the clinical laboratory (18, 40, 42, 49, 64). Anytime an identification system is added to the laboratory, it is necessary to document that the system performs as described by the manufacturer. The first evidence of acceptable performance should be found in published reports by other laboratories that have evaluated the system in a sound, scientific manner (64). The next evidence of acceptable performance by a new identification instrument should be in-laboratory verification of performance by the purchasing laboratory. Verification is the documentation of test accuracy in the laboratory where the instrument will be used (40). The Clinical Laboratory Improvement Amendments of 1988 (21) specify the conditions for systems placed into service (see chapter 2).

Smaller laboratories will have fewer resources than larger laboratories for verification of the accuracy of an identification system. Laboratory size, however, has no bearing on the need to ensure the accuracies of laboratory identification methods and of the work performed by a laboratory in support of patient care. The role of verification by the purchasing laboratory should be to ensure that the personnel using the system can make it perform at the levels of accuracy already documented by the manufacturer and published in the literature. The laboratorian should expect a level of 95% agreement with the existing system or reference method and accept, in the final analysis, no less than 90% agreement. This takes into account the fact that the new system may be more accurate than the old one.

As of early 1998, the Food and Drug Administration (FDA) no longer does premarket [510(K)] evaluations to "clear" automated or manual phenotypic identification systems, nor does it receive or approve quality control protocols from these devices to meet the 1988 Clinical Laboratory Improvement Amendment requirements. Laboratorians must be aware that the identification component of the new or modified system that they are using is not cleared by the FDA because this approval is no longer required. This makes it even more important for laboratorians to search the literature for valid evaluations of their chosen instrument and to conduct their own in-house validation to make sure that the instrument meets the claims of the manufacturer regarding identification.

TABLE 5 Database entries of the *Enterobacteriaceae* (human isolates) manual systems

Organism	API 20E, version 4.0	BBL Crystal E/NF, version 4.0	RapID ONE, version 1.93	Microbact 24E[e]	MIDI, version 5.0
Budvicia aquatica				×	
Buttiauxella agrestis	×			×	
Cedecea davisae	×	×	×	×	
Cedecea lapagei	×	×	×	×	
Cedecea neteri		×	×	×	
Cedecea sp. 3			×	×	
Cedecea sp. 5			×	×	
Citrobacter amalonaticus	× (*Citrobacter koseri*)[a]	×	×	×	×
Citrobacter braakii	×			×	×
Citrobacter farmeri	× (*Citrobacter koseri*)			×	×
Citrobacter freundii	×	×	×	×	×
Citrobacter gillenii					
Citrobacter koseri	× (*Citrobacter amalonaticus*)	×	×	×	×
Citrobacter murliniae					
Citrobacter sedlakii				×	
Citrobacter werkmanii				×	
Citrobacter youngae	×			×	
Edwardsiella hoshinae	×	×	×	×	
Edwardsiella ictaluri				×	
Edwardsiella tarda	×	×	×	×	×
Enterobacter aerogenes	×	×	×	×	×
Enterobacter amnigenus group 1	×		×		
Enterobacter amnigenus group 2	×				
Enterobacter asburiae	×	×	×		
Enterobacter cancerogenus	×	× (*Enterobacter taylorae*)[c]	×		×
Enterobacter cloacae	×	×	×	×	×
Enterobacter gergoviae	×	×	×	×	×
Enterobacter hormaechei					
Enterobacter intermedius	×		×		×
Enterobacter sakazakii	×	×	×	×	×
Escherichia coli	×	×[d] (O111, O157)	×	×	×
Escherichia fergusonii	×	×	×		
Escherichia hermannii	×	×	×		×
Escherichia vulneris	×	×	×	×	×
Ewingella americana	×	×	×	×	
Hafnia alvei	×	×	×	×	×
Klebsiella oxytoca	×	×	×	×	×
Klebsiella planticola			×	×	
Klebsiella pneumoniae subsp. *ozaenae*	×	×	×	×	×
Klebsiella pneumoniae subsp. *pneumoniae*	×	×	×	×	×
Klebsiella pneumoniae subsp. *rhinoscleromatis*	×	×	×	×	×
Klebsiella terrigena	×			×	×
Kluyvera ascorbata	#[b]	×	×	×	×

(Continued on next page)

TABLE 5 Database entries of the *Enterobacteriaceae* (human isolates) manual systems *(Continued)*

Organism	API 20E, version 4.0	BBL Crystal E/NF, version 4.0	RapID ONE, version 1.93	Microbact 24E[e]	MIDI, version 5.0
Kluyvera cryocrescens	#[b]	×	×	×	×
Leclercia adecarboxylata	×	×	×	×	×
Leminorella grimontii			×	×	
Leminorella richardii			×	×	
Moellerella wisconsensis	×	×	×	×	
Morganella morganii	×	×	×	×	×
Pantoea agglomerans	#[b]	×	×		×
Pantoea dispersa	#[b]				
Proteus mirabilis	×	×		×	×
Proteus penneri	×	×	×	×	×
Proteus vulgaris	×	×	×	×	×
Providencia alcalifaciens	× (*Providencia rustigianii*)	×	×	×	×
Providencia heimbachae				×	×
Providencia rettgeri	×	×	×	×	×
Providencia rustigianii	× (*Providencia alcalifaciens*)	×	×	×	×
Providencia stuartii	×	×	×	×	×
Rahnella aquatilis	×	×	×	×	×
Raoultella ornithinolytica	× (*Klebsiella*)[c]	× (*Klebsiella*)[c]	× (*Klebsiella*)[c]	× (*Klebsiella*)[c]	
Salmonella	× (six groups)	× (four groups)	× (seven groups)	× (eight groups)	× (four groups)
Serratia ficaria	×	×		×	
Serratia fonticola	×	×		×	×
Serratia liquefaciens	×	×	×	×	×
Serratia marcescens	×	×	×	×	×
Serratia odorifera group 1	×	×	×	×	×
Serratia odorifera group 2	×	×	×	×	
Serratia plymuthica	×	×	×	×	×
Serratia rubidaea	×	×	×	×	×
Shigella sp.	× (*Shigella sonnei*)	× (two groups)	#[b] (*Shigella sonnei*)	× (two groups)	× (six groups)
Tatumella ptyseos		×	×	×	
Yersinia enterocolitica	×	× (group)[f]	×	×	×
Yersinia frederiksenii	× (*Yersinia intermedia*)		×	×	×
Yersinia intermedia	× (*Yersinia frederiksenii*)		×	×	×
Yersinia kristensenii	×		×	×	×
Yersinia pestis	×			×	×
Yersinia pseudotuberculosis	×	×	×	×	×
Yersinia ruckeri				×	
Yokenella regensburgei		×	×	×	

[a] Some products give a choice between two species; the alternate species is indicated in parentheses.
[b] #, genus-level identification only.
[c] Previous taxonomic designation.
[d] Able to differentiate O111 from O157.
[e] Also contains 10 unnamed enteric groups in the database.
[f] "Group" indicates *Yersinia* group.

TABLE 6 Database entries of the *Enterobacteriaceae* (human isolates) for automated systems[a]

Organism	Vitek GNI+, version 10.01	Vitek 2 GN, version 4.01	Dade Behring MicroScan		MicroLog, version 6.01/6.02	Sensititre, version 2.6	Phoenix
			Conventional LabPro, version 1.6	Rapid LabPro, version 1.6			
Budvicia aquatica	×				×		
Buttiauxella agrestis		×			×		
Cedecea davisae	×	×	×	×	×	×	×
Cedecea lapagei	×	×	×	×	×	×	×
Cedecea neteri			×	×	×	×	×
Cedecea sp. 3			×	"3/5"			
Cedecea sp. 5			×	"3/5"			
Citrobacter amalonaticus	×	×	×	×	×	×	×
Citrobacter braakii	×	×		× (*Citrobacter braaki/freundii/sedlakii*)	×		×
Citrobacter farmeri		×		×	×		×
Citrobacter freundii	×	×	×	× (*Citrobacter braaki/freundii/sedlakii*)	×	×	×
Citrobacter gillenii					×		
Citrobacter koseri	×	×	×	×	×	×	×
Citrobacter murliniae					×		
Citrobacter sedlakii		×		× (*Citrobacter braaki/freundii/sedlakii*)	×		×
Citrobacter werkmanii				× (*Citrobacter werkmanii/C. youngae*)	×		×
Citrobacter youngae		×		× (*Citrobacter werkmanii/C. youngae*)	×		×
Edwardsiella hoshinae	×	×		×	×	×	×
Edwardsiella ictaluri					×	×	×
Edwardsiella tarda	×	×	×	×	×	×	×
Enterobacter aerogenes	×	×	×	×	×	×	×
Enterobacter amnigenus group 1	×	×	×	×	×	×	×
Enterobacter amnigenus group 2	×	×	×	×			×
Enterobacter asburiae		×	×	×	×	×	×
Enterobacter cancerogenus	×	×	×	×	×	×	×
Enterobacter cloacae	×	×	×	×	×	×	×
Enterobacter gergoviae	×	×	×	×	×	×	×
Enterobacter hormaechei	×			×	×		×
Enterobacter intermedius	×	×	×	×	×	×	×
Enterobacter sakazakii	×	×	×	×	×	×	×
Escherichia coli	×	× (O157)[b]	×	× (O157:H7)[b]	×	×	×
Escherichia fergusonii	×	×	×	×	×		×
Escherichia hermannii	×	×	×	×	×	×	×
Escherichia vulneris	×	×	×	×	×	×	×
Ewingella americana	×	×	×	×	×	×	×
Hafnia alvei	×	×	×	×	×	×	×

(Continued on next page)

TABLE 6 Database entries of the *Enterobacteriaceae* (human isolates) for automated systems[a] *(Continued)*

Organism	Vitek GNI+, version 10.01	Vitek 2 GN, version 4.01	Dade Behring MicroScan		MicroLog, version 6.01/6.02	Sensititre, version 2.6	Phoenix
			Conventional LabPro, version 1.6	Rapid LabPro, version 1.6			
Klebsiella oxytoca	× (*Klebsiella pneumoniae*)	×	×	×	×	×	×
Klebsiella pneumoniae subsp. *ozaenae*	×	×	×	×	×	×	×
Klebsiella pneumoniae subsp. *pneumoniae*		×	×	×	×	×	×
Klebsiella pneumoniae subsp. *rhinoscleromatis*	×	×	×	×	×	×	×
Kluyvera ascorbata	#[c]	×	×	#	×	×	×
Kluyvera cryocrescens	#	×	×	#	×	×	×
Leclercia adecarboxylata	×	×	×	×	×	×	×
Leminorella grimontii			#	#	×	#	×
Leminorella richardii			#	#	×	#	×
Moellerella wisconsensis	×	×	×	×	×	×	×
Morganella morganii		×	×	×	×	×	×
Pantoea agglomerans	×	×	× (*Enterobacter agglomerans*)	× (*Enterobacter agglomerans*)	×	×	×
Pantoea dispersa					×		
Pragia fontium					×		×
Proteus mirabilis	×	×	×	×	×	×	×
Proteus penneri	× (*Proteus vulgaris*)	× (*Proteus vulgaris*)	×	×	× (*Proteus vulgaris*)	×	×
Proteus vulgaris	× (*Proteus penneri*)	× (*Proteus penneri*)	×	×	× (*Proteus penneri*)	×	×
Providencia alcalifaciens	×	×	×	×	×	×	×
Providencia heimbachae					×		
Providencia rettgeri	×	×	×	×	×	×	×
Providencia rustigianii		×	×	×	×		×
Providencia stuartii	×	×	×	×	×	×	×
Rahnella aquatilis	×	×			×		×
Raoultella ornithinolytica	×	×	×	×	× (*Raoultella planticola*)		×
Raoultella planticola					× (*Raoultella ornithinolytica*)	×	
Raoultella terrigena					×	×	
Salmonella	#[a] *and Salmonella enterica* serovar Typhi	× (five groups)	× (four groups)	× (four groups)	× (twelve groups)	× (thirteen groups)	× (seven groups)
Serratia ficaria		×		×	×		×
Serratia fonticola	×	×	×	×	×	×	×
Serratia liquefaciens	×	×	×	×	× (*Serratia grimesii*)	×	×
Serratia marcescens	×	×	×	×	×	×	×
Serratia odorifera group 1	×	×	×	×	×	×	×
Serratia odorifera group 2			×	×			×

Serratia plymuthica	×	×	×		×		×		×		×	
Serratia rubidaea	×	×	×		×		×		×		×	
Shigella sp.	× (four groups)	× (two groups)	× (*Shigella sonnei*), #		× (*Shigella sonnei*) #		× (four groups)		× (four groups)		× (four groups)	
Tatumella ptyseos			×		×		×		×		×	
Trabulsiella guamensis							×					
Yersinia enterocolitica	×	×	× (group)[d]		× (group)		×		×		×	
Yersinia frederiksenii	×		× (group)		× (group)		×		×		×	
Yersinia intermedia	×		× (group)		× (group)		×		×		×	
Yersinia kristensenii	×		× (group)		× (group)		×		×		×	
Yersinia pestis	×	×	×		×		×		×			
Yersinia pseudotuberculosis	×	×	×		×		×		×		×	
Yersinia ruckeri		×	×		×		×		×		×	
Yokenella regensburgei	×	×	×		×		×		×		×	

[a] Some products give a choice between two or more species; the alternate species is in parentheses.
[b] Includes the ability to differentiate between serogroups O111 and O157.
[c] #, genus designation only.
[d] "Group" indicates *Yersinia* group.

Devices and methods incorporating probes, nucleic acid amplification, and other genetic methods, as well as the susceptibility test component of commercial instruments, will continue to be reviewed by the FDA for clearance.

LIMITATIONS OF MICROORGANISM IDENTIFICATION SYSTEMS

The databases of microbial identification systems must be revised frequently to accommodate newly named species. For example, had *Enterobacter sakazakii* (the yellow-pigmented variant of *Enterobacter cloacae*) not been added to the databases of these instruments, the clinical correlation of *E. sakazakii* with neonatal meningitis would likely be obscured if only *E. cloacae* was reported. Laboratorians must be aware that the accuracy of a system is limited to the claims of the manufacturer for the version of the database currently in the instrument and that the database may be outdated.

The laboratory procedure manual must stipulate the action to be taken when a result is questionable either because of the unusual biochemical profile of the organism or because of the appearance of an unexpected susceptibility profile. A backup method must be used to achieve an accurate identification profile. Otherwise the isolate should be sent to a reference laboratory for analysis.

The biochemical properties of closely related species may make it difficult or impossible for the algorithms of the identification process to separate these organisms accurately; however, the inability to distinguish all species within a genus does not always have a negative effect on patient outcome. For example, accurate identification of all of the newly recognized *Citrobacter* species may not be possible for some of the systems. In this case, the effect on patient outcome because of the inability of a system to recognize *Citrobacter werkmanii* may be negligible, and a simple report of "*Citrobacter* species" may provide adequate data for patient management. Where such distinctions may be critical is in the recognition of a potential agent of bioterrorism. This has implications not only for the individual patient, but also for public health. Although some commercial systems have these organisms in their databases (Table 9), the inability to identify members of some of the genera to the species level (e.g., *Yersinia* spp.) makes these systems unreliable for identification (36). Users of these systems should be aware of the limitations of commercial products with respect to their biopreparedness plans and substitute other tests for presumptive diagnosis per recommended guidelines (1). Suspicious pathogens should be referred to a public health or other reference laboratory for definitive identification. Table 9 summarizes the agents of bioterrorism included in the databases of the more frequently used identification systems.

As pathogens continue to evolve and taxonomic classifications are revised, laboratorians must pay attention to the manufacturer's communications about products, such as letters, notices, or test exclusions regarding the accuracy of their methods, as well as the published literature describing the potential problems encountered by others using these identification systems. Likewise, the user has a responsibility to report continued problems with a system or product where poor performance may lead to adverse patient outcomes.

TABLE 7 Database entries of the gram-positive organisms (human isolates) for bioMerieux and Dade MicroScan products[b]

Organism	API		VITEK GPI, version 10.01	VITEK 2 GP, version 4.01	MicroScan	
	Staph, version 4.0	20 Strep, version 6.0			Conventional Pos ID, LabPro version 1.6	Rapid Pos ID, LabPro version 1.6
Listeria monocytogenes		×	#[a]	×	×	×
Micrococcaceae						
Kocuria (*Micrococcus*) *kristinae*	×			×	×	×
Micrococcus luteus	*Micrococcus* species			× (*Micrococcus lylae*)	*Micrococcus* species	*Micrococcus* species
Micrococcus lylae				× (*Micrococcus luteus*)	*Micrococcus* species	*Micrococcus* species
Kocuria (*Micrococcus*) *rosea*	× (*Micrococcus varians*)			×	*Micrococcus* species	×
Kocuria varians	× (*Micrococcus rosea*)			×	*Micrococcus* species	*Micrococcus* species
Micrococcus sedentarius					*Micrococcus* species	*Micrococcus* species
Rothia dentocariosa						
Rothia mucilaginosa				×		
Staphylococcus arlettae				×		×
Staphylococcus aureus	×		×	×	×	×
Staphylococcus auricularis	×		×	×	×	×
Staphylococcus capitis subsp. *capitis*	×		×	×	×	×
Staphylococcus capitis subsp. *ureolyticus*					×	
Staphylococcus caprae	×			×		×
Staphylococcus carnosus	×			×		×
Staphylococcus caseolyticus						
Staphylococcus chromogenes	×					× (*Staphylococcus hyicus*)
Staphylococcus cohnii subsp. *cohnii*	×		×	×	×	×
Staphylococcus cohnii subsp. *urealyticum*	×			×	×	
Staphylococcus epidermidis	×		×	×	×	×
Staphylococcus equorum				×		×
Staphylococcus felis						
Staphylococcus gallinarum				×		×
Staphylococcus haemolyticus	×		×	×	×	×
Staphylococcus hominis subsp. *hominis*	×		×	×	×	×
Staphylococcus hominis subsp. *novobiosepticus*					×	
Staphylococcus hyicus	×		×	×	×	× (*Staphylococcus chromogenes*)
Staphylococcus intermedius			×	×	×	×
Staphylococcus kloosii				×		×
Staphylococcus lentus	×		×	×		×
Staphylococcus lugdunensis	×			×	×	×
Staphylococcus pasteuri						
Staphylococcus saccharolyticus						

Staphylococcus saprophyticus	×		×	×	×	×
Staphylococcus schleiferi	×			×	×	×
Staphylococcus sciuri	×		×	×	×	×
Staphylococcus simulans	×		×	×	×	×
Staphylococcus vitulinus				×		
Staphylococcus warneri	×		×	×	×	×
Staphylococcus xylosus	×		×	×	×	×
Streptococcaceae						
Abiotrophia adiacens		×				
Abiotrophis defectiva		×	×	×		
Aerococcus urinae			×	×		
Aerococcus viridans		×	×	×	×	×
Alloiococcus otitidis			×	×		
Dermacoccus nishinomiyaensis			×	×		
Enterococcus avium		×	×	×	×	×
Enterococcus casseliflavus			×	×	×	×
Enterococcus durans		×	×	×	× (*Enterococcus hirae*)	×
Enterococcus faecalis		×	×	×	×	×
Enterococcus faecium		×	×	×	×	×
Enterococcus gallinarum		×	×	×	×	
Enterococcus hirae				×	× (*Enterococcus durans*)	
Enterococcus malodoratus						
Enterococcus raffinosus					×	
Facklamia hominis						
Gemella haemolysans		×		×		
Gemella morbillorum		×	×	×		×
Globicatella sanguinis				×		
Granulicatella sp.						
Helcoccus kunzii				×		
Lactococcus species		×		×		
Leuconostoc species		×		×	×	
Pediococcus species				×	×	
Streptococcus acidominimus		×	×			
Streptococcus agalactiae		×	×	×	×	×
Streptococcus anginosus			×	×		
Streptococcus β-hemolytic non-A, non-B					×	
Streptococcus bovis		×	×		×	×
Streptococcus constellatus			×	×		
Streptococcus criceti						
Streptococcus cremoris/thermophilus						
Streptococcus cristatus			×			
Streptococcus dysgalactiae/subsp. *dysgalactiae*/subsp. *equisimilis equisimilis*		×	×	×		

(Continued on next page)

TABLE 7 Database entries of the gram-positive organisms (human isolates) for bioMerieux and Dade MicroScan products[b] *(Continued)*

Organism	API		VITEK GPI, version 10.01	VITEK 2 GP, version 4.01	MicroScan	
	Staph, version 4.0	20 Strep, version 6.0			Conventional Pos ID, LabPro version 1.6	Rapid Pos ID, LabPro version 1.6
Streptococcus equi subsp. *equi*		×	×	×		× (*Streptococcus equisimilis*)
Streptococcus equi subsp. *zooepidemicus*						×
Streptococcus equinus		×	×	×		×
Streptococcus gordonii			× (*Streptococcus sanguis*)			
Streptococcus groups E, G, L, P, and U		×	× Group G			
Streptococcus intermedius		×	×	×		
Streptococcus lactis/diacetylacis						
Streptococcus milleri group			×		×	×
Streptococcus mitis group		×	×	×	×	×
Streptococcus mutans		×	×	×	×	×
Streptococcus oralis		×	×	× (*Streptococcus mitis*)		
Streptococcus parasanguis				×		
Streptococcus pneumoniae		×	×	×	×	×
Streptococcus porcinus		×		×		
Streptococcus pyogenes		×	×	×	×	×
Streptococcus salivarius		×	×	×	×	×
Streptococcus sanguinis			×	×		
Streptococcus sanguis		×	×		×	×
Streptococcus sobrinus				×		
Streptococcus uberis		×	×	×		
Streptococcus vestibularis				×		
Vagococcus fluvialis				×		
Weissella confusus						

[a] #, genus identification only.
[b] Some products give a choice between two species; the alternate species is in parentheses.

TABLE 8 Database entries of the gram-positive organisms for BD Diagnostics, Remel, TREK, Biolog, and MIDI products[c]

Organism	BBL Crystal		Remel RapID STR	Oxoid Microbact Staph 12S	TREK Sensititre GPID	Biolog GP2 version 6.11/6.12	MIDI version 5.0	BD Diagnostics Phoenix-100 GPID PMIC/II-100
	Gram-Pos ID	Rapid Gram-Pos ID						
Listeria species	× (four species)	× (three species)	× (*Listeria monocytogenes*)	*[a]	× (four species)	× (seven species)	× (five species)	× (five species)
Micrococcaceae								
Kocuria (*Micrococcus*) *kristinae*	#[b]	× (*Micrococcus*)			×	×	×	×
Kocuria (*Micrococcus*) *rosea*	#	× (*Micrococcus*)			×	×	×	×
Kocuria varians						×	×	×
Kytococcus sedentarius	#				×	×		×
Macrococcus (*Staphylococcus*) *caseolyticus*						×	×	×
Micrococcus luteus	#	×			×	×	×	×
Micrococcus lylae	#				×	×	×	×
Rothia dentocariosa	×					×	×	
Rothia mucilaginosus	× (*Stomatococcus*)	× (*Stomatococcus*)				×	×	×
Staphylococcus arlettae						×	×	
Staphylococcus aureus	×	×		×	×	×	×	×
Staphylococcus auricularis	×			×		×	×	×
Staphylococcus capitis subsp. *capitis*	×			×	×	×	×	×
Staphylococcus capitis subsp. *ureolyticus*	×			×				×
Staphylococcus caprae	×			×		×		×
Staphylococcus carnosus	×			×		×	×	×
Staphylococcus chromogenes				×		×	×	×
Staphylococcus cohnii subsp. *cohnii*	×			×		×	×	×
Staphylococcus cohnii subsp. *urealyticum*	×			×				×
Staphylococcus epidermidis	×	×		×	×	×	×	×
Staphylococcus equorum	×					×	×	×
Staphylococcus felis	×					×		×
Staphylococcus gallinarum	×	×				×	×	×
Staphylococcus haemolyticus	×	×		×	×	×	×	×
Staphylococcus hominis subsp. *hominis*	×	×		×	×	×	×	×
Staphylococcus hominis subsp. *novobiosepticus*						×		×
Staphylococcus hyicus				×	×	×	×	×
Staphylococcus intermedius	×	×		×	×	×	×	×
Staphylococcus kloosii	×					×	×	×

(Continued on next page)

TABLE 8 Database entries of the gram-positive organisms for BD Dianostics, Remel, TREK, Biolog, and MIDI products[c] *(Continued)*

Organism	BBL Crystal		Remel	Oxoid Microbact	TREK	Biolog CP2	MIDI	BD Diagnostics
	Gram-Pos ID	Rapid Gram-Pos ID	RapID STR	Staph 12S	Sensititre GPID	version 6.11/6.12	version 5.0	Phoenix-100 GPID PMIC/II-100
Staphylococcus lentus	×	×		×		×	×	×
Staphylococcus lugdunensis	×	×		×		×	×	×
Staphylococcus pasteuri	×					×		×
Staphylococcus saccharolyticus	×	×						×
Staphylococcus saprophyticus	×	×		×	×	×	×	×
Staphylococcus schleiferi	× (two subspecies)					×	×	×(two subspecies)
Staphylococcus sciuri	×	×		×		×	×	×
Staphylococcus simulans	×	×		×		×	×	×
Staphylococcus vitulinus	×							×
Staphylococcus warneri	×	×		×	×	×	×	×
Staphylococcus xylosus	×	×		×	×	×	×	×
Streptococcaceae								
Abiotrophia species								
Aerococcus urinae	×					×		×
Aerococcus viridans	×	×	#		×	×	×	×
Alloiococcus otitidis	×					×		×
Dermacoccus nishinomiyaensis						×		×
Enterococcus avium	×	×	×		×	×	×	×
Enterococcus casseliflavus	× (*Enterococcus gallinarum*)	× (*Enterococcus gallinarum*)	× (*Enterococcus mundtii*)			×	×	×
Enterococcus cecorum						×	×	
Enterococcus columbae						×	×	
Enterococcus durans	×	×	× (*Enterococcus hirae*)		×	×	×	×
Enterococcus faecalis	×	×	×		×	×	×	×
Enterococcus faecium	×	×	×		×	×	×	×
Enterococcus gallinarum	× (*Enterococcus casseliflavus*)	× (*Enterococcus casseliflavus*)	×			×	×	×
Enterococcus hirae	×		× (*Enterococcus durans*)			×	×	×
Enterococcus malodoratus			×			×	×	
Enterococcus mundtii						×	×	
Enterococcus raffinosus	×	×	×			×		×
Enterococcus solitarius	×					×		
Gemella haemolysans	×	×			×	× (*Gemella morbillorum*)	×	×
Gemella morbillorum	×	×	×			× (*Gemella haemolysans*)		×
Globicatella sanguinis	×					×		×

Helcoccus kunzii	×					×		×
Lactococcus species	× (five species)	× (four species)				× (seven species)		× (six species)
Leuconostoc species	× (four species)	× (three species)	× (three species)			× (eight species)		× (five species)
Pediococcus species	× (three species)	× (three species)	× (two species)			× (five species)	×(one species)	× (five species)
Streptococcus acidominimus	×	×	×			×		×
Streptococcus agalactiae	×	×	×		×	×	×	×
Streptococcus anginosus	×	×	×		×	×	×	×
Streptococcus bovis	× (bovis I and II)	× (bovis I and II)	×		×	×	×	
Streptococcus constellatus	×	×	×		×	×		×
Streptococcus cricetus	×	×				×		
Streptococcus cristatus	×					×		×
Streptococcus crista	× (included in *Streptococcus sanguis* group)	×						×
Streptococcus dysgalactiae/ subsp. *dysgalactiae*/ subsp. *equisimilis*					×	×		×
Streptococcus equi subsp. *equi*/ subsp. *zooepidemicus*	×	×	Group C streptococcus		×	×	×	×
Streptococcus equinus	×	×	×			×	×	×
Streptococcus gordonii	×	×	× (*Streptococcus sanguis*)			×	×	×
Streptococcus intermedius	×	×	×			×	×	×
Streptococcus milleri group	×	×			×			
Streptococcus mitis	×	×	×		×	×	×	×
Streptococcus mutans	×	×	×		×	× (*Streptococcus ratti*)	×	×
Streptococcus oralis	×	×				×	×	×
Streptococcus parasanguis	×					×	×	×
Streptococcus pneumoniae	×	×			×	×	×	×
Streptococcus porcinus	×					×	×	×
Streptococcus pyogenes	×	×	×		×	×	×	×
Streptococcus salivarius	×	×	× (*Streptococcus vestibularis*)		×	×	×	×
Streptococcus sanguinis						×		×
Streptococcus sanguis	×	×	× (*Streptococcus gordonii*)		×		×	
Streptococcus sobrinus	×	×				×	×	×
Streptococcus uberis	×	×			×	×	×	×
Streptococcus vestibularis	×	×	× (*Streptococcus salivarius*)			×	×	×
Weisella confusus			×					

[a] Oxoid has a similar product for *Listeria* identification called the Listeria Identification System 12 S. It identifies six species.
[b] #, Genus-level identification only.
[c] Some products give a choice between two species; the alternate species is indicated in parentheses.

TABLE 9 Database entries for highly pathogenic organisms for select assays or systems

System	Category A agents			Category B agents						
	Bacillus anthracis	*Yersinia pestis*	*Francisella tularensis*	*Brucella* sp.	*Salmonella*	*Shigella*	Shiga toxin-producing *Escherichia coli*	*Burkholderia mallei*	*Burkholderia pseudomallei*	*Vibrio cholerae*
API 20 E and NE		×			×	×				×
BBL Crystal E/NF					×	×	× (O157, O111)[c]		×	×
GNI+ (Vitek)		×			×	×		×	×	×
GN (Vitek 2)		×	×	×	×	×	× (O157)[c]	×	×	×
MicroLog[a]	×	×	×	×	×	×	× (O157)[c]	×	×	×
Microbact 24E		×			×	×			×	×
MicroScan Conventional		×			×	×				×
MicroScan Rapid		×			×	×				×
MIDI (BTR2.0)[a,b]	×		×	×	×	×		×	×	×
Phoenix GNID		×			×	×				×
Sensititre		×			×	×				×

[a] This commercial system has a dangerous-pathogens database.
[b] The MIDI system has been awarded "official methods status" for confirmatory identification of *Bacillus anthracis* (59).
[c] Systems are able to presumptively identify these serotypes.

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Molecular Detection and Identification of Microorganisms

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Since the publication of the eighth edition of this Manual, significant changes have occurred in the practice of diagnostic molecular microbiology. Nucleic acid amplification techniques are now commonly used to diagnose infectious diseases and manage patients with infectious diseases. The growth in the number of commercially available test kits and analyte-specific reagents (ASRs) has facilitated the use of this technology in the clinical laboratory. Technological advances in real-time PCR techniques, nucleic acid sequencing, DNA microarrays, and proteomics have invigorated the field and created new opportunities for growth.

Molecular microbiology is the leading area in molecular pathology in terms of both the numbers of tests performed and clinical relevance. This technology has reduced the dependency of the clinical microbiology laboratory on culture-based methods and created new opportunities for the clinical laboratory to affect patient care. This chapter covers amplified and nonamplified probe techniques, postamplification detection and analysis, clinical applications of these techniques, and the special challenges and opportunities that these techniques provide for the clinical laboratory. Molecular methods used in epidemiological investigations are covered in chapter 11. A more comprehensive discussion of many of the topics covered in this chapter is found in Persing et al., *Molecular Microbiology: Diagnostic Principles and Practice* (96).

NONAMPLIFIED NUCLEIC ACID PROBES

Nucleic acid probes are segments of DNA or RNA labeled with radioisotopes, enzymes, or chemiluminescent reporter molecules that can bind to complementary nucleic acid sequences with high degrees of specificity. Although probes can range from 15 to thousands of nucleotides in size, synthetic oligonucleotides of <50 nucleotides are most commonly incorporated into commercial kits. The probes can be designed to identify microorganisms at any taxonomic level. A number of commercially available DNA probes have been developed for direct detection of pathogens in clinical specimens and identification of pathogens after isolation by culture.

The commonly used formats for probe hybridization include liquid-phase, solid-phase, and in situ hybridization. The leading method used in clinical microbiology laboratories is a liquid-phase hybridization protection assay (Gen-Probe, Inc., San Diego, Calif.). In this method, a single-stranded DNA probe labeled with an acridinium ester is incubated with the target nucleic acid. Alkaline hydrolysis follows the hybridization step, and probe binding is measured in a luminometer after the addition of peroxides. For a positive sample, the acridinium ester on the bound probe is protected from hydrolysis and, upon the addition of peroxides, emits light. The hybridization protection assay can be completed in several hours and does not require removal of unbound single-stranded probe or isolation of probe-bound double-stranded sequences (3).

In solid-phase hybridization, target nucleic acids are bound to nylon or nitrocellulose and are hybridized with a probe in solution (122). The unbound probe is washed away, and the bound probe is detected by means of fluorescence, luminescence, radioactivity, or color development. Although solid-phase hybridization is a powerful research tool, the length of time required and the complexity of the procedure limit its application in clinical practice.

In situ hybridization is another type of solid-phase hybridization in which the nucleic acid is contained in tissues or cells which are affixed to microscope slides and is governed by the same basic principles described previously (44). In most clinical applications, formalin-fixed, paraffin-embedded tissue sections are used. The sensitivity of in situ hybridization is often limited by the accessibility of the target nucleic acid in the cells.

In general, due to the poor analytical sensitivities of nonamplified probe techniques, the application of these techniques to direct detection of pathogens in clinical specimens is limited to those situations in which the number of organisms is large. Such situations include cases of group A streptococcal pharyngitis and genital tract infections with *Neisseria gonorrhoeae* and *Chlamydia trachomatis*. These techniques are used most effectively in culture confirmation assays for mycobacteria and systemic dimorphic fungi. These culture confirmation tests have a positive effect on patient management by providing rapid and accurate detection of these slowly growing, often difficult to identify pathogens.

Nucleic acid probes for direct detection of group A streptococci, *C. trachomatis*, and *N. gonorrhoeae* are available from Gen-Probe. Probes for identification of *Blastomyces dermatitidis, Coccidioides immitis, Histoplasma capsulatum, Campylobacter* spp., enterococci, group A streptococci, group B streptococci, *Haemophilus influenzae, Listeria monocytogenes,* mycobacteria, *N. gonorrhoeae, Staphylococcus aureus,* and

Streptococcus pneumoniae isolated in culture are also available from Gen-Probe.

Peptide nucleic acid (PNA) probes are DNA mimics in which the negatively charged sugar phosphate backbone of DNA is replaced with a noncharged polyamide or "peptide" backbone. PNA probes contain the same nucleotide bases as DNA and follow standard Watson-Crick base pairing rules when hybridizing to complementary nucleic acid sequences (115). Because PNA probes are noncharged, they do not have to overcome the destabilizing electrostatic repulsion that occurs when two negatively charged DNA molecules hybridize. As a result, PNA probes bind more rapidly and tightly to nucleic acid targets. In addition, the relatively hydrophobic character of the PNA probes enables them to penetrate the hydrophobic cell membrane following preparation of a standard smear.

PNA probes have been used for identification of *S. aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida albicans* directly from positive blood culture bottles (90, 102, 112) and direct detection of *Mycobacterium tuberculosis* in smear-positive sputum samples (116). PNA probes for direct identification of *S. aureus, Enterococcus faecalis*, and *C. albicans* from positive blood culture bottles are available from AdvanDx, Woburn, Mass.

AMPLIFIED NUCLEIC ACID TECHNIQUES

The development of the PCR by Saiki et al. (108) was a milestone in biotechnology and heralded the beginning of molecular diagnostics. PCR had its 20th birthday in 2005 and has stood the test of time. Although PCR is the best-developed and most widely used nucleic acid amplification strategy, other strategies have been developed, and several have clinical utility. These strategies are based on signal, target, or probe amplification. Examples of each category will be discussed in the sections that follow. These techniques have sensitivity unparalleled in laboratory medicine, have created new opportunities for the clinical laboratory to have an effect on patient care, and have become the new "gold standards" for laboratory diagnosis of several infectious diseases.

SIGNAL AMPLIFICATION TECHNIQUES

In signal amplification methods, the concentration of the probe or target does not increase. The increased analytical sensitivity comes from increasing the concentration of labeled molecules attached to the target nucleic acid. Multiple enzymes, multiple probes, multiple layers of probes, and reduction of background noise have all been used to enhance target detection (59). Target amplification systems generally have greater analytical sensitivity than signal amplification methods, but technological developments, particularly in branched DNA (bDNA) assays, have lowered the limits of detection to levels that may rival those of target amplification assays in some applications (57).

Signal amplification assays have several advantages over target amplification assays. In signal amplification systems, the number of target molecules is not altered, and as a result, the signal is directly proportional to the amount of the target sequence present in the clinical specimen. This reduces concerns about false-positive results due to cross contamination and simplifies the development of quantitative assays. Since signal amplification systems are not dependent on enzymatic processes to amplify the target sequence, they are not affected by the presence of enzyme inhibitors in clinical specimens. Consequently, less cumbersome nucleic acid extraction methods may be used. Typically, signal amplification systems use either larger probes or more probes than target amplification systems and, consequently, are less susceptible to errors resulting from target sequence heterogeneity. Finally, RNA levels can be measured directly without the synthesis of a cDNA intermediate.

bDNA Assays

The bDNA signal amplification system is a solid-phase, sandwich hybridization assay incorporating multiple sets of synthetic oligonucleotide probes (86). The key to this technology is the amplifier molecule, a bDNA molecule with 15 identical branches, each of which can bind to three labeled probes.

The bDNA signal amplification system is illustrated in Fig. 1. Multiple target-specific probes are used to capture the target nucleic acid onto the surface of a microtiter well. A second set of target-specific probes also binds to the target. Preamplifier molecules bind to the second set of target probes and up to eight bDNA amplifiers. Three alkaline phosphatase-labeled probes hybridize to each branch of the amplifier. Detection of bound labeled probes is achieved by incubating the complex with dioxetane, an enzyme-triggerable substrate, and measuring the light emission in a luminometer. The resulting signal is directly proportional to the quantity of the target in the sample. The quantity of the target in the sample is determined from an external standard curve.

Nonspecific hybridization of any of the amplification probes and nontarget nucleic acids leads to amplification of the background signal. In order to reduce potential hybridization to nontarget nucleic acids, isocytidine (isoC) and isoguanosine (isoG) were incorporated into the preamplifier and labeled probes were used in the third-generation bDNA assays (21). IsoC and isoG form base pairs with each other but not with any of the four naturally occurring bases (97). The use of isoC- and isoG-containing probes in bDNA assays increases target-specific signal amplification without a concomitant increase in the background signal, thereby greatly enhancing the detection limits without loss of specificity. The detection limit of the third-generation bDNA assay for human immunodeficiency virus type 1 (HIV-1) RNA is 75 copies/ml. bDNA assays for the quantitation of hepatitis B virus (HBV) DNA, hepatitis C virus (HCV) RNA, and HIV-1 RNA are commercially available (Bayer HealthCare, Diagnostics Division, Tarrytown, N.Y.). The System 340 analyzer for bDNA assays automates the incubation, washing, reading, and data-processing steps.

Hybrid Capture Assays

The hybrid capture system is a solution hybridization-antibody capture assay that uses chemiluminescence detection of the hybrid molecules. The target DNA in the specimen is denatured and then hybridized with a specific RNA probe. The DNA-RNA hybrids are captured by antihybrid antibodies that are used to coat the surface of a tube. Alkaline phosphatase-conjugated antihybrid antibodies bind to the immobilized hybrids. The bound antibody conjugate is detected with a chemiluminescent substrate, and the light emitted is measured in a luminometer. Multiple alkaline phosphatase conjugates bind to each hybrid molecule, amplifying the signal. The intensity of the emitted light is proportional to the amount of target DNA in the specimen. Hybrid capture assays for detection of *N. gonorrhoeae, C. trachomatis,* human papillomavirus (HPV) (23), and cytomegalovirus (CMV) (76) in clinical specimens are commercially available (Digene Corp., Gaithersburg, Md.).

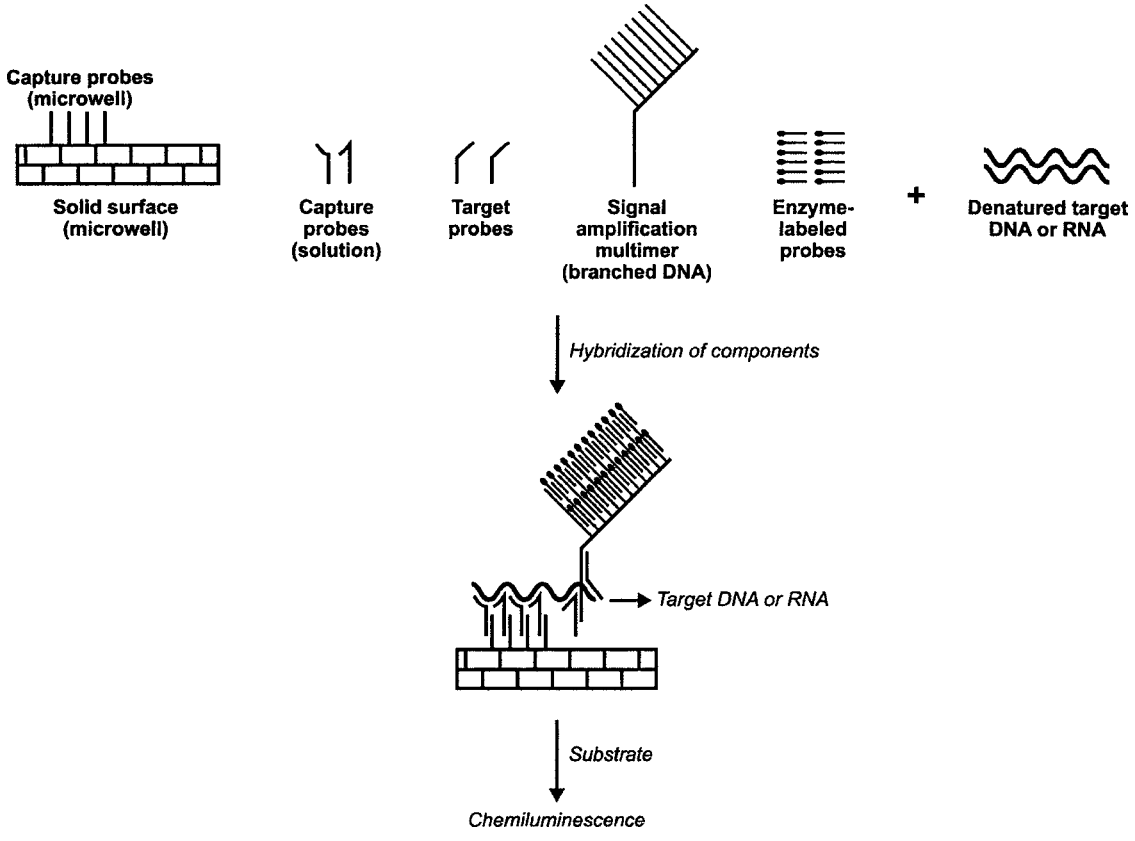


FIGURE 1 bDNA signal amplification (reprinted with permission of Elsevier from reference 134).

TARGET AMPLIFICATION TECHNIQUES

All of the target amplification systems share certain fundamental characteristics. They use enzyme-mediated processes, in which a single enzyme or multiple enzymes synthesize copies of target nucleic acid. In all of these techniques, the amplification products are detected by two oligonucleotide primers that bind to complementary sequences on opposite strands of double-stranded targets. All the techniques result in the production of millions to billions of copies of the targeted sequence in a matter of hours, and in each case, the amplification products can serve as templates for subsequent rounds of amplification. Because of this, all of the techniques are sensitive to contamination with product molecules that can lead to false-positive reactions. The potential for cross contamination is real and should be adequately addressed before any of these techniques are used in the clinical laboratory. However, the occurrence of false-positive reactions can be reduced through special laboratory design, practices, and workflow.

PCR

PCR is a simple, in vitro, chemical reaction that permits the synthesis of essentially limitless quantities of a targeted nucleic acid sequence. This is accomplished through the action of a DNA polymerase that, under the proper conditions, can copy a DNA strand (Fig. 2). At its simplest, a PCR consists of target DNA, a molar excess of two oligonucleotide primers, a heat-stable DNA polymerase, an equimolar mixture of deoxyribonucleotide triphosphates (dNTPs; dATP, dCTP, dGTP, and dTTP), $MgCl_2$, KCl, and a Tris-HCl buffer. The two primers flank the double-stranded DNA sequence to be amplified, typically <100 to several hundred bases, and are complementary to opposite strands of the target.

To initiate a PCR, the reaction mixture is heated to separate the two strands of target DNA and is then cooled to permit the primers to anneal to the target DNA in a sequence-specific manner. The DNA polymerase then initiates extension of the primers at their 3′ ends toward one another. The primer extension products are dissociated from the target DNA by heating. Each extension product, as well as the original target, can serve as a template for subsequent rounds of primer annealing and extension.

At the end of each cycle, the PCR products are theoretically doubled. Thus, after n PCR cycles the target sequence can be amplified 2^n-fold. The whole procedure is carried out in a programmable thermal cycler that precisely controls the temperature at which the steps occur, the lengths of time that the reaction mixture is held at the different temperatures, and the number of cycles. Ideally, after 20 cycles of PCR a 10^6-fold amplification is achieved and after 30 cycles a 10^9-fold amplification occurs. In practice, the amplification may not be completely efficient due to failure to optimize the reaction conditions or the presence of inhibitors of the DNA polymerase. In such cases, the total amplification is best described by the expression $(1 + e)^n$, where e is the amplification efficiency ($0 \leq e \leq 1$) and n is the total number of cycles.

RT-PCR

As it was originally described, PCR was a technique for DNA amplification. Reverse transcriptase PCR (RT-PCR) was

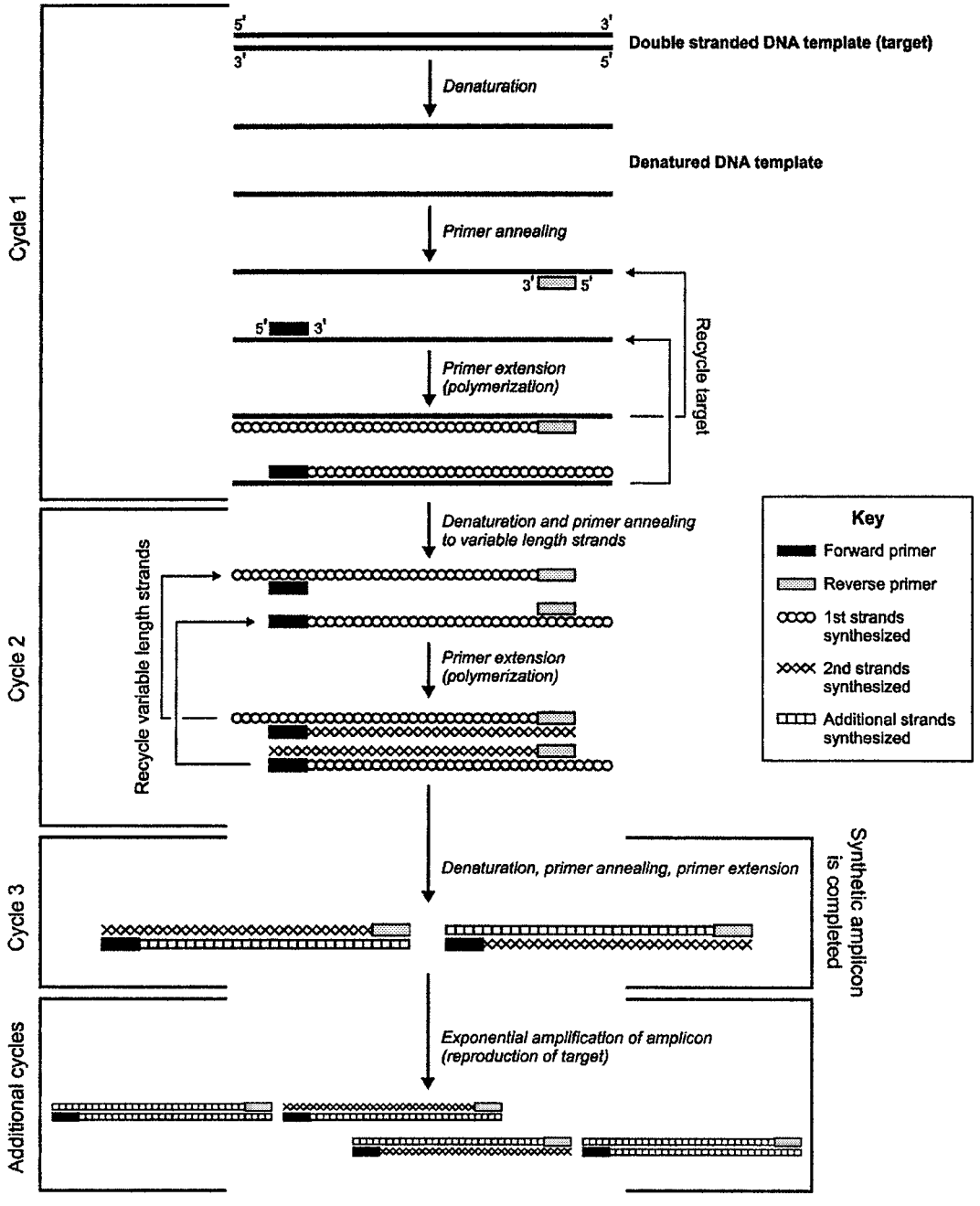


FIGURE 2 PCR target amplification (reprinted with permission of Elsevier from reference 134).

developed to amplify RNA targets. In this process, cDNA is first produced from RNA targets by reverse transcription and then the cDNA is amplified by PCR. As it was originally described, RT-PCR used two enzymes, a heat-labile RT such as avian myeloblastosis virus RT and a thermostable DNA polymerase. Because of the temperature requirements of the heat-labile enzyme, cDNA synthesis had to occur at temperatures below the optimal annealing temperatures of the primers. This presented problems in terms of both nonspecific primer annealing and inefficient primer extension due to the formation of RNA secondary structures. These problems have largely been overcome by the development of a thermostable DNA polymerase derived from *Thermus thermophilus* that under the proper conditions can function efficiently as both an RT and a DNA polymerase (82). RT-PCRs with this enzyme are more specific and efficient than previous protocols with conventional, heat-labile RT enzymes. Commercially available kits (Roche Diagnostics, Indianapolis, Ind.) that use this single-enzyme technology are available for detection of HCV RNA and for quantitation of HIV-1 and HCV RNA in clinical specimens.

Nested PCR

Nested PCR was developed to increase both the sensitivity and the specificity of PCR (45). It uses two pairs of amplification primers and two rounds of PCR. Typically, one primer pair is used in the first round of PCR for 15 to 30 cycles. The products of the first round of amplification are then subjected to a second round of amplification with the second set of primers that anneal to a sequence internal to the sequence amplified by the first primer set. The increased sensitivity arises from the high total cycle number, and the increased specificity arises from the annealing of the second primer set to sequences found only in the first-round products, thus verifying the identity of the first-round product. The major disadvantage of nested PCR is the high rates of contamination that can occur during the transfer of first-round products to the second tube for the second round of amplification. This contamination can be avoided either by physically separating the first- and second-round amplification mixtures with a layer of wax or oil or by designing single-tube amplification protocols. In practice, the enhanced sensitivity afforded by nested PCR protocols is rarely required in diagnostic applications, and the identity of an amplification product is usually confirmed by hybridization with a nucleic acid probe.

Multiplex PCR

In multiplex PCR, two or more primer sets designed for amplification of different targets are included in the same reaction mixture (12). By this technique, more than one target sequence in a clinical specimen can be coamplified in a single tube. The primers used in multiplexed reactions must be carefully selected so that they have similar annealing temperatures and lack complementarity. Multiplex PCRs have proved to be more complicated to develop and are usually less sensitive than PCRs with single primer sets.

Many multiplex assays have been developed, especially for the detection of central nervous system (9, 24) and respiratory (58, 121) pathogens. Multiplex PCR assays for bacterial and viral respiratory pathogens are commercially available from Prodesse, Inc., Waukesha, Wis.

A promising new platform for multiplex PCR analysis is the LabMAP system (Luminex Corp., Austin, Tex.). The LabMAP system incorporates a proprietary process to internally dye polystyrene microspheres with two spectrally distinct fluorochromes. By using precise ratios of these fluorochromes, an array is created consisting of 100 different microsphere sets with specific spectral addresses. Each microsphere set can possess a different reactant on its surface. For nucleic acid analysis, oligonucleotide probes would be covalently bound to the microsphere surface by carbodiimide coupling. Since each microsphere set can be distinguished by its spectral address, the sets can be combined, allowing up to 100 different analytes to be measured simultaneously in a single reaction vessel. A third fluorochrome coupled to a reporter molecule quantifies the biomolecular interaction that occurs at the microsphere surface.

Microspheres are investigated individually in a rapidly flowing liquid stream as they pass by two separate lasers in the Luminex 100 flow cytometer. High-speed digital signal processing classifies each microsphere based on its spectral address and quantifies the reaction on its surface. Thousands of microspheres are investigated per second, resulting in an analysis system capable of analyzing and reporting up to 100 different reactions in a single reaction vessel in a few seconds. The technology has been adapted to a wide variety of applications in bacteriology (27), mycology (25), and virology (111, 129).

Real-Time (Homogeneous, Kinetic) PCR

The term real-time PCR describes methods in which the target amplification and detection steps occur simultaneously in the same tube (homogeneous). These methods require special thermal cyclers with precision optics that can monitor the fluorescence emission from the sample wells. The computer software supporting the thermal cycler monitors the data throughout the PCR at every cycle and generates an amplification plot for each reaction (kinetic).

Figure 3 shows a representative amplification plot and defines the terms used in real-time PCR quantitation. The amplification plot shows the normalized fluorescence signal from the reporter at each cycle number. In the initial cycles of PCR, there is little change in the fluorescence signal. This initial signal level defines the baseline for the plot. An increase above the baseline indicates the detection of accumulated PCR product. A fixed fluorescence threshold can be set above the baseline. The cycle threshold (C_T) is defined as the cycle number at which the fluorescence passes the fixed threshold. A plot of the log of the initial target concentration versus C_T for a set of standards is a straight line (49). The amount of the target in an unknown sample is determined by measuring the sample C_T and using a standard curve to determine the starting copy number. Alternatively, the cycle number corresponding to the maximal change in fluorescence, the second derivative maximum, has a similar relationship to the initial target concentration.

In its simplest format, the PCR product is detected as it is produced by using fluorescent dyes that preferentially bind to double-stranded DNA. SYBR Green I is one such dye that has been used in this application (81). In the dye's unbound state, the fluorescence is relatively low, but when the dye is bound to double-stranded DNA, the fluorescence is greatly enhanced. The dye will bind to both specific and nonspecific PCR products. The specificity of the detection can be improved through melting curve analysis. As the temperature is slowly raised, the two strands of the amplicon will melt apart and the amount of fluorescence will decrease. The data are transformed and analyzed by plotting the first derivative of the fluorescence on the y axis and the temperature on the

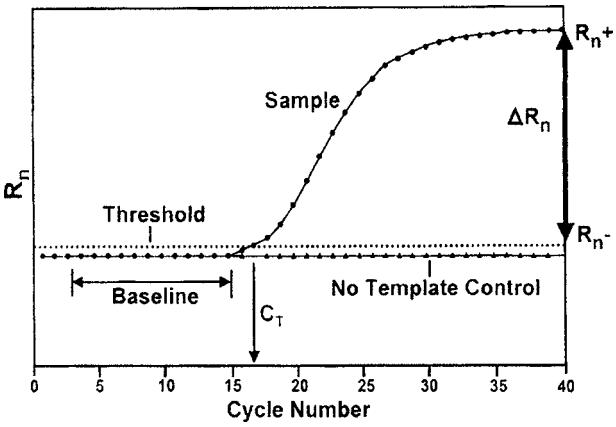


FIGURE 3 Real-time PCR amplification plot with commonly used terms and abbreviations. R_n, normalized fluorescent signal from reporter dye. (From *TaqMan Universal PCR Master Mix Protocol*, Foster City, Calif.: Applied Biosystems; 2002:5–94. Reprinted with permission.)

x axis. The specific amplified product will have a characteristic melting peak at its predicted melting temperature (T_m), whereas the primer dimers and other nonspecific products should have different T_ms or give broader peaks (103).

The specificity of real-time PCR can also be increased by including fluorescent resonance energy transfer (FRET) probes in the reaction mixture. These probes are labeled with fluorescent dyes or with combinations of fluorescent and quencher dyes. In 5′ exonuclease PCR (TaqMan) assays, the 5′ to 3′ exonuclease activity of *Taq* DNA polymerase is used to cleave a nonextendable hybridization probe during the primer extension phase of PCR (50). This approach uses dual-labeled fluorogenic hybridization probes and is illustrated in Fig. 4. One fluorescent dye serves as a reporter, and its emission spectrum is quenched by the second fluorescent dye. The nuclease degradation of the hybridization probe releases the

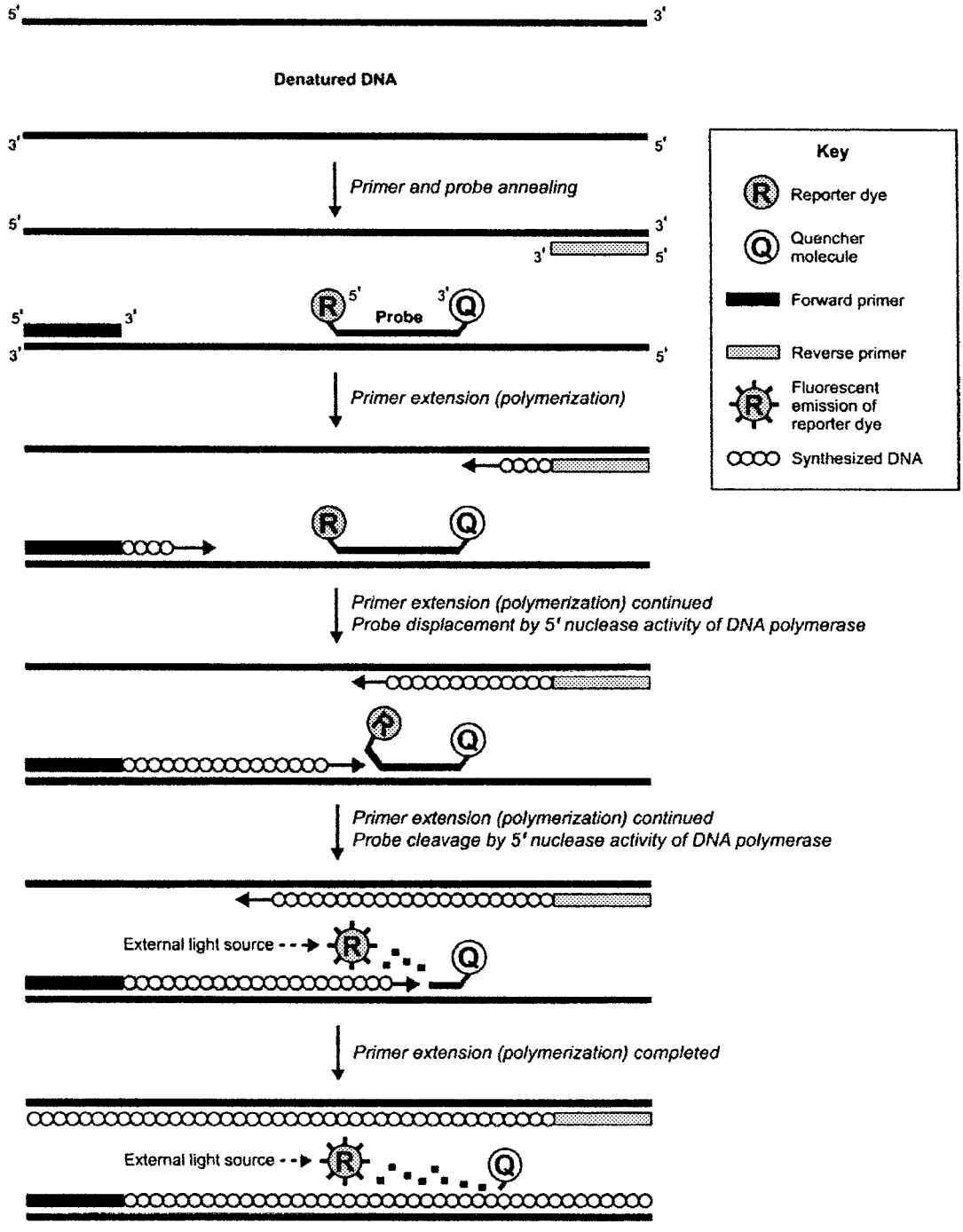


FIGURE 4 5′ exonuclease chemistry for real-time PCR applications (reprinted with permission of Elsevier from reference 134).

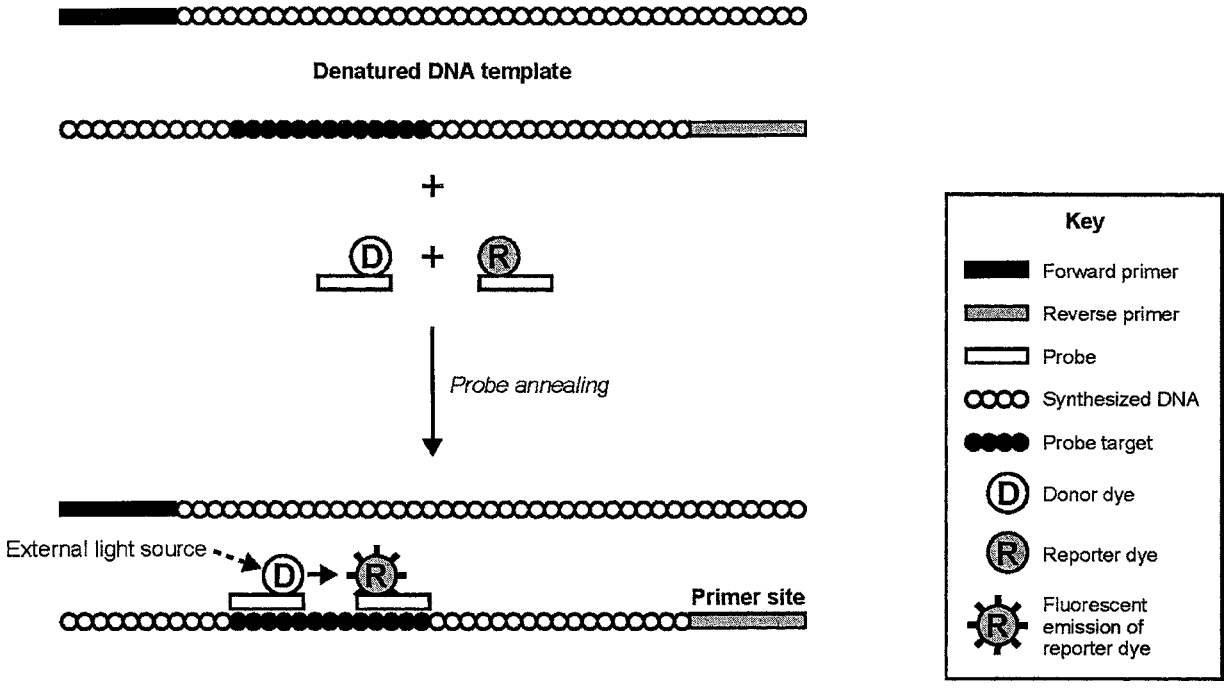


FIGURE 5 Dual hybridization probes for real-time PCR applications (reprinted with permission of Elsevier from reference 134).

reporter dye, resulting in an increase in the peak fluorescent emission. The increase in fluorescent emission indicates that specific PCR product has been made, and the intensity of fluorescence is related to the amount of the product (46). The specificity is increased because a signal is generated only when the primer and probe are bound to the same template strand.

The use of dual hybridization probes is another approach to real-time PCR (65). This method uses two specially designed sequence-specific oligonucleotide probes (Fig. 5). These hybridization probes are designed to hybridize within 1 to 5 nucleotides apart on the product molecule. The 3′ end of the anchor probe is labeled with a donor dye, and the 5′ end of the reporter probe is labeled with an acceptor dye. The 3′ end of the reporter probe is phosphorylated to prevent extension during PCR. The donor dye is excited by an external light source, and instead of emitting light, it transfers its energy to the acceptor dye by FRET. The excited acceptor dye emits light at a longer wavelength than the unbound donor dye, and the intensity of the acceptor dye light emission is proportional to the amount of PCR product.

Real-time detection and quantitation of amplification products can also be accomplished with molecular beacons (126). Molecular beacons are hairpin-shaped oligonucleotide probes with an internally quenched fluorophore whose fluorescence is restored when the probes bind to a target nucleic acid (Fig. 6). The probes are designed in such a way that the loop portion of each probe molecule is complementary to the target sequence. The stem is formed by the annealing of complementary arm sequences on the ends of the probe. A fluorescent dye is attached to one end of one arm, and a quenching molecule is attached to the end of the other arm. The stem keeps the fluorophore and quencher in close proximity such that no light emission occurs. When the probe encounters a target molecule, it forms a hybrid that is longer and more stable than the stem and undergoes a conformational change that forces the stem apart, causing the fluorophore and the quencher to move away from each other, restoring the fluorescence.

Scorpion probes combine a PCR primer with a molecular beacon (124, 131). Intramolecular hybridization of the loop structure to a downstream portion of the amplification product separates the reporter and quencher dyes. The hybridization kinetics of Scorpion probes are generally faster than those of molecular beacons because the primer and probe are located on the same molecule.

Dark quencher probes are also used in real-time PCR applications (Nanogen, Bothell, Wash.). Dark quencher probes contain a fluorophore on the 5′ end and a nonfluorescent

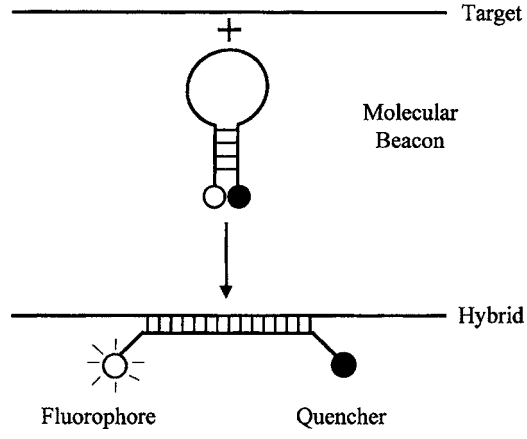


FIGURE 6 Molecular beacon probes for real-time amplification applications (reprinted with permission of Elsevier from reference 134).

quencher molecule on the 3′ end (62). The fluorescence is quenched when the probe is a random coil and emitted when the probe anneals to the target sequence. Unlike fluorogenic 5′ nuclease probes, these probes are not degraded by the DNA polymerase during target amplification. Since the dark quencher is not fluorescent, it does not contribute to the background signal. This trait has the advantage of improving the signal-to-noise ratio for the detection system, which may improve sensitivity. These probes also incorporate a hybridization-stabilizing compound, known as a minor groove binder. It is a small, crescent-shaped molecule that is covalently linked to the 3′ end of the probe that spans about 3 to 4 nucleotides and snugly fits into the minor groove of DNA, where it forms hydrogen bonds with the template. Minor groove binders will increase the T_m of the probe. The minor groove binder allows for the use of shorter probes because of the increased T_ms and enables improved T_m leveling, which increases the specificity of the detection reaction.

Real-time PCR methods decrease the time required to perform nucleic acid assays because there are no post-PCR processing steps. Also, since amplification and detection occur in the same closed tube, these methods eliminate the postamplification manipulations that can lead to laboratory contamination with the amplicon. In addition, real-time PCR methods lend themselves well to quantitative applications because analysis is performed early in the log phase of product accumulation and, as a result, is less prone to error resulting from differences in sample-to-sample amplification efficiency.

Transcription-Based Amplification Methods

Nucleic acid sequence-based amplification (NASBA) and transcription-mediated amplification (TMA) are both isothermal RNA amplification methods modeled after retroviral replication (22, 39, 63). The methods are similar in that the RNA target is reverse transcribed into cDNA and then RNA copies are synthesized with an RNA polymerase. NASBA uses avian myeloblastosis virus RT, RNase H, and T7 bacteriophage RNA polymerase, whereas TMA uses an RT enzyme with endogenous RNase H activity and T7 RNA polymerase.

Amplification involves the synthesis of cDNA from the RNA target with a primer containing the T7 RNA polymerase promoter sequence (Fig. 7). The RNase H then degrades the initial strand of target RNA in the RNA-cDNA hybrid. The second primer then binds to the cDNA and is extended by the DNA polymerase activity of the RT, resulting in the formation of double-stranded DNA containing the T7 RNA polymerase promoter. The RNA polymerase then generates multiple copies of single-stranded, antisense RNA. These RNA product molecules reenter the cycle, with subsequent formation of more double-stranded cDNA molecules that can serve as templates for more RNA synthesis. A 10^9-fold amplification of the target RNA can be achieved in less than 2 h by this method.

The single-stranded RNA products of TMA in the Gen-Probe tests are detected by modification of the hybridization protection assay. Oligonucleotide probes are labeled with modified acridinium esters with either fast or slow chemiluminescence kinetics so that signals from two hybridization reactions can be analyzed simultaneously in the same tube. The NASBA products in the bioMérieux (Durham, N.C.) tests are detected by hybridization with probes labeled with tris(2,2′-bispyridine)ruthenium and electrochemiluminescence. NASBA has also been used with molecular beacons to create a homogeneous, kinetic amplification system similar to real-time PCR (68).

Transcription-based amplification systems have several strengths, including no requirement for a thermal cycler, rapid kinetics, and a single-stranded RNA product that does not require denaturation prior to detection. Also, single-tube clinical assays and a labile RNA product may help minimize contamination risks. Limitations include the poor performance with DNA targets and concerns about the stability of complex multienzyme systems. Gen-Probe has developed TMA-based assays for detection of *Mycobacterium tuberculosis, C. trachomatis, N. gonorrhoeae,* HCV, and HIV-1. NASBA-based kits (bioMérieux) for the detection and quantitation of HIV-1 RNA and CMV RNA transcripts and detection of enterovirus and respiratory syncytial virus RNA are commercially available. A basic NASBA kit is also available for the development of other applications defined by the user.

Strand Displacement Amplification

Strand displacement amplification (SDA) is an isothermal template amplification technique that can be used to detect trace amounts of DNA or RNA of a particular sequence. SDA, as it was first described, was a conceptually straightforward amplification process with some technical limitations (128). Since its initial description, however, it has evolved into a highly versatile tool that is technically simple to perform but conceptually complex. SDA is the intellectual property of Becton Dickinson and Company, Sparks, Md.

In its current iteration, SDA occurs in two discrete phases, target generation and exponential target amplification (70). Both are illustrated in Fig. 8. In the target generation phase, a double-stranded DNA target is denatured and hybridized to two different primer pairs, designated as bumper and amplification primers. The amplification primers include the single-stranded restriction endonuclease enzyme sequence for BsoB1 located at the 5′ end of the target binding sequence. The bumper primers are shorter and anneal to the target DNA just upstream of the region to be amplified. In the presence of BsoB1, an exonuclease-free DNA polymerase, and a dNTP mixture consisting of dUTP, dATP, dGTP, and thiolated dCTP (C_s), simultaneous extension products of both the bumper and amplification primers are generated. This process displaces the amplification primer products, which are available for hybridization with the opposite-strand products with the opposite-strand bumper and amplification primers.

The simultaneous extension of opposite-strand primers produces strands complementary to the product formed by extension of the first amplification primer with C_s incorporated into the BsoB1 cleavage site. This product enters the exponential target amplification phase of the reaction. The BsoB1 enzyme recognizes the double-stranded site, but because one strand contains C_s, it is nicked rather than cleaved by the enzyme. The DNA polymerase then binds to the nicked site and begins synthesis of a new strand while simultaneously displacing the downstream strand. This step re-creates the double-stranded species with the hemimodified restriction endonuclease recognition sequence, and the iterative nicking and displacement process repeats. The displaced strands are capable of binding to opposite-strand primers, which produces exponential amplification of the target sequences.

These single-stranded products also bind to detector probes for real-time detection. The detector probes are single-stranded DNA molecules with fluorescein and rhodamine labels. The region between the labels includes a stem-loop structure. The loop contains the recognition site for the BsoB1 enzyme. The target-specific sequences are located 3′ of

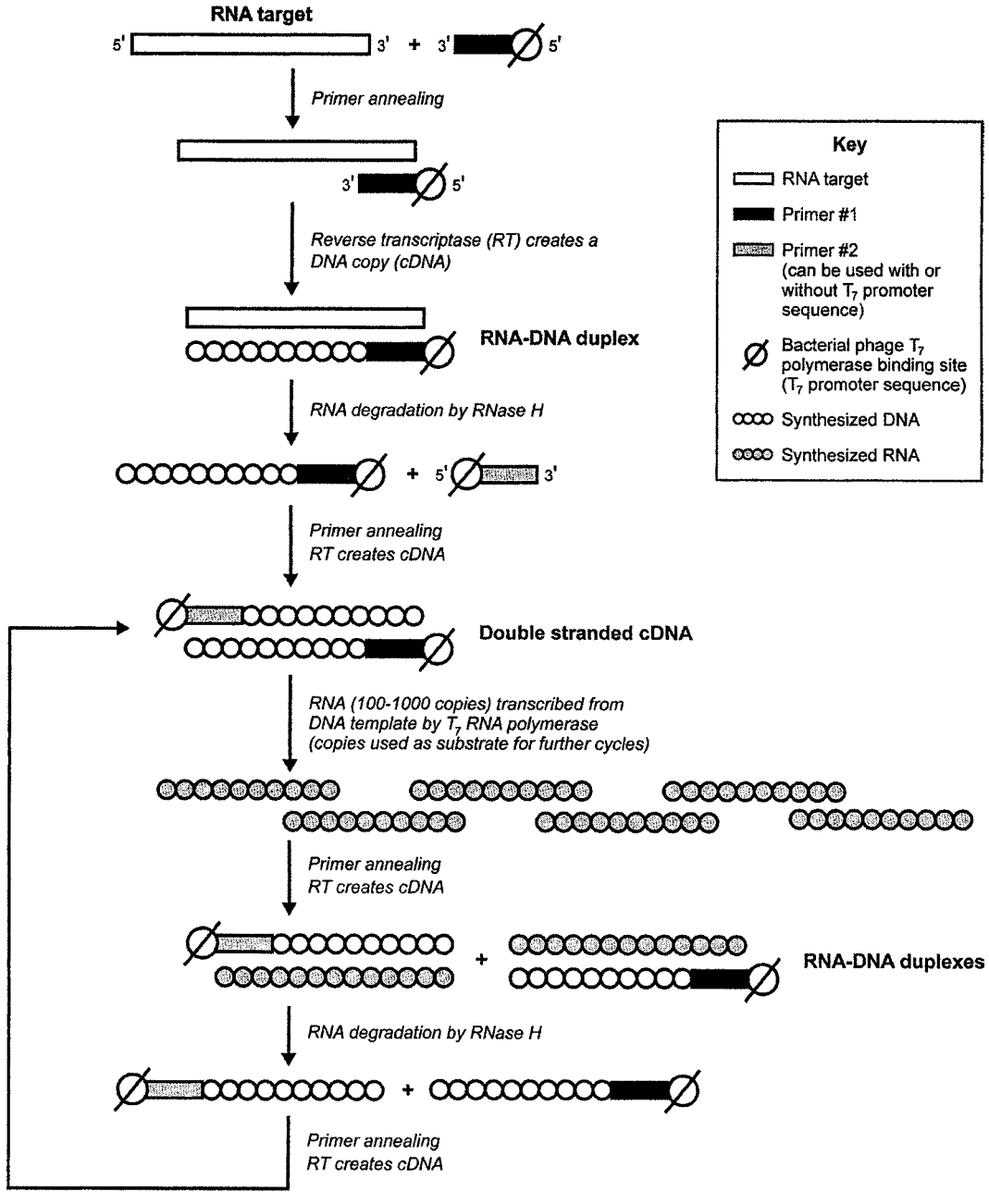


FIGURE 7 Transcription-based target amplification. NASBA and TMA are examples of transcription-based amplification systems (reprinted with permission of Elsevier from reference 134).

the rhodamine label. In the absence of a specific target, the stem-loop structure is maintained with the fluorescein and rhodamine labels in close proximity. The net effect is that very little emission for the fluorescein is detected after excitation. After SDA, the probe is converted to a double-stranded species, which is cleaved by BsoB1. The cleavage causes physical separation of the fluorescein and rhodamine labels, which results in an increase in emission from the flourescein label.

The diagnostic applications of SDA include the direct detection of *M. tuberculosis, C. trachomatis,* and *N. gonorrhoeae* in clinical specimens. SDA has a reported sensitivity high enough to detect as few as 10 to 50 copies of a target molecule (128). By using a primer set designed to amplify a repetitive sequence with 10 copies in the *M. tuberculosis* genome, the assay is sensitive enough to detect 1 to 5 genome copies from the bacterium. Recently, SDA has been adapted to quantitate RNA by adding an RT step (RT-SDA). In this case, a primer hybridizes to the target RNA and an RT synthesizes a cDNA molecule. This cDNA can then serve as a template for primer incorporation and strand displacement. The products of this strand displacement then feed into the amplification scheme described above. RT-SDA has been used for the determination of HIV viral load (88).

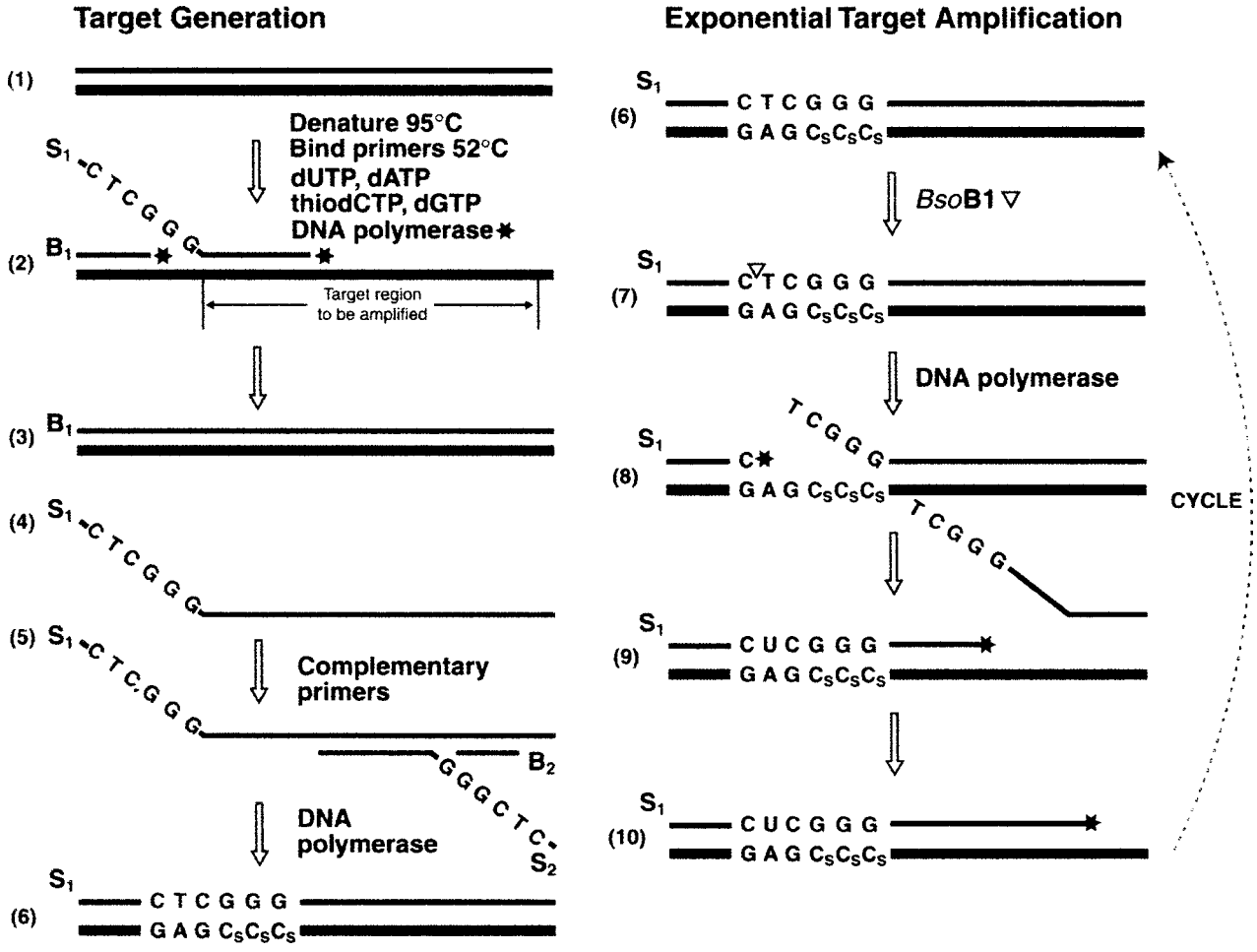


FIGURE 8 Strand displacement target amplification. The process is shown for only one strand of a double-stranded DNA target, but amplification occurs on both strands simultaneously. B_1 and B_2, bumper primers; S_1 and S_2, amplification primers. (Modified from reference 70.)

The main advantage of SDA is that it is an isothermal process that, unlike PCR, can be performed at a single temperature after initial target denaturation. This eliminates the need for expensive thermal cyclers. Furthermore, samples can be subjected to SDA in a single tube, with amplification times varying from 30 min to 2 h. The main disadvantage of SDA lies in the fact that, unlike those at which PCR is performed, the relatively low temperature at which SDA is carried out (52.5°C) can result in nonspecific primer hybridization to sequences found in complex mixtures such as genomic DNA. Hence, when the target is in low abundance compared to background DNA, nonspecific amplification products can swamp the system, decreasing the sensitivity of the technique. However, the use of organic solvents to increase stringency at low temperatures and the recent introduction of more thermostable polymerases capable of strand displacement have alleviated much of this problem.

PROBE AMPLIFICATION TECHNIQUES

Probe amplification methods differ from target amplification methods in that the amplification products contain only a sequence present in the initial probes. Ligase chain reaction (136), cycling probe technology (31), and cleavase-invader technology (73) are all examples of probe amplification methods for which diagnostic applications have been developed. However, diagnostic tests based on ligase chain reaction and cycling probe technology are no longer available in the United States.

Cleavase-Invader Technology

Invader assays (Third Wave Technologies, Madison, Wis.) are based on a probe amplification method that relies upon the specific recognition and cleavage of particular DNA structures by cleavase, a member of the FEN-1 family of DNA polymerases. These polymerases will cleave the 5′ single-stranded flap of a branched base-paired duplex. This enzymatic activity likely plays an essential role in the elimination of the complex nucleic acid structures that arise during DNA replication and repair. Since these structures may occur anywhere in a replicating genome, the enzyme recognizes the molecular structure of the substrate without regard to the sequence of the nucleic acids making up the DNA complex (69).

In the invader assays, two primers are designed which hybridize to the target sequence in an overlapping fashion

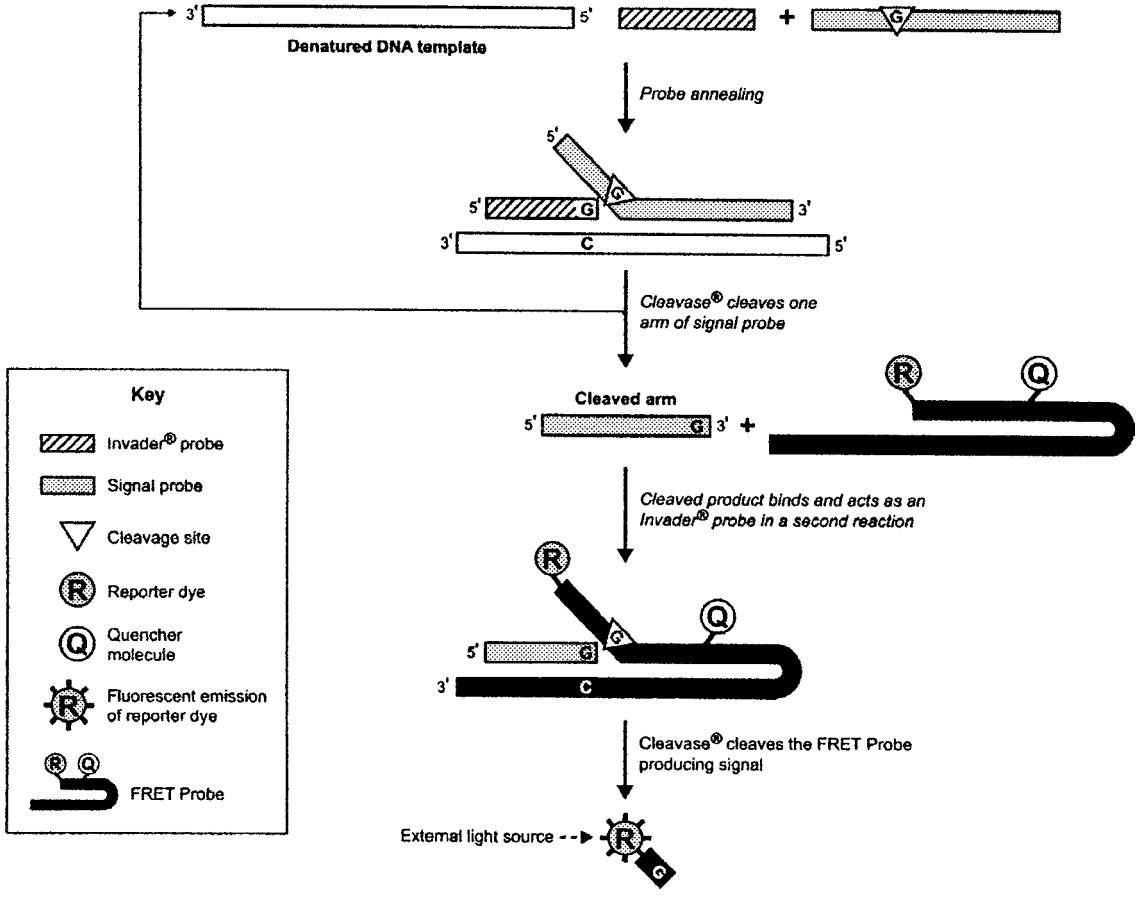


FIGURE 9 Cleavase-invader probe-based amplification (reprinted with permission of Elsevier from reference 134).

(Fig. 9). Under the proper annealing conditions, the probe oligonucleotide binds to the target sequence. The invader oligonucleotide is designed such that it hybridizes upstream of the probe with a region of overlap between the 3′ end of the invader and the 5′ end of the probe. Cleavase cleaves the 5′ end of the probe and releases it. It is in this way that the target sequence acts as a scaffold upon which the proper DNA structure can form. Since the DNA structure necessary to serve as a cleavase substrate will occur only in the presence of the target sequence, the generation of cleavage products indicates the presence of the target. Use of a thermostable cleavase enzyme allows reactions to be run at temperatures high enough for a primer exchange equilibrium to exist. This allows multiple cleavase products to form off of a single target molecule.

Various methods can be used to detect the cleavage products. Third Wave Technologies uses FRET probes and a second invasive cleavage reaction to detect the target-specific products. Invader technology can be used for genotyping, detection of mutations, and viral load testing. An ASR for HCV genotyping is currently available from Third Wave Technologies. ASRs are described in detail in the "Regulatory and Reimbursement Issues" section of this chapter.

The invader assay has several inherent advantages. Because the overlap in the invader probe need be only 1 bp, this technology can easily be adapted to detect point mutations of interest by designing the overlap region to encompass the mutation to be detected (74). The detection of these point mutations would not require postreaction restriction digestion, since the primers would be differentially cleaved on the basis of the presence or the absence of the mutation in question. This feature could be exploited to track mutations in pathogens associated with drug resistance or virulence. In addition, unlike amplification techniques such as PCR, SDA, and TMA, in which the target sequence itself is amplified, the invader assay does not increase the amount of the target sequence. As a consequence, invader assays are less prone to problems of false-positive results due to amplicon cross contamination.

POSTAMPLIFICATION DETECTION AND ANALYSIS

Gel Analysis

Visualization of amplification products in agarose gels after electrophoresis and ethidium bromide staining was the earliest detection method. After gel electrophoresis, DNA is often transferred onto a nitrocellulose or nylon membrane and hybridized to a specific probe to increase both the sensitivity and the specificity of detection. Membranes with bound radiolabeled probes are placed in proximity to X-ray film, and the hybrids are visualized as dark bands. Enzyme-labeled probes can be visualized through either light or color production after the addition of the appropriate chemiluminescent or

chromogenic substrates. Many of these nonisotopic approaches are at least as sensitive as isotopic methods and are faster. In addition, the enzyme-labeled probes are more stable. Although gel electrophoresis and blotting remain important research tools, these techniques are being replaced by faster and simpler methods in the clinical laboratory.

Single-strand conformation polymorphism (SSCP) analysis and restriction fragment length polymorphism (RFLP) analysis have been used to ascertain information about the base compositions of the amplification products visualized in a gel. In SSCP analysis, the PCR product is denatured and then subjected to electrophoresis in a nondenaturing gel (92). Variations in the physical conformations of the PCR products are related to the base compositions and are detected by differential gel migration. This technique has successfully been used to detect mutations causing rifampin resistance in *M. tuberculosis* (120).

RFLP analysis uses restriction endonucleases to cleave amplification products at specific recognition sites. The fragments are separated by electrophoresis, and the resulting banding pattern provides information about the nucleic acid sequence. When coupled with a hybridization reaction, RFLP analysis can also provide information about the location and number of loci homologous to the probe. Both SSCP analysis and RFLP analysis of short products have largely been replaced by direct DNA sequencing as this technology has improved and the costs have decreased.

Colorimetric Microtiter Plate Systems

Colorimetric microtiter plate (CMP) systems are convenient alternatives to traditional gel and blotting techniques for detection of amplified products. In these systems, the amplified product is captured in microtiter plate wells by specific oligonucleotide probes coating the plastic surface. Bound product is detected by a color change that takes place after addition of an enzyme conjugate and the appropriate substrate. These systems resemble enzyme immunoassays and use microtiter plate washers and readers commonly found in clinical laboratories. CMP systems are more practical and faster than the traditional membrane hybridization techniques described above.

Several variations of CMP systems are commercially available. In one popular approach, biotinylated primers are used to amplify the target, and the biotin-containing PCR product is denatured and added to the microtiter well. After hybridization with a capture probe, the bound product is detected with a streptavidin-enzyme conjugate and a chromogenic substrate (71). Enzyme-conjugated antibodies directed against double-stranded DNA have also been used to detect PCR products in CMP systems (75). Another approach uses digoxigenin-dUTP to label the PCR product and enzyme-conjugated antidigoxigenin antibodies to detect the captured product (98).

Allele-Specific Hybridization

Line probe assays are manufactured by Innogenetics (Ghent, Belgium) for genotyping of HCV and HBV, identification of mycobacteria, and analysis for drug resistance mutations in HIV-1, HBV, *M. tuberculosis,* and *Helicobacter pylori* (105, 117, 118). The HCV line probe assays are distributed by Bayer. In these assays, a series of probes with poly(T) tails are attached to nitrocellulose strips. Biotin-labeled PCR product is then hybridized to the immobilized probes on the strip. The labeled PCR product hybridizes only to the probes that give a perfect sequence match under the stringent hybridization conditions used. After hybridization, streptavidin labeled with alkaline phosphatase is added and binds to the biotinylated hybrids. Incubation with a chromogen results in a purple precipitate. The pattern of hybridization provides information about the nucleic acid sequence of the amplicon. This method is capable of detecting single-nucleotide polymorphisms.

Direct Sequencing

The combination of PCR and dideoxynucleotide chain termination methods can be used to determine DNA sequences in clinical samples (52). The use of fluorescent dye terminator chemistry and laser scanning in a polyacrylamide gel electrophoresis format has been the standard in electrophoretic separation technology. However, the recent application of capillary electrophoresis techniques to the separation of PCR and dideoxy chain termination products has streamlined the sequencing process by eliminating some of the labor-intensive steps, which makes the technology a better fit for diagnostic applications (30). The Clinical and Laboratory Standards Institute (CLSI) has recently developed guidelines for nucleic acid sequencing in clinical laboratories (19, 85).

CLIP (a coupled amplification and sequencing method) uses oligonucleotide primers labeled with different fluorescent dyes, standard dideoxynucleotide termination reagents, and PCR to produce extension products that end with a chain-terminating nucleotide. The nucleic acid sequence is deduced from the electrophoretic mobilities of the different extension products from a set of four reactions, each product containing a different chain-terminating nucleotide. A unique feature of CLIP sequencing is that one reaction produces sequence information for both nucleic acid strands. CLIP sequencing serves as the basis for commercially available assays for HIV-1 drug resistance and HCV genotyping (Bayer).

The ViroSeq HIV-1 genotyping assays (Celera Diagnostics, Alameda, Calif.; distributed by Abbott Molecular Diagnostics, Des Plains, Ill.) also use dideoxy chain-terminating sequencing, but each dideoxynucleotide is labeled with a different fluorescent dye. Each reaction mixture contains one primer but all four uniquely labeled dideoxynucleotides. Separation of the terminated PCR products is done by capillary electrophoresis.

Although direct sequencing of PCR products by electrophoresis is a powerful research tool, its routine use in the clinical laboratory depends upon the development of high-throughput systems with integrated databases and data analysis software. Such systems are available for HIV-1 and HCV genotyping and for identification of bacteria and fungi by rRNA gene sequence analysis.

Pyrosequencing (Biotage, Uppsala, Sweden) represents an alternative approach to conventional sequencing and is useful for genotyping and short-read-length sequencing (26). Pyrosequencing is based on the luminometric detection of pyrophosphate that is generated during DNA synthesis.

A sequencing primer is hybridized to a single-stranded PCR amplicon and incubated with the enzymes DNA polymerase, ATP sulfurylase, luciferase, and apyrase and the substrates adenosine 5′ phosphosulfate and luciferin. The first of four dNTPs is added to the reaction mixture. DNA polymerase catalyzes the incorporation of the dNTP into the DNA strand. Each incorporation event is accompanied by release of pyrophosphate (PP_i) in a quantity equimolar to the amount of incorporated nucleotide. The ATP sulfurylase quantitatively converts PP_i to ATP in the presence of adenosine 5′ phosphosulfate. This ATP drives the luciferase-mediated conversion of luciferin to oxyluciferin that generates light in amounts that are proportional to the amount of ATP. The light produced in the reaction is detected by a charge-coupled device camera.

A program is produced in which the height of each peak is proportional to the number of nucleotides incorporated. Apyrase, a nucleotide-degrading enzyme, continuously degrades ATP and unincorporated dNTPs. This degradation switches off the light and regenerates the reaction solution. The next dNTP is added, and the process is repeated.

Pyrosequencing has been used in microbiology to detect drug resistance mutations and to identify and type bacteria, viruses, and fungi (2, 37, 40, 91). Unlike conventional sequencing strategies, pyrosequencing provides reliable data for sequences adjacent to the sequencing primer termini. Pyrosequencing provides a simple-to-use and robust platform for short-read-length sequencing.

Hybridization Arrays

DNA hybridization arrays are produced by attaching or synthesizing hundreds or thousands of oligonucleotides on a solid support in precise patterns. A labeled amplification product is hybridized to the probes, and hybridization signals are mapped to various positions within the array. If the number of probes is sufficiently large, the sequence of the PCR product can be deduced from the pattern of hybridization. A number of manufacturers are developing DNA microarrays and the instrumentation required to acquire and analyze the data. Hybridization arrays have a number of applications in microbiology, including microbial and host gene expression profiling and diagnostic sequencing. The CLSI has published a guideline for the use of diagnostic nucleic acid microarrays (18).

One of the most developed approaches brings together advances in synthetic nucleic acid chemistry with photolithography, a process used in the manufacture of semiconductors for the computer industry. This approach uses light to direct the synthesis of short oligonucleotides on a silica wafer (94). On a 15-mm-square chip, thousands of individual sites or features can be established. At each feature, specific oligonucleotides are assembled one nucleotide at a time by light-activated chemistry.

The DNA chip is incubated in a flow cell with DNA product that has been fragmented and labeled with a fluorophore. After hybridization, a scanning laser confocal microscope evaluates the surface fluorescence intensity of the chip. Automated scanning by the microscope takes only a few minutes to acquire an image of the entire surface of the chip, and computer software analyzes the fluorescent image and determines the nucleic acid sequence of the PCR product. A DNA chip based on this technology for the detection of HIV-1 drug resistance mutations is commercially available (Affymetrix, Santa Clara, Calif.).

Another method of producing DNA hybridization arrays involves the precise micropipetting of premade double-stranded DNA probes (typically 200 to 2,000 bp in length) onto glass slides with a robotic device (110). These arrays are not suitable for mutation detection due to the size and density of the arrayed DNA probes but have facilitated gene expression profiling. DNA arrays of this type can be used to determine the activation states (mRNA levels) of thousands of genes simultaneously. Gene expression profiling of pathogens by use of arrays may provide new insights into pathogenic mechanisms and help identify new therapeutic and vaccine targets.

Nanogen (San Diego, Calif.) has developed a bioelectronic chip with 100 individually addressable electrodes. The silicon chip is manufactured by using the same type of photolithographic and deposition techniques used in the microelectronics industry. The technology uses electrical fields to move biological samples through the chip and direct the samples to the electrodes. Cheng and colleagues (13) used a microelectrode array to separate *E. coli* cells from a whole-blood sample and then to lyse the isolated *E. coli* cells on the chip. The lysate was then transferred to another bioelectronic chip for an electronically enhanced hybridization assay developed previously (114). The development of fully integrated analytical systems that function as a laboratory, the so-called "lab on a chip," is the ultimate goal of microchip designers.

DNA microarrays and bioelectronics hold much promise for molecular diagnostics. However, the current technology has several limitations, including the complexity of fabricating the microarrays, limited availability, and high test cost.

QUANTITATIVE METHODS

Many of the methods discussed above can be used to quantify the amount of RNA or DNA in a clinical sample. The most commonly used methods include PCR and RT-PCR, transcription-based amplification, bDNA assays, and hybrid capture. The principle of quantitative molecular methods is that there is a linear relationship between the quantity of the input template and the amount of the product or signal generated. Competitive PCR (cPCR) is a reliable and robust method that is commonly used in commercial and laboratory-developed assays. The basic concept behind cPCR is the coamplification in the same reaction tube of target and calibrator templates with equal or similar lengths and with the same primer binding sequences. Since both templates are amplified with the same primer pair, identical thermodynamics and amplification efficiencies are ensured. The amount of the calibrator must be known, and after amplification, products from both templates must be distinguishable from each other. Different types of calibrators have been used in cPCR, but in general those calibrators similar in size and base composition to the target work most effectively. RNA competitors should be used in quantitative RT-PCRs to address the problem of variable RT efficiency. This competitive amplification approach has also been used effectively with transcription-based amplification methods using RNA targets and RNA calibrators.

For cPCR, the concentration of the target template in the clinical sample can be determined by a simple calculation. The yield of the PCR product is described by the equation $Y = I(1 + e)^n$, where Y is the quantity of the PCR product, I is the quantity of the template at the beginning of the reaction, e is the efficiency of the reaction, and n is the number of cycles. In cPCR, this equation is written for both templates, as follows: competitor, $Y_c = I_c(1 + e)^n$; target, $Y_t = I_t(1 + e)^n$. Since e and n are the same for both the competitor and the target, the relative product ratio Y_c/Y_t directly depends on the initial concentration ratio I_c/I_t and the function $Y_c/Y_t = I_c/I_t$ is linear.

An alternative method of quantification is to run multiple concentrations of the calibrator in parallel with the reaction mixtures containing the target molecules. The signal generated from these external calibrators is used to generate a calibration curve, and the amount of the target in the original specimen is calculated based on comparison to the curve. The use of external calibration with the target amplification methods offers the advantage that the calibrators do not compete with the target for assay components. However, since the final amount of the amplified product depends on exponential amplification of the initial quantity of the template, minor differences in amplification efficiency may lead to very large and unpredictable differences in the final product yield (16). The

sample-to-sample differences may depend on sample preparation, nucleic acid purification procedures, the presence of inhibitors, and thermal cycler performance. For these reasons, simple quantitation of the amplified product and the use of external standard reference curves may not provide reliable quantitation of the template initially present in the sample.

Real-time amplification and detection methods are particularly well suited for quantification of nucleic acid because the amount of the fluorescent signal generated is proportional to the concentration of the target DNA or RNA in the original sample. Real-time PCR and transcription-based amplification methods are the most commonly used quantitative methods. For real-time PCR, the fluorescent signal is measured during the exponential phase of amplification, which is where the amplification plot crosses the threshold (Fig. 3). This is in contrast to standard PCR methods that measure the end point signal. There are advantages to measuring the fluorescent signal during the exponential phase of amplification; the reaction components are not limiting, and the assay is less sensitive to the effects of inhibitors. As a result, real-time PCR assays are more reproducible than standard PCR assays. Both internal and external calibrators can be used with real-time assays, but the improved precision of real-time assays allows more reliable results to be obtained with an external calibration curve than would be obtained with standard PCR. When external calibrators are used, a calibration curve is generated by plotting the $\log_{10}$ concentration of the external calibrator versus the C_T and this plot is used to calculate the concentration of nucleic acid in the sample. The concentration of nucleic acid in the sample is inversely related to the C_T: the higher the concentration of the nucleic acid, the lower the C_T (49). In general, quantitative real-time PCR assays are not more sensitive than standard PCR assays; however, they have a much broader linear range, typically 6 to 7 orders of magnitude.

The CLSI has published guidelines for quantitative molecular methods for infectious diseases that address the development and application of quantitative PCR assays and other nucleic acid amplification methods (84).

AUTOMATION AND INSTRUMENTATION

Molecular assays consist of three major steps: specimen processing, nucleic acid amplification, and product detection. Sample processing is usually the most labor-intensive step and has represented the biggest challenge for manufacturers of automated test systems. However, in the past several years there have been considerable advances in this area with the availability of both semiautomated and fully automated systems. Automation of the nucleic acid extraction process offers laboratories several advantages, including ease of use, limited handling of the sample, improved reproducibility, reduced opportunity for cross contamination, and for some systems, postelution functions such as adding samples into the master mix. These advantages need to be weighed against the costs of automated systems, the inflexibility of batch size, and the large sizes of many of the automated instruments. The systems vary in the types of nucleic acid extraction methods that they provide and include total nucleic acid, DNA-only, and RNA-only protocols. Other features of automated extraction systems to consider are the availability of protocols for various specimen types and volumes, variable elution volumes, the availability of target-specific and/or generic target extraction methods, and specimen throughput. The available automated systems range from fully automated high-throughput systems such as the MagNA Pure system (Roche) and *m*2000 generic extractor (Abbott) to those designed for a small number of specimens with random access capabilities, such as BioRobot EZ1 (Qiagen).

There are a few automated systems available for the conventional amplification methods, such the COBAS system (Roche) for PCR and the System 340 platform for bDNA assays (Bayer). Considerable advances in automation have been made with the availability of real-time amplification and detection systems.

Several instruments are commercially available for real-time PCR testing. These instruments vary as to speed, capacity of samples per test run, reaction volume, optics, and support for different fluorescence probe types. The time required for analysis depends to a great extent on the time required for thermocycling, and the speed of thermocycling depends on how quickly the instrument can change temperature over time. For example, some instruments can change the temperature at 20°C per s, permitting instrument analysis of up to 32 samples in as little as 30 min. Capacity may offset thermocycling speed. Although a higher-capacity instrument may have a longer thermocycling time than a lower-capacity instrument, potentially more samples may be analyzed by the high-capacity instrument in a specific time period than by the low-capacity instrument.

The reaction mixture volume assayed may also vary from one system to another. If target nucleic acid is present in extremely small amounts in a sample, an instrument that permits higher-volume analysis may be preferred.

Real-time PCR instruments utilize a variety of optics for fluorescence detection. A tungsten source lamp for excitation and selectable filters for excitation and emission wavelength detection are used in a number of instruments. Light-emitting diodes or laser excitation devices coupled with emission wavelength detection may also be used. The new real-time PCR instruments allow up to five different fluorogenic dyes to be used simultaneously in one reaction. Until recently, real-time PCR instruments were designed for research applications. The ABI Prism series of sequence detection systems (Celera), LightCycler (Roche), and SmartCycler (Cepheid, Sunnyvale, Calif.) are examples of research instruments that find widespread use in molecular diagnostics laboratories. The COBAS TaqMan analyzer (Roche) and the *m*2000 system (Abbott) are the first real-time instruments designed specifically for use in clinical laboratories (5).

Many manufacturers are coupling automated nucleic acid extraction instruments with amplification and detection systems to create high-throughput, fully automated nucleic acid analyzers. The TIGRIS system (Gen-Probe) (77), the AmpliPrep-COBAS TaqMan system (Roche), and the *m*2000 system (Abbott) are examples of fully automated and integrated systems designed to perform sample processing, nucleic acid amplification, and product detection. The TIGRIS system was developed for screening the blood supply for HCV and HIV RNA and can process up to 500 nucleic acid detection tests in 8 h. The GeneXpert system (Cepheid) represents the other end of the automation spectrum in which a single sample is added to a disposable fluidic cartridge that fully automates and integrates sample preparation, amplification, and real-time detection. The instrument is a random access design, raising the possibility of on-demand molecular testing.

PROTEOMICS

The term proteome was coined in 1994 to describe the set of proteins encoded by the genome (132). Proteomics, the study of the proteome, has come to encompass the identification,

characterization, and quantification of the complete set of proteins expressed by the entire genome in the lifetime of a given cell, tissue, or organism. Development of vaccines, therapeutics, and diagnostics directed at a particular infectious disease commonly requires analysis of organisms at the protein level. Genome level analysis of an organism, as valuable as that has been, is incomplete because only a fraction of the genome is translated into mRNA. Similarly, analysis of an organism at the mRNA level (transcriptome) may also be misleading because proteins translated from mRNA can undergo posttranslational modifications.

Two-dimensional gel electrophoresis and mass spectrometry are the tools used to define an organism's proteomic signature. Comparison of the proteomic signature of a reference state to a proteomic signature derived under experimental conditions or a disease state can reveal important information about the biology of the pathogen and the host's immune response to it (11, 54, 56, 127). Proteomic approaches have been used successfully to identify diagnostic markers for gastric disease caused by *Helicobacter pylori* (41), Lyme disease (55), severe acute respiratory disease (101), and tuberculosis (4).

The same approaches that are used to find biomarkers can also be used to develop rapid, sensitive, and high-throughput multimarker assays. The premise is to first establish composite fingerprint profiles for both disease and nondisease states and then use the composite profiles to make a diagnosis with an unknown patient sample. The development of chip-based protein differential display systems will facilitate the development of diagnostics based on protein expression analysis (33).

CURRENT APPLICATIONS

Molecular methods have created new opportunities for the clinical microbiology laboratory to impact patient care in the areas of initial diagnosis, disease prognosis, and monitoring of response to therapy. Over time the methods have become more automated, the cost of testing has decreased, and clinical utility has been proven for the diagnosis and management of a variety of infectious diseases. As a result, molecular testing is now routinely performed in many clinical microbiology laboratories and clinical applications will continue to expand in the future.

Initial Diagnosis

With the development of molecular methods, the clinical microbiology laboratory is no longer reliant solely on the traditional culture methods for detection of pathogens in clinical specimens. Culture-based methods have long been the gold standard for infectious disease diagnosis, but for several diseases, nucleic acid-based tests have replaced culture as the gold standard. Hepatitis C infection, enteroviral meningitis, pertussis, herpes simplex virus (HSV) encephalitis, and genital infections due to *C. trachomatis* are some examples of infectious diseases in which nucleic acid-based tests are the new gold standards for diagnosis. This technology has been used to best advantage in situations in which traditional methods are slow, insensitive, expensive, or not available. These techniques work particularly well with fragile or fastidious microorganisms that may die in transit or be overgrown by contaminating flora when cultured. *N. gonorrhoea*e is an example in which the nucleic acid can be detected under circumstances in which the organism cannot be cultured. The use of improper collection media, inappropriate transport conditions, or delays in transport can reduce the viability of the pathogen but may leave the nucleic acid still detectable. It is beyond the scope of this chapter to review all of the possible applications or to provide a compendium of methods for detection of various pathogens. The reader is directed to another excellent resource for this information (96).

Opportunities to actually replace culture for bacterial pathogens in routine practice are limited by the need to isolate the organisms for antibiotic susceptibility testing. In those applications in which culture has actually been replaced by nucleic acid testing, the pathogens are of predictable susceptibilities and, consequently, routine susceptibility testing is not performed, or the genetics of resistance are well defined and simple to detect, such as methicillin resistance in *S. aureus*.

Molecular methods have had the biggest impact in clinical virology in which the molecular approaches are often faster, more sensitive, and more cost-effective than the traditional methods. The diagnoses of enteroviral meningitis, HSV encephalitis, and CMV infections in immunocompromised patients are examples of clinically relevant and cost-effective applications of nucleic acid-based tests. There are greater opportunities to replace the conventional methods in virology than in bacteriology because the culture-based methods are costly and antiviral susceptibility testing is not routinely performed. In those situations in which antiviral susceptibility testing is required, such as identification of ganciclovir-resistant CMV, it may also be more amenable to molecular approaches. A limitation of molecular tests for viral diagnostics is the clinical need for simultaneous identification of multiple pathogens, for example, respiratory viruses. Multiplex PCR can be difficult to optimize, so detection of common respiratory pathogens may require multiple tests.

Perhaps the greatest impact of molecular methods has been in the discovery of previously unrecognized or uncultivable pathogens. During the past 15 years, a number of infectious agents were first identified directly from clinical material by using molecular methods**.** HCV, the principal etiologic agent of what was once known as non-A, non-B hepatitis, was discovered in 1989 through the application of molecular cloning techniques by investigators from the Centers for Disease Control and the Chiron Corporation (15). Cloning and analysis of the HCV genome led to production of viral antigens that now serve as the basis of the specific serological tests used to screen the blood supply and to diagnose hepatitis C. To date, HCV has resisted all attempts at sustained in vitro propagation. As a result, RT-PCR is used to detect, quantitate, and genotype HCV in infected individuals.

Tropheryma whippelii, the causative agent of Whipple's disease, is another example of an uncultivable microorganism which was initially identified by molecular methods (100). It was discovered by the use of broad-range PCR, in which primers are directed against conserved sequences in the bacterial 16S rRNA gene. Sequence analysis of the PCR product and comparison with known 16S rRNA gene sequences were used to characterize the organism and establish its disease association. This approach provides a new paradigm for discovery of unrecognized pathogens that is of value in other diseases with features that suggest an infectious etiology.

Recently, molecular methods were used to rapidly identify the etiologic agent of severe acute respiratory syndrome as a coronavirus (60, 95). Within a few months of the recognized outbreak, the virus was identified and sequenced and the molecular assays were developed that played an essential role in diagnosing the infection and defining the epidemiology of the infection.

Identification of Bacteria and Fungi by Nucleic Acid Sequencing

Nucleotide sequence analysis of the 16S bacterial rRNA gene has expanded our knowledge of the phylogenetic relationships among bacteria and is the new standard for bacterial identification. rRNA contains several functionally different regions, with some regions having highly conserved and others having highly varied nucleic acid sequences (133). The sequence of the 16S rRNA gene is a stable genotypic signature that can be used to identify an organism at a genus or species level. The 16S gene sequence can be determined rapidly and provides objective results independent of phenotypic characteristics. As discussed in the preceding section, it can also be used to characterize previously unrecognized species. A similar approach that targets the nuclear large subunit of the rRNA gene can be used for the identification of fungi (61). This gene is universally found in all fungi and contains sufficient variation to identify most fungi accurately to the species level.

The DNA sequencing approach to microbial identification involves extraction of the nucleic acids, amplification of the target sequence by PCR, sequence determination, and a computer software-aided search of an appropriate sequence database. The major limitations of this approach to microbial identification include the high cost of automated nucleic acid sequencers, the lack of appropriate analysis software, and limited databases.

Applied Biosystems (Foster City, Calif.) has developed ribosomal gene sequencing kits for bacteria and fungi. A sequence from an unknown bacterium is compared with either full or partial 16S rRNA sequences from over 1,000 type strains by using the MicroSeq analysis software (119). The software analysis provides percent base pair differences between the unknown bacterium and the 20 most closely related bacteria, alignment tools to show differences between the related sequences, and phylogenetic tree tools to verify that the unknown bacterium actually clusters with the 20 closest bacteria in the database. The MicroSeq fungal identification system is similar to the bacterial identification system but targets D2 large-subunit rRNA (42, 43). Continued improvements in automation, refinements of analysis software, and decreases in cost should lead to more widespread use of nucleic acid sequence-based approaches to microbial identification.

Disease Prognosis

Molecular techniques have created opportunities for the laboratory to provide important information that may predict disease progression. Probably the best example is HIV-1 viral load as a predictor of progression to AIDS and death in infected individuals. This predictive value was first demonstrated in 1996 as part of a multicenter AIDS cohort study (79). The investigators showed that the risk of progression to AIDS and death was directly related to the magnitude of the viral load in plasma at study entry. The viral load in plasma was a better predictor of disease progression than the number of $CD4^+$ lymphocytes. Subsequent studies have confirmed that baseline viral load critically influences disease progression.

Subtyping of certain viruses by molecular methods may also have prognostic value. Subtyping of respiratory syncytial viruses may provide information about the severity of infection in hospitalized infants, with those infected with group A viruses having poorer outcomes (130). HPV causes dysplasia, intraepithelial neoplasia, and carcinoma of the cervix in women. HPV types 16 and 18 are associated with a high risk of progression to neoplasia, and types 6 and 11 are associated with a low risk of progression (99). The clinical utility of molecular testing for high-risk HPV DNA has been established for managing women with the cervical cytologic diagnosis of atypical squamous cells of undetermined significance. Women with this condition can be referred for colposcopy based on the detection of high-risk HPV DNA (113). HPV DNA testing was recently approved by the Food and Drug Administration (FDA) for use as an adjunct to cytology for cervical cancer screening in women aged 30 years or more (135).

CMV viral load testing has recently been shown to be useful for deciding when to initiate preemptive therapy in organ transplant recipients and distinguishing active disease from asymptomatic infection. Qualitative CMV DNA PCR assays have been unable to distinguish patients with asymptomatic CMV infection from those with active CMV disease (36, 89). Recently, studies have shown that the level of CMV DNA can predict the development of active CMV disease. With the availability of standardized commercial assays, it is possible to establish viral load cutoffs for predicting the development of CMV disease and initiating preemptive therapy (1, 51). It is likely that quantitative assays will be useful in distinguishing disease from infection with other herpesviruses such as Epstein-Barr virus and human herpesvirus type 6.

Response to Therapy

Molecular methods have been developed to detect the genes responsible for resistance to single antibiotics or classes of antibiotics in bacteria and in many cases are superior to the phenotypic, growth-based methods. The detection of methicillin resistance in staphylococci, vancomycin resistance in enterococci, and rifampin resistance in *M. tuberculosis* provide examples of where molecular methods are used to supplement the growth-based methods (123). However, it is difficult to imagine, given our current state of knowledge of the molecular genetics of antimicrobial resistance and the technological limitations, that a genotypic approach to routine antimicrobial susceptibility testing of bacteria could rival the phenotypic methods in terms of information content and cost.

Molecular techniques are playing an increasing role in predicting and monitoring patient response to antiviral therapy. The laboratory may have a role in predicting response to therapy by detecting specific drug resistance mutations, determining viral load, and genotyping. Both viral load and genotype are independent predictors of response to combination therapy with pegylated interferon and ribavirin in chronic HCV infections (32, 78). Those patients with high pretreatment viral loads of >2 million copies/ml or with genotype 1 infections have poor sustained response rates. Both of these virological parameters are used in conjunction with other factors to determine the duration of therapy (83).

Quantitative tests for HIV-1 RNA are the standard of practice for guiding clinicians in initiating, monitoring, and changing antiretroviral therapy. Several commercially available HIV-1 viral load assays have been FDA approved, and guidelines for their use in clinical practice have been published (107). Viral load assays have also been used in monitoring response to therapy in patients chronically infected with HBV and HCV (32, 47, 67). In organ transplant recipients, the persistence of CMV viral load after several weeks of antiviral therapy is associated with the development of resistant virus (10).

LABORATORY PRACTICE

The unparalleled analytical sensitivity of nucleic acid amplification techniques coupled with their susceptibility to cross

contamination presents unique challenges to the routine application of these techniques in the clinical laboratory. There are special concerns in the areas of specimen processing, workflow, quality assurance, and interpretation of test results. Additional information can be found in the CLSI documents MM3-A2, *Molecular Diagnostic Methods for Infectious Diseases; Approved Guideline,* 2nd ed. (20), and MM13-A, *Collection, Transport, Preparation, and Storage of Specimens and Samples for Molecular Methods; Approved Guideline* (17).

Specimen Collection, Transport, and Processing

Proper collection, transport, and processing of clinical specimens are essential to ensure reliable results from molecular assays. Nucleic acid integrity must be maintained throughout these processes. Important issues to consider in specimen collection are the timing of specimen collection in relationship to disease state and the proper specimen type. Other factors that come into play include the use of the proper anticoagulant, transport and storage temperatures, and time to processing of the specimen. HIV-1 viral load testing is an example in which the proper conditions for specimen collection, transport, and processing have been well described and has provided insight into the importance of these factors. For HIV-1 viral load testing, the plasma needs to be separated from the cells within 6 h of collection to minimize degradation of RNA. Once the plasma has been separated, it can be stored at 4°C for several days, but −70°C is recommended for long-term storage (107). Most types of specimens are best stored at −20 to −70°C prior to processing.

Molecular methods have several advantages over conventional culture with regard to specimen collection. It may be easier to maintain the integrity of nucleic acid than the viability of an organism. Molecular tests for the detection of *C. trachomatis* and *N. gonorrhoeae* are an example in which DNA is stable on dry cervical swabs for a week at room temperature or refrigeration temperatures, which is in stark contrast to the conditions required to maintain organism viability for culture. Nucleic acid persists in specimens after initiation of treatment (35, 66), thus allowing detection of a pathogen even though the organism can no longer be cultured. Also, due to the increased sensitivity of molecular assays, it may be possible to test a smaller volume of specimen or use a specimen that is collected using a less invasive method.

The major goals of specimen processing are to release nucleic acid from the organism, maintain the integrity of the nucleic acid, render the sample noninfectious, remove inhibiting substances, and in some instances concentrate the specimen. These processes need to be balanced with minimizing manipulation of the specimen. Complex specimen processing methods are time-consuming and may lead to the loss of target nucleic acid or result in contamination between specimens. Care must be taken to avoid carrying over inhibitory substances, such as phenol or alcohol, from the nucleic acid isolation step to the amplification reaction.

There are several general methods for nucleic acid extraction. Different methods may be used depending on whether the desire is to purify RNA or DNA or both. Another factor to consider when deciding on a nucleic acid extraction method is the type of pathogen sought. Some pathogens, such as viruses, can be very easy to lyse, and mycobacteria, staphylococci, and fungi can be very difficult to lyse. Enzyme digestion or harsh lysis conditions may be required to disrupt the cell walls of these organisms.

DNA isolation methods often use detergents to solubilize the cell wall or membranes, a proteolytic enzyme, such as proteinase K, to digest proteins, and EDTA to chelate divalent cations needed for nuclease activity (7, 38). The lysate can be used directly in amplification assays, or additional steps may follow to purify the nucleic acid. These additional steps remove proteins and traces of organic solvents and concentrate the specimen. In order to successfully use a crude lysate, the target DNA must be present in a relatively high concentration and there must be minimal inhibitors of amplification in the sample. If these criteria are not met, additional purification steps should be used.

Another commonly used method of nucleic acid isolation involves disruption of cells or organisms with the chaotropic agent guanidinium thiocyanate and a detergent (14). After a short incubation, the nucleic acid can be precipitated with isopropanol. Guanidinium thiocyanate denatures proteins and is also a strong inhibitor of ribonucleases, making it a very useful method of RNA isolation, although it is also used for purification of DNA. The Boom extraction method is also based on the lysing and nuclease-inactivating properties of guanidinium thiocyanate but utilizes the acid-binding properties of silica or glass particles to purify nucleic acid (8). Over the past several years, various manufacturers have developed commercially available reagents using one of these basic methods or a modification of these methods. Many of these methods rely on the use of spin column technology, are easy to use, and provide a rapid, reproducible method for purification of nucleic acid from a wide variety of clinical specimens. In recent years, further advances have been made with the introduction of magnetic silica particles which are coupled with instruments providing various degrees of automation, thus further simplifying nucleic acid extraction and purification. These reagents tend to be expensive, but the additional cost can be offset by labor savings.

Tissue samples need to be disrupted prior to the nucleic acid extraction process. This can be accomplished by cutting the tissue into small pieces or mechanically homogenizing the tissue prior to proceeding with one of the above-described extraction methods. Preserved tissue specimens require removal of the paraffin with solvents and slicing into fine sections prior to processing.

Removing inhibitors of amplification is a key function of the nucleic acid extraction process. Simple methods of nucleic acid extraction that involve boiling of the specimen have been used for relatively acellular specimens such as cerebrospinal fluid (CSF). Though the boiling method is fast and easy, there are problems with inhibitors of amplification in CSF that are not inactivated by boiling (80). The inhibition rate can be reduced to <1% by using a silica-based extraction method. Similarly, crude lysates of urine and cervical swab specimens are commonly used for the detection of *C. trachomatis* and *N. gonorrhoeae*. Specimens containing amplification inhibitors have been reported to range from 1 to 5% for urine to as much as 20% for cervical swabs (104). Common inhibitory substances include hemoglobin, crystals, β-human chorionic gonadotropin, and nitrates. Blood samples are used commonly for detection and/or quantification of a variety of viral pathogens including HIV-1, HCV, and CMV. HIV-1 viral load testing is an example in which the effects of different anticoagulants have been well studied. HIV-1 viral RNA is most stable when collected in EDTA, and heparin has been shown to be inhibitory to amplification and should be avoided (6, 53). In addition, very small volumes of whole blood (1%) can be inhibitory to *Taq* DNA polymerase (48). Other compounds such as acidic polysaccharides, which are components of glycoproteins present in sputum and cervical specimens and bile salts found in stool,

can also inhibit polymerase (34). With the recognition of such a wide array of inhibitors of amplification and the availability of simple, reliable, semiautomated and automated nucleic acid extraction methods, the use of crude lysates for testing becomes more difficult to justify. Regardless of the nucleic acid extraction method employed, the laboratory should monitor inhibition rates for different specimen types and nucleic acid extraction methods (see "Quality Control and Assurance").

Contamination Control

Several types of contamination can occur with molecular testing: cross contamination of specimens during the nucleic acid extraction step, contamination of specimens with positive control material, and carryover contamination of amplified products. Contamination with amplified products can occur with DNA or RNA target amplification and with probe amplification methods. It does not occur with signal amplification assays since no nucleic acid molecules are synthesized with these methods. Cross contamination that occurs during specimen processing or handling of positive control material can occur with all amplification methods. The approach to the control of contamination due to amplified products has changed dramatically with the widespread use of real-time amplification and detection methods. Since the reaction tube is not opened after amplification, there is minimal risk of contamination from the amplified product. Many laboratories using real-time methods continue to use a variety of good laboratory practices to control for contamination, but the focus is on minimizing cross contamination between specimens rather than contamination from the amplified product. Refer to CLSI document MM3-P2, *Molecular Diagnostic Methods for Infectious Diseases; Proposed Guideline,* 2nd ed. (20), for a detailed description of good laboratory practices to minimize contamination.

Clinical microbiologists have long been concerned about minimizing contamination between samples with microorganisms during specimen processing. Molecular methods have raised the level of concern considerably, and for good reason, as current methods can detect a few molecules. The previously undetected low levels of contamination that occurred in processing specimens for routine culture can lead to false-positive results in molecular assays. Prevention of contamination due to target DNA is best done by careful handling of specimens to avoid splashing, opening only one specimen tube at a time, pulse-spinning tubes prior to opening, using screw-top tubes rather than snap-cap tubes to minimize aerosolization, bleaching work surfaces, and using plugged pipette tips. Some of these approaches can be difficult for high-volume laboratories, which is why automated extraction systems can be very useful. Care must be taken with these systems to ensure that there is no cross contamination during the automated process. This is often done by alternating negative and high-titer specimens in a checkerboard arrangement and monitoring for carryover of sample into the negative specimens. These experiments should be designed with an understanding of the concentration of the organism in the clinical specimen. For example, the concentration of HSV in CSF from patients with meningitis is quite low compared to the concentration of BK virus in the urine of a patient with nephropathy.

Preventing contamination of the laboratory with DNA from a clinical specimen or positive control material is very important, because eliminating contamination with target DNA once it occurs can be very difficult. This is why care should be taken to use a positive control at the lowest concentration that consistently amplifies. The enzymatic and photochemical inactivation methods used to control carryover contamination of amplified products are not effective in preventing contamination with target DNA.

Enzymatic inactivation of amplified product can be accomplished with uracil-*N*-glycosylase (UNG), a DNA repair enzyme found in a variety of bacterial species. During the PCR, dTTP is replaced with dUTP so that dUTP is incorporated into the newly synthesized DNA products. This allows for a distinction between starting template DNA and amplified products; only newly synthesized PCR products will contain deoxyuracil. If UTP-containing amplification products are present as contaminants, the addition of UNG to the reaction mixture will result in the cleavage of deoxyuracil residues, thus destroying the contaminating DNA (72). The use of UNG increases the amount of carryover DNA needed to contaminate the reaction mixture by several orders of magnitude (93). When UNG is used, it is important to keep the annealing temperature above 55°C so that the UNG remains inactive, thus avoiding degradation of newly synthesized product. For the same reason, after completion of amplification, the reaction mixture should be held at 72°C (125). UNG can be inactivated at 94°C, but prolonged inactivation at 94°C may also affect the activity of the polymerase enzyme. UNG will not remove uracil from RNA molecules and is therefore ineffective in controlling contamination in RNA amplification assays, such as TMA and NASBA.

When UTP and UNG are used, the PCR reaction conditions should be reoptimized as the magnesium requirement may increase. The efficiency of amplification may be reduced when UTP is substituted for TTP. This can be overcome by adding a mixture of dUTP and dTTP into the master mix. The efficiency of inactivation using UNG depends on the size of the amplified product and its G+C content. Inactivation may not be effective with amplified products of fewer than 100 bp, as maximum UNG efficiency requires the DNA molecule to be 150 bp (28).

Quality Control and Assurance

Verification and validation are terms that are often used interchangeably; however, they are very different processes. Verification is the process by which assay performance is determined; parameters such as sensitivity, specificity, positive and negative predictive value, and accuracy are established. The verification of an assay is completed before the assay is used for patient testing. Validation is the ongoing process of proving that the assay is performing as expected and achieves the intended results.

The verification of an assay includes analytical verification and clinical verification. The analytical verification provides information on the performance characteristics of the assay, and the clinical verification determines the clinical utility of the assay. Determining the clinical utility of a molecular assay can be difficult when the molecular assay is more sensitive than the gold standard. This situation was seen with the commercial assays designed to detect *C. trachomatis* in genital specimens. Molecular assays proved to be much more sensitive than the gold standard method of culture. An insensitive gold standard can make a molecular assay appear to have a falsely low specificity. In this situation, an expanded gold standard can be used. For *C. trachomatis,* this included direct fluorescent-antibody testing and/or another molecular method (35, 66, 109). There are additional challenges in determining the clinical utility of molecular assays that detect rare pathogens. These assays are usually laboratory developed, and any given medical center may see very few cases of

the disease, making clinical verification difficult. Moreover, standards and control material can be difficult to obtain for rare pathogens. Several companies now provide control material for the more common molecular assays such as those for *C. trachomatis*, *N. gonorrhoeae*, HIV-1, HCV, and CMV.

A positive control is designed to ensure that the test can consistently detect a concentration of target nucleic acid at or near the limit of detection of the assay. The positive control should be at the lowest concentration that can be reproducibly amplified. A positive control that is significantly greater than the cutoff of the assay may not detect small decreases in amplification efficiency. In addition, large amounts of target DNA can increase problems with contamination in the laboratory. Depending on the availability of material, the positive control may be purified nucleic acid or lysed or intact organisms. An extraction control tests the ability of the nucleic acid extraction or purification method to successfully free nucleic acid from the organism. The extraction control, which should be intact organisms, can also serve as a positive control if it is used at the appropriate concentration.

Monitoring for the presence of inhibitors in a specimen is important, particularly for complex specimens such as blood or sputum. Several methods can be used to control for inhibition. A commonly used method is to amplify two aliquots of a clinical specimen, one directly and the second spiked with an aliquot of positive control DNA. For a specimen to be considered negative for the target analyte, testing results for the direct specimen must be negative and those for the spiked specimen must be positive. If an inhibitor of amplification was present, the spiked specimen would be negative. The concentration of positive control used for the spike must be near the limit of detection of the assay to ensure that low-level inhibition of amplification is detected.

Another approach to monitoring for inhibition of amplification is adding an internal control to the clinical specimen prior to nucleic acid extraction. As discussed in "Quantitative Methods," the internal control molecule may be designed with the same primer binding sites as the target molecule but modified in some manner so as to allow detection separate from the target based on size or sequence. An internal control is an effective way to monitor for inhibition, but it may decrease the sensitivity of the assay due to competition for assay components. Amplification of a human housekeeping gene such as that for β-globin may also be used as an internal control, but the gene should not be present in vast excess of the target molecule or inhibition of amplification of the target molecule can occur without evidence of inhibition of the housekeeping gene. Inhibition controls should be included in assays that use a new specimen extraction method or specimen type. However, a cost-effective approach is to discontinue these controls once the inhibition rate is determined to be less than 1 to 2%.

Under certain conditions, there may be a need to determine if there is adequate nucleic acid in a specimen, for example, when using paraffin-embedded tissue or when evaluating the quality of a specimen. In these situations, amplification of housekeeping genes can be used to determine if the specimen contains human DNA. The absence of amplifiable human DNA from the specimen raises concern about whether the specimen quality is adequate.

Negative controls should be included in all assays and processed in a manner similar to the processing of the clinical specimens. The negative control should be taken through all steps of the assay, including the nucleic acid extraction process. However, no amplification in the negative control does not ensure that there is not contamination in the run, as contamination is often low level and sporadic. Including multiple negative controls in the run may provide additional assurance that there is no contamination, but this approach may be cost prohibitive. Ideally, the negative control should be a clinical specimen that does not contain the analyte of interest. These types of controls may be difficult to obtain, so water or buffer is often substituted.

Currently, the College of American Pathologists is the only Centers for Medicare and Medicaid Services-approved proficiency program for molecular testing for infectious diseases. The College of American Pathologists provides proficiency testing for many common pathogens for which routine testing is done in the clinical laboratory. The Quality Control for Molecular Diagnostics proficiency program, which is jointly sponsored by the European Society for Clinical Virology and the European Society for Clinical Microbiology and Infectious Diseases (Glasgow, Scotland, United Kingdom), also provides testing for a variety of pathogens. The Centers for Disease Control and Prevention also offer a model performance evaluation program for HIV-1 twice per year. When formal external proficiency testing programs are not available, laboratories may split sample testing with other laboratories, split samples between a new method and an established laboratory-developed method, or clinically validate the test result by clinical diagnosis. When exchanging specimens between laboratories for proficiency testing, it is important that both laboratories use the same method, particularly for quantitative methods, as viral load values will differ among the various assays.

Reporting and Interpretation of Results

The interpretation of molecular assays requires a basic understanding of the strengths and limitations of these technologies. There are unique problems in interpreting molecular testing results that are not routinely encountered with traditional microbiologic assays, such as culture and serology. Some of the problems that may occur in interpreting molecular assays include recognizing false-positive results, distinguishing viable from nonviable organisms, and correlating nucleic acid detection with the presence of disease.

For interpretation of a positive test result, the issues that need to be considered are assay specificity and contamination. The specificities of most molecular assays are established by the primers and probes used during amplification and detection steps; if they cross-react with other pathogens, then false-positive results are possible. For example, primers designed to detect *Mycobacterium pneumoniae* from respiratory specimens must not cross-react with organisms that are part of normal oral flora or other common respiratory pathogens, such as *Streptococcus pneumoniae*. Although uncommon, problems with primer specificity do occur; the primers designed to amplify the 5′ untranslated region of enteroviruses have been reported to cross-react with rhinoviruses (106). This would not be a problem for testing of CSF specimens but would preclude using the assay on respiratory specimens. Problems with primer specificity have also been reported for a commercially available PCR assay designed to detect *N. gonorrhoeae*. The primers used in this assay cross-react with *Neisseria subflava*, a nonpathogenic organism found in the oropharynx (29). False-positive results can also be due to contamination, which may occur during specimen processing or as a result of carryover contamination of previously amplified products.

The interpretation of a negative result requires consideration of assay sensitivity, specimen quality, nucleic acid extraction efficacy, and amplification efficiency. Problems with any of these factors can lead to a false-negative result,

which is why measures to control for each of these parameters should be included in assays whenever feasible. Another source of false-positive results is sequence variation, which may prevent binding of either primers or probes. To minimize this problem, one should perform a thorough search of known sequences before designing the assay and occasionally reexamine the available databases after the assay is put into clinical use.

Molecular assays detect pathogen nucleic acid but cannot determine whether that nucleic acid is found in a viable or nonviable organism. Pathogen nucleic acid can be detected for long periods of time after appropriate treatment is initiated. For example, *C. trachomatis* DNA can be found in the urine of patients for up to 3 weeks after completion of a course of therapy (35). Similar results have been reported for the detection of HSV DNA in the CSF of patients with encephalitis. DNA can persist for 2 weeks or longer after the initiation of acyclovir therapy (64). Due to the persistence of pathogen DNA after initiation of therapy, qualitative molecular assays should not be used to monitor response to therapy. One notable exception is the use of a qualitative HCV RNA RT-PCR assay to monitor the response to therapy with pegylated interferon and ribavirin. In this instance, the absence of detectable viral RNA from plasma is used to define treatment response (32).

The detection of pathogen nucleic acid does not ensure that the organism is the cause of disease. The organism may be present as part of the normal flora, as a colonizer of a particular area, or as a cause of infection. Distinguishing between colonization and infection may be more difficult when molecular techniques that are more sensitive than culture are used. Organisms present in very low concentrations, which may have gone undetected by routine culture methods, may be detected by using molecular techniques.

Distinguishing colonization from infection is easier when testing a specimen from a normally sterile site such as CSF or blood; however, this factor alone does not ensure that the organism is a true pathogen. This distinction is a concern with the detection of herpesviruses, which cause lifelong latent infections. An important example of distinguishing these two states is monitoring transplant recipients for CMV disease using molecular methods. Initial studies used very sensitive qualitative PCR assays (36, 87), and it was clear that CMV DNA could be detected in the blood of patients that never went on to develop symptomatic disease.

Reporting the results of a qualitative molecular assay is usually straightforward; results are often reported as DNA detected or not detected. Several key parameters that may also be reported are the limit of detection of the assay, data pertaining to the rate of inhibition for a given sample type, the gene target, and the amplification method used for testing. Reporting results from quantitative assays is more complex and requires consideration of several parameters including dynamic range, units, and precision. Results of quantitative assays can be expressed as copies, weight (nanograms or picograms), or international units of the target nucleic acid in a defined volume, such as milliliters of plasma or blood, grams of tissue, or number of leukocytes. When the results of quantitative assays are reported, the precision of the assays needs to be considered. For the currently available HIV-1 viral load assays, the assay and biological variability are approximately 0.5 $\log_{10}$ (107). Therefore, changes in viral load must exceed 0.5 $\log_{10}$ (threefold) in order to represent a biologically significant change in viral replication. For these assays, values should be reported as $\log_{10}$ rather than integers to avoid the overinterpretation of small changes in viral load. Quantitative assays have a defined linear or dynamic range. Values below the lower limit of quantification should be reported as less than the lower limit of the linear range, rather than as negative. Values above the upper limit of quantification should be reported as greater than the upper limit of the linear range. For values above the limit of detection but below the limit of quantitation, results may be reported as detectable, less than the lower limit of the linear range. For example, if the lower limit of quantitation of an HIV-1 viral load assay is 400 copies/ml, a value of 250 copies/ml could be reported as detectable, <400 copies/ml. Inclusion of the amplification method and specimen type in the report is particularly important for quantitative assays, as values from different assay types are not always comparable.

Regulatory and Reimbursement Issues

The medical needs for new molecular microbiology tests have exceeded the capacity of the diagnostic industry to provide FDA-cleared test kits to fill these needs. Table 1 lists the FDA-cleared nucleic acid-based tests for infectious diseases. Notably absent from the list are tests that have become a standard of care in a variety of diseases such as HSV encephalitis, enteroviral meningitis, and pertussis. Many laboratories have developed tests to fill these unmet needs. These laboratory-developed tests must be appropriately verified and validated as specified in the Centers for Medicare and Medicaid Services final rule for laboratory requirements, 42 CFR part 493 (88a). Such tests are eligible for reimbursement by Medicare and other payers if they are determined to be part of a standard of care or to be of proven clinical benefit.

Laboratory-developed tests often utilize a combination of reagents from different manufacturers, some of which are ASRs. ASRs are chemical substances, for example, antibodies or nucleic acid sequences, that are used in diagnostic tests to detect another specific substance in a specimen and are purchased from manufacturers under this label. ASRs do not include a protocol for use or information on analytical performance or clinical indication. The FDA requires a disclaimer on reports for laboratory-developed tests using ASRs, and it reads: "This test result was developed and its performance characteristics determined by [laboratory name]. It has not been cleared or approved by the U.S. Food and Drug Administration." This disclaimer was not intended to cover laboratory-developed tests not using ASRs or the off-label uses of FDA-cleared products.

A laboratory may want to include clarifying statements in the reports of results from laboratory-developed tests employing ASRs. These statements may point out that FDA clearance is not necessary for these tests and that they are used for clinical purposes. Additional information may include that the laboratory is certified under the Clinical Laboratory Improvement Amendments of 1988 to perform high-complexity testing and that pursuant to the requirements of the amendments the laboratory has established and verified the test's accuracy and precision.

Correct current procedural terminology (CPT) coding of molecular microbiology tests is essential to coverage and reimbursement by payers. In 1998, many analyte-specific codes for tests using direct probes, amplified probes, and amplified probes with quantification were established in the microbiology section of the CPT coding manual, and this list of available codes continues to expand (1a). Prior to 1998, molecular microbiology tests were billed using multiple-component CPT codes selected from the molecular pathology section of the manual. The introduction of analyte-specific codes has simplified the coding process and in many cases

TABLE 1 FDA-cleared or -approved molecular diagnostic tests for infectious diseases[a]

Test objective	Manufacturer (distributor)	Test name	Method[b]
C. trachomatis detection (single organism)	Digene Corp., Gaithersburg, Md.	HC2 CT ID	HC
	Gen-Probe, Inc., San Diego, Calif.	APTIMA CT assay	TMA
	Gen-Probe, Inc.	PACE 2 CT and PACE 2 CT probe competition assay	HPA
	Roche Diagnostics, Indianapolis, Ind.	AMPLICOR CT/NG test for *C. trachomatis*	PCR
	Roche Diagnostics	COBAS AMPLICOR CT/NG test for *C. trachomatis*	PCR
N. gonorrhoeae detection (single organism)	Digene Corp.	HC2 GC ID	HC
	Gen-Probe, Inc.	APTIMA GC assay	TMA
	Gen-Probe, Inc.	PACE 2 GC and PACE 2 GC probe competition assay	HPA
	Roche Diagnostics	AMPLICOR CT/NG test for *N. gonorrhoeae*	PCR
	Roche Diagnostics	COBAS AMPLICOR CT/NG test for *N. gonorrhoeae*	PCR
C. trachomatis and *N. gonorrhoeae* detection	Becton Dickinson and Company, Sparks, Md.	BD ProbeTec ET *C. trachomatis* and *N. gonorrhoeae* amplified DNA assay	SDA
	Digene Corp.	HC2 CT/GC combo test	HC
	Gen-Probe, Inc.	APTIMA combo 2 assay	TMA
	Gen-Probe, Inc.	PACE 2C CT/GC	HPA
	Roche Diagnostics	AMPLICOR CT/NG test	PCR
	Roche Diagnostics	COBAS AMPLICOR CT/NG test	PCR
Gardnerella spp. *Trichomonas vaginalis,* and *Candida* spp. detection	Becton Dickinson and Company	BD Affirm VPIII microbial identification test	Hybridization
Group A *Streptococcus* detection	Gen-Probe, Inc.	GASDirect	HPA
Group B *Streptococcus* detection	GeneOhm Sciences, Inc., San Diego, Calif. (Cepheid, Sunnyvale, Calif.)	IDI-Strep B assay	Real-time PCR
Legionella pneumophila detection	Becton Dickinson and Company	BD ProbeTec ET *Legionella pneumophila* amplified DNA assay	SDA
Methicillin-resistant *S. aureus detection*	GeneOhm Sciences, Inc. (Cepheid)	IDI-MRSA assay	Real-time PCR
M. tuberculosis detection	Gen-Probe, Inc.	AMPLIFIED *M. tuberculosis* direct test	TMA
	Roche Diagnostics	AMPLICOR *M. tuberculosis* test	PCR
Culture confirmation of *Mycobacterium* spp., various fungi, and other bacteria[c]	Gen-Probe, Inc	AccuProbe culture identification tests	HPA
CMV detection	Digene Corp.	HC1 CMV DNA test	HC
	bioMérieux, Inc., Durham, N.C.	CMV pp67 mRNA	NASBA
HCV detection	Gen-Probe, Inc. (Bayer HealthCare, Diagnostics Division, Tarrytown, N.Y.)	VERSANT HCV RNA	TMA
	Roche Diagnostics	AMPLICOR HCV test, version 2.0	RT-PCR
	Roche Diagnostics	COBAS AMPLICOR HCV test, version 2.0	RT-PCR
HCV quantitation	Bayer HealthCare, Diagnostics Division	VERSANT HCV RNA 3.0 bDNA assay	bDNA assay
HIV-1 drug resistance detection	Celera Diagnostics, Alameda, Calif. (Abbott Molecular Diagnostics, Des Plains, Ill.)	ViroSeq HIV-1 genotyping system	Sequencing
	Bayer HealthCare, Diagnostics Division	TruGene HIV-1 genotyping system	Sequencing

(Continued on next page)

TABLE 1 *(Continued)*

Test objective	Manufacturer (distributor)	Test name	Method[b]
HIV-1 quantitation	Bayer HealthCare, Diagnostics Division	VERSANT HIV-1 RNA 3.0 bDNA assay	bDNA assay
	bioMérieux, Inc.	NucliSens HIV-1 QT	NASBA
	Roche Diagnostics	AMPLICOR HIV-1 MONITOR test, version 1.5	RT-PCR
	Roche Diagnostics	COBAS AMPLICOR HIV-1 MONITOR test, version 1.5	RT-PCR
HBV, HCV, and HIV-1 screening for blood donations	Gen-Probe, Inc. (Chiron, Emeryville, Calif.)	Procleix HIV-1/HCV assay	TMA
	National Genetics Institute, Los Angeles, Calif.	UltraQual HCV RT-PCR assay	RT-PCR
	National Genetics Institute	UltraQual HIV-1 RT-PCR assay	RT-PCR
	Roche Diagnostics	COBAS AmpliScreen HBV test	PCR
	Roche Diagnostics	COBAS AmpliScreen HCV test, version 2.0	RT-PCR
	Roche Diagnostics	COBAS AmpliScreen HIV-1 test, version 1.5	RT-PCR
HPV detection	Digene Corp.	HC2 HR and LR	HC
	Digene Corp.	HC2 HPV HR	HC
	Digene Corp.	HC2 DNA with Pap	HC

[a] Information was current as of 15 August 2005. Modified from www.ampweb.org.
[b] HC, hybrid capture; HPA, hybridization protection assay.
[c] Mycobacteria include *M. avium*, *M. intracellulare*, the *M. avium* complex, *M. gordonae*, *M. kansasii*, and the *M. tuberculosis* complex. Fungi include *Blastomyces dermatitidis, Coccidioides immitis, Cryptococcus neoformans,* and *Histoplasma capsulatum*. Other bacteria include *Campylobacter* spp., *Enterococcus* spp., group A *Streptococcus*, group B *Streptococcus*, *Haemophilus influenzae, Listeria monocytogenes, N. gonorrhoeae, Streptococcus pneumoniae,* and *S. aureus*.

increased the reimbursement for molecular microbiology procedures, although there continues to be considerable regional variation in reimbursement rates for the codes. The analyte-specific codes cover all aspects of testing, including the interpretation of the test result, and the use of these specific codes precludes the use of the component codes.

Credentials

Staffing a molecular diagnostics laboratory with individuals who have an appropriate knowledge base and skill set remains a challenge. Until recently, molecular diagnostics was not part of the core curriculum in medical technology programs. However, the situation is changing, and the acquisition of credentials in this area is now available for medical technologists and technicians from the American Board of Bioanalysts, the National Credentialing Agency, and the American Society for Clinical Pathology. Laboratory directors may receive credentials in molecular diagnostics through the American Board of Bioanalysts (physicians and clinical laboratory scientists), the American Board of Clinical Chemistry (physicians and clinical laboratory scientists), and jointly through the American Boards of Pathology and Medical Genetics (physicians only).

FUTURE DIRECTIONS

Nucleic acid testing will continue to be one of the leading growth areas in laboratory medicine. The number of applications of this technology in diagnostic microbiology will continue to increase, and the technology will increasingly be incorporated into routine clinical microbiology laboratories as it becomes less technically complex and more accessible. More clinical and financial outcome data will be needed to justify the use of this expensive technology in an era of declining reimbursement and increased cost consciousness.

Despite its growth, molecular diagnostics largely remains a cottage industry, with the proliferation of tests developed by individual laboratories to satisfy new medical needs not met by the diagnostic test industry. As a result, one of the biggest concerns for the future is the development of effective proficiency testing programs that will help ensure that the results of these tests are reliable and reproducible among laboratories.

To a great extent, the future of molecular microbiology depends on automation. Many of the available tests are labor-intensive, with much of the labor devoted to tedious sample processing methods. Sample processing remains the greatest challenge to automation, but the recent development of fully automated systems for molecular diagnostics offers hope for the future. Perhaps the most exciting prospects for automation come from the biochip and microfluidics sectors. With the currently available technology, it is not difficult to imagine the development in the near future of a small chip that could automate several functions of the microbiology laboratory.

The use of multiplex nucleic acid-based assays to screen at-risk patients for panels of probable pathogens remains a goal for molecular microbiology. Success to date has been limited by technical difficulties, but the development of such assays is key to providing molecular tests with the same broad diagnostic range provided by culture and other conventional methods.

Advances in human genomics will be exploited in the future to develop tests for immunogenetic factors that may influence the risk of becoming infected with certain pathogens or the progression of disease. Human gene expression profiling with microarrays may be important in defining patterns of host gene expression associated with different pathogens or disease states. Better understanding of pathogen genomics, gene expression, and proteomics will lead to the discovery of new diagnostic and therapeutic targets.

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Susceptibility Testing Instrumentation and Computerized Expert Systems for Data Analysis and Interpretation*

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Commercial antimicrobial susceptibility testing (AST) systems were introduced into clinical microbiology laboratories during the 1980s and have been used in the majority of laboratories since the 1990s (46). Manual and semiautomated broth microdilution systems are utilized for small volumes of susceptibility testing, while larger laboratories often choose an automated broth microdilution system. Most AST systems also perform organism identification as described in chapter 15. Semiautomated systems available for the disk diffusion method are marketed primarily outside the United States. The AST systems include data management software that may be interfaced with a laboratory information system (LIS) and offer various levels of expert system analysis. Epidemiology and pharmacy software packages are also available.

The U.S. Food and Drug Administration (FDA) provides regulatory oversight for AST systems marketed in the United States. Susceptibility test systems are classified as class II medical devices (subject to general and special controls) and require premarket notification with a 510(k) submission for FDA clearance (30, 31). A 510(k) submission must demonstrate that a device is substantially equivalent to other devices marketed in the United States. The FDA recommends a multicenter comparison of an AST system to the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) reference method (16, 17). The level of performance considered acceptable for each antimicrobial agent-organism combination is >89.9% categorical agreement (same susceptible, intermediate, or resistant classification), >89.9% essential agreement (MIC results within 1 dilution of the reference method), ≤1.5% very major errors (VME, false susceptibility based on the number of resistant organisms), and ≤3% major errors (ME, false resistance based on the number of susceptible isolates) (31). Any antimicrobial agent-organism combination not meeting these standards must be listed as a limitation in the package insert with a recommendation to use an alternative method. Limitation statements are also required if the evaluation did not include a sufficient number of resistant organisms, showed unacceptable (<95%) reproducibility, or showed an elevated "no growth" rate (>10%) for an organism group (31). The FDA allows reporting of AST results only if the antimicrobial agent has known clinical efficacy against the organism (31). Current information describing FDA regulations and a list of approved devices can be found online (http://www.fda.gov/cdrh/consumer/mda/index.html).

This chapter will focus primarily on commercial susceptibility testing systems currently available in the United States. The broth microdilution AST systems are manufactured by four companies: bioMerieux (Durham, N.C.; http://www.biomerieux-usa.com), Dade Behring, Inc. (Sacramento, Calif.; http://www.dadebehring.com), Becton Dickinson Diagnostics (Sparks, Md.; http://www.bd.com), and TREK Diagnostic Systems (Cleveland, Ohio; http://www.trekds.com). Only one disk diffusion system, manufactured by Giles Scientific (Santa Barbara, Calif.; http://www.biomic.com), has FDA clearance. Readers should be aware that susceptibility testing system components are constantly changing in response to new technology and problems that are discovered.

SEMIAUTOMATED INSTRUMENTATION FOR DISK DIFFUSION TESTING

The advantages of the disk diffusion method of susceptibility testing include simplicity, reliability, low cost, and a high degree of flexibility in the selection of agents tested (46). Semiautomated systems available for reading and interpreting disk diffusion inhibition zones are listed in Table 1. For all systems, agar plates are manually inserted into an instrument after incubation for image acquisition and measurement of the zone of inhibition. Despite advances in imaging technology, a visual review of plates for faint growth or pinpoint colonies within the zone is recommended to assess the need for manual adjustment of the diameter measurement. Data management software determines the categorical interpretation (susceptible, intermediate, or resistant) and may be interfaced with an LIS. Although linear regression may be used to generate an MIC from a zone measurement, the validity of this method for some antimicrobial agent-organism combinations has been questioned (93). Expert system analysis and epidemiology software are available for the systems. The primary advantages of these instruments are (i) less variability in zone measurement (in comparison to caliper readings by different technologists), (ii) reduced transcription errors,

*This chapter contains information presented in chapter 15 by Mary Jane Ferraro and James H. Jorgensen in the eighth edition of this Manual.

TABLE 1 Overview of manual and semiautomated susceptibility testing instrumentation

Type	Features	Manufacturer(s)[a]	System	Reference(s)
Semiautomated disk diffusion	Assistance in reading, recording, and interpreting zones of inhibition; data management with expert and epidemiology software.	Giles Scientific i2a Oxoid Mast Bio-Rad	BIOMIC V3 SIRSCAN[b] Aura Image[b] Mastacan Elite[b] Osiris[b]	4, 33, 54 63, 66 1 97 5, 6, 52, 66, 74
Manual broth microdilution	Devices to facilitate visual interpretation, recording, and reporting.	Dade Behring Becton Dickinson TREK Diagnostics	MicroScan LabPro PASCO data management Sensititre SensiTouch	
Semiautomated broth microdilution	Automated devices read and report results after off-line incubation of tray or strip.	Dade Behring TREK Diagnostics bioMerieux	Microscan AutoSCAN-4 Sensititre AutoReader Mini API[b]	

[a] Bio-Rad, Hercules, Calif., http://bio-rad.com; i2a, Montleier, France; Mast, Bootle, United Kingdom, http://www.mastascan.com; Oxoid, Basingstoke, United Kingdom, http://www.oxoid.com; see text for other manufacturers.
[b] These systems are not currently available in the United States.

(iii) labor savings, (iv) improved data management capabilities, and (v) expert review to ensure correct reporting of results that are consistent with known resistance phenotypes. In general, these instruments provide reproducible and accurate results. In evaluations of the systems, organisms with faint growth accounted for most discrepancies (33, 63, 66).

MANUAL BROTH MICRODILUTION SYSTEMS

The manual broth microdilution systems listed in Table 1 facilitate the visual reading and recording of MICs. The panels are frozen (Pasco system, TREK custom plates) or dehydrated microwell trays (MicroScan, Sensititre). Devices for rehydration and inoculation of dehydrated trays include the manual RENOK device (MicroScan), the microprocessor-controlled Sensititre Autoinoculator, and the Sensititre multichannel electronic pipette.

After off-line incubation, results are recorded on the Pasco Reader with a light pen and transferred to a data management system. The MicroScan data management system (LabPro) displays an image of the tray configuration for recording manual results directly on the computer. The Sensititre SensiTouch reader sequentially illuminates a liquid crystal display superimposed on the tray to guide the reading of endpoints that are recorded via a keypad and transferred to the data management system (SWIN, Sensititre Windows software) with expert analysis. The SWIN data management system is also available for recording of manual results without the SensiTouch reader.

Most manufacturers offer standard gram-positive, gram-negative, *Streptococcus pneumoniae*, and extended-spectrum β-lactamase (ESBL) confirmatory panels. TREK Diagnostic Systems also offers FDA-cleared yeast panels (Sensititre YeastOne) and "research use only" Sensititre panels for mycobacteria (rapid and slow growers) and anaerobes that can be read manually or on the SensiTouch.

SEMIAUTOMATED BROTH MICRODILUTION SYSTEMS

The semiautomated broth microdilution systems listed in Table 1 utilize automated devices to read endpoints after overnight off-line incubation. The results are transferred to a data management system that may include expert system analysis using the same software available for the automated systems. The Sensititre AutoReader and miniAPI read susceptibility and identification tests, while the MicroScan AutoSCAN-4 reads only susceptibility results from overnight panels. Further information regarding MicroScan and Sensititre panels is presented in the section on automated systems.

AUTOMATED BROTH MICRODILUTION SYSTEMS

Automated AST systems do not require further manual intervention to obtain results after placement of the test panel in an instrument where incubation and reading of endpoints occur. An overview of the automated systems currently available in the United States is presented in Table 2. The VITEK 1, VITEK 2, MicroScan WalkAway, and Phoenix systems provide AST results after short-term incubation (<16 h); the currently available Sensititre ARIS panels and some MicroScan WalkAway panels require overnight incubation. Manufacturers should be consulted regarding the current antimicrobial agents available for each system.

VITEK 1

The first VITEK instrument developed for the provision of rapid MIC results was introduced in the 1980s. The identification of common gram-positive and gram-negative bacteria may be determined simultaneously by running a separate ID card. The AST panels are thin plastic 45-well cards with one to five concentrations of 15 to 19 antimicrobial agents. After manual preparation of the inoculum, cards are placed in a vacuum module for card inoculation. Cards are then manually lifted to a sealing device and placed in a carousel within the incubator-reader unit. A robotic system moves cards to a photometer every 15 min for turbidimetric measurement of growth. Linear regression analysis is used to determine algorithm-derived MICs that are reported in 4 to 16 h. The system includes a computer with monitor and printer, an expert system, and a data management system (DataTrac) to archive test data and generate reports.

TABLE 2 Overview of automated broth microdilution susceptibility testing instrumentation[a]

Manufacturer	System	Panel capacity	Panels	Types of panels (no.)	Instrument features	Software
Becton Dickinson	BD Phoenix	100	Two-sided polystyrene tray: 85-well AST/ 51-well ID	Gram pos (2), gram neg (8), *S. pneumoniae* (1)	AST panels available as MIC +/− ID substrates. Turbidimetric and redox indicator readings every 20 min up to 16 h. Full-range MICs.	BDXpert, BD EpiCenter
bioMerieux	VITEK 1	32, 60, 120, 240, 480	45-Well cards	Gram pos (6), gram neg (36)	Turbidimetric reading every 15 min. MICs derived from 1–5 antimicrobial agent dilutions.	DataTrac, Expert System, Stellara
	VITEK 2 VITEK 2 XL	60 120	64-Well cards	Gram pos (2), gram neg (9), *S. pneumoniae* (1)	Most automated system with reduced time for initial setup. Also automated AST dilution and filling/ sealing of cards. Turbidimetric readings every 15 min. MICs derived from 1–6 antimicrobial agent dilutions.	DataTrac, AES, Stellara
	VITEK 2 Compact	30 or 60	Same as VITEK 2	Same as VITEK 2	Less automated, more affordable than VITEK 2. Windows-based DMS and expert system has improved visual aesthetics.	Observa, AES, Stellara
Dade Behring	MicroScan WalkAway SI	40 or 96	Standard 96-microwell trays	Overnight (23), *S. pneumoniae* (1) Rapid (7), Synergies Plus (6)	Panels available as full-range MIC or breakpoint. Combo panels include ID substrates. MIC readings: ON, turbidimetric; "read when ready," colorimetric; rapid panels (4.5–15 h), fluorometric.	LabPro, LabPro Alert, PharmLink
TREK	Sensititre ARIS 2x	64	Standard 96-microwell trays	Gram pos (4), gram neg (6), *S. pneumoniae* (1)	Fluorometric readings after ON incubation of full-range MIC trays. Nonfermenter, *Haemophilus*/ *S. pneumoniae*, custom MIC panels also available.	SWIN

[a] Abbreviations: DMS, data management system; ID, identification; neg, negative; ON, overnight; pos, positive.

VITEK 2

The VITEK 2 received FDA clearance in 2000 and is the most automated AST instrument currently available. The cards are slightly thicker than VITEK 1 cards, with 64 wells that contain one to six concentrations of 9 to 20 antimicrobial agents. An identification card for common gram-positive or gram-negative bacteria may be run simultaneously with each AST card. The Smart Carrier Station includes a bar code scanner and base unit with microprocessor that holds a cassette with a capacity of 15 cards. A memory chip on the cassette allows the transfer of scanned information to the reader-incubator unit. The workflow for large laboratories can be optimized by placement of Smart Carrier Stations at multiple locations. After placement of a cassette into the VITEK 2 loading station, it is automatically moved through stations for bar code reading, AST dilution preparation, card inoculation, and card sealing. The transport system then places cards on a carousel with a 60-card capacity for incubation. The VITEK 2XL instrument has a capacity of 120 cards. Each card is moved to an optic station every 15 min for measurement of light transmittance that is proportional to growth. The Advanced Expert System (AES) is discussed in a later section.

The VITEK 2 Compact was introduced in 2005 for smaller labs and uses the same cards as the VITEK 2 system. The instrument is available in two sizes that accommodate 30 or 60 cards. The VITEK 2 Compact is less automated and more affordable than the VITEK 2. The initial inoculum and dilution preparation is manual, but a bar code reader replaces the hand labeling required with VITEK 1. Cards are initially placed into the vacuum unit (on the left side of the instrument) for filling followed by manual transfer to the right side of the instrument for automated sealing and transfer to the incubator-reader unit. The VITEK 2 Compact data management system (Observa) is PC Windows-based with better visual aesthetics and a more layered presentation of the same expert system (AES) analysis originally offered with the VITEK 2. Observa software may be used for preparing epidemiologic reports with data from VITEK 2 Compact and the manufacturer's blood culture instrument, BacT/ALERT. Observa software is expected to become available for VITEK 2 in 2006.

All of the VITEK systems connect to Stellara, a new system component launched in 2005 utilizing wireless technology to allow Health Insurance Portability and Accountability Act-compliant real-time communication of lab results to pharmacists and clinicians. The results are compared to patient records, allowing clinicians to be notified via a personal digital assistant of inappropriate antimicrobial therapy with suggested changes. Other lab results (chemistry or hematology) may also be reported with Stellara.

MicroScan WalkAway

The MicroScan WalkAway system was developed in the late 1980s and until recently offered two major types of AST panels: conventional panels read turbidimetrically after overnight incubation and rapid panels read fluorometrically after 4.5 to 15 h of incubation. These panels, all conventional 96-well microdilution trays, include (i) MIC panels (a broad range of antimicrobial agent dilutions); (ii) MIC combo panels (some wells used for identification); and (iii) breakpoint combo panels (identification with a limited range of antimicrobial agent dilutions for a categorical result of susceptible, intermediate, or resistant). A third type of MicroScan panel, Synergies plus, became available in 2005. Synergies plus combines three methods in one panel: "read-when-ready" AST results available as quickly as 4.5 h (colorimetric reading), overnight results for drugs requiring longer incubation (turbidimetric reading), and identification (fluorometric results within 2.5 h). Synergies plus gram-negative panels contain 19 to 25 antimicrobial agents; FDA clearance for the gram-positive panel is pending.

The WalkAway system includes an incubator-reader unit, a personal computer with an LIS interface, and a printer. "Prompt" is available for preparation of the inoculum for overnight panels without measurement of turbidity and is stable for 4 h. A manual device (RENOK) rehydrates and inoculates panels. The humidified incubator-reader unit has a bar code scanner, rotating carousel, and robotics to position panels under a central photometer or fluorometer for readings. An updated data management system, LabPro, interprets results, generates patient reports, and archives data to allow production of user-defined reports (antibiograms, trend analysis, and epidemiology reports). Since 2002, the data management system may be coupled with an expert system (LabPro Alert) that incorporates >100 rules that may be customized. Two instrument sizes accommodate 40 or 96 panels. A new SI version of the WalkAway instrument available since 1999 has eliminated the need for manual addition of identification reagents; older instruments may be upgraded to SI capability.

BD Phoenix

The BD Phoenix System was launched in Europe in 2001 and the United States in 2004. The instrument holds up to 100 test panels. The panels are polystyrene trays containing 136 wells divided into a 51-well identification (ID) side and an 85-well AST side with 16 to 22 antimicrobial agents. After preparation of the AST inoculum, a drop of redox indicator is added. The suspension is manually poured into the AST side of the panel, sealed with a plastic cap, and bar coded prior to placement on the instrument. The instrument reads panels every 20 min using both the colorimetric change in the redox indicator and turbidity to determine organism growth. Growth (metabolic activity) causes the redox indicator to change from an oxidized (blue) state to a reduced (pink) form. A full range of antimicrobial agent concentrations and a "growth" or "no growth" reading for each well allow the system to provide direct rather than calculated MICs.

The BDXpert system applies rules based on CLSI guidelines to analyze AST results. AST results will not be reported for an organism-antimicrobial agent combination if clinical efficacy is unknown, there are no approved MIC interpretive criteria, or the organism has intrinsic resistance. The BD EpiCenter is data management software for analyzing epidemiologic trends and generating reports using information from multiple BD instruments (Phoenix, BACTEC blood culture, and MGIT 960 systems). Features of BD EpiCenter include an LIS interface, reporting of inferred results for antimicrobial agents not on panels, and the capability to apply BDXpert system analysis to manual off-line AST results.

Sensititre ARIS 2x

The Sensititre ARIS (Automated Reading and Incubation System) was introduced in the United States in 1992 and provides overnight AST results (13, 14). The latest ARIS 2x version with hardware and software upgrades was released in 2004. The ARIS 2x instrument fits on the Sensititre

AutoReader and holds up to 64 plates (standard 96-microwell trays) available as MIC panels or separate identification plates. Plates are rehydrated and inoculated with the Sensititre AutoInoculator or a handheld multichannel electronic pipette before placement in the instrument's carousel. An internal bar code scanner identifies the plate type to assign the appropriate time of incubation. After 16 to 24 h of incubation, each AST plate is transported to the AutoReader for fluorometric reading of endpoints. The data management software (SWIN) provides expert analysis of results.

ADVANTAGES OF AUTOMATED SYSTEMS

Advantages of automated AST systems include labor savings, reproducibility, data management with expert system analysis, and the opportunity to generate results more rapidly. A workflow and performance evaluation of the VITEK 2 and Phoenix systems reported a longer mean setup time per isolate for Phoenix (3 min) than for VITEK 2 (1.5 min) but more monthly maintenance time for VITEK 2 (63.2 min) than for Phoenix (21.2 min) (24). The mean time to generate AST results for *Enterobacteriaceae* isolates was higher for Phoenix (11.7 ± 2.6 h) than for VITEK 2 (7.5 ± 1.3 h) (24). Ligozzi et al. reported that the time required for VITEK 2 AST for gram-positive cocci was 6 to 17 h, with 90% of results available as follows: 8 h, *S. aureus;* 11 h, coagulase-negative staphylococci (CoNS); 9 h, enterococci; 7 h, *Streptococcus agalactiae;* 9 h, *S. pneumoniae* (56). The average times required for AST results in a Phoenix study were as follows: for *Enterococcus* spp., 5.5 h; for *Staphylococcus* spp., 7 h; for *Enterobacteriaceae*, 6.5 h; and for nonfermenting gram-negative bacilli, 12 h (T. Wiles, D. Turner, W. B. Brasso, J. Hong, and J. Reuben, Abstr. 99th Gen. Meet. Am. Soc. Microbiol., abstr. C-94, p. 123, 1999). There are limited data showing financial and clinical benefits in association with the rapid provision of AST results. Doern et al. reported lower mortality rates and cost savings (fewer diagnostic tests and days in intensive care) associated with the rapid reporting of AST results (22). Barenfanger et al. also demonstrated reduced lengths of stay and cost savings for patients with rapid reporting of AST results that were attributed to earlier adjustments in antimicrobial therapy (2). Effective communication of the results to clinicians and pharmacists is essential to realizing the potential benefits of rapid testing. Communication may be enhanced by software packages that interface with medication records and alert clinicians or pharmacists when adjustments in antimicrobial therapy are needed.

DISADVANTAGES OF AUTOMATED SYSTEMS

Disadvantages of automated systems include a higher cost for equipment and consumables than manual methods, predetermined antimicrobial panels, an inability to test all clinically relevant organisms, and problems with detection of some resistance phenotypes (47). Reports of AST performance for detecting problematic resistance phenotypes are discussed below. The current performance of a system may not be accurately reflected by studies utilizing panels and software that are no longer available. A higher error rate should be accepted for evaluations using challenge strains with difficult to detect phenotypes than for studies that test populations of isolates usually encountered in the clinical laboratory.

Vancomycin Resistance in Enterococci

Problems with the detection of low-level vancomycin resistance *(vanB* and *vanC)* among enterococci by automated systems have been demonstrated in multiple studies (72, 89). The change from a 30-well to a 45-well VITEK 1 card in 1999 increased the testing of vancomycin from 3 wells to 4 wells and improved sensitivity for *vanB* isolates (26, 41, 67). A VITEK 2 evaluation reported difficulty detecting only *vanC2 (Enterococcus casseliflavus)* strains (94). Other VITEK 2 and Phoenix studies have demonstrated accurate detection of vancomycin-resistant enterococci, but rigorous studies comparing systems are lacking (27, 32). MicroScan overnight panel studies reported detection of all isolates except those containing *vanC,* which are difficult to detect for all AST systems since their MICs (4 to 16 μg/ml) span susceptible and intermediate categories (15, 19).

HLAR in *Enterococcus* spp.

The detection of high-level aminoglycoside resistance (HLAR) in enterococci by overnight and short-incubation AST systems has been improved by changes in growth medium and extended incubation (95, 98). Initial problems detecting high-level streptomycin resistance (HLSR) in MicroScan overnight panels appear to have been resolved after a broth reformulation, since two subsequent studies have demonstrated detection of HLAR that compared favorably to that obtained with reference and molecular methods (15, 65). A study reporting a higher VME rate for MicroScan detection of HLSR (19) may not have performed the recommended read at 48 h for isolates that appear streptomycin susceptible after overnight incubation; in one study that read improved detection of HLSR by 6.2% (15). Separate VITEK 2 and Phoenix evaluations testing different strains reported VME rates of 0 to 5.2% and ME rates of 0.9 to 7.3% for the detection of high-level streptomycin or gentamicin resistance (27, 32).

Oxacillin Resistance in Staphylococci

Most of the studies discussed below used *mec*A PCR as the "gold standard" when evaluating the accuracy of a system for detection of oxacillin resistance in staphylococci. Multiple studies have demonstrated excellent sensitivity and specificity of automated systems for detecting methicillin-resistant *Staphylococcus aureus* (MRSA) (27, 56, 73, 100). However, an evaluation focusing on low-level MRSA isolates reported problems detecting heterogeneous MRSA strains that are often undetected by routine oxacillin testing (29). After assessing the detection of heterogeneous oxacillin-resistant strains among *S. aureus* challenge organisms by eight methods including VITEK 1, MicroScan conventional, and MicroScan rapid panels, Swenson et al. concluded that no phenotypic system was totally reliable and suggested using several methods (86).

For the detection of oxacillin resistance among CoNS, VITEK 1, VITEK 2, and Phoenix evaluations have demonstrated excellent sensitivities (95.7 to 99.4%) with lower specificities (64.9 to 95.5%) (27, 37–39, 56, 61, 62, 80, 99). Isolates with false-resistant results often have MICs of 0.5 to 2 μg/ml that would have been considered susceptible under previous CLSI CoNS oxacillin breakpoints, which were lowered to ≤0.25 μg/ml in 1999 (38). Some of the major errors involved *Staphylococcus lugdunensis* isolates with oxacillin MICs now considered susceptible based on the CLSI 2005 decision to apply *S. aureus* breakpoints (≤2 μg/ml) to this species of CoNS (37, 38).

The lower CoNS oxacillin breakpoint is most accurate for detecting *mecA* carriage in *Staphylococcus hominis*, *Staphylococcus haemolyticus,* and *Staphylococcus epidermidis* isolates but may overcall oxacillin resistance for other CoNS species (40, 62, 69, 99). Current CLSI guidelines promote *mecA* or PBP 2a assays as the most accurate means of detecting oxacillin resistance and suggest the use of these assays for testing CoNS isolates (not *S. epidermidis*) with oxacillin MICs of 0.5 to 2 μg/ml causing serious infections (18).

Reduced Glycopeptide Susceptibility in Staphylococci

The inability of automated AST systems or disk diffusion to reliably detect vancomycin-intermediate and vancomycin-resistant *S. aureus* (11, 70, 90, 91) led to the current CLSI recommendation to supplement those methods with a 6-μg/ml vancomycin agar screening plate incubated 24 h (18). AST system detection of CoNS with reduced glycopeptide susceptibility is also unreliable (20, 70). Any staphylococcal isolate with a reproducible vancomycin MIC ≥4 μg/ml requires confirmation at a reference laboratory (18, 70).

Inducible Clindamycin Resistance

Routine testing of macrolide-resistant staphylococci and nonpneumococcal streptococci to detect inducible clindamycin resistance by use of a disk approximation test is recommended by CLSI (18). The inoculum purity plates from an automated broth AST system may be used for the induction test and should replace the practice of reporting all macrolide-resistant staphylococci or streptococci as resistant to clindamycin (49). An investigation of *S. aureus* isolates with VITEK 1 erythromycin-intermediate results showed that all were resistant by broth microdilution (87). VITEK 2 and Phoenix revealed accurate results (erythromycin resistant) for 96 to 100% of the isolates (87). Disk diffusion testing found that 95% of the isolates had inducible clindamycin resistance, but expert rules did not override the clindamycin-susceptible results for any of the systems (87). Use of the disk approximation test in nonpneumococcal streptococci is supported by the inability of VITEK 1 and VITEK 2 to accurately detect macrolide resistance phenotypes among group B streptococci (88).

Pneumococcal Resistance

The VITEK 2 *S. pneumoniae* panel represents the first commercial method for rapid AST of pneumococci. Initial testing of the VITEK 2 *S. pneumoniae* panel demonstrated provision of reliable results in a mean time of 8.1 h, with most errors classified as minor (35, 45). VITEK 2 detected 86.7% of 60 gatifloxacin-resistant and 95.6% of 23 moxifloxacin-resistant pneumococci without any major errors (48). A Phoenix pneumococcal panel recently received FDA approval. Overnight *S. pneumoniae* trays are available from MicroScan and Sensititre, with accurate results reported for antimicrobial agents other than trimethoprim-sulfamethoxazole (TMP/SMX), for which the discrepancies were attributed to trailing endpoints (36).

ESBL-Producing *Enterobacteriaceae*

Confirmatory ESBL tests that typically measure the inhibitory effect of clavulanate on ceftazidime and cefotaxime are available for all of the automated systems listed in Table 2. The first automated ESBL test was available for VITEK 1 and demonstrated reliable performance (99.5% sensitivity and 100% specificity) for organisms that are the most common ESBL producers *(Escherichia coli, Klebsiella oxytoca,* and *Klebsiella pneumoniae)* (75). A comparative evaluation of ESBL detection among multiresistant *E. coli* and *Klebsiella* spp., using three AST systems, revealed sensitivities of 83% (VITEK 1), 74% (VITEK 2), and 92% (Phoenix) with specificities of 82 to 85% (55). Evaluations of a single AST system (VITEK 2 or Phoenix) have reported sensitivities of 95.8 to 100% and specificities of 96.2 to 99.3% for ESBL detection (78, 79, 81). A recent evaluation of VITEK 1 and MicroScan ESBL confirmation tests reported sensitivities of 99 and 100%, respectively, and a specificity of 98% for both systems (58). The MicroScan ESBL confirmation overnight panels accurately detected ESBL-positive strains of *Proteus mirabilis, E. coli,* and *Klebsiella* spp. (53, 85). False-positive ESBL results for K1-hyperproducing *K. oxytoca* isolates have been reported for MicroScan (85) and Phoenix systems (78, 84).

Pseudomonas Resistance

Automated commercial AST systems are contraindicated for testing isolates of *Pseudomonas aeruginosa* from cystic fibrosis patients due to high VME rates and poor correlation with reference methods attributed to slow growth and mucoid strains (10). Problems with false-intermediate and false-resistant results for cefepime-susceptible *P. aeruginosa* isolates tested on VITEK 1 and MicroScan WalkAway systems have been reported (7). Problems with the VITEK 1 system overcalling *P. aeruginosa* resistance to piperacillin, ticarcillin-clavulanic acid, and cefepime led to software adjustments; a subsequent study reported acceptable piperacillin results, but elevated VME and ME rates for ticarcillin-clavulanic acid and increased minor errors with cefepime (12). Susceptibility testing of *P. aeruginosa* isolates by use of VITEK 2 identified only a small number of agents with categorical agreement <90% (cefepime, cefotaxime, and gentamicin) that were predominantly minor errors (50). A Phoenix study reported low categorical agreement for *P. aeruginosa* isolates primarily due to minor and major errors with β-lactams (23).

Other Gram-Negative Resistance

An assessment of ciprofloxacin resistance detection using a challenge set of *Enterobacteriaceae* isolates revealed elevated error rates for both MicroScan WalkAway and VITEK 1 systems (83). In evaluations testing different collections of gram-negative isolates with a single system (VITEK 2 or Phoenix), antimicrobial agents with low essential or categorical agreement reported by at least one study were aztreonam, cefotaxime, ceftazidime, cefepime, imipenem, piperacillin, piperacillin-tazobactam, ciprofloxacin, and TMP/SMX (25, 57, 81).

Outbreaks reported in New York City of infections by ESBL-positive *K. pneumoniae* organisms that also possess the carbapenem-hydrolyzing β-lactamase KPC-2 are cause for concern (9). A surveillance study identified three clinical labs that each failed to detect an ESBL/KPC-positive isolate with MicroScan WalkAway or VITEK systems; thus, an alternative method to confirm carbapenem susceptibility in *Enterobacteriaceae* that are resistant to third-generation cephalosporins may become necessary (9).

More common is the problem of false resistance when determining carbapenem susceptibility. The CDC could confirm only 8.9% of 123 *Enterobacteriaceae* and 74.2% of 325 *P. aeruginosa* isolates initially reported as imipenem nonsusceptible by 44 U.S. hospital laboratories during 1996–99 (82). Most isolates had been tested locally using VITEK or MicroScan, but retesting at CDC using the systems showed

minimal errors except for a 20% ME rate for VITEK 1 testing of imipenem against *P. aeruginosa* (82). The lack of a clear explanation for this overdetection of carbapenem resistance (82) emphasizes the need for confirmation of any unusual AST results. Testing of five *K. pneumoniae* VIM-1-producing challenge isolates by using VITEK 2, Phoenix, and MicroScan Autoscan-4 resulted in false carbapenem resistance determinations by VITEK 2 and Phoenix (34). Tsakris et al. reported a problem with VITEK 1 overcalling imipenem resistance for *Acinetobacter baumannii* isolates in Greece (92). Antimicrobial agent deterioration in test panels (96) and technical errors are factors that may contribute to the overdetection of carbapenem resistance by AST systems (82, 92). Results of false resistance obtained by VITEK 1 for *P. aeruginosa* and *Enterobacteriaceae* have been attributed to heavy inoculum density—particularly for agents active against the cell wall (piperacillin, mezlocillin, aztreonam, ticarcillin, ticarcillin-clavulanic acid, ampicillin-sulbactam, and imipenem) (21, 43).

COMPUTERIZED EXPERT SYSTEMS

Expert systems to assist in the critical review of AST results are available for all commercial susceptibility systems currently marketed in the United States. Expert systems can enhance workflow by identifying the subset of results that require human expert attention and may also improve the quality of AST results reported from smaller labs that may lack a human expert (77). By continuous monitoring, the algorithms allow more rapid recognition of incorrect results and more uniform reporting. However, the software must be frequently updated to reflect the emergence of new resistance and changes in reporting guidelines recommended by national organizations such as CLSI. Users must be aware of what rules and comments are activated in their system and work closely with manufacturer-provided specialists to customize the expert system for their laboratory. Ideally an expert system will report actual MICs with categorical interpretation before and after recommended changes.

Most expert systems use a rules-based approach focusing on AST results for only one drug at a time without considering results for other agents tested simultaneously. The VITEK 2 AES differs from rules-based expert systems by performing an "interpretive reading" that compares the MICs for multiple agents to a large database of known resistance phenotypes and MIC distributions for different species (59). The rationale for interpretative reading with phenotype assignment is that a single mechanism typically mediates resistance to multiple agents (60). An evaluation of the VITEK 2 AES assignment of β-lactam phenotypes for *Enterobacteriaceae* and *P. aeruginosa* provides insight into the process of phenotype analysis (76). The VITEK 2 AES also deduces the susceptibility of an isolate to agents not tested and detects inconsistencies between organism identification and AST results (77).

Excellent concordance of VITEK 2 AES interpretive reading with resistance genotypes has been reported in multicenter studies (3, 59). When AES was compared to human expert analysis of VITEK 2 results for 259 consecutive clinical isolates in a university-based microbiology laboratory, there was disagreement for only 5 of the 65 isolates (7.7%) with AES corrections (77). A limitation that has been noted for the AES is an inability to interpret multiple inconsistent results as being caused by a single problem (8, 77).

CRITICAL REVIEW OF AST RESULTS

Regardless of whether a laboratory is using a commercial expert system, it is important to be aware of unusual "resistant" (Table 3) and "susceptible" (Table 4) results that require verification of the organism's identification and repeat of the susceptibility test by the same or a different method (18, 60). An example of an unprecedented phenotype that should prompt retesting is an *Enterobacteriaceae* or *P. aeruginosa* isolate that appears more resistant to piperacillin-tazobactam than to piperacillin (51). There are a number of antimicrobial agents that may appear active in vitro but lack clinical efficacy (Table 5) and to which the organisms should be reported as resistant. The most recent CLSI M100 document should be consulted for current recommendations regarding agents to test for specific organisms, methodology, interpretive criteria, results that may be inferred without testing a specific agent, antimicrobial agents to report based on the site of infection, and unusual results requiring verification.

SELECTING AN AST SYSTEM

Factors to consider when selecting an AST system include cost, performance, workflow, data management capabilities, and manufacturer technical support (44, 64). Performance may be assessed by comparing dilutions of FDA-cleared antimicrobial agents and limitations (antimicrobial agent/organisms listed in package inserts that require an alternative method) of panels from different manufacturers.

TABLE 3 Resistance phenotypes that are rare or have not been detected

Organism	Antimicrobial agent(s) to which resistance requires verification
Gram positive	
Enterococcus faecalis	Linezolid, daptomycin, ampicillin
Enterococcus faecium	Linezolid, daptomycin, quinupristin-dalfopristin
S. pneumoniae	Vancomycin, linezolid, fluoroquinolone
Viridans group streptococci	Vancomycin, linezolid, daptomycin
Beta-hemolytic streptococci	Vancomycin, linezolid, daptomycin, ampicillin, penicillin, cephalosporins
Coagulase-negative staphylococci	Vancomycin, linezolid, daptomycin, quinupristin-dalfopristin
S. aureus	Vancomycin, linezolid, daptomycin, quinupristin-dalfopristin
Gram negative	
Enterobacteriaceae (all)	Carbapenem
Neisseria gonorrhoeae, N. meningitidis	Extended-spectrum cephalosporin
Haemophilus influenzae	Third-generation cephalosporin, aztreonam, carbapenem, fluoroquinolone

TABLE 4 Gram-negative organisms with expected resistance to commonly tested antimicrobial agents

Organism	Agent(s) to which organism is usually resistant
Enterobacteriaceae	
Citrobacter, Enterobacter, Klebsiella, Morganella, Proteus penneri, Proteus vulgaris, Providencia, Serratia, Yersinia	Ampicillin
Citrobacter freundii, Enterobacter, Morganella, Proteus penneri, Proteus vulgaris, Providencia, Serratia, Yersinia	Cefazolin, cephalothin
Klebsiella	Ticarcillin
C. freundii, Enterobacter, Serratia	Cefoxitin, cefotetan
C. freundii, Enterobacter, Proteus vulgaris, Serratia	Cefuroxime
Citrobacter, Enterobacter, Serratia	Amoxicillin-clavulanic acid, ampicillin-sulbactam
Non-*Enterobacteriaceae*	
Acinetobacter, Burkholderia cepacia, Pseudomonas aeruginosa, Stenotrophomonas maltophilia	Ampicillin, 1st- and 2nd-generation cephalosporins
Burkholderia cepacia, Stenotrophomonas maltophilia	Aminoglycosides
Stenotrophomonas maltophilia	Carbapenems
P. aeruginosa	Trimethoprim-sulfamethoxazole

TABLE 5 Antimicrobial agents that may appear active in vitro but lack clinical efficacy

Organism	Antimicrobial agents to which the organisms should be reported as resistant
Oxacillin-resistant staphylococci	All β-lactam agents including β-lactam/β-lactamase inhibitor combinations, cephems, and carbapenems
Enterococcus spp.	Aminoglycosides (other than high-level), cephalosporins, clindamycin, trimethoprim-sulfamethoxazole
ESBL-producing *E. coli, Klebsiella* spp., and *Proteus mirabilis*	Aztreonam, cephalosporins (except cephamycins), penicillins
Listeria spp.	Cephalosporins
Salmonella and *Shigella* spp.	Aminoglycosides, 1st- and 2nd-generation cephalosporins
Yersinia pestis	All β-lactam agents

Current users of systems should be consulted, and publications in peer-reviewed journals should be reviewed. Manufacturers should be asked if problems reported for particular antimicrobial agent-organism combinations have been resolved and what is under development. Extensive exhibits at the annual American Society for Microbiology general meeting provide demonstrations of systems and a convenient venue for interaction with manufacturer representatives. Poster presentations at national meetings may provide new information and the opportunity to interact with recent users of systems.

Another method of assessing the performance of AST systems is participation in proficiency testing surveys such as that of the College of American Pathology (42, 68), whose final critiques of susceptibility testing challenges provide information regarding AST methods used and problem antimicrobial agent-organism combinations with high error rates.

An AST system's ability to perform identification is also important because expert rules are linked to organism identity. Additional information regarding the selection of an AST system and laboratory verification of performance as required by the Clinical Laboratory Improvement Amendments of 1988 (28) is in the *Clinical Microbiology Procedures Handbook* (64).

SUMMARY

AST systems provide accurate and reproducible results for many antimicrobial agent-organism combinations. Expert analysis may improve workflow as well as the quality of reported results. The labor savings attributed to automated AST systems is particularly important for laboratories in regions with current or projected technologist shortages. In addition, the provision of more rapid AST results with a short incubation system may improve patient care and lower health care costs. Future advances in AST system development will likely increase their clinical impact with the incorporation of real-time PCR growth detection (71) or gene arrays for common resistance determinants that dramatically shorten the time required for results.

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Immunoassays for the Diagnosis of Infectious Diseases*

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Immunoassays are laboratory tests that employ antibodies as analytical reagents (8, 22, 24, 29, 30, 48). They have become increasingly used in the diagnosis of infectious diseases either as a primary means of diagnosis or as a confirmation of culture results. Overall immunoassays are specific, sensitive, and relatively inexpensive. They are used in all parts of the clinical laboratory. Due to the high specificity of the antigen-antibody reaction and the ease of use, immunoassays are leading the way for an increasing number of laboratory tests that are available at the patient bedside (point-of-care testing), in doctors' offices, and even in at-home testing. This chapter summarizes the variety of different assays available and their particular application in the field of infectious disease. The discussion emphasizes general assay design with important caveats relevant to test interpretation and development. Relevant examples are given as they relate to testing for particular infectious disease agents; however, for in-depth discussions, the reader is directed to the particular chapters on the specific agents.

HISTORY OF DEVELOPMENT OF IMMUNOASSAYS

Immunoassays have changed significantly over time with improvements in the types of antibodies and antigens available as well as improved detection systems (8, 22, 24, 27, 30, 48). With immunoassays, any analyte can be measured if an antibody can be raised to it or an antigenic form is available. The first immunoassays available measured milligram to microgram quantities of antibodies and relied primarily upon precipitation reactions between antigen and antibody. In the 1960s, the advent of radioimmunoassay (RIA) heralded techniques with greater sensitivity and greatly expanded the repertoire of analytes available for testing. By use of RIA, previously undetectable analytes were now easily available for testing in the clinical laboratory. The discovery of monoclonal antibodies led to assays with greater specificities and further expanded the repertoire of analytes available for measurement. Concerns about utilization of radioactivity and the desire for greater sensitivities led to the development of chemiluminescence (CL) immunoassays and use of the avidin-biotin detection system. Increasing automation of laboratory testing has expanded into the area of immunoassays, with many tests requiring only limited technologist input. As immunoassays have evolved, there has been increased utilization of various solid-phase matrices for adherence of either antigens or antibodies. Initially polypropylene test tubes were used. This has evolved to microtiter plates, and with the increased use of automated systems, smaller solid phases such as tiny disks or spheres are being increasingly used. Thus, immunoassays have significantly advanced in both the level of sensitivity detected and the breadth of their utilization, so they are now some of the most popular and most widely used of all laboratory tests.

DEFINITION OF TERMS

The array of terms used for immunoassays can be a confusing alphabet soup. This chapter discusses some widely used conventions in terminology; however, the reader may find some references in which the terms are used differently. Overall, most assays utilize the term "immuno" coupled with a second term which describes the type of assay or label used. For example, immunoprecipitation is an immunoassay utilizing a precipitation reaction. RIA is an immunoassay that utilizes radioactivity as the label. The term enzyme immunoassay (EIA) is a more general term that can be applied to any immunoassay which uses an enzyme label, although often EIA is used to refer to reagent-limited competitive type assays. The term enzyme-linked immunosorbent assay (ELISA) can also be used as a general term for any assay utilizing an enzyme label; however, it is most often used to refer to assays in which the antigen or antibody is adsorbed to a solid-phase matrix, often then employing a second enzyme labeled antibody, the so-called "sandwich" assay format. Immunometric is an additional term used and generally refers to any reagent excess assay. For the purposes of this chapter, the term EIA is used to refer to any assay using an enzyme, while ELISA refers only to solid-phase "sandwich"-type assays.

GENERAL CONCEPTS OF ASSAY DESIGN

There are a number of ways to characterize immunoassays. One useful classification scheme looks at the amount of label and reagent available (18). There are three major

*This chapter contains information presented in chapter 16 by Niel T. Constantine and Dolores P. Lana in the eighth edition of this Manual.

groups of immunoassays: label free, reagent excess, and reagent limited. The assays which are label free rely upon the ability of antigen and antibodies to bind and form a detectable agglutination or precipitation. There are many classic agglutination assays used in the diagnosis of infectious disease such as the Widal test for typhoid fever. The reagent excess methods require an excess of labeled antigen or antibody, use either one or two sites, and include immunoblotting and solid-phase ELISA. These are commonly employed immunoassays in microbiology today. Reagent-limited assays are competitive-based tests and employ a limited amount of either antigen or antibody and either require separation or are separation free. These include classic RIA and EIA and are less often used in diagnosis of infectious disease.

Another commonly used classification scheme looks at immunoassays as either heterogeneous (solid phase) or homogeneous (free-solution assays) (8, 24). Heterogeneous assays are ones in which the bound and free components must be separated, whereas homogeneous assays do not require a separation step. In addition, heterogeneous assays involve some type of solid phase to which the immunoreactants are attached. Homogeneous assays generally are free-solution methods. While this is a useful and commonly employed classification scheme, there are assays that do not strictly fit into this classification scheme. For example, agglutination assays and particle-enhanced light-scattering methods are considered homogeneous assays; however, the antibody is bound to a solid phase, and there is no required separation of the bound from the free components.

ASSAY INTERPRETATION

When choosing an assay for the laboratory and in-patient diagnosis, it is critical to understand the concepts of sensitivity, specificity, and predictive values (48). Sensitivity is the proportion of individuals *with* a disease that are correctly identified with a particular test. Sensitivity defines the true positives (TP), which are the number of patients with disease detected by the assay. Conversely, false negatives (FN) are the patients with disease who are not detected by the test. The formula for sensitivity is as follows: Sensitivity = $[TP/(TP + FN)] \times 100$. Specificity is the proportion of those *without* the disease that are correctly classified. Conversely, specificity is a measure of the true negatives (TN), which are the number of patients without disease not detected by the assay. False positives (FP) are the proportion of patients without disease who test positive. The formula for specificity is as follows: Specificity = $[TN/(TN + FP)] \times 100$. With a highly sensitive test, the majority of diseased individuals are picked up, and thus the number of false-negative results is very low. In contrast, with a highly specific test, the majority of individuals without the disease test negative, so the number of false-positive results is very low. When an assay is developed, the diagnostic cutoffs can be modified to alter both the sensitivity and the specificity. For example, if one moves the cutoff to a lower level, the assay sensitivity is increased with a resulting decrease in specificity. The optimal balance of these two components must be evaluated for each laboratory test and depends on multiple factors such as the utility of the test and the prevalence of the disease in the population.

The probability of having the disease, given the results of a test, is called the predictive value of the test. Positive predictive value (PPV) determines the percentage of patients with positive results who are diseased: PPV = $[TP/(TP + FP)] \times 100$; the negative predictive value (NPV) calculates the percentage of patients with negative test results who do not have the disease: NPV = $[TN/(TN + FN)] \times 100$. The predictive value of a test combines the prevalence of disease in a particular population with the sensitivity and specificity. Positive and negative predictive values are important because they assess the ability of a test to predict the presence or absence of disease in a patient from a particular population. In this context, the disease prevalence is a critical component. Prevalence is the proportion of the population with the disease in question. If a disease state has a low prevalence in the target population, a positive result will most likely be a false-positive result, whereas the opposite is true in a high prevalence population. A potential use of a high negative predictive value is that a negative test can exclude disease. In addition to the values discussed above, there are a variety of other statistical methods that can be used to evaluate laboratory tests such as odds ratio, receiver-operator curve analysis, and likelihood ratios, among others. It is beyond the scope of this chapter to discuss these, and the reader is referred to other sources for a more complete discussion (43).

SCREENING VERSUS DIAGNOSTIC ASSAYS

An important component of assay design is based upon the ultimate use of the test, i.e., whether it will be used as a screening or diagnostic test (37, 45). Screening tests are designed to pick up disease in asymptomatic individuals who may have early disease or precursors of disease, whereas diagnostic tests are performed for persons with specific indications of possible disease. However, the screening procedure itself does not diagnose the illness; those individuals with a positive result from the screening test need further evaluation with additional diagnostic tests. If the individual has a previous positive screening test, the diagnostic test acts as a confirmatory test. The ideal screening test should be both highly specific and sensitive; however, this may be difficult to achieve. As there is such a variety of screening tests available, there is not a particular sensitivity target value which is suggested; nevertheless, the sensitivity should be as high as possible without sacrificing specificity. It is not advisable to use a test with low specificity as a screening test, since many people without the disease will screen positive and potentially receive unnecessary diagnostic procedures. Moreover, for an effective screening test, the prevalence of disease in the population should be high; for if the prevalence of the disease is low, then a positive test will most likely be a false positive, leading to further unnecessary testing. Other considerations in regard to screening tests include weighing the cost of the test versus the impact of early detection. Overall, good screening tests should be easy to perform, inexpensive, and performed in high disease prevalence populations. In addition, early detection of disease should have a measurable impact on patient outcomes.

SEROLOGICAL ASSAYS

Traditionally, serological assays referred to the use of serum or plasma samples for the detection of antibodies to a variety of antigens. This concept has been broadened to refer to a variety of patient samples such as cerebrospinal fluid, urine, and other body fluids. In addition, it refers to the detection of both antibodies and antigen. There are a variety of clinical scenarios in which serological assessment is

the test of choice. For the identification of organisms for which culture is difficult or requires prolonged incubation, the determination of antibody titers or antigen detection can often give a quick answer. Although molecular techniques have sometimes supplanted serology in these situations, often cost issues make serology a more viable technique. There are frequent clinical situations where it is unnecessary to perform a culture if an antigen test is positive. One of the most common situations is the diagnosis of group A beta-hemolytic streptococcal throat infection. A rapid immunoassay for the detection of the group A streptococcal antigen is performed. If this is positive, then treatment can be instituted. Only when this quick test is negative is it necessary to perform a culture.

Basic Immunologic Reactions

In order to facilitate understanding of antibody titers, a brief review of basic immunologic reactions is provided (1, 2). Upon initial exposure to an infectious disease (primary antibody response), there are four phases in the subsequent response: an initial lag (or window) phase, when there is no antibody detected; a log phase, when the antibody titer increases in a logarithmic fashion; a plateau phase, in which the amount of antibody stabilizes; and a decline phase, during which the antibody is cleared or catabolized. The actual time course and ultimate maximum antibody titer depend on the antigen and the host. In the primary response, the initial antibody response is the production of immunoglobulin M (IgM), which usually appears after 10 days. The period after initial exposure, but before antibody is produced or is at sufficient levels to be detected, is called the "window period." This can vary, depending on the infectious agent, from as short as 10 days to as long as 6 months. IgG antibody production usually begins 10 days after exposure but is much less than the IgM response. As the IgM antibody level decreases, the IgG level increases, so that usually by the end of the first month, only IgG antibody is detectable. If there is a repeat infection with the same infectious agent, the kinetics of the response are different, with a lag phase of only 1 to 3 days, and IgG antibody is the primary isotype produced. In the months following antigen exposure, the IgG level reaches a plateau, and the antibody may remain detectable for life, even if there is no further exposure to the antigen. B lymphocytes utilize membrane-bound antibodies to recognize a wide array of antigens. In the case of infectious disease agents, the antigens are often expressed on the microbial surfaces. The particular parts of the expressed antigens that are bound by antibodies are referred to as epitopes; the strength of the binding of one epitope to one antibody is called the affinity. Upon repeated infection with a microbe, there is an increase in the strength of the antigen-antibody binding, a phenomenon called affinity maturation. However, depending upon the immunoglobulin molecule present, there are more than one antigen binding sites on each immunoglobulin (IgG, 2 sites; polymerized IgA, 4 sites; IgM, 10 sites); therefore, the total strength of the antigen-antibody binding is much greater than the affinity of a single interaction. This is called the avidity. Just as with affinity maturation, there is an increase in avidity with additional exposure to an antigen. Upon initial exposure to an antigen, the avidity of the IgG is low; upon secondary exposure, there is an increase in IgG avidity. Although there is exquisite specificity in the antigen-antibody reaction, there can be a spectrum of antibodies produced in response to a particular antigen; they can have differing affinities and avidities with a particular antigen and thus can be responsible for cross-reactions. Recognizing cross-reacting antibodies can be critical to assay specificity. Often, initial screening assays are set up with crude antigen preparations in which false-positive reactions can occur. Secondary confirmatory or diagnostic assays utilize purified and more expensive antigen preparations which confer greater specificity. Recognition of cross-reactivity is critical in tests used in infectious disease, because often organisms within the same genus and species share multiple antigenic determinants, making cross-reactivity a common problem. Although assays are designed to obviate these problems, laboratorians and clinicians should just be cognizant of the potential for cross-reactions.

Caveats in Serologic Interpretation

In general, a positive IgG titer means only that an individual has been exposed to a particular infectious agent and thus is "immune." For each infectious agent, the laboratory result is usually set up so that a positive result is the minimum amount of IgG antibody present which makes the individual "immune." For purposes of this discussion, the term "immune" is used; however, this does not necessarily mean that the level of antibody is protective against reinfection. The actual amount of antigen-specific antibody present in the serum of a particular individual is host determined and is controlled by immune-response genes which are products of the human histocompatibility system. The level of the IgG titer from a single serum sample to a particular infectious agent may not be used to determine if the infection is recent or remote. For example, person A may be a high responder to certain antigens and a low responder to others. Therefore, if a high titer of IgG is obtained for an individual, it may be tempting to think that this may represent a more recent exposure; however, this may indicate only that the individual is a high responder to that particular antigen. Therefore, a positive IgG titer establishes only that the individual has been exposed to a particular agent at some time in the past and has detectable IgG. In addition, the nature of the antigen is important, as some antigens are more effective than others in stimulating the immune system. Moreover, the ability of the immune system to respond to antigens can be affected by a variety of factors, such as age. For example, the very young may be unable to respond to certain types of antigens (e.g., carbohydrates) (1, 2).

Using serologic methods, there are several ways to determine if the infection is recent. The most useful and frequently used method is assessment of IgM antibody to a particular infectious disease agent. In general, a positive IgM titer to a particular organism is evidence of an active (i.e., recently acquired) infection with that agent. However, there are several considerations to keep in mind in the interpretation of this test. First of all, a positive IgM titer does not always mean that the infection is recent. There have been reports of persistent elevations of IgM antibody for a year or more. This has been seen with multiple organisms, including cytomegalovirus, *Mycoplasma pneumoniae,* hepatitis A virus, and *Toxoplasma gondii,* among others (9, 33, 45). Conversely, a negative IgM titer does not exclude a recent exposure. The amount of IgM may have been small and resolved quickly; thus, it was not detected at the time of the assay. The second way to establish a primary infection is to determine acute- and convalescent-phase titers. This requires drawing two sets of antibody titers: one set early in the exposure to the infectious agent and a second set 2 to 3 weeks later. Evidence of an acute infection can be confirmed if there is a fourfold increase in antibody titer between the first and second titers.

While this can sometimes provide information on the pathogenesis of a disease state, it requires at least 2 to 3 weeks for definitive results, thus obviating its use for early clinical management. Also, a false-negative reaction is not uncommon due to the fact that it requires drawing the specimen at a point low enough on the log part of the antibody response curve to obtain the required fourfold increase in antibody titer. Therefore, the lack of a fourfold increase does not rule out a primary infection.

At present, many assays report results as absorbance values or international units, making it problematic to apply the concept of a fourfold increase in titer. While it is possible to develop an approximate equivalency between titers and absorbance values, this has to be individually developed for each assay. One method to do this involves collecting multiple pairs of acute- and convalescent-phase sera to use as reference sera. They must demonstrate a fourfold increase in titer on traditional assays. They are then run on the EIA for the agent in question, and reference ranges are reported in optical density units. Assuming that the EIA provides distinctly different absorbance ranges to adequately separate acute- and convalescent-phase sera, pairs of test sera can then be run on the EIA, and the values can be compared to the reference ranges. While this can theoretically provide an adequate way to evaluate acute- and convalescent-phase titers by using the newer assay formats, it can have multiple problems. First of all, establishment of the reference range has to be performed separately on assays for each infectious agent, which can be expensive and time-consuming. Secondly, the lab has to have multiple sets of positive acute- and convalescent-phase paired sera for each organism tested, which can be quite difficult to obtain. In addition, depending on the EIA used and the range of the standard curve, titer values may not be easily converted into equivalent and meaningful absorbance values. Considering the cost and difficulties associated with this type of analysis, it is not generally recommended. Instead, it is preferable to test for the presence of an acute infection by using an IgM assay or an IgG avidity test (see below) or to directly test for the organism by using one of the increasingly available molecular techniques.

An additional test to perform, which can address some of the concerns with IgM testing and acute- and convalescent-phase titers, is an IgG avidity test (28, 33, 39). IgG antibodies produced early in infection have a low avidity, but a much greater avidity is seen with a secondary exposure. Using an avidity assay, in conjunction with the assessment of IgG and IgM antibody levels to a pathogen, can provide a much clearer indication of acute infection. Avidity tests are performed with a modification of the standard IgG EIA, in which IgG antibodies are exposed to a dissociating agent (usually high concentrations of urea). The serum IgG avidity is estimated by comparing the treated sample with one untreated. While this test can be quite useful, there are several caveats with its use. First of all, low avidity does not always mean that the infection is recent because low avidity antibodies can persist for months to years. In addition, there can be quality control issues in this testing with variability in test results related to the type of assay plates used, the antigens employed, and the type of dissociating agent used. This test has special utility in testing for some of the pathogens associated with pre- and perinatal infections (toxoplasmosis, rubella, and cytomegalovirus) (28, 33). For example, one algorithm suggested for prenatal toxoplasmosis testing follows all positive IgG antibody assays with an avidity test. If the avidity test is high, an acute infection is ruled out; however, if the avidity test is low, an IgM test is then performed. If the IgM test is positive, then a recent IgM infection is highly suspected. However, considering the implications for pregnancy, the FDA recommends that sera with positive IgM results obtained at a nonreference laboratory should then be sent to a toxoplasma reference laboratory for confirmatory testing.

SPECIFIC IMMUNOASSAYS

The spectrum of immunologic assays is discussed in detail in the following sections. Table 1 lists selected assays in order of relative sensitivities and provides approximate levels of detection.

Precipitation Reactions

When soluble antigens and antibodies are in equimolar concentrations, they bind and form insoluble antigen-antibody complexes which form a visible precipitate (24, 29). There are a number of laboratory tests available that utilize this reaction. Immunodiffusion is the simplest of the precipitation assays and involves putting the immune reactants in an inert semisolid material and then viewing the visible precipitation line. There are several variants of immunodiffusion. Radial immunodiffusion is designed to provide protein quantitation, whereas double immunodiffusion (Ouchterlony analysis) allows characterization of the relationship between different antigens. Overall, immunodiffusion reactions are simple to perform, easy to evaluate, and inexpensive and can be adapted to a variety of health care settings. The drawbacks include low sensitivity, as the level of detection is microgram quantities of antibody or antigen; requirements for relatively large amounts of antigens and antibody; and long assay times. Immunodiffusion is also routinely used for determination of antibody titers to a variety of agents, most commonly antifungal antibodies *(Coccidioides, Aspergillus, Histoplasma,* and *Entamoeba histolytica)*.

Agglutination Reactions

Agglutination reactions require a particulate antigen and its antibody with the resultant visible clumping as evidence of a positive reaction (21, 24, 29, 30). A test involving the particulate antigen which agglutinates the antibody present in the patient sample is termed a direct agglutination assay. To enhance the visibility of the agglutination reaction, an indirect assay format can be used, in which the antigen is coupled with a variety of particles that serve as an inert matrix. Various materials which have been employed include gelatin, latex, erythrocytes, polypeptides, and silicates. In addition, soluble antigen can be detected in a patient sample by absorption of a specific antibody to a particle; this is termed

TABLE 1 Sensitivity of immunoassays (34, 41)

Technique	Approximate sensitivity (per ml)
Precipitation, tube	100 mg
Immunodiffusion	1–3 mg
Agglutination	1 mg
Hemagglutination, passive	15–30 mg
Complement fixation	0.001 mg
Particle immunoassay	30–50 ng
EIA	<1 ng

reverse agglutination. Due to the large IgM molecule with its pentameric structure, IgM antibodies are several hundred times more efficient at agglutination than IgG and thus give more consistent and stable agglutination reactions. If the immune response involves primarily IgG antibody, the reaction may require some type of chemical enhancement or an antiglobulin reagent. Flocculation assays are another variant of agglutination assays, in which the particles are suspended. The most frequently used assays are the Venereal Disease Research Laboratory and rapid plasma reagin tests for syphilis.

Many agglutination assays, called hemagglutination assays, employ red blood cells (RBC) and use either a direct or an indirect assay format. Direct agglutination of RBC is commonly used in the blood bank for ABO typing. For infectious disease diagnosis, one of the most frequently ordered direct hemagglutination assays is the monospot test. This test detects the presence of a heterophile antibody which is produced in infectious mononucleosis and happens to spontaneously agglutinate equine RBC. The indirect hemagglutination assay is a commonly used format in which antigen is adsorbed to RBC, thus testing for the presence of specific antibody in the patient serum. Alternatively, the assay can be modified to test for antigen, in which case it is called reverse agglutination assay. For infectious disease testing, hemagglutination (especially indirect) is a popular assay format, as it is sensitive and simple to perform and does not require sophisticated equipment. For these reasons, it has been used in many third world countries for testing of a variety of infectious disease agents such as human immunodeficency virus (HIV), hepatitis virus (A, B, and C), and *Treponema pallidum.* There is a unique type of hemagglutination assay format used primarily in viral serology called hemagglutination inhibition. It is most commonly used for detection and quantitation of antiinfluenza antibodies. It is based on the principle that some viruses have surface proteins that will agglutinate RBC, so the assay uses the ability of antiviral antibodies in the patient sample to inhibit the spontaneous agglutination of the test RBC. The titer of antiviral antibodies is reported as the last dilution of the patient serum still able to inhibit the agglutination reaction.

Specialized types of agglutination assays that require optical counting are called particle immunoassays (8, 15, 16, 26). They involve primarily the measurement of scattered light which occurs upon the antigen-antibody reaction, and this is measured by either turbidimetry or nephelometry. They can be used for testing a wide range of proteins and analytes. Particle immunoassays are 3 orders of magnitude more sensitive than standard agglutination. One additional assay is the particle-counting immunoassay, which is used for quantitating haptens, antigens, and antibody. It is also available in a fully automated immunoassay format. In this assay, optical cell counting is employed, and there is an assessment of the decrease in agglutination following the immunoreaction. These assays are sensitive to a level of nanograms per milliliter. The patient sample is mixed with latex beads coated with antibody. As the antigen-antibody reaction occurs, the antigen particles are no longer dispersed in the solution; therefore, the antigen concentration is inversely proportional to the amount of antigen particles remaining in solution. Antibody can also be quantitated in this assay. The use of a particle-counting immunoassay has been reported for quantitation of hepatitis B virus surface antigen, along with quantitation of antibodies to hepatitis C virus, *T. pallidum,* and *T. gondii* (15, 16, 20).

Overall, basic agglutination assays are easy to perform and inexpensive and can be done in a variety of clinical settings such as the doctor's office, the emergency room, and the hospital bedside and in the field. They are performed either on a card, in tubes, or in microtiter plates. Often they provide only qualitative results, although an antibody or antigen titer can be obtained through serial dilutions of the sample. Direct assays continue to be performed for rare pathogens such as *Francisella* and *Brucella.* They utilize an inactivated source of the whole organism mixed with the patient sera. Although agglutination assays suffer from both limited sensitivity and limited specificity, they continue to be utilized because they are easy to perform and relatively inexpensive. If a more quantitative assay is needed, the assay can be adapted to light-scattering equipment such as a nephelometer to provide more quantitative and sensitive results. Overall the major drawback to direct agglutination assays is their limited sensitivity. They detect only to a level of microgram to milligram quantities of analytes per ml. However, greater sensitivities can be achieved with many of the variants of direct agglutination. For example, microtiter passive hemagglutination assays for infectious agents can achieve a sensitivity equivalent to that of a conventional EIA. The more sensitive hemagglutination assays for measuring antigen can measure as low as 15 to 30 μg/ml. If an agglutination assay is read visually, it is reported as a titer value. While these are fairly sensitive assays, titer values are always plus or minus one tube dilution, so that a titer of 16 could actually represent a titer of either 8 or 32. With latex-enhanced nephelometry or turbidimetry, sensitivity ranges in the area of 30 to 50 ng/ml.

There are several problems that can affect both sensitivity and specificity. The first problem affecting sensitivity is called the prozone effect (8, 24, 29). This refers to a lack of agglutination due to an excessive amount of antibodies in the patient sample. The high concentration of antibody inhibits agglutination, giving a false-negative result. This can be easily overcome by simply diluting the sample. In regard to specificity, the major concern is false-positive reactions from IgM rheumatoid factor (RF) (8, 11, 24, 29, 32). This occurs most commonly in assays in which the latex beads are coated with IgG antibody. This has been commonly reported for the latex agglutination test for cryptococcal antigen (24, 46). IgM RF, which is specific for the Fc portion of the IgG molecule, binds and gives a false-positive reaction. RF has also been reported to bind to other serum proteins nonspecifically, also giving a false-positive reaction. It is crucial that the clinician notify the laboratory if the patient has a known RF. There are several measures that could be taken. First of all, if a false-positive reaction is suspected, the sample result can be compared to the reaction using control particles coated with normal IgG. If this indicates that there is a false-positive reaction, the sample can be treated with a reducing agent such as 2-mercaptoethanol or it can be treated with pronase. Both of these treatments have been shown to reduce the majority of false-positive reactions due to IgM RF. Alternatively, the sample could be pretreated with aggregated IgG to remove the IgM RF; however, this can result in loss of antigen or specific antibodies and give a false-negative result. Also, an alternate test method could be used for assessment of the ordered analyte. Most importantly, communication of pertinent clinical information to the laboratory is critical to ensure the most accurate diagnostic information.

CF Test

Another traditional immunoassay is the complement fixation (CF) test, which is based upon the interaction of immune complexes with complement (29). As antigen-antibody complexes form, the complement cascade is activated and complement components are "fixed" or consumed. Conversely, if there is no antigen-antibody complex formation, there will be no activation of the complement cascade. This two-step test is primarily used to determine the titer of antibodies to specific antigens. For example, to set up a CF test for anti-*Mycoplasma pneumoniae* antibodies, patient serum would be incubated with *M. pneumoniae* antigen and a defined quantity of guinea pig complement. If the patient sample contains *M. pneumoniae* antibody, immune complexes will form and fix the complement. RBC coated with antierythrocyte antibodies are then added to the tube. The final readout for the assay is the release of hemoglobin from any lysed RBC. If the patient sample is positive for *M. pneumoniae,* then there will be no complement remaining, so there will be no release of hemoglobulin. The opposite will occur if the patient sample is negative for anti-*M. pneumoniae* antibody. Although CF assays are relatively sensitive and inexpensive, they can be technically demanding and time-consuming. Therefore, many of these assays have been converted to ELISA formats. However, a number of laboratories still use them as a confirmatory test for the presence of antibodies to a variety of infectious agents such as *Coccidioides, Histoplasma capsulatum,* adenovirus, herpesvirus, influenza virus, *M. pneumoniae,* and rickettsias.

Neutralization Assays

Neutralization assays are traditional laboratory tests used to determine if an antibody which can neutralize the infectivity of a particular virus is present (29). The classic assay involves mixing patient serum antibody samples with virus and then using this mix to inoculate either a cell line or a preparation of peripheral blood mononuclear cells. The readout involves either the assessment of viral cytopathic effect in the cell line or some other measure of viral replication, such as that obtained by a classic immunoassay of viral protein. For example, in the case of HIV testing, one can perform a p24 antigen test or reverse transcriptase assay and look for lower values. Evidence of decreased viral replication confirms the presence of neutralizing antibody. Although these assays are relatively simple, they can be expensive and can take days to complete. In addition, they can be difficult to standardize, especially when comparing results from different laboratories. To decrease the assay length, the quantitation of viral products can be assessed using PCR; however, this technique can be expensive and also difficult to standardize between laboratories. A blocking ELISA can also be performed, in which viral antigen and serum are mixed, after which a standard ELISA for virus is performed and the decrease in the amount of antibody detected is assessed. An additional traditional neutralization assay is a reverse passive hemagglutination, as previously discussed. In the field of HIV vaccine development, there is interest in developing new and better assays for neutralization, since it is crucial in the assessment of vaccine efficacy to demonstrate that a putative virus can initiate antibody production to prevent infection (35).

IFAs

The immunofluorescence assay (IFA) uses a histochemical technique to detect either antigen or serum antibody, utilizing a fluorescent-compound-labeled detector antibody (24, 29, 30). There are two types of IFA, direct and indirect (Fig. 1). Direct assays are used to detect the presence of antigens in tissue or body fluids. For example, to detect the presence of influenza virus in a nasal wash specimen, it is applied to a slide, and then it is overlaid with a fluorescent-compound-labeled anti-influenza antibody. If there is influenza antigen present, there is emission of fluorescent light, which is evaluated with a fluorescent microscope. Indirect assays are two-step tests used primarily for the detection of antibodies in serum or a body fluid. The patient sample is applied to a slide containing the target antigen; this is allowed to incubate, and specific antibody in the patient sample forms immune complexes with the antigen present on the slide. Any unbound reagent is then washed away, and the slide is overlaid with a fluorescent-compound-labeled anti-immunoglobulin. Positive staining is the emission of fluorescent light. Overall, IFAs are useful tests that are relatively easy to perform and inexpensive. In addition, they allow the localization of the antibody to a specific antigen location in the tissue. For example, the IFA for antibody to Epstein-Barr virus early antigen allows visualization of a specific pattern of staining of the virus-infected cell line. The disadvantages of this assay are that it is relatively time-consuming and requires both the purchase of an expensive fluorescence microscope and the presence of trained and experienced personnel for interpretation.

EIAs

EIAs are taking on increasingly more prominent roles in laboratory medicine (7, 8, 22, 24, 30, 47, 48). They are found in all areas of the clinical laboratory, in physicians' offices, and in at-home testing and are being increasingly used in molecular pathology laboratories. EIAs have taken the place of the RIAs in many laboratories, as they offer comparable sensitivity without the problems of disposal and the short half-life associated with radioactive materials. They are also replacing a variety of other techniques in the laboratory such as immunofluorescence and agglutination because EIAs provide greater objectivity, the potential to automate, and the ability to process large numbers of samples with less hands-on technician time. As a single unit of enzyme label can amplify a reaction product manyfold, many EIAs are optimized for detection at the pico- or attomole level. EIAs can be broadly classified as either homogeneous or heterogeneous assays. In homogeneous assays, the enzyme activity is altered as part of the immunologic reaction itself. In these assays, there is no requirement to separate the bound from the free immunoreactants. Although this technique is especially suited for the measurement of drugs and haptens, homogeneous assays

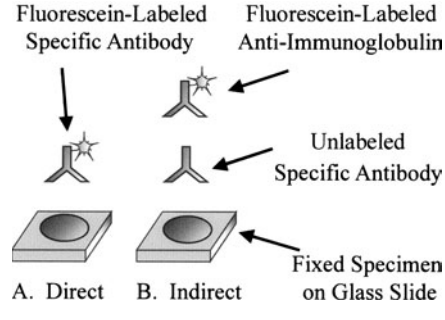


FIGURE 1 Direct and indirect immunofluorescence assays.

have not achieved widespread use in microbiology laboratories. In contrast, heterogeneous immunoassays are widely used in microbiology. In these assays, the enzyme activity of the labeled immunoreactant is not directly involved in the immunologic reaction itself.

The basic principle of the heterogeneous EIA is the use of an antibody or antigen conjugated with an enzyme which, upon reacting with its substrate, forms a measurable reaction product. Often a color reaction product is produced. The color change is monitored visually or with the use of a spectrophotometer to determine the proportionality between the amount of color and the amount of analyte present. An essential component of these assays is the separation of the bound enzyme-labeled component from the free labeled reagent. Assays can be competitive or noncompetitive and can be used to measure antigens or antibodies. The presence of all antibody isotypes can be quantitated depending on the specificity of the antibodies used. Whenever antibody or antigen is absorbed to the solid phase, the assay is referred to as an ELISA and also as a "sandwich" assay.

EIAs can be set up primarily as competitive or noncompetitive (7). Competitive assays most commonly measure antigens and are set up with either antibody or antigen on the solid phase. They are often termed "limited reagent" methods because the antigens and antibodies are used in measured and limited amounts. When the assay design uses specific antibody with which the solid phase is coated, the patient sample containing the putative antigen and the labeled antigen are added simultaneously and compete for binding to this matrix (Fig. 2). It is critical that the avidity of the antibody for both the labeled and unlabeled antigens be the same. In addition, a separate reaction is set up using enzyme-labeled antigen and buffer alone, which are added to the antibody-adsorbed solid phase. The substrate for the enzyme is added, and the color reaction is assessed. If the patient sample contains the antigen in question, it will effectively compete for binding to the solid phase, thus preventing any enzyme-labeled antigen from binding, thus giving no or minimal color. This reaction is compared to a reaction well to which buffer alone is substituted for the patient sample. The separation of the bound reactant from the free reactant is achieved through the washing steps. As is true with all competitive assays, the amount of labeled immunoreactant detected through the enzymatic reaction is inversely proportional to the amount of antigen present in the sample.

Antigens present in a patient sample can also be measured by using the coating of the solid phase with antigen. For this technique, the test sample containing the antigen in question is mixed with a limited amount of enzyme-labeled antibody. If the patient's sample contains the antigen, it will bind the labeled antigen, thus preventing this antibody from binding to the antigen with which the solid phase is coated. Following the washing step, the color reaction is developed and again, no color is seen if the sample contains the antigen. This technique can be modified by using unlabeled antibody in the first step and then adding a secondary enzyme-labeled anti-Ig.

Another variant of a competitive technique uses a two-step procedure. In the first step, test antigen is preincubated with its specific antibody. Any antigen-antibody complexes that have formed are removed during a wash step. Enzyme-labeled antigen (ag*) is then added to bind any remaining free antibody not bound by test antigen in the initial reaction. In the second step, beads coated with anti-immunoglobulin are added. These beads will bind any ag*-antibody complexes which formed in the previous step, and they can be quantitated in the pellet following centrifugation.

Compared to noncompetitive assays, competitive tests often provide greater specificity with less sensitivity; however, this is dependent on the affinity and purity of the immunological reagents and the design of the particular system. Competitive assays are ideal for measuring relatively small molecules which can be obtained in relative purity and in large enough amounts to be labeled with an enzyme. As they generally require small amounts of antibody, competitive assays are ideal for use in systems which have a limited amount of antibody available.

Noncompetitive ELISAs

The next major type of assay is the noncompetitive indirect solid-phase ELISA. This method is one of the most frequently employed immunoassays in the clinical laboratory. As with competitive assays, the two major variants involve using either antigen or antibody on the solid phase. When antigen is used for coating, specific antibodies in the sample bind to the solid phase and are detected with an enzyme-labeled anti-immunoglobulin secondary antibody (Fig. 3). Isotype-specific, enzyme-labeled anti-Ig antibodies can be used to determine the specific Ig class present. This type of assay is commonly used in the measurement of immune status to infectious agents and for autoantibody testing. A variety of solid-phase supports are used, including microtiter plates, nitrocellulose, and beads. One common variant, which uses nitrocellulose membranes, is the dot blot assay (12, 29, 40). In this system, the antigen or antibody is coupled to the membrane, and usually the reaction is assessed

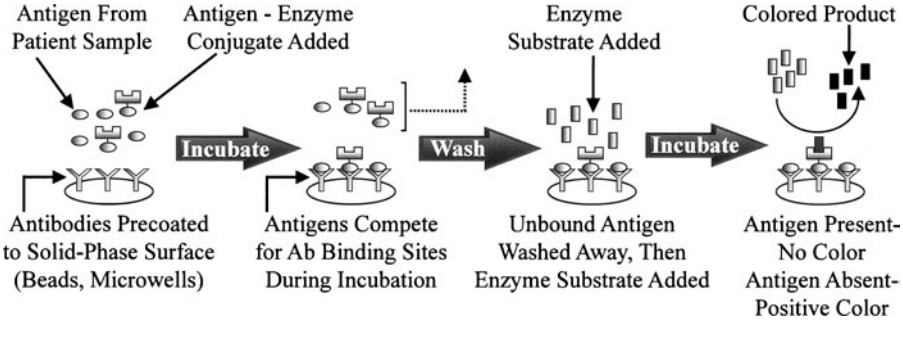


FIGURE 2 Competitive EIA.

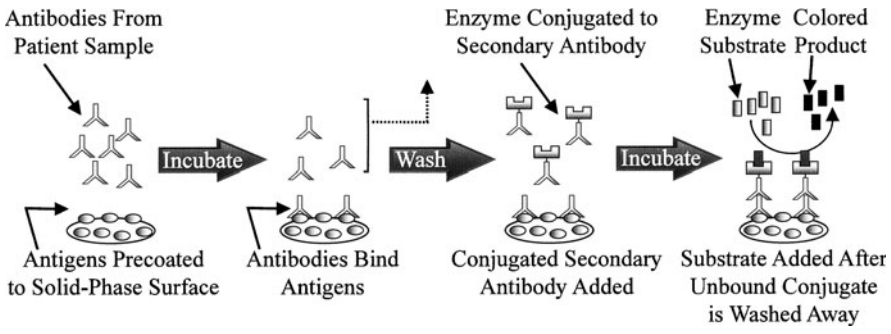


FIGURE 3 Noncompetitive indirect solid-phase ELISA.

visually by a colored reaction production, providing a qualitative assay. Many of the at-home testing kits (e.g., pregnancy kits) use a variant of this technique. This assay can be modified for increased sensitivity with such variants as can be made semiquantitative by using a densitometer for reading the color reaction.

When the antibody is coupled to the solid phase, these assays are often termed "capture" or "sandwich" assays, because the antigen in the sample is captured by an antibody-coated matrix. An enzyme-labeled secondary antibody directed to a different antigenic epitope is then added, completing the sandwich. There are numerous variations of this type of assay. The antigen captured can be an immunoglobulin, a viral protein, or any antigen that has at least two epitopes.

Noncompetitive ELISAs can also be modified by incorporating additional layers of immune reactants. This increases the sensitivity of the assay, but it also increases both the cost and time requirements. The most frequent application is the so-called avidin-biotin-peroxidase complex (ABC method) which can significantly improve the level of detectability. Biotinylated anti-Ig is generally used as the second antibody of the sandwich. This is then reacted with a preformed mixture of avidin and biotinylated peroxidase (ABC). The peroxidase can be developed with chemiluminescent reagents for increased sensitivity. Other variants include peroxidase anti-peroxidase methods and the incorporation of lectins as bridging molecules.

Microparticle Enzyme Immunoassay

The microparticle enzyme immunoassay is a variant of the ELISA that utilizes tiny beads (1-mm-diameter or less) that can be coupled with antibody or antigens (24, 28). The small size leads to a greater surface area for binding of antibody or antigen, which results in a decrease in reaction time. The particles act as the solid phase, but the reaction can be performed in suspension. These types of assays can be easily adapted to automated analyzers.

Analytical Interferences and Technical Issues

As in all laboratory tests, there are always factors that can affect test validity. Overall, immunoassays are affected more than routine chemistry and microbiology assays (6, 11, 22, 31, 32, 42, 47, 48). There are various clues that should alert one to the possibility of erroneous results. These include test results that are inconsistent with the clinical findings and/or an unexplained change in a test result from a previous assessment. These findings should prompt consideration of the possibility of technical issues or some type of test interference.

Plate Variability

There are several issues to consider with solid-phase microtiter plates that are often used for reagent excess sandwich-type ELISA (7, 47, 48). First, there can be variability between readings on adjacent wells of a microtiter plate. This variability is expressed as the well coefficient of variation, which should not be greater than 3 to 5% between wells. Secondly, there is the "edge effect," which refers to the variability between the readings on the outer wells of a microtiter plate and the readings on the inside wells. Although manufacturing variability in the plates must be evaluated as a possible cause, this occurs primarily due to differences in temperature between outer wells and interior ones. This can affect both the antigen-antibody and enzyme-substrate reactions. There are several ways to deal with the edge effect. One easy solution is to use smaller break-apart wells that can be placed in a larger plate. Simply being careful to protect the plate from exposure to light can also easily solve this problem. It is crucial that different plates from several manufacturers be screened for this effect when initially setting up an assay. In addition, when the lot of a plate is changed, the plates should be reevaluated to ensure that it is not necessary to modify any of the assay parameters.

Hook Effect

The hook effect refers to an unexpected fall in the amount of an analyte at the high end of the dose-response curve, resulting in a gross underestimation of the analyte (6, 11, 22, 31, 32, 42, 47, 48). This is particularly a problem in sandwich immunoassays with patient samples which contain an extremely high level of an analyte. The patient sample gives a low to moderately high result when using the standard assay dilution. However, upon further dilution of the sample, either the result is out-of-range high or, if it is diluted far enough, the sample gives an extremely elevated value. Therefore, if the laboratory ran the sample only at the routine dilution, a significant underestimation of the value would be reported.

The explanation for this phenomenon has not been completely established. Many investigators feel that it is caused by antigen excess, in which a majority of the antigen binding sites are filled, preventing completion of the sandwich. It has also been suggested to arise from low-affinity antibody, inadequate washing, and suboptimal concentrations of labeled antibody. Tests that are especially susceptible to problems with the hook effect include ones in which there may be samples with extremely high levels of the measured substance. These include IgE, human chorionic gonadotropin, tumor markers, ferritin, infectious antigens, and antibodies.

Numerous suggestions have been made regarding ways that laboratories can deal with the hook effect. One obvious strategy is to run all patient samples at 2 dilutions to screen for this problem. If the sample provides an answer with the first dilution, while the more dilute sample is out-of-range high, then the laboratory is alerted to the possibility of the hook effect. Although this is an effective approach, many laboratories are concerned about the time, cost, and labor involved in running 2 dilutions for every sample to avoid problems with only a small minority of patients. Thus, there are other strategies which can lessen the probability of the hook effect occurring.

First of all, always ensure that adequate washing is performed between all steps of the ELISA, especially between the steps following the addition of each antibody. Automatic plate washers are relatively inexpensive and can simplify this task. In addition, good communication with the kit manufacturers can also lessen the frequency of this problem. A number of companies have established the level at which the hook effect occurs and will readily share this information. Also, when completing new kit evaluations, testing specimens with high levels of the analyte is also crucial, as the frequency of the hook effect with different kits may be variable. Lastly, good communication with the clinician is also important; include discussion of the hook effect with a suggestion of notifying the laboratory when patients are expected to have very high levels of the analyte ordered.

Antibody Interference

There are a number of endogenous antibodies in patients' sera that may cause either positive or negative interferences in immunoassays (6, 11, 22, 31, 32, 42, 47, 48). There are multiple types of antibody interferences; they can be caused by antibodies binding to the actual analyte (e.g., antiviral antibodies), binding to components of the detection system (e.g., anti-alkaline phosphatase), and binding to reagent antibodies (e.g., anti-immunoglobulin antibodies). The last category is the most common and involves three types of antibodies. First, there are heterophile antibodies, which are weak antibodies to immunoglobulins from multiple species with no known or obvious identifiable immunogen. Secondly, RF can have a known effect on a variety of immunoassays and is most often found in patients with connective tissue diseases. Thirdly, there are various types of anti-animal antibodies; the most commonly reported are human anti-mouse antibodies (HAMA). Estimates of the prevalence of anti-mouse antibodies in normal sera vary greatly, from 0.5% to as high as 40%, depending on the sensitivity of the testing assay. There are both iatrogenic and noniatrogenic causes for the development of HAMA. In regard to iatrogenic causes, the culprit appears to be the increasing use of mouse monoclonal antibodies for therapeutic and imaging purposes. In regard to noniatrogenic causes, there are a number of suggested etiologies including environmental exposure to mice, maternal transfer across the placenta, passage of dietary antigens across the gut wall in inflammatory conditions, such as celiac disease, and association with a number of disease states, such as cardiomyopathy (25). While mouse anti-human antibodies can affect a variety of immunoassays, they are most often reported in two-site murine monoclonal antibody assays which often require only a small serum dilution. The presence of these antibodies can have a variable effect on immunoassays. If the analyte is present, they may cause either an over- or an underestimation. However, if the analyte is not present, a false-positive result may arise from the anti-mouse antibody cross-linking the two mouse monoclonal antibodies of the sandwich (the coating and conjugate antibodies). There have been a number of techniques advocated for decreasing the interference caused by heterophile antibodies and HAMA. These include heating the sample to 70°C, precipitating with polyethylene glycol, blocking with mouse IgG, and blocking with solid-phase anti-human IgG or mouse IgG. Caution should be observed in using heat treatment. The most popular method is the addition of nonimmune mouse immunoglobulin; however, the amount and source of the mouse serum may be crucial. Some studies with interfering anti-mouse antibodies demonstrated that the serum had to be from the same strain of mouse as the monoclonal antibody used in the assay. Therefore, it is recommended that a pool of mouse immunoglobulin from various strains be used to increase the probability of blocking as many patients' samples as possible. Most studies use approximately 10% mouse serum added to the reaction buffer; however, a few patient samples required a high concentration (>20%) of normal serum coupled with a long incubation time to correct the interference. To obviate problems with the majority of samples, laboratorians should consider routinely adding normal pooled heterologous sera to the dilution buffer of sandwich assays. In addition, special attention should be given to any sample for which the lab result is discordant with the clinical presentation, as this may represent a heterophile antibody resistant to the standard protocols.

Measurement of IgM

Quantitation of the IgM isotype of specific antibody poses special technical problems (7, 24, 28, 48). False positivity is common due to the presence of IgM RF in the patient sample. In addition, false negativity can occur from competitive inhibition of IgM binding in the presence of high levels of specific IgG. Previously, assays for IgM used a standard indirect solid-phase ELISA with the antigen immobilized and an IgM-specific secondary antibody. However, these assays were frought with the problems of false positivity and negativity. To obviate these problems, an IgM capture assay was developed (Fig. 4). In this procedure, a polyclonal anti-IgM antibody is bound to the solid phase. Upon incubation of the patient sample, all IgM is captured on the plate. The test antigen is then added, binding any specific IgM present on the plate. An enzyme-labeled secondary antibody is then added, and the reaction is completed. This assay obviates the problems with false-negative results due to competitive inhibition with IgM, as all the IgG in the patient sample is washed away in the first step. False-positive results, however, may still occur due to bound IgM RF reacting with either the IgG conjugate or binding any antigen-specific IgG in the sample. One way to avoid the problem with conjugate binding is to use $F(ab')_2$-conjugated capture antibodies. Alternatively, the assay can be modified to a direct technique by employing enzyme-labeled antigen in the second step, thus eliminating any Ig which could bind RF. Even with these modifications, problems can still occur with borderline and low positive IgM results. For this reason, all IgM-specific antibody results should be evaluated cautiously. As mentioned above, often running an IgG avidity assay can help in the evaluation of IgM results.

RIA

RIA was the original immunoassay technique and ushered in the area of improved and more sensitive immunoassays. Basically, all principles of assay design for EIA were based upon the experience gleaned from the RIA. Although RIA

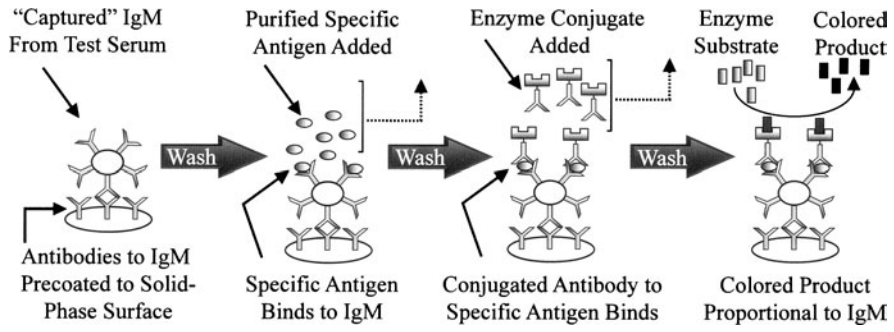


FIGURE 4 IgM capture assay.

is still a viable technique, it has largely been replaced by CL and EIA in most clinical laboratories. A variety of radioisotopes are utilized, most commonly ^{125}I, ^{3}H, and ^{14}C. Both CL and EIA offer more stable reagents and comparable to more sensitive detection limits, and they present no problems with hazardous waste disposal.

FIAs

The fluorescence immunoassay (FIA) uses fluorescence as the detection end point, and this method can be used in either homogeneous or heterogeneous assays (8, 24, 29). Fluorescence is the emission of photons of light as electrons go from an excited singlet state to the original ground state. The system requires a light source, excitation and emission filters, and a detection system utilizing photo multiplier tubes. A mercury lamp is the most frequently used light source, although xenon, halogen, and laser can also be used as excitation light. Fluorescein isothiocyanate and rhodamine are two of the most popular fluorochromes; however, there are a variety of other compounds used which have unique properties making them especially suited to a particular assay design.

There are numerous homogeneous assays which are performed in the liquid phase and do not require a separation of the bound from the free components. One popular homogeneous technique is the fluorescence polarization immunoassay. Some popular clinical analyzers utilize this methodology. This technique gives a measure of the bound/free ratio of the analyte without requiring a separation step. Polarization of light is measured by illuminating a sample with two polarizers in the same plane as the incident light and then at 90° to each other. The assay is based on an increase in light polarization which occurs when a fluorescent-tag antigen binds antibody and forms an immune complex. The labeled antigen is small and thus can rotate rapidly, causing depolarization of light. When the antigen-antibody complex forms, the increase in molecular weight causes a slower rotation and an increased emission of highly polarized light. This technique is primarily utilized for measurement of drugs and some hormones; however, it has utility for the detection of infectious disease. Its use has been described for the detection of antibodies to a variety of organisms such as gram-negative bacteria (*Brucella* spp. and *Salmonella* spp.) and equine infectious anemia virus (20, 44). There are a number of variants of both homogeneous and heterogeneous assays; however, it is beyond the scope of this chapter to discuss these and the reader is referred to other sources (24). FIAs have a number of advantages including high sensitivity and speed, and they are at least as sensitive as RIA. In addition, the reagents are stable and the assays are easily performed. For the fluorescence polarization immunoassay, one limitation is that the antigen used must be relatively small (i.e., with an MW no greater than 2,000) to allow a significant difference in the polarization when it forms an immune complex. Another important drawback in the use of fluorescent assays is the problem of autofluorescent compounds both in the patient sample and in the reaction mixture. This can be a significant problem in homogeneous assays where no washing steps occur and sample components are present during the entire assay. To circumvent this problem, samples can be treated with proteolytic enzymes, oxidizing agents, or denaturing reagents which will limit the amount of autofluorescence. In solid-phase assays, the majority of inferring substance will be washed away.

CL Immunoassays

The chemiluminescence (CL) immunoassay is a very popular technique which is widely utilized in many different assay formats (8, 24). Chemiluminescence is the emission of light which occurs when a substrate decays from an excited state to a ground state. In contrast to the fluorescence reaction which utilizes incident radiation for energy, chemiluminescence derives energy from the chemical reaction itself, which most often is an oxidation reaction. It is one of the most sensitive of all immunoassays, with detection limits down to the attomole (10^{18}) or zeptomole (10^{21}) level. CL substrates are used as the end point in both homogeneous and heterogeneous assays, in addition to their use in immunoblotting and multianalyte detection. Either chemiluminescence is used as a direct label on an antigen or antibody in a reaction which is catalyzed by adding a substrate, or a chemiluminescence compound is used as the substrate for an enzyme-labeled immunoreactant. The acridinium ester labels most commonly employed are derivatives of isoluminol and acridinium esters. The latter is a popular label which is the most sensitive and widely used. It can be conjugated to antigen and antibody by using standard techniques. Detection is relatively simple with the addition of sodium hydroxide and hydrogen peroxide. This reaction results in a flash of light which is read using a luminometer. In addition, the light signal can be captured on photographic film.

Western Blot and Immunoblot

Western blot and immunoblot are two solid-phase assays which combine the separation of proteins, using separation by denaturing gel electrophoresis followed by transfer to a filter (Fig. 5), and the determination of reactivity of the patient sera with the individually separated proteins, using a

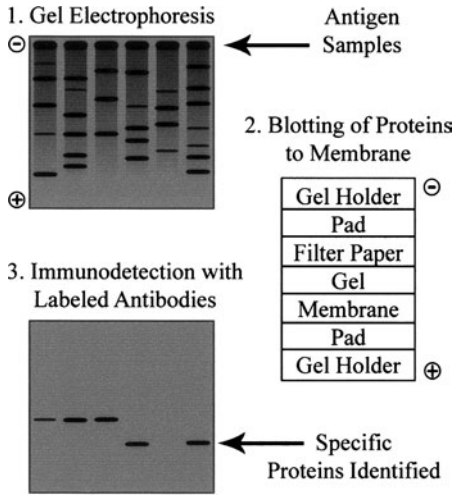


FIGURE 5 Western blot procedure.

typical sandwich-type ELISA. Immunoblotting utilizes a solid-support membrane filter containing antigens which are identified by a specific reaction with antibody. Most commonly, this technique is utilized for identifying the specific pattern of antibody to various infectious disease agents. One common application is for confirmation of antibody to HIV. Immunoblotting patterns can be read visually, using radiolabeled isotopes or using a CL substrate which is then developed on X-ray or photographic film or a charged-coupled device camera.

IPCR

The immuno-PCR (IPCR) is a novel technique that combines traditional ELISA with PCR (3, 4, 5, 11, 13, 27, 36). It uses antibodies labeled directly with nucleic acids (Fig. 6). It is an ultrasensitive technique which has been used for the detection of a variety of viruses. It has the advantage of being able to detect prion proteins where there is no nucleic acid present. In addition, it can detect viral proteins not associated with nucleic acids. This technique has been reported for ultrasensitive detection of a variety of infectious agents such as *Streptococcus,* HIV, and rotavirus, among others. In the case of rotavirus, it has been reported to detect as few as 100 viral particles/ml (versus 100,000 particles detected by ELISA). For detection of p24 HIV antigen, IPCR is a very sensitive test for determination of HIV type 1 viral load for p24 antigen. Although IPCR is a powerful technique, there can be technical issues when combining nucleic acids to proteins, and there can be problems with high assay backgrounds. An alternative approach which uses an indirect double-stranded DNA substrate for alkaline phosphatase has also been published (4). Overall, these highly sensitive methodologies represent the wave of the future, and there will be an increasing number of applications in infectious disease testing.

Rapid Immunoassays

The development of a multitude of rapid immunoassays has revolutionized diagnostic testing, for many tests that were previously available only in specialized or reference laboratories are now easily performed and can usually be completed in less than 30 min (10, 17, 29, 37, 38). There are a variety of formats utilized for these assays which can detect both antigens and antibodies along with products of nucleic acid amplification tests. One of the most popular formats is the lateral-flow immunoassay or immunochromatography. This has the advantage of being a one-step assay. One common format uses a chromatographic pad with three zones: sample application area, conjugate pad, and capture line (Fig. 7). The conjugate pad can use a variety of types of conjugates to generate a signal including colloidal gold, dye, or latex beads. The sample is applied to the sample pad and flows laterally by capillary action. Upon reaching the conjugate pad, if the analyte is present, it binds to the conjugate, forming an immune complex. The complex then continues to flow laterally along the pad by capillary action and is captured by the second antibody or antigen impregnated in the capture line. The presence of a colored line is a positive reaction. There is a positive control line also included in the test to make sure that the test was properly performed. There are variations to this assay format with systems which require no separate venipuncture, combining collection of fingerstick samples with testing in a one-step lateral-flow assay. Another frequently used type of rapid test is the dot blot immunoassay, which has been previously discussed. These rapid assay formats have been especially useful for HIV testing and have been used extensively in testing for HIV in underdeveloped countries. Overall, rapid assays are simple and easy to perform, can be used in field conditions, and can be performed by individuals with little training. While many are not as sensitive as conventional EIAs, there are reports of some with sensitivities approaching the traditional assays (10). Problems with rapid assays include the facts that they cannot be automated, that interpretation can be subjective, and that performance by individuals with no formal laboratory training can result in erroneous results. However, considering their overall advantages and low cost, their use will continue and be expanded as more analytes are adapted to this type of testing.

Automated Technologies

With the expansion of EIAs available, the immunoassay market of automated analyzers and the repertoire of tests available have exploded. Recently, 36 immunoassay analyzers that can run a wide spectrum of tests important in infectious disease testing were profiled (14). The machines run the gamut of serological assays (both antigen and antibody determinations) for infectious disease including HIV infection, hepatitis (A, B, and C), *T. gondii* infection, cytomegalovirus infection, rubella, and *Chlamydia trachomatis* infection, among others. Many of the systems listed are walk-away machines, requiring only limited technologist input. There are also robotic systems available which are cost-effective for even moderate-size hospital laboratories. For detailed information about each analyzer, the complete table can be downloaded from the Internet (http://www.cap.org).

An exciting new area is the development of multiplex immunoassay systems (19, 23). These are laboratory instruments which combine several technologies and allow rapid and simultaneous tests of multiple analytes in a single sample. There are many different assay designs which combine an array of technologies including CL, FIA, flow cytometry, and molecular diagnostics (PCR and use of oligonucleotides and nanoparticles). As these assays provide rapid results and can be performed with very small sample sizes, they have wide applicability to epidemiological studies and vaccine trials. These assays are also especially suited to the assessment of multiple biological agents in a variety of samples.

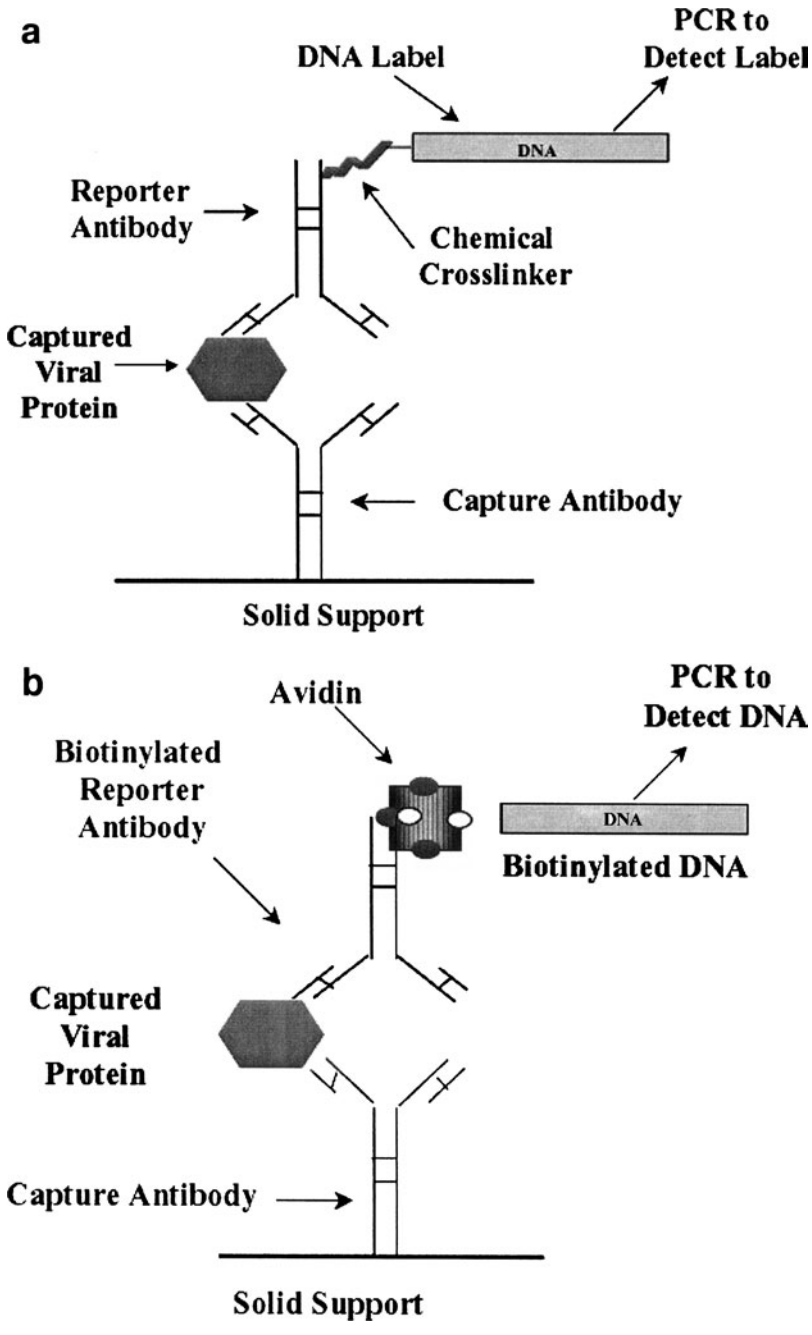


FIGURE 6 Principle of immuno-PCR. (a) DNA directly conjugated to reporter antibody; (b) DNA conjugated via an avidin-biotin reaction.

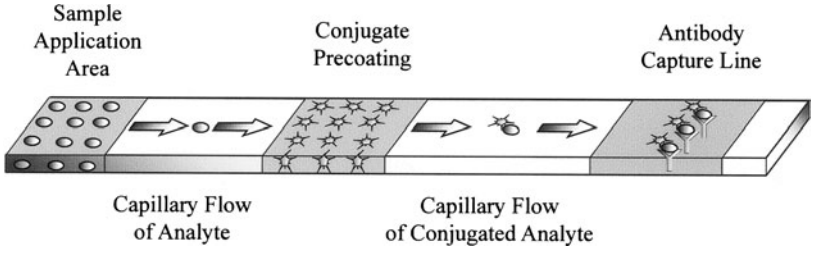


FIGURE 7 Lateral-flow immunoassay.

With this advent of extensive tests easily performed by automated systems, the potential for both improper test utilization and incorrect interpretation of results will increase. This results in even greater pressure on laboratorians and infectious disease specialists to provide information to both their colleagues and patients about the proper use and interpretation of laboratory tests for diagnosis of infectious disease agents.

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BACTERIOLOGY

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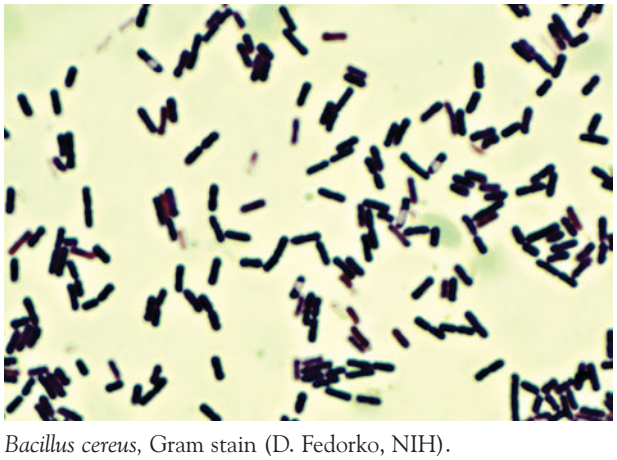
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Taxonomy and Classification of Bacteria

PETER A. R. VANDAMME

19

Taxonomy is written by taxonomists for taxonomists; in this form the subject is so dull that few, if any, non-taxonomists are tempted to read it and presumably even fewer try their hand at it. It is the most subjective branch of any biological discipline, and in many ways is more of an art than a science.

With these words, S. T. Cowan introduced a sparkling essay on the sense and nonsense in bacterial taxonomy in 1971 (18). His contributions to the practice of bacterial taxonomy, written in the 1960s and 1970s (15–18), should be read by everyone interested in this field. Taxonomy is generally considered a synonym of systematics and is traditionally divided into classification (the orderly arrangement of organisms into taxonomic groups on the basis of similarity), nomenclature (the labeling of the units), and identification (the process of determining whether an unknown belongs to one of the units defined) (16). During the past decade, it became generally accepted that bacterial classification should reflect as much as possible the natural relationships between bacteria, which are considered the phylogenetic relationships as encoded in highly conserved macromolecules such as 16S or 23S rRNA genes (68, 132). Nowadays, whole-genome comparisons offer new and exciting opportunities for the study of these natural relationships.

It is nevertheless true that every classification is artificial and that boundaries are made by humans. However, classification serves very practical purposes, i.e., the recognition of organisms that were encountered previously and the categorization of new ones into a logical and tractable system. In this era of whole-genome sequence analysis, it is more than ever obvious that the genomes of microbes undergo change, sometimes considerably. Although the extent of lateral gene transfer is controversial, it does not alter our need to identify organisms, particularly in the context of epidemiological studies and surveillance, as identification bears a tremendous amount of accompanying information. Science indeed has a way of making itself useful, and the useful application of classification is identification (15).

CLASSIFICATION OF BACTERIA

The process of species delineation in bacterial systematics underwent drastic modifications as the species concept evolved in parallel with technical progress. Early classification systems used mainly morphological and biochemical criteria to delineate the species of bacteria. This type of classification was monothetic, as it was based on a unique set of characteristics necessary and sufficient to delineate groups. This early classification concept was replaced by theories of so-called natural concepts, which were the phenetic and phylogenetic classifications (36). In the former, relationships between bacteria were based on the overall similarity of both phenotypic and genotypic characteristics. Phenetic classifications demonstrate the relationships between organisms as they exist, without reference to ancestry or evolution. In phylogenetic classifications, relationships are described by ancestry, not according to their present properties.

Special-purpose and general-purpose classification systems are the main categories of classification systems. Special-purpose classification systems are objectively determined and do not fit a preconceived idea. For instance, the separation between the very closely related species *Escherichia coli* and *Shigella dysenteriae* or between *Bordetella pertussis* and *Bordetella bronchiseptica* does not conform to the general ideas of present-day species delineation (see below) but fits primarily a practical and historical purpose (36). Yet, nowadays, most taxonomists favor a general-purpose classification system that is stable, objective, and predictive and that can be applied to all bacteria. The classifications obtained with a general-purpose classification system do not fit a single purpose but attempt to reflect the natural diversity among bacteria. The best way to generate such general-purpose classifications is by combining the strengths of both phenetic and phylogenetic studies, a practice now often referred to as polyphasic taxonomy (118).

Criteria for Species Delineation

The criteria used to delineate species have developed in parallel with technology. The early classifications were based on morphology and biochemical data. When evaluated by means of our present views, many of these early phenotype-based classifications generated extremely heterogeneous assemblages of bacteria. Individual species were characterized by a common set of phenotypic characters and differed from other species in one or a few characters which were considered important. The introduction of computer technology allowed comparison of large sets of characteristics for large numbers of strains, forming the basis for phenetic taxonomy (96, 98). Such numerical analyses of phenotypic characters

yielded superior classifications in terms of objectivity and stability. Gradually, chemotaxonomic and genotypic methods were introduced into classification systems. Numerous different chemical compounds were extracted from bacterial cells, and their suitability for use in the classification of bacteria and the definition of species has been analyzed.

In 1987, the Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics (125) stated that taxonomy should be determined phylogenetically and that the complete genome sequence should therefore be the standard for species delineation. In present-day practice, whole-genome DNA-DNA hybridization analysis approaches the sequence standard and represents the best applicable procedure. A bacterial species was therefore defined as a group of strains, including the type strain, that share 70% or greater DNA-DNA relatedness with a ΔT_m of 5°C or less (T_m is the melting temperature of the hybrid, as determined by stepwise denaturation; ΔT_m is the difference in T_m [in degrees Celsius] between the homologous hybrid and the heterologous hybrid formed under standard conditions [135]). This species definition was based on a large amount of empirical data including both DNA-DNA hybridization data and other characteristics. The designated type strain of a species serves as the name bearer of the species and as the reference specimen (104). It was also recommended that phenotypic and chemotaxonomic features should agree with this definition. Preferentially, several simple and straightforward tests should endorse the species delineation based on DNA-DNA hybridization values. Groups of strains which were delineated by means of DNA-DNA hybridization studies as distinct species but which could not be distinguished by phenotypic characteristics should not be named. The term "genomovar" was subsequently introduced for such phenotypically similar genomic species (110); however, this term may be somewhat misleading, as these taxa are given an infraspecific rank, whereas by definition they are to be considered species that cannot be reliably differentiated by phenotypic tests.

The level of DNA-DNA hybridization thus plays a key role in our present species concept as defined by Wayne et al. (125). Although that seems to suggest that the species definition has become less vague, the practice of DNA-DNA hybridization is very complex (see below).

The Polyphasic Species Concept

A wide variety of cellular components have been used to study relationships between bacteria and to design classifications. The information present at the DNA level has been analyzed by estimations of the DNA base composition and the genome size, whole-genome DNA-DNA hybridization, restriction enzyme analysis, and, increasingly more, by direct sequence analysis of various genes. rRNA fractions have been studied intensively, particularly because they serve as phylogenetic markers (68). Various chemical compounds including fatty acids, mycolic acids, polar lipids, polysaccharides, sugars, polyamines, and respiratory quinones, as well as, again, a tremendous number of expressed features (data derived from, e.g., morphologic, serologic, and enzymologic studies), were all used to characterize bacteria. Several of these approaches have been applied to taxonomic analyses of virtually all bacteria. Others, such as amino acid sequencing, were performed with a limited number of organisms because they are laborious, time-consuming, or technically demanding or because they were relevant only for a particular group.

The term "polyphasic taxonomy" was coined by Colwell (12) in 1970 and described the integration of all available genotypic, phenotypic, and phylogenetic information into a consensus type of general-purpose classification. It departs from the assumption that the overall biological diversity cannot be encoded in a single molecule and that the variability of characters is group dependent. Therefore, it integrates several generally accepted ideas for the classification and reclassification of bacteria. Polyphasic taxonomy is phylogeny based and uses sequence analysis and signature features of rRNA for the deduction of a phylogenetic framework for the classification of bacteria (43, 118, 121). Several other macromolecules such as the beta subunit of ATPase, elongation factor Tu, chaperonin, various ribosomal proteins, RNA polymerases, and tRNAs (43, 68) have similar potential. The next step in the process of classification is the delineation of individual species—and other taxa—within these phylogenetic branches. Despite its drawbacks, DNA-DNA hybridization forms the cornerstone of species delineation. However, the threshold value for species delineation should be allowed considerable variation. This polyphasic approach is pragmatic; for instance, *B. pertussis, Bordetella parapertussis,* and *B. bronchiseptica,* which share DNA-DNA hybridization levels of over 80%, are considered three distinct species because they differ in many phenotypic and chemotaxonomic aspects (114). In other genera which are phenotypically more homogeneous, such as *Acidovorax* (131), species are defined as groups of strains that have DNA-DNA hybridization levels of at least 40%. It is essential that the boundaries of species demarcation be flexible in order to achieve a classification scheme that facilitates identification.

The application of numerous other types of analyses of genotypic, chemotaxonomic, and phenotypic characteristics of bacteria to the delineation of bacteria at various hierarchical levels represents the third component of polyphasic taxonomy (118). The goal is to collect as much information as possible and to evaluate all results in relation to each other in order to draw useful conclusions. An additional advantage is that, once the taxonomic resolution of these approaches has been established for a particular group of bacteria through the analysis of a set of taxonomically well-characterized strains, they may be used as alternative tools to classify new isolates at different taxonomic levels. A typical example is the application of one-dimensional whole-cell protein electrophoresis to replace DNA-DNA hybridization experiments for identification to the species level of *Helicobacter* species (117). It should be noted that the resolution of these alternative methods is often group dependent. For instance, cellular fatty acid analysis is useful for the accurate identification of strains of many bacterial species to the species level. In certain bacterial groups, however, the cellular fatty acid profile may be indicative of the genus or a group of phylogenetically related genera but not of a particular species within one of these genera (118). Within the group of the gram-negative nonfermenting bacteria, this is nicely illustrated by the characteristic fatty acid profiles of members of the genera *Chryseobacterium, Empedobacter, Ornithobacterium,* and *Riemerella* (the last two genera are of veterinary interest), which are characterized by extremely high percentages (80 to 90% of the total fatty acid content) of saturated branched-chain fatty acids in the *iso* and *anteiso* configurations (119, 120).

The contours of a polyphasic bacterial species are obviously less clear than the ones defined by Wayne et al.

(125), and this lack of a rigid definition has been contested as it allows too many interpretations (135). Polyphasic classification is empirical and contains elements from both phenetic and phylogenetic classifications. There are no strict rules or guidelines, and the approach integrates any significant information on the organisms, resulting in a consensus type of classification. Its main weakness is indeed that it relies on common sense to draw its conclusions. The bacterial species appears as a group of isolates in which a steady generation of genetic diversity resulted in clones characterized by a certain degree of phenotypic consistency, by a significant degree of DNA-DNA hybridization, and by a high level of 16S rRNA sequence similarity (118).

Obviously, the species is the most important and, at the same time, the central element of bacterial taxonomy. There are at present no rules for the delineation of higher hierarchical ranks such as genus, family, and order. Although there is an expectation that at the generic level taxa should be supported by phenotypic descriptions (76), in practice higher ranks are mostly delineated on the basis of 16S rRNA sequence comparison and stability analyses of the clusters that are obtained. Undoubtedly, the latter has weakened the emphasis on phenotypic descriptions of taxa (135). In polyphasic taxonomy, attempts are made to endorse these phylogeny-driven demarcations by other data. An example is the subdivision of the former genus *Campylobacter* into the revised genus *Campylobacter* and the novel genera *Arcobacter* and *Helicobacter* (38, 116). Although this subdivision was mainly phylogeny based, it was supported by differences in respiratory quinone components and ultrastructural properties.

A New Species Concept?

In 2002, a new Ad Hoc Committee for the reevaluation of the species definition in bacteriology made various recommendations regarding the species definition in light of developments in methodologies available to systematists (99). As stated by the Ad Hoc Committee, the introduction of innovative methods is providing new opportunities for prokaryotic systematics. One of the particularly interesting developments includes the analysis of complete genome sequences. There is a growing interest in using these genome sequence data to assess evolutionary relationships among prokaryotic species. It has become clear that, in addition to nucleotide substitutions, other genetic forces such as gene loss, gene duplication, horizontal (or lateral) gene transfer, and chromosomal rearrangements shape the genome and that considerable fractions of the genome of any particular strain may be unique to that strain (9, 59). With an increasing number of species for which multiple whole-genome sequences are available, a range of novel approaches for assessing taxonomic relationships within and between closely related species become available. These novel approaches include analysis of gene content and gene order, comparative sequence analysis of conserved and other macromolecules or of complete genomes, presence-absence analyses, nucleotide signature composition analyses, and even metabolic pathway reaction content analyses (reviewed in reference 9). Despite the documented enormous strain-to-strain variation in genome content, these novel taxonomic analytical tools generally substantiate that bacterial species delineated by DNA-DNA hybridization experiments and ordered in a phylogenetic backbone through comparative 16S rRNA sequence analysis represent coherent biological entities, although, in terms of population genetics, they still encompass considerable ecological and genetic heterogeneity (10).

Studies by Konstantinidis and Tiedje (59) revealed the average nucleotide identity of the shared genes between two strains to be a robust means to compare genetic relatedness among strains and that average nucleotide identity values of approximately 94% corresponded to the traditional 70% DNA-DNA reassociation standard of the current species definition. At the 94% average nucleotide identity cutoff, current species included only moderately homogeneous strains, apparently as a result of the strains having evolved in different ecological settings. A large fraction of the differences in gene content within species was associated with bacteriophage and transposase elements, revealing an important role of these elements during bacterial speciation. These findings were consistent with a definition for species that would include a more homogeneous set of strains than that provided by the current definition and one that considers the ecology of the strains in addition to their evolutionary distance (10).

Multilocus sequence analysis (MLSA) is another novel approach that holds great promise. In contrast to multilocus sequence typing, a specific tool designed for molecular epidemiology and for defining strains within named species, whereby similarities and differences are usually measured as differences in allelic profiles, MLSA aims to amplify and sequence gene fragments of strains representing different species within and between genera. Gene sequences are subsequently studied by traditional software used for phylogenetic analyses. Examples of such studies have recently been published for two groups of *Burkholderia* species, i.e., *Burkholderia mallei, Burkholderia pseudomallei,* and *Burkholderia thailandensis* (34) and the *Burkholderia cepacia* complex (2), and for *Yersinia* species (61). Especially for depicting relationships within and between closely related species, this approach promises a resolution superior to the traditional 16S rRNA gene sequence analysis. The deduced phylogenetic trees not only provide a phylogenetic backbone but also reveal intraspecies relationships at a level where comparative 16S rRNA sequence analysis is no longer discriminatory. It is for instance noteworthy that the DNA-DNA hybridization results which demonstrated that *Yersinia pestis* and *Yersinia pseudotuberculosis* on the one hand and *B. mallei* and *B. pseudomallei* on the other hand represented a single species each were mirrored in the MLSA trees where *Y. pestis* clusters among *Y. pseudotuberculosis* strains and *B. mallei* clusters among *B. pseudomallei* strains. Two studies of complete genomes have suggested a universal set of protein-coding genes that may be useful for such an MLSA-based approach to microbial taxonomy (91, 138).

Major Groups of Bacteria

The tree of life based on comparative small subunit rRNA studies comprises three lines of descent that are nowadays referred to as the domains *Bacteria, Archaea*, and *Eucarya* (132). The *Bacteria* have been grouped into 23 phyla, which are further subdivided into 28 classes (62). Three phyla, the *Proteobacteria,* the *Firmicutes* (gram-positive organisms with low G+C contents, including *Bacillus, Clostridium, Staphylococcus, Mycoplasma,* and the classical lactic acid bacteria such as *Enterococcus, Streptococcus,* and *Lactobacillus*), and the *Actinobacteria* (gram-positive organisms with high G+C contents, including *Bifidobacterium, Mycobacterium,* and *Corynebacterium*), comprise the large majority of the clinically relevant species. The *Bacteroidetes* (*Bacteroides,* flavobacteria,

and sphingobacteria), the *Spirochaetes* (spirochetes and leptospiras), and the *Chlamydiae* (chlamydias) represent some of the other phyla. A detailed overview is given by Krieg and Garrity (62) and Ludwig and Klenk (66) in their introductory chapters to the second edition of *Bergey's Manual of Systematic Bacteriology.* That edition is structured in an order based on the topology of the 16S rRNA phylogenetic tree.

The largest phylum by far is the *Proteobacteria,* which contains five main clusters (classes) of genera that are referred to with the Greek letters alpha, beta, gamma, delta, and epsilon (102). The *Proteobacteria* comprise the majority of the known gram-negative bacteria of medical, industrial, and agricultural significance. This phylum includes *Brucella, Ehrlichia*, and *Rickettsia (Alphaproteobacteria); Burkholderia*, *Bordetella,* and *Neisseria (Betaproteobacteria); Aeromonas, Legionella,* and *Vibrio* and the family *Enterobacteriaceae (Gammaproteobacteria)*; and *Campylobacter* and *Helicobacter (Epsilonproteobacteria)*. The *Deltaproteobacteria* comprise a variety of mainly sulfate-reducing bacteria that have little clinical relevance.

Unculturable Bacteria

The classification and nomenclature of unculturable bacteria that are only minimally characterized by morphological characteristics or by differences in a molecular sequence (77) are outstanding challenges in bacterial classification. The members of the International Committee on Systematic Bacteriology have agreed to recognize a category that formally classifies incompletely described prokaryotes (78). This action was useful and timely because of the increasing involvement of sequencing technology in the characterization of prokaryotes that are difficult to cultivate. *Candidatus* is considered a taxonomic status for uncultured "candidate" species for which relatedness has been determined (for instance, for which phylogenetic relatedness has been determined by amplification and sequence analysis of prokaryotic RNA genes by use of universal prokaryotic primers) and authenticity has been verified by in situ probing or a similar technique for cell identification. In addition, it is also mandatory that information concerning phenotypic, metabolic, or physiological features be made available. The latter data may serve as a starting point for further investigation and eventual description and naming. A detailed list of items for inclusion in the codified record of a *Candidatus* taxon is provided elsewhere (78). With the increasing application of molecular methods to the assessment of the diversity of prokaryotic populations in nature and to the study of complex symbioses, it was anticipated that numerous *Candidatus* organisms would be recorded (78). There are several caveats. Information derived from 16S rRNA gene sequence analysis may not be sufficient to ensure that the uncultured organism represents a novel species (see below). Also, 16S rRNA gene sequences are not available for all known bacteria for comparison. Alternatively, morphological characteristics, for example, may not be sufficiently reliable to conclude that uncultured cells represent a novel organism (118).

CLASSIFICATION METHODS

In principle, all genotypic, phenotypic, and phylogenetic information can be used to classify bacteria. Genotypic information is derived from the nucleic acids (DNA and RNA) present in the cell, whereas phenotypic information is derived from proteins and their functions, different chemotaxonomic markers, and a wide range of other expressed features.

When working one's way through lists of methods, it is of primary interest to understand at which level these methods carry information and to realize their technical complexity, i.e., the amount of time and work required to analyze a certain number of isolates. The list of methods given below is not meant to be complete or to describe all of their aspects. It comprises the major categories of taxonomic techniques required to study bacteria at different taxonomic levels and roughly describes general concepts and applications of those techniques, as well as some other considerations. Detailed descriptions of such methods can be found in handbooks such as those by Goodfellow and O'Donnell (36) or Priest and Austin (89).

Genotypic Methods

DNA-DNA Hybridization Studies

At present, DNA-DNA hybridization is acknowledged as the reference method to establish relationships within and between species. Different DNA-DNA hybridization procedures have been described: the hydroxyapatite method (4), the optical renaturation method (20), and the S1 nuclease method (19, 41) have mostly been used. These classical techniques, however, need considerable amounts of DNA and are time-consuming. New quick methods that consume less DNA have been described (6, 24, 51) and have partially replaced the classical methodologies.

Many DNA-DNA hybridization protocols have been described, and it is often not clear if hybridizations were performed under optimal, stringent, or suboptimal conditions. The stringency of the reaction is determined by the salt and formamide concentration and by the temperature and the mol percent G+C contents of the DNAs used. DNA-DNA hybridizations are often performed under standard conditions that are not necessarily optimal or stringent for all bacterial DNAs. As a standard, optimal conditions for hybridizations should be preferred because the optimal temperature curve for hybridization is rather broad (about 5°C) (118).

Obviously, quantitative comparisons of DNA hybridization values generated with different techniques should be handled with caution. When different methodologies are used, it is safer to distinguish categories of DNA-DNA relatedness, such as "high DNA-DNA relatedness" (denoting relationships between strains of a single species), "low but significant DNA-DNA relatedness" (comprising the significant hybridization levels below the cutoff for a separate species; the depth of this range depends primarily on the technique used), and finally, "non-significant DNA-DNA relatedness" (denoting that the degree of DNA hybridization is too low to be measured by the method used).

rRNA Similarity Studies

It is now generally accepted that rRNA is the best target for studying phylogenetic relationships because it is present in all bacteria, it is functionally constant, and it is composed of highly conserved as well as more variable domains (43, 62, 66, 93, 100, 132). The components of the ribosome (rRNA and ribosomal proteins) have been the subjects of phylogenetic studies for several decades. The gradual development of molecular techniques enabled microbiologists to focus on the comparative study of the rRNA molecules, and direct sequencing of parts of, or nearly entire, 16S or 23S rRNA molecules by the PCR technique with a selection

of appropriate primers has become common practice. These sequences provide a phylogenetic framework that serves as the backbone for modern microbial taxonomy (62, 66). The larger the conserved elements examined, the more information they bear and the more reliable the conclusions become. International databases comprising published and unpublished partial or complete sequences have been constructed (82, 121). The presence of universal sequence motifs in the bacterial rRNA genes allowed taxonomists to classify unculturable organisms and to perform phylogenetic identifications and in situ detection of individual cells without cultivation (1).

More recently, however, it has been found that rRNA sequence analysis is no longer exclusively used to determine relationships between genera, families, and other higher ranks but often replaces DNA-DNA hybridization studies for the delineation of species in taxonomic practice. In many cases, such application of rRNA similarity data is not appropriate. In 1992, Fox et al. (28) reported that 16S rRNA sequence identity is not always sufficient to guarantee species identity. Indeed, three phenotypically similar *Bacillus* strains exhibited more than 99.5% rRNA sequence similarity, while DNA-DNA hybridization experiments indicated that they belonged to two distinct species. Stackebrandt and Goebel (100) reported on the place for 16S rRNA gene sequence analysis and DNA-DNA reassociation in the present species definition in bacteriology. Their extensive literature review revealed that organisms sharing more than 97% rRNA similarity may or may not belong to a single species and that the resolution of 16S rRNA sequence analysis for determination of the degree of relatedness between closely related organisms is generally low. There is obviously no threshold value of 16S rRNA similarity for species recognition (100). However, they reported that organisms with less than 97% 16S rRNA sequence similarity do not give a DNA-DNA reassociation level of more than 60%, no matter which DNA-DNA hybridization method is used. In fact, rRNA sequence analysis seemed to rightfully replace DNA-DNA hybridization studies as part of the description of new species, provided that the rRNA similarity level was below 97% and provided that rRNA sequence data for all relevant taxa were available for comparison. However, more recent studies extended the observations on intraspecies 16S rRNA divergence considerably, as differences in 16S rRNA gene sequence of up to 4.5% were reported among strains of several species belonging to the *Epsilonproteobacteria* (46, 117).

Clearly, one should be prudent in drawing conclusions based on analysis of a single sequence. In 1995, Clayton and colleagues (8) presented a detailed comparison of duplicate rRNA sequences present in the GenBank database with remarkable results. Unexpectedly high levels of intraspecies variation (within and between strains) of 16S rRNA sequences were found. The variability was thought to represent interoperon variation within a single strain, strain-to-strain variation within a species, misidentification of strains, sequencing error, or other laboratory errors. Critical selection and the use of sequences from databases are required. More recently, studies by Jaspers and Overmann (53) and Gonzales-Escalona et al. (35) provided new examples of diversity among 16S rRNA genes within single organisms and sequence identity in bacteria with highly divergent genomes and ecophysiologies. Alternatively, numerous other macromolecules have been examined for their potential as microbiological clocks. Among others, various ribosomal proteins (81), the beta subunit of ATPase (66, 67), elongation factor Tu (66, 67), chaperonin (123), RNA polymerases (58, 140), and manganese-dependent superoxide dismutase (29) were shown to be valuable molecular chronometers in bacterial systematics. These alternative macromolecules should be universally distributed among bacteria, they should not be transmitted horizontally, and their molecular evolution rate should be comparable to, or somewhat higher than, that of 16S rRNA, which would render them more suitable for differentiation of closely related organisms (for example, see reference 29).

Other Applications of rRNA in Taxonomy

The interesting taxonomic properties of rRNA or rRNA gene molecules have been exploited in several alternative ways (5, 40, 44, 48, 128). An rRNA operon typically consists of the following components (5′ to 3′): 16S, spacer, 23S, spacer, and 5S rRNA sequences. Amplification of part of this operon by means of PCR assays, followed by digestion of the amplicon by means of restriction enzymes and the electrophoretic separation of the resulting array of DNA fragments, is referred to as amplified rRNA restriction analysis or rRNA restriction fragment length polymorphism analysis (44). Depending on the target selected, the banding pattern is useful for species level discrimination (for target sequences that are highly conserved) or for strain typing (for target sequences that are variable). The technique has most of the advantages inherent in the rRNA approach; in addition, it is clearly less expensive and more rapid than direct sequence analysis and large numbers of strains can easily be examined. This not only renders the method a useful screening tool but also provides a better view on the intraspecies variability of the rRNA operon.

Another rRNA-based approach for the identification and classification of bacteria is ribotyping (3, 40). By this procedure, genomic DNA is digested with a restriction enzyme (or with a set of restriction enzymes). The digest is separated by electrophoresis, and the bands are transferred to a membrane and hybridized with a labeled rRNA probe. This probe may be based on 16S rRNA, 23S rRNA, or both, with or without the spacer region, or on a conserved fragment of one of the rRNA genes. Although designed and mostly used to determine interstrain relationships (40), a fully automated procedure for identification of bacteria to the species level is commercially available (RiboPrinter; Dupont Qualicon Inc., Wilmington, Del.).

Finally, terminal restriction fragment length polymorphism analysis of 16S rRNA genes has been used in several studies for the characterization of microbial diversity in natural specimens and for identification of the members therein (65, 90). The technique employs a PCR assay in which one of the two primers used is fluorescently labeled at the 5′ end and is used to amplify a selected region of bacterial genes encoding 16S rRNA from total community DNA. The PCR product is digested with restriction enzymes, and the fluorescently labeled terminal restriction fragment is precisely measured by using an automated DNA sequencer. Computer-simulated analysis of terminal restriction fragment length polymorphisms for 1,002 bacterial sequences showed that with proper selection of PCR primers and restriction enzymes, 686 sequences could be PCR amplified and classified into 233 unique terminal restriction fragment lengths or "ribotypes" (65).

Other Genotypic Methods for Bacterial Classification

A range of different genotypic techniques has been used to characterize bacteria at various taxonomic levels. The molar percentage of guanosine plus cytosine (the DNA base ratio

or percent G+C value) is one of the classical genotypic characteristics and is part of the standard description of bacterial taxa. Generally, the range observed within a species should not be more than 3%, and within a genus it should not be more than 10% (101). It varies between 24 and 76% in the bacterial world.

During the past decade, a tremendous number of molecular diagnostic methods, most of which are PCR based, have been developed. Most of these generate arrays of DNA fragments that are separated and detected in various ways, and appropriate software has been developed for pattern recognition and analysis and for database construction.

One of these DNA fingerprinting methods, amplified-fragment length polymorphism (AFLP) analysis (136), was shown to be very useful for the classification of strains at the species and genus levels. The basic principle of AFLP is restriction fragment length polymorphism analysis, modified by using a PCR-mediated amplification to select particular DNA fragments from the pool of restriction fragments. AFLP analysis screens for AFLPs by selective amplification of restriction fragments. The restriction is performed with two restriction enzymes, which yield DNA fragments with two different types of sticky ends that are randomly combined. To these ends, short oligonucleotides (adapters) are ligated to form templates for the PCR. The selective amplification reaction is performed by using two different primers that contain the same sequence as the adapters but whose sequences are extended to include one or more selective bases next to the restriction site of the primer. Only those fragments that completely match the primer sequence are amplified. The amplification process results in an array of about 30 to 40 DNA fragments, some of which are group specific, while others are strain specific (52).

PCR-based typing methods that use random or repetitive elements as primers have been applied to strain characterization of a wide variety of bacteria (47, 70, 112, 127, 128). In several of these studies, species-specific DNA fragments or patterns have been generated (e.g., for species belonging to the genera *Campylobacter*, *Capnocytophaga*, *Enterococcus,* and *Naegleria* [21, 31]). These specific DNA fragments may be useful as probes to rapidly screen and identify other isolates. Although primarily applied for infraspecies strain comparisons, these techniques are useful in classification as well.

Phenotypic Methods

Phenotypic methods comprise all those that are not directed toward DNA or RNA and therefore also include the chemical or chemotaxonomic techniques. As the introduction of chemotaxonomy is generally considered one of the essential milestones in the development of modern bacterial classification, it is often treated as a separate unit in taxonomic reviews. The classical phenotypic tests traditionally constituted the basis for the formal description of bacterial species, subspecies, genera, and families. While genotypic data are used to allocate taxa on a phylogenetic tree and to draw the major borderlines in classification systems, phenotypic consistency is required to generate useful classification systems and may therefore influence the depth of a hierarchical line (118, 125). The paucity or variability of phenotypic characteristics for certain bacterial groups regularly causes problems in describing or differentiating taxa. For such bacteria, alternative chemotaxonomic or genotypic methods are often required to reliably characterize strains.

The classical phenotypic characteristics of bacteria comprise morphological, physiological, and biochemical features. Individually, many of these characteristics were shown to be poor parameters for genetic relatedness, yet as a whole, they provide descriptive information for the recognition of taxa. The morphology of a bacterium comprises both cellular (shape; the presence of an endospore, flagella, and inclusion bodies; and Gram staining characteristics) and colonial (color, dimensions, and form) characteristics. The physiological and biochemical features comprise data on growth at different temperatures; growth in the presence of different pH values, salt concentrations, or atmospheric conditions; and growth in the presence of various substances such as antimicrobial agents and data on the presence or activities of various enzymes, utilization of compounds, etc. Very often, highly standardized procedures are required to obtain reproducible results within and between laboratories (for examples, see references 83 and 84).

In taxonomic practice, phenotypic characterization became compromised and sometimes more of a burden than a useful taxonomic activity. Frequently, phenotypic data are compared with literature data, which were obtained using other conditions or methods. The need for continued phenotypic characterization at every taxonomic level not only to delineate taxa and appreciate their phenotypic coherence but also to evaluate their physiological and ecological functions cannot be denied. A minimal phenotypic description is not only the identity card of a taxon but also a key to its biology. Although accepted as necessary, differential phenotypic characters are often hard to find with a reasonable amount of effort and time.

Numerical Analysis

Phenotypic data were the first to be analyzed by means of computer-assisted numerical comparison. In the 1950s, numerical taxonomy arose in parallel with the development of computers (96, 98) and allowed comparison of large numbers of phenotypic traits for large numbers of strains. Data matrices showing the degree of similarity between each pair of strains and cluster analyses resulting in dendrograms revealed a general picture of the phenotypic consistency of a particular group of strains. As such large numbers of characteristics reflect a considerable amount of genotypic information, it soon became evident that numerical analysis of large numbers of phenotypic characteristics was indeed taxonomically relevant.

Semiautomated Systems

A large number of miniaturized semiautomated phenotypic test systems are commercially available and partially replace classical phenotypic analyses. These microtest galleries can be used for both classification and identification (see chapter 15). It should be noted that the outcomes of a particular test obtained with a commercial system and by a classical procedure may be different. This, however, may occur with two classical procedures of the same test as well.

Chemical Methods

The term "chemotaxonomy" refers to the application of analytical methods to the collection of information on various chemical constituents of the cell to classify bacteria. As for the other phenotypic and genotypic techniques, some of the chemotaxonomic methods have been widely applied on vast numbers of bacteria, whereas others were so specific that their application was restricted to particular taxa. Similarly, a high degree of automation was introduced

with the development of novel technologies such as matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF [MS]) or Fourier transform infrared (FTIR) spectroscopy.

Apart from the chemical markers discussed in more detail below, a range of other chemical markers have been examined in bacterial classification. They include cytochromes, polyamines, pigments, particular enzymes, sterols, and hopanoids (37, 107). Very often, analytical difficulties have been the main restrictions to their wide-scale application.

Cellular Fatty Acid Analysis

Over 300 fatty acids and related compounds are present in bacterial cells. Polar lipids are the constituents of the lipid bilayer of bacterial membranes and have been frequently studied for classification and identification purposes. Other types of lipids, such as sphingophospholipids, occur in only a restricted number of taxa and were shown to have taxonomic value within these groups (54). Fatty acids are the major constituents of lipids and lipopolysaccharides and have been used extensively for taxonomic purposes. Variabilities in chain lengths, double-bond positions, and substituent groups are very useful for the characterization of bacterial taxa (105). Mostly, the total cellular fatty acid fraction is extracted, but particular fractions, such as the polar lipids, have also been analyzed.

The cellular fatty acid methyl ester composition is a stable parameter, provided highly standardized culture conditions are used. The methylated fatty acids are typically separated by gas-liquid chromatography, and both the occurrence and the relative amounts of methylated fatty acids characterize bacterial fatty acid profiles.

Cellular fatty acid analysis offers many advantages over other phenotype-based identification systems; however, it has several limitations as well. First, the result of the analysis is culture dependent. Strains must be grown under identical conditions so that their fatty acid compositions can be compared. Although the conditions recommended by the manufacturer allow cultivation of a large number of bacteria, different sets of conditions and databases are used for different groups of bacteria (e.g., the aerobic bacteria, anaerobic bacteria, and mycobacteria). In addition, the level of resolution is organism dependent. Many bacteria may be adequately characterized and identified at the species level by means of their cellular fatty acid profile. However, others are not, and often species of the same genus or even different genera have highly similar fatty acid compositions. Whole-cell fatty acid analysis is widely used both in taxonomic studies and in identification analyses. The applications and restraints of the technique were extensively discussed and documented by Welch (126). In the framework of polyphasic taxonomy, cellular fatty acid analysis is often very useful as a rapid and fairly inexpensive screening method. The method allows the comparison and clustering of large numbers of strains with a minimal effort and yields descriptive information to characterize the organisms.

MALDI-TOF (MS)

The first reports involving the use of MALDI-TOF (MS) were published in the late 1980s, and its application increased exponentially during the past 15 years. The two main research areas are the field of proteomics, where it is used as an instrument to identify proteins, and the detection of biomarkers of several diseases including cancer, Alzheimer's disease, arthritis, and allergy (72, 137). In MALDI-TOF (MS), the sample is mixed with a matrix that is chosen such that it specifically absorbs a laser beam. The resulting high-energy impact is followed by the formation of ions that are extracted through an electric field and that are subsequently focused and detected as an m/z (mass/charge) spectrum. The potential to desorb high-molecular-weight thermolabile molecules, the extremely high precision and sensitivity, and the large mass range (1 to 300 kDa) indicate that MALDI-TOF (MS) is a promising tool for the study of biomolecules in complex biological samples. The simplicity and speed of analysis represent part of its strength. Sample processing is potentially restricted to adding matrix to a bacterial sample, and all measurements can be performed within a few minutes. The whole process can be highly automatized. These features make the approach particularly attractive to research laboratories that routinely deal with the analysis and identification of large numbers of bacterial isolates. In microbiology, MALDI-TOF (MS) has been used to distinguish antimicrobial-resistant isolates from susceptible ones and to differentiate strains within a single species, thus highlighting its potential as a tool for strain quality control and identity check (57, 111). The most challenging diagnostic problem, i.e., the differentiation of closely related species through the analysis of an appropriate number of reference strains of multiple closely related species, is gradually being explored (23, 57, 103, 111).

Surface enhanced laser desorption ionization (SELDI) is distinguished from MALDI in its use of an active sample probe—the ProteinChip array—which has an adsorptive surface that allows bacterial lysates to be subjected directly, without prior treatments, to on-chip sample preparation steps, such as selective washing and desalting. This procedure minimizes sample losses, while speeding up and simplifying sample preparation, compared to the standard methods normally employed prior to the use of MALDI. Furthermore, the active capture of the proteins by the ProteinChip array ensures nondiscriminatory binding of target proteins, which in turn improves the reproducibility and allows both peak mass-to-charge (m/z) ratios and intensity to be used in sample characterization. SELDI-TOF-MS was used by Lundquist et al. (69) to discriminate between the four subspecies of *Francisella tularensis*.

FTIR Spectroscopy

FTIR spectroscopy is used for the identification of substances in chemical analyses. In general, the wave number, the reciprocal of the wavelength, is used as the physical unit. FTIR spectroscopy involves the observation of vibrations of molecules that are excited by an infrared beam. Molecules are able to absorb the energy of distinct light quanta and start a rocking or rotation movement. The FTIR spectrum uses only vibrations that lead to a change in the dipole moment. An infrared spectrum represents a fingerprint which is characteristic for any chemical substance. The composition of biological material and thus of its FTIR spectrum is exceedingly complex, representing a characteristic fingerprint. Naumann and coworkers suggested identifying microorganisms by FTIR spectroscopy (79). In principle, a reference spectrum library is assembled based on well-characterized strains and species. The FTIR spectrum of any unidentified isolate is then measured under the same conditions as those used for the reference spectra and is compared to spectra in the reference spectrum library. The application of FTIR spectroscopy has been reported for a limited number of strains of some species of the genera *Lactobacillus*, *Actinomyces*, *Listeria*, *Streptococcus*, and *Clostridium* (63). More extensive studies have been

published for yeasts (63, 130) and coryneform bacteria (80); these organisms display a certain degree of overlapping distribution of different taxonomical markers, leading to a limited differentiation capacity of nongenotypical identification methods. The easy handling, rapid identification (within 25 h starting from a single colony), satisfactory differentiation capacity, and low cost have shown FTIR technology to be superior to routine methods for the identification of coryneform bacteria and related taxa.

Whole-Cell Protein Analysis

The comparison of whole-cell protein patterns obtained by a highly standardized procedure, sodium dodecyl sulfate polyacrylamide gel electrophoresis, has proved to be extremely reliable for comparison and grouping of large numbers of closely related strains, and numerous studies document the application of this procedure in taxonomic studies (14, 56, 88). Bacterial strains cultivated under highly standardized conditions have characteristic protein compositions that can be separated and visualized by electrophoretic techniques. Mostly, a high similarity in whole-cell protein content is an indication of extensive DNA-DNA hybridization (14, 56, 88). Therefore, an obvious advantage of this technique is that, once the correlation between percentage similarity in whole-cell protein composition and DNA-DNA relatedness for a particular group has been established, it can replace DNA-DNA hybridization experiments. Provided highly standardized conditions are used throughout the procedures of cultivation and electrophoresis, computer-assisted numerical comparisons of protein patterns are feasible, and databases can be created for identification purposes. This allows comparison of large numbers of strains and grouping of the strains in clusters of closely related strains (14, 56, 88). For some bacteria, numerical analysis is strongly hindered by the presence of distorted protein profiles or hypervariable (often immunogenic) dense protein bands. In these cases, visual comparison is essential to interpret the similarity of protein patterns.

Cell Wall Composition

The distinction between gram-negative and gram-positive types of bacteria is still one of the characteristics that is first analyzed in order to guide subsequent characterization and identification steps. The determination of the cell wall composition has traditionally been important for gram-positive bacteria. The peptidoglycan type of cell wall of gram-negative bacteria is rather uniform and provides little information. The cell walls of gram-positive bacteria, in contrast, contain various peptidoglycan types which may be genus or species specific (92, 105). The most valuable information is derived from the type and composition of the peptide cross-link between adjacent chains in the polymer network. A variable that received little attention is the degree of N and O acetylation of the amino sugars of the glycan chain. The analytical procedure is time-consuming, although a rapid screening method has been proposed. Membrane-bound teichoic acid is present in all gram-positive species, but cell wall-bound teichoic acid is present in only some gram-positive species. Teichoic acids can easily be extracted and purified and can be analyzed by gas-liquid chromatography (25).

Isoprenoid Quinones

Isoprenoid quinones occur in the cytoplasmic membranes of most prokaryotes and play important roles in electron transport, oxidative phosphorylation, and, possibly, active transport (11, 105). Two major structural groups, the naphthoquinones and the benzoquinones, are distinguished. The former can be further subdivided into two main types, the phylloquinones, which occur less commonly in bacteria, and the menaquinones. The large variability of the side chains (e.g., differences in length, saturation, and hydrogenation) can be used to characterize bacteria at different taxonomic levels (11).

IDENTIFICATION

Identification is part of taxonomy. It is the process whereby an organism is recognized as belonging to a known taxon (species, genus) and designated accordingly. It relies on a comparison of the characters of an unknown with those of established units in order to name it appropriately. This implies that identification depends on adequate characterization.

Identification Strategy

In routine diagnostic laboratories, the majority of isolates are identified using classical biochemical tests and a combination of intuition and stepwise analysis of results that are obtained. However, if an organism is not readily identified in a minimal amount of time and at minimal expense, it often remains unidentified. Such strains must be identified without a clue to their phylogenetic affiliation. Comparison of (nearly) entire 16S rRNA gene sequences is arguably one of the most powerful tools for establishment of the phylogenetic neighborhood of an unknown organism, and commercial identification systems based on analysis of rRNA gene sequences have become available (e.g., MicroSeq 500 and 16S rDNA Bacterial Sequencing Kit [Perkin-Elmer Applied Biosystems, Foster City, Calif.]). A fraction of the 5′-terminal region of the 16S rRNA gene (positions 60 to 110 of the *Escherichia coli* numbering system) is one of the most informative or discriminating regions for closely related organisms (66). Similar variable regions (flanked by highly conserved regions) occur in the 23S rRNA gene (113). A review by Clarridge recently covered this topic in detail (7) but struggled with the rRNA sequence similarity level as a limit for species delineation. As outlined above, use of the DNA-DNA hybridization level as a threshold for species delineation is more than just a mere "proposal" (7) and is now supported by a range of whole-genome-based analyses. Comparison of 16S rRNA gene sequences will lead to correct identification to the species level in many bacterial genera, but it is equally true that many taxonomic studies have revealed that comparative rRNA sequence analysis is often not sensitive enough to identify strains to the species level. There is clearly a lack of knowledge not only of the strain-to-strain variation within a species but also of the interoperon variation within a single strain. Therefore, concluding that an unidentified isolate belongs to a particular species because it shares a high percentage of its 16S rRNA gene sequence with particular species or concluding that it represents a novel species because it occupies a unique position in the phylogenetic tree or because it shares only 97% of its 16S rRNA sequence with its closest neighbor is premature in the absence of appropriate complementary data. This is even more true for partial sequence data, as partial rRNA gene sequences carry only limited information of the molecule and different parts of the gene may carry information for different taxonomic levels (66, 68). Nevertheless, erroneous claims that 16S rRNA gene sequencing represents the general "gold

standard" for identification of new isolates appear regularly in medical (and other) microbiological literature (27).

Alternative Approaches

As part of identification strategies, dichotomous keys based on morphological and biochemical characteristics have only partly been replaced by other methods. As described above, taxonomic analyses provided an impressive array of alternative techniques derived from analytical biochemistry and molecular biology for examination of numerous cellular compounds (36, 37, 89). Each of these parameters is useful for characterization and hence identification of bacteria. Databases of rRNA sequences, whole-cell fatty acid components, ribotyping profiles, MALDI-TOF mass spectra and FTIR spectra, or miniaturized series of phenotypic characteristics are available (see above and chapter 15) and allow identification of many isolates. Yet, the success of these databases also depends on the exactness of the methods and how carefully the individual entries have been delineated. The classification of new or unusual isolates will, however, often require a polyphasic approach, whereby an unknown is allocated to a certain phylogenetic neighborhood (typically by using 16S rRNA gene sequence analysis), followed by a comparison of characteristics of the unknown with those of its closest phylogenetic neighbors by using appropriate taxonomic tools in order to assign it a particular species rank.

Molecular Diagnostics

The information content of the rRNA cistrons and other genomic information have been used in several alternative ways for the identification of bacteria by the development of a range of DNA or RNA probes and amplification assays. Although the overall rRNA sequence similarity may be very high, the presence of variable regions in 16S or 23S RNA genes can provide the basis for specific and sensitive targets for identification purposes (for examples, see references 68, 94, and 113). During the last 20 years, DNA technology has emerged, and the tools of molecular biology are now used for the detection, characterization, and identification of bacteria. The first applications were labeled probes intended to hybridize with specific nucleic acid fragments. Later, in vitro nucleic acid amplification procedures were developed. It was thought that this enzymatic duplication and amplification of specific nucleic acid sequences would gradually replace culture-based approaches. However, it became rapidly clear that the molecular diagnostic approach has its own difficulties in terms of sensitivity, specificity, turn-around time, and cost. As a consequence, its application was restricted to the solution of these problems in cases in which it is superior to the conventional approach (50). Molecular diagnostic techniques are indicated for the detection of organisms that cannot be grown in vitro, for which current culture techniques are too insensitive, or that require sophisticated media or cell cultures and/or prolonged incubation times.

The basic principle of any molecular diagnostic test is the detection of a specific nucleic acid sequence by hybridization to a complementary sequence of DNA or RNA, a probe, followed by detection of the hybrid (68). There are two types of molecular diagnostic techniques: those in which the hybrids are not amplified prior to detection and those in which they are. With the probe technology not involving amplification, the probes used for the detection of complementary nucleic acid sequences are labeled with enzymes, chemiluminiscent moieties, radioisotopes, or antigenic substrates (49). This technology was first applied in the field of infectious diseases for the detection of enterotoxigenic *E. coli* in stool samples by DNA hybridization after growth on MacConkey agar plates (74). This application illustrated the major advantage of the technology: in a mixture of pathogenic and nonpathogenic bacteria belonging to the same species, present in the same specimen, and difficult to distinguish by traditional culture and identification techniques, the former bacteria were easily detected and identified (49).

Alternatively, any DNA fragment can be copied by using a DNA polymerase, provided that some sequence data are known for the design of appropriate primers. In vitro DNA replication was made possible in 1959 when Kornberg (60) discovered DNA polymerase I, but only in 1986 did Mullis and coworkers (75) introduce the idea of PCR, i.e., reiteration of the process of DNA polymerization, leading to an exponential increase of the nucleic acid. Alternative nucleic acid amplification techniques have been developed by using different enzymes and strategies, but they are all based on reiterative reactions. In most of these the target nucleic acid is amplified; in some the probe is multiplied (13, 33, 42, 64, 124). The advantages of these amplification systems are obvious: they can be highly specific and rapid, and they have very high sensitivities. However, there are many practical problems as well (chapter 16; see also references 49 and 50).

Immunological Techniques

Immunoassays are procedures which measure antigen or antibody levels to determine whether patients are infected or are immunologically responding to infection or immunization. Two general approaches are distinguished: testing for specific microbial antigens and testing for specific microbial antigen-specific antibodies. These immunological techniques are described in detail in chapter 18. An important advantage of immunoassay tests is that they provide information even when culture and Gram staining results are negative for patients who received antimicrobial therapy.

Conclusion

At present, the scientifically and economically ideal identification technique remains beyond reach. Cowan's (15) intuitive approach (which is used when the identity of the unknown is anticipated) and the stepwise method (which involves the use of dichotomous keys) suffice for numerous isolates and require only simple, rapid, and cheap biochemical tests. Cowan's views are easily adapted to modern methodology. If this first-line approach fails, alternative procedures are required and available. At present, complete 16S rRNA gene sequence analysis is the most straightforward and obvious choice to establish a rough identity of an isolate, although in the present species concept with DNA-DNA hybridization levels as the cornerstones, it often fails to differentiate closely related species. Much of its superiority is based on its capacity to reveal the phylogenetic neighborhood of the organism studied, which is information not provided by any of the other current identification protocols. This information will direct the additional analyses required for final identification to the species level.

NOMENCLATURE

Valid Publication of Bacterial Names

The *International Code of Nomenclature of Bacteria* (97) includes rules on how to name bacteria at different taxonomic ranks. The aim of nomenclature is to ensure that

an organism is tagged with a unique name that carries valuable information. Prior to 1980, a proposal of a new bacterial taxon could be validly published in any microbiological book or journal, and the authors of the relevant sections of the successive editions of *Bergey's Manual of Determinative Bacteriology* had to attempt to give a complete list of the members of any particular genus or group of genera. The unavailability of type strains and the fact that microbiologists from different disciplines were not always familiar with one another's work caused great difficulty. All too often a worker would discover several years later that "his" or "her" organism had in fact been described earlier under a different name.

To overcome such problems and others, 1 January 1980 was chosen as a new starting date for bacterial nomenclature. At that time, Approved Lists of Bacterial Names were published on behalf of the Judicial Commission of the International Committee on Systematic Bacteriology (95). Only those names included on these lists had standing in bacterial nomenclature, and names of taxa were to be included only if they were adequately described and if a type strain was available. From then onward, all new names were validly published only in the *International Journal of Systematic Bacteriology* (now the *International Journal of Systematic and Evolutionary Microbiology*). Names could effectively be published in other journals and then validated subsequently by announcement in Validation Lists in the *International Journal of Systematic and Evolutionary Microbiology*. A number of organisms were involuntarily omitted from the Approved Lists and were revived later (for instance, see reference 72). After 1980 several updates of these lists were published in the form of Validation Lists, and in 1989, an update of all names validly published between 1 January 1980 and 1 January 1989 was published by Moore and Moore (73). Nowadays, complete overviews of validly published names can easily be obtained through Internet sites such as http://www.bacterio.cict.fr/ or http://www.dsmz.de/bactnom/genera1.htm. Proposals of new taxa can continue to be made in any journal, but their names are only validated the moment that they are included in one of the Validation Lists, published regularly in the *International Journal of Systematic and Evolutionary Microbiology*. In case different names for the same organism are validly published, nomenclatural priority goes to the name that was validated first. As a result of this practice, all validly named species in any particular group can easily be traced and reference strains are available.

Why Do Names Change?

There are more important causes for the modification of bacterial names than the occasional detection of synonymy. As described above, our present view on bacterial classification is phylogeny based. With the advent of rRNA-DNA hybridization and, subsequently, the various rRNA gene sequencing methods, taxonomists had a new framework in which they could revise classification schemes. The classical—and extreme—example is the revision of the taxonomy and nomenclature of the genus *Pseudomonas,* which has been proceeding painstakingly slowly during the past 3 decades. The most important reason for this slow progress is that, through the work of De Vos and De Ley (22), it became clear not only that the genus *Pseudomonas* consisted of five major species clusters (87) but also that these clusters formed a polyphyletic part of a major group of bacteria now known as the *Proteobacteria.* Revision of the taxonomy of the pseudomonads had to consider the relationships of the various subbranches toward their numerous respective neighbors (55).

The modification of our view on classification is by far the most important reason for name changes. However, various forms of poor taxonomic practice also invoke a lot of changes, and hence irritation. As observed long ago (18), nomenclature often is "the generator of heat, bad temper and ill-will among taxonomists and every kind of microbiologists." The classification (and identification) of *Helicobacter* species represents a fine example of the difficulties encountered in our present-day view on taxonomy. Although this view is often challenged, it is the level of DNA-DNA hybridization and not the level of 16S rRNA gene sequence similarity that is the most critical parameter for species delineation. Relatively few laboratories have the experience required to perform DNA-DNA hybridization experiments in a highly standardized way. Yet, because of the tremendous clinical relevance of these bacteria, numerous investigators study all sorts of *Helicobacter*-like organisms from human and animal hosts. The biodiversity within *Helicobacter* is very high, and there is a need for many DNA-DNA hybridization data to delineate the species. Regrettably, helicobacters mostly are fastidious organisms, and many are difficult (and some nearly impossible) to culture in vitro; in addition, the preparation of sufficient DNA for the hybridization experiments is a hardy, if not impossible, task. In practice, new species have often been described on the basis of 16S rRNA gene sequence data and a limited number of differential phenotypic characteristics. A recent study that involved various taxonomic approaches, including DNA-DNA hybridization experiments, demonstrated that comparison of nearly complete 16S rRNA gene sequences combined with minimal biochemical characterization does not provide conclusive evidence for identification to the species level and may prove highly misleading (117).

Another example is the ongoing revision of the classification and nomenclature of group II pseudomonads. In 1992, Yabuuchi et al. (133) reclassified several rRNA group II pseudomonads as *Burkholderia* species. However, in this study only some of the rRNA group II pseudomonads were examined and the conclusions were based on data for only a limited set of strains. As a consequence, several additional rounds of name changes were required to reclassify the remaining rRNA group II pseudomonads as *Burkholderia* species (32, 109, 134, 139). This group of bacteria also serves as an example to illustrate other causes for name changes: the lack of criteria for genus delineation (two of the *Burkholderia* species *[B. solanacearum* and *B. pickettii]* were again reclassified into the new genus *Ralstonia* [32, 134], and some of the *Ralstonia* species were then further reclassified into the novel genus *Wautersia* [122]) and the intrinsically inadequate description of species that comprise only a single isolate. The lack of precise guidelines for genus level delineation was discussed recently by Young (135), who argued strongly that phenotypic coherence at the genus level should have priority over phylogenetic information.

The *Ralstonia* example also illustrates a tedious problem raised by Clarridge (7), namely, the challenges in the nomenclature for organisms named before their correct taxonomy was revealed by 16S rRNA gene sequence comparisons. Shortly after the reclassification of several *Ralstonia* species into the novel genus *Wautersia* (122), Vandamme and Coenye (115) reported that *Wautersia eutropha,* the type species of the genus *Wautersia,* is a junior

synonym of *Cupriavidus necator,* the type (and only) species of the genus *Cupriavidus,* which was validly named in 1987, i.e., long before 16S rRNA gene sequence studies were performed routinely (71). The only possible consequence—if one does not decide to bend the rules each time they turn out to be inconvenient—was to replace the name *Wautersia* by *Cupriavidus* and to consider all species of the genus *Wautersia* species of the genus *Cupriavidus*. While renaming and subsequent further renaming of bacterial species cause confusion and—not the least—irritation in the wider microbiological community, adhering to the rules of nomenclature is essential for establishing a truly systematic taxonomy.

The so-called "one strain taxa" (species or genera that are proposed on the basis of data for only one strain) have probably caused more problems than they have solved, and this is definitely the case in the context of diagnostic microbiology. It is not possible to estimate the variability of the phenotype in the case of a species with one strain or in the case of a genus with one species and one strain, for which many recent examples exist. The question of whether such strains can be validly named has been the subject of many debates. There are different views, each with advantages and disadvantages. In diagnostic microbiology, it is well known that a species is characterized by a certain degree of variability. This variability can be measured by both phenotypic and genotypic criteria and may be revealed by simple biochemical testing or sophisticated genomic fingerprinting techniques. In the absence of sufficient strains for quantitation of the range of divergence within a species, it will be difficult or impossible to identify new isolates of this organism without DNA-DNA hybridization experiments. A classification based on results obtained with a single strain cannot be stable. Indeed, the detection of a second strain will inevitably necessitate revision of the original species description.

Clearly, some of the instances of nomenclatural modifications described above could have been avoided, and that is the main reason why they jeopardize the credibility of taxonomists in the microbiology community. As a concluding remark, it should be mentioned that there is no "undo" function in bacterial nomenclature. A name that was validly published remains valid regardless of the number of modifications it undergoes thereafter. For instance, the changes of the name *Pseudomonas maltophilia* to *Xanthomonas maltophilia* (106) and finally to *Stenotrophomonas maltophilia* (85) or the changes of the name *Pseudomonas acidovorans* to *Comamonas acidovorans* (108) and finally to *Delftia acidovorans* (129) may be reasonable to some taxonomists, but the changes, particularly the most recent, have been refuted by many clinical microbiologists. As these six names were all proposed according to the rules of bacterial nomenclature, they were all validated, and the use of each of them is correct and valid. Use of the original *Pseudomonas* names could imply that the user disagrees with the phylogenetic rationale for present-day genus level classification. Use of the names *X. maltophilia* or *C. acidovorans* may simply indicate that one disagrees with the most recent modification, whether the reason is scientific or practical, or it may be a simple statement of discord with successive and excessive name changes.

CONCLUSION

A much broader range of taxonomic studies of bacteria has gradually replaced the former reliance upon morphological, physiological, and biochemical characterization. This polyphasic taxonomy takes into account all available phenotypic and genotypic information and integrates it in a consensus type of classification, framed in a general phylogeny derived from 16S rRNA gene sequence analysis. The bacterial species appears as a group of isolates which originated from a common ancestor population in which a steady generation of genetic diversity resulted in clones that had different degrees of recombination and that were characterized by a certain degree of phenotypic consistency, a significant degree of DNA-DNA hybridization, and a high degree of 16S rRNA gene sequence similarity (118).

The majority of bacteria in routine diagnostic laboratories will continue to be identified by classical methods, as these methods are adequate, cheap, readily available, and easy to handle. In the case of new or atypical isolates, or for many research groups in which, for example, bacteria are isolated from new sources, a straightforward means of identification of microorganisms by a single method is often not possible, and several methods are needed. The most direct approach is first to allocate such isolates in the phylogenetic framework and then to determine the finer relationships by means of an appropriate approach, which may be polyphasic. This tendency of identification to become polyphasic is an unavoidable reality.

In some cases, the consensus classification is a compromise that contains a minimum of contradictions. It is thought that the more parameters that become available in the future, the more polyphasic classification will gain stability. Although the idea is purely speculative at present, insight into the vast amount of data that are potentially available could be the basis for a perfectly reliable and stable classification system. However, already with our present data, it is sometimes unclear if it makes sense to order bacteria into a classification system. Undoubtedly, there is a huge amount of biodiversity, which can be handled in a practical manner only if it is arrayed in an ordered structure, artificial or not, with appropriate terms for communication.

Our present view on classification reflects the best science of this time. The same was true in the past, when only data from morphological and biochemical analyses were available. The main perspective in bacterial taxonomy is that technological progress will dominate and drastically influence methodology, as it always has. More data will become available, more bacteria will be detected (whether they can be cultivated or not), there will be more automation, and bioinformatics will have to address the combination and linking of databases. Most important, the increasing access to whole-genome sequences will generate proposals for novel species concepts. These future concepts may be based on whole-genome sequences, on a shared core of genes, on a certain type of genes such as housekeeping or informational genes (26, 45), or on a well-balanced selection of genes included in an MLSA type of analysis (9, 30). It will be a formidable challenge to translate such information into classification and identification schemes and to evaluate classifications that have been carefully designed.

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Specimen Collection, Transport, and Processing: Bacteriology*

RICHARD B. THOMSON, JR.

20

The use of specimens for bacteriological analysis requires that specific clinical material be collected, stabilized, and transported according to exacting specifications to ensure valid results. Poor specimen quality contributes to misdiagnosis and inappropriate antimicrobial therapy. Communication between the laboratory representative and clinician is essential to the proper selection of bacteriology tests and interpretation of their results. Laboratory personnel are responsible for monitoring and educating those collecting and transporting specimens. The use of professional journals is an excellent approach to the continuing education of all who work in and use the laboratory. Laboratories are required by accrediting agencies to provide specimen collection and transport manuals. A useful alternative to printed manuals is an electronic version available over a local area network (13, 100).

Specimens for bacteriological culture should be collected as soon as possible after the onset of disease and before the initiation of antimicrobial therapy. A second specimen may be necessary because of poor specimen quality or inadequate transport conditions that affected the first specimen, but is otherwise rarely required for diagnosis of an acute infectious disease. Exceptions include the collection of multiple blood specimens for culture and those obtained for the detection of fastidious or unusual pathogens not originally suspected.

SELECTION AND COLLECTION OF SPECIMENS

Material for bacteriological testing should be collected from a site representative of the active disease process. Sites of an inflammatory process and free of contaminating flora are optimal. In practice, most specimen collection sites are contaminated with various quantities of commensal bacteria. As examples, urethral floras contaminate urine collected by micturition, skin floras contaminate blood collected by percutaneous venipuncture, vaginal and cervical floras contaminate endometrial specimens collected through the endocervix, skin and environmental floras contaminate cutaneous fistulas and deep wounds that are open to skin or mucous membrane surfaces, floras in the external ear canal contaminate middle ear fluid following rupture of the tympanic membrane, floras in the nasopharynx and nasal passages contaminate specimens from the nasal sinuses, and the floras in the upper respiratory tract contaminate sputum and other lower respiratory tract specimens. Table 1 lists common diseases with appropriate and inappropriate clinical specimens (11, 224). General specimen selection and collection guidelines include the following (122).

1. Select the proper anatomic site from which to collect the specimen.
2. Avoid contamination with indigenous flora. Growth and reporting of normal flora can be mistaken by the physician or caregiver as the cause of the infectious process. In addition, the flora can overgrow and obscure the true etiology.
3. Surface disinfection followed by aspiration and biopsy of tissue are appropriate methods for specimen collection when anaerobic bacteria are suspected (Table 2). Collection with a swab is not recommended because of the relatively small amount of specimen sampled, difficulty of actually obtaining anaerobes on the swab, aeration of swabs, and the ease with which the swab can be contaminated with adjacent members of the normal flora. Unless they are in a completely airtight container, specimens for anaerobic culture should be stored at room, not refrigeration, temperature, since oxygen diffuses into cold specimens more readily.
4. Collect sufficient volume of material to enable all test requests to be performed satisfactorily. Insufficient material may yield false-negative results.
5. Label each specimen with the patient's name and identification number, source of specimen, date and time of collection, and initials of the collector.
6. Use a specimen container designed to promote survival of pathogenic bacteria, eliminate leakage of specimen, and allow safe handling during transport and processing. Do not transport specimens in syringes.

Specific specimen collection guidelines are summarized in Table 3.

TRANSPORT OF SPECIMENS

Specimens for bacterial culture should be transported to the laboratory immediately. Excessive delay or exposure to temperature extremes compromises results and must be avoided.

* This chapter contains information presented in chapter 20 by Richard B. Thomson, Jr., and J. Michael Miller in the eighth edition of this Manual.

TABLE 1 Selection of common clinical specimens for bacterial culture[a]

Anatomic site	Clinical specimen(s)	
	Appropriate	Inappropriate
Lower respiratory tract	Freshly expectorated mucus and inflammatory cells (pus), sputum	Saliva, oropharyngeal secretions, sinus drainage from nasopharynx
Sinus	Secretions collected by direct sinus aspiration, or washes, curettage, and biopsy material collected during endoscopy	Nasal or nasopharyngeal swab, nasopharyngeal secretions, sputum, and saliva
Urinary tract	Midstream urine, urine collected by "straight" catheterization, urine collected by suprapubic aspiration, urine collected during cystoscopy or other surgical procedure	Urine from Foley catheter collection bag, "bagged" urine from infants
Superficial wound	Aspirations of pus or local irrigation fluid (nonbacteriostatic saline), swab of purulence originating from beneath the dermis	Swab of surface material or specimen contaminated with surface material, irrigation with saline containing preservative
Deep wound	Purulence, necrosis, or tissue from deep subcutaneous site	Specimen contaminated with surface material
Gastrointestinal tract	Freshly passed stool, washes, or feces collected during endoscopy; rectal swab (in selected cases)	Rectal swab, specimen for bacterial culture if diarrhea developed after patient was in hospital for >3 days
Venous blood	Two or three blood specimens collected from separate venipunctures, before initiation of antibiotics, each containing approx 20 ml of blood for patients >90 lb (see Table 7 for pediatric volumes); antisepsis with iodine-containing compound or chlorhexidine	Clotted blood; one or more than three blood specimens collected within a 24-h period; vol of blood <20 ml per culture (i.e., per venipuncture); antisepsis with alcohol only (adults)

[a] Reprinted from reference 195 with permission.

TABLE 2 Suitability of various specimens for anaerobic culture

Acceptable material (method of collection)	Unacceptable material
Aspirate (by needle and syringe)	Bronchoalveolar lavage washing
Bartholin's gland inflammation/secretions	Cervical secretions
Blood (venipuncture)	Endotracheal secretions (aspirate)
Bone marrow (aspirate)	Lochia secretions
Bronchoscopic secretions (protected specimen brush)	Nasopharyngeal swab
Culdocentesis fluid (aspirate)	Perineal swab
Fallopian tube fluid or tissue (aspirate/biopsy)	Prostatic or seminal fluid
IUD, for *Actinomyces* spp.	Sinus washings or swabs
Nasal sinus (aspirate)	Sputum (expectorated or induced)
Placenta tissue (via cesarean delivery)	Stool or rectal swab samples
Stool, for C. *difficile*	Tracheostomy secretions
Surgical site (aspirate, tissue)	Urethral secretions
Transtracheal aspirate	Urine (voided or from catheter)
Urine (suprapubic aspirate)	Vaginal or vulvar secretions (swab)

TABLE 3 Bacteriology collection, transport, and storage guidelines[a]

Specimen type (reference)	Collection guidelines	Transport device and/or minimum vol	Transport[b] time and temp	Storage time and temp	Replica limits	Comments
Abscess (17)						
General	Remove surface exudate by wiping with sterile saline or 70% alcohol.					Tissue or aspirate is always superior to a swab specimen. If swabs must be used (aerobic culture only), collect two, one for culture and one for Gram staining. Preserve swab material by placing in Stuart's or Amies medium.
Open	Aspirate if possible or pass a swab deep into the lesion to firmly sample the lesion's "fresh border."	Swab transport system	≤2 h, RT	≤24 h, RT	1/day/source	Samples of the base of the lesion and abscess wall are most productive.
Closed	Aspirate abscess material with needle and syringe. Aseptically transfer *all* material into anaerobic transport device.	Anaerobic transport system, ≥1 ml	≤2 h, RT	≤24 h, RT	1/day/source	Contamination with surface material will introduce colonizing bacteria not involved in the infectious process. Do not use syringe for transport.
Bite wound	See Abscess					Do not culture animal bite wounds ≤12 h old (agents are usually not recovered) unless signs of infection are present.
Blood (15, 160)	Disinfect culture bottle; apply 70% isopropyl alcohol or phenolic disinfectant to rubber stoppers and wait 1 min. Palpate vein before disinfection of venipuncture site. Disinfection of venipuncture site: 1. Cleanse site with 70% alcohol. 2. Swab concentrically, starting at the center, with tincture of iodine or chlorhexidine. 3. Allow the disinfectant to dry. 4. *Do not palpate vein at this point without sterile glove.*	Blood culture bottles for bacteria; adult, 20 ml/set (higher vol most productive); infant and child, 1–20 ml/set depending on wt of patient (see Table 7)	≤2 h, RT	≤2 h, RT or per instructions	3 sets in 24 h	Acute febrile episode: 2 sets[c] from separate sites, all within 10 min (before antimicrobials) Nonacute disease, antimicrobials will not be started or changed immediately: 2 or 3 sets from separate sites, all within 24 h at intervals no closer than 3 h (before antimicrobials) Endocarditis, acute: 3 sets from 3 separate sites, within 1–2 h, before antimicrobials if possible Fever of unknown origin: 2 or 3 sets from separate sites ≥1 h apart during 24-h period. If negative at 24–48 h, obtain 2 or 3 more sets. Some data indicate that an additional aerobic or fungal bottle is more productive than the anaerobic bottle.

(Continued on next page)

TABLE 3 Bacteriology collection, transport, and storage guidelines[a] *(Continued)*

Specimen type (reference)	Collection guidelines	Transport device and/or minimum vol	Transport[b] time and temp	Storage time and temp	Replica limits	Comments
	5. Collect blood. 6. After venipuncture, remove iodine from the skin with alcohol.					Pediatric: collect immediately; rarely necessary to document ccontinuous bacteremia with hours between cultures.
Bone marrow aspirate	Prepare puncture site as for surgical incision.	Inoculate blood culture bottle or a 1.5-ml lysis centrifugation tube.	≤24 h, RT, if in culture bottle or tube	≤24 h, RT	1/day	Small volumes of bone marrow may be inoculated directly onto culture media. Routine bacterial culture of bone marrow is rarely useful.
Burn	Clean and debride the burn.	Tissue is placed into a sterile screw-cap container; aspirate or swab exudates; transport in sterile container or swab transport system	≤24 h, RT	≤24 h, RT	1/day/source	A 3- to 4-mm punch biopsy specimen is optimum when quantitative cultures are ordered. Process for aerobic culture only. Quantitative culture may or may not be valuable. Cultures of surface samples of burns may be misleading.
Catheter i.v. (109)	1. Cleanse the skin around the catheter site with alcohol. 2. Aseptically remove catheter and clip 5 cm of distal tip directly into a sterile tube. Some elect to culture the 5-cm intracutaneous portion to evaluate for soft tissue infection. 3. Transport immediately to microbiology laboratory to prevent drying.	Sterile screw-cap tube or cup	≤15 min, RT	≤2 h, 4°C	None	Acceptable i.v. catheters for semiquantitative culture (Maki method): central, CVP, Hickman, Broviac, peripheral, arterial, umbilical, hyperalimentation, Swan-Ganz
Foley	Do *not* culture, since growth represents distal urethral flora.					Not acceptable for culture.
Cellulitis, aspirate from area of (17)	1. Cleanse site by wiping with sterile saline or 70% alcohol.	Sterile tube (syringe transport not recommended)	≤15 min, RT	≤24 h, RT	None	Yield of potential pathogens in minority of specimens cultured

	2. Aspirate the area of maximum inflammation (commonly the center rather than the leading edge) with a needle and syringe. Irrigation with a small amount of sterile saline may be necessary. 3. Aspirate saline into syringe and expel into sterile screw-cap tube.					
CSF	1. Disinfect site with iodine preparation. 2. Insert a needle with stylet at L3-L4, L4-L5, or L5-S1 interspace. 3. Upon reaching the subarachnoid space, remove the stylet and collect 1–2 ml of fluid into each of 3 leakproof tubes.	Sterile screw-cap tubes Minimum amt required: bacteria, ≥1 ml	Bacteria: never refrigerate; ≤15 min, RT	≤24 h, RT	None	Obtain blood for culture also. If only 1 tube of CSF is collected, it should be submitted to microbiology first; otherwise submit tube 2 to microbiology. Aspirate of brain abscess or a biopsy sample may be necessary to detect anaerobic bacteria or parasites.
Decubitus ulcer (17)	A swab is not the specimen of choice (see comments). 1. Cleanse surface with sterile saline. 2. If a biopsy sample is not available, aspirate inflammatory material from the base of the ulcer.	Sterile tube/container (aerobic) or anaerobic system (for tissue)	≤2 h, RT	≤24 h, RT	1/day/source	Since a swab specimen of a decubitus ulcer provides no clinical information, it should not be submitted. A tissue biopsy sample or needle aspirate is the specimen of choice.
Dental culture: gingival, periodontal, periapical, Vincent's stomatitis	1. Carefully cleanse gingival margin and supragingival tooth surface to remove saliva, debris, and plaque. 2. Using a periodontal scaler, carefully remove subgingival lesion material and transfer it to an anaerobic transport system. 3. Prepare smear for staining with specimen collected in the same fashion.	Anaerobic transport system	≤2 h, RT	≤24 h, RT	1/day	Periodontal lesions should be processed only by laboratories equipped to provide specialized techniques for the detection and enumeration of recognized pathogens.

(Continued on next page)

TABLE 3 Bacteriology collection, transport, and storage guidelines[a] *(Continued)*

Specimen type (reference)	Collection guidelines	Transport device and/or minimum vol	Transport[b] time and temp	Storage time and temp	Replica limits	Comments
Ear						
Inner (25)	Tympanocentesis reserved for complicated, recurrent, or chronic persistent otitis media 1. For intact eardrum, clean ear canal with soap solution and collect fluid via syringe aspiration technique (tympanocentesis). 2. For ruptured eardrum, collect fluid on flexible shaft swab via an auditory speculum (aerobic culture only).	Sterile tube, swab transport medium, or anaerobic system	≤2 h, RT	≤24 h, RT	1/day/source	Results of throat or nasopharyngeal swab cultures are not predictive of agents responsible for otitis media and should not be submitted for that purpose.
Outer (168)	1. Use moistened swab to remove any debris or crust from the ear canal. 2. Obtain a sample by firmly rotating swab in the outer canal.	Swab transport	≤2 h, RT	≤24 h, 4°C	1/day/source	For otitis externa, *vigorous* swabbing is required since surface swabbing may miss streptococcal cellulitis.
Eye						
Conjunctiva (4, 86)	1. Sample both eyes with separate swabs (premoistened with sterile saline) by rolling them over each conjunctiva. 2. Medium may be inoculated at time of collection. 3. Smear may be prepared at time of collection. Roll swab over 1- to 2-cm area of slide.	Direct culture inoculation: BAP and CHOC Laboratory inoculation: swab transport	Plates: ≤15 min, RT Swabs: ≤2 h, RT	≤24 h, RT	None	If possible, sample both conjunctivas, even if only one is infected, to determine indigenous microflora. The uninfected eye can serve as a control with which to compare the agents isolated from the infected eye. If cost prohibits this approach, rely on the Gram stain to assist in interpretation of culture.
Corneal scrapings (4, 86)	1. Specimen collected by ophthalmologist 2. Using sterile spatula, scrape ulcers or lesions, and inoculate scraping directly onto medium. 3. Prepare 2 smears by rubbing material from spatula onto 1- to 2-cm area of slide.	Direct culture inoculations: BHI with 10% sheep blood, CHOC, and inhibitory mold agar	≤15 min, RT	≤24 h, RT	None	If conjunctival specimen is collected, do so before anesthetic application, which may inhibit some bacteria. Corneal scrapings are obtained after anesthesia. Include fungal media.

Vitreous fluid aspirates	Prepare eye for needle aspiration of fluid.	Sterile screw-cap tube or direct inoculation of small amount of fluid onto media	≤15 min, RT	≤24 h, RT	1/day	Include fungal media. Anesthetics may be inhibitory to some etiologic agents.
Feces						
Routine culture (63)	Pass specimen directly into a clean, dry container. Transport to microbiology laboratory within 1 h of collection or transfer to Cary-Blair holding medium.	Clean, leakproof, widemouthed container or use Cary-Blair holding medium (>2 g)	Unpreserved: ≤1 h, RT Holding medium: ≤24 h, RT	≤24 h, 4°C ≤48 h, RT or 4°C	1/day	Do not perform routine stool cultures for patients whose length of hospital stay is >3 days and the admitting diagnosis was not gastroenteritis without consultation with physician. Tests for *C. difficile* should be considered for these patients. Swabs for routine pathogens are not recommended except for infants (see Rectal swabs).
C. difficile (84)	Pass liquid or soft stool directly into a clean, dry container. Soft stool is defined as stool assuming the shape of its container. Swab specimens are not recommended for toxin testing.	Sterile, leakproof, widemouthed container, >5 ml	≤1 h, RT; 1–24 h, 4°C; >24 h, −20°C or colder	2 days, 4°C, for culture; 3 days at 4°C or longer at −70°C for toxin test	1 or 2 specimens may be necessary to detect low toxin levels.	Patients should be passing ≥5 liquid or soft stools per 24 h. Testing of formed or hard stool is not recommended. Freezing at −20°C or above results in rapid loss of cytotoxin activity.
E. coli (O157:H7) and other Shiga toxin-producing serotypes (5, 50)	Pass liquid or bloody stool into a clean, dry container.	Sterile, leakproof, widemouthed container, or Cary-Blair holding medium (>2 g)	Unpreserved. ≤1 h, RT Swab transport system: ≤24 h, RT or 4°C	≤24 h, 4°C ≤24 h, RT	1/day	Bloody or liquid stools collected within 6 days of onset from patients with abdominal cramps have the highest yield. Shiga toxin assay for all EHEC serotypes is better than sorbitol MacConkey or chromogenic agar culture for O157:H7 only.
Leukocyte detection (68) (not recommended for use with patients who have acute infectious diarrhea)						This procedure should be discouraged because it provides results of little clinical value.

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TABLE 3 Bacteriology collection, transport, and storage guidelines[a] *(Continued)*

Specimen type (reference)	Collection guidelines	Transport device and/or minimum vol	Transport[b] time and temp	Storage time and temp	Replica limits	Comments
Rectal swab	1. Carefully insert a swab approx 1 in. beyond the anal sphincter. 2. Gently rotate the swab to sample the anal crypts. 3. Feces should be visible on the swab for detection of diarrheal pathogens.	Swab transport	≤2 h, RT	≤24 h, RT	1/day	Reserved for detecting *N. gonorrhoeae, Shigella, Campylobacter,* herpes simplex virus, and anal carriage of group B *Streptococcus* and other beta-hemolytic streptococci, or for patients unable to pass a specimen.
Fistula	See Abscess					
Fluids: abdominal, amniotic, ascites, bile, joint, paracentesis, pericardial, peritoneal, pleural, synovial, thoracentesis (17)	1. Disinfect overlying skin with iodine preparation. 2. Obtain specimen via percutaneous needle aspiration or surgery. 3. Always submit as much fluid as possible; *never* submit a swab dipped in fluid.	Anaerobic transport system, sterile screw-cap tube, or blood culture bottle for bacteria; transport immediately to laboratory Bacteria, >1 ml	≤15 min, RT	≤24 h, RT; pericardial fluid and fluids for fungal cultures, ≤24 h, 4°C	None	Amniotic and culdocentesis fluids should be transported in an anaerobic system and need not be centrifuged prior to Gram staining. Other fluids are best examined by Gram staining of a cytocentrifuged preparation. One aerobic blood culture bottle inoculated at bedside is highly recommended.
Gangrenous tissue	See Abscess					Discourage sampling of surface or superficial tissue. Tissue biopsy or aspirates should be collected.
Gastric						
Wash or lavage for mycobacteria (24)	Collect in early morning before patients eat and while they are still in bed. 1. Introduce a nasogastric tube to the stomach. 2. Perform lavage with 25–50 ml of chilled sterile, distilled water. 3. Recover sample and place in a leakproof, sterile container.	Sterile, leakproof container	≤15 min, RT, or neutralize within 1 h of collection	≤24 h, 4°C	1/day	The specimen must be processed promptly, since mycobacteria die rapidly in gastric washings. Neutralize with sodium bicarbonate when holding for > 1 h.
Biopsy for *H. pylori*	Collected by gastroenterologist during endoscopy.	Sterile tube with transport medium	<1 h, RT	≤24 h, 4°C	None	Culture may be needed for antimicrobial testing.
Genital, female						
Amniotic fluid (209)	Aspirate via amniocentesis, or collect during cesarean delivery.	Anaerobic transport system, ≥1 ml	≤2 h, RT	≤24 h, RT	None	Swabbing or aspiration of vaginal secretions is *not* acceptable because of the potential for contamination with commensal vaginal flora.

Bartholin gland secretions	1. Disinfect skin with iodine preparation. 2. Aspirate fluid from ducts.	Anaerobic transport system, ≥1 ml	≤2 h, RT	≤24 h, RT	1/day	
Cervical secretions (6)	1. Visualize the cervix using a speculum without lubricant. 2. Remove mucus and secretions from the cervical os with swab and discard the swab. 3. Firmly yet gently sample the endocervical canal with a new sterile swab.	Swab transport	≤2 h, RT	≤24 h, RT	1/day	See text for collection and transport need for *C. trachomatis* and *N. gonorrhoeae*.
Cul-de-sac fluid	Submit aspirate or fluid.	Anaerobic transport system, >1 ml	≤2 h, RT	≤24 h, RT	1/day	
Endometrial tissue and secretions	1. Collect transcervical aspirate via a telescoping catheter. 2. Transfer entire amount to anaerobic transport system.	Anaerobic transport system, ≥1 ml	≤2 h, RT	≤24 h, RT	1/day	
Products of conception	1. Submit a portion of tissue in a sterile container. 2. If obtained by cesarean delivery, immediately transfer to an anaerobic transport system.	Sterile tube or anaerobic transport system	≤2 h, RT	≤24 h, RT	1/day	Do not process lochia, culture of which may give misleading results.
Urethral secretions (6)	Collect at least 1 h after patient has urinated. 1. Remove old exudate from the urethral orifice. 2. Collect discharge material on a swab by massaging the urethra against the pubic symphysis through the vagina.	Swab transport	≤2 h, RT	≤24 h, RT	1/day	If no discharge can be obtained, wash the periurethral area with Betadine soap and rinse with water. Insert a small swab 2–4 cm into the urethra, rotate swab, and leave swab in place for at least 2 s to facilitate absorption.
Vaginal secretions (6)	1. Wipe away old secretions/discharge. 2. Obtain secretions from the mucosal membrane of the vaginal wall with a sterile swab or pipette. 3. If a smear is also needed, use a second swab.	Swab transport	≤2 h, RT	≤24 h, RT	1/day	For IUDs, place entire device into a sterile container and submit at RT. Gram stain, not culture, is recommended for diagnosis of BV.

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TABLE 3 Bacteriology collection, transport, and storage guidelines[a] *(Continued)*

Specimen type (reference)	Collection guidelines	Transport device and/or minimum vol	Transport[b] time and temp	Storage time and temp	Replica limits	Comments
Genital, female or male lesion	1. Clean with sterile saline and remove lesion's surface with a sterile scalped blade. 2. Allow transudate to accumulate. 3. While pressing the base of the lesion, *firmly* rub base with a sterile swab to collect fluid.	Swab transport	≤2 h, RT	≤24 h, RT	1/day	For dark-field examination to detect *T. pallidum*, touch a glass slide to the transudate, add coverslip, and transport immediately to the laboratory in a humidified chamber (petri dish with moist gauze). *T. pallidum* cannot be cultured on artificial media.
Genital, male						
Prostate	1. Cleanse urethral meatus with soap and water. 2. Massage prostate through rectum. 3. Collect fluid expressed from urethra on a sterile swab.	Swab transport or sterile tube for >1 ml of specimen	≤2 h, RT	≤24 h, RT	1/day	Pathogens in prostatic secretions may be identified by quantitative culture of urine before and after massage. Ejaculate may also be cultured.
Urethra	Insert a small swab 2–4 cm into the urethral lumen, rotate swab, and leave it in place for at least 2 s to facilitate absorption.	Swab transport	≤2 h, RT	≤24 h, RT	1/day	
Pilonidal cyst	See Abscess					
Respiratory, lower						
Bronchoalveolar lavage, brush or wash, endotracheal aspirate	1. Collect washing or aspirate in a sputum trap. 2. Place brush in sterile container with 1 ml of saline.	Sterile container, >1 ml	≤2 h, RT	≤24 h, 4°C	1/day	A total of 40–80 ml of fluid is needed for quantitative analysis. For quantitative analysis of brushings, place brush into 1.0 ml of saline.
Sputum, expectorated (10)	1. Collect specimen under the direct supervision of a nurse or physician. 2. Have patient rinse or gargle with water to remove excess oral flora. 3. Instruct patient to cough deeply to produce a lower respiratory specimen (not postnasal fluid). 4. Collect in a sterile container.	Sterile container, >1 ml Minimum amt: bacteria, >1 ml	≤2 h, RT	≤24 h, 4°C	1/day	For pediatric patients unable to produce a sputum specimen, a respiratory therapist should collect a specimen via suction. The best specimen from all patients should have ≤10 squamous cells/100× field (10× objective and 10× ocular).

Sputum, induced (10)	1. Have patient rinse mouth with water after brushing gums and tongue. 2. With the aid of a nebulizer, have patients inhale approx 25 ml of 3–10% sterile saline. 3. Collect in a sterile container.	Sterile container, >1 ml	≤2 h, RT	≤24 h, RT	1/day	Same as above for sputum, expectorated.
Respiratory, upper						
Oral	1. Remove oral secretions and debris from the surface of the lesion with a swab. Discard this swab. 2. Using a second swab, vigorously sample the lesion, avoiding any areas of normal tissue.	Swab transport	≤2 h, RT	≤24 h, RT	1/day	Discourage sampling of superficial tissue for bacterial evaluation. Tissue biopsy specimens or needle aspirates are the specimens of choice.
Nasal	1. Insert a swab, premoistened with sterile saline, approx 1–2 cm into the nares. 2. Rotate the swab against the nasal mucosa.	Swab transport	≤2 h, RT	≤24 h, RT	1/day	Anterior nose cultures are reserved for identifying staphylococcal carriers or for nasal lesions.
Nasopharynx (25)	1. Gently insert a small swab (e.g., calcium alginate) into the posterior nasopharynx via the nose. 2. Rotate swab slowly for 5 s to absorb secretions.	Direct medium inoculation at bedside or examination table, swab transport	Plates: ≤15 min, RT Swabs: ≤2 h, RT	≤24 h, RT	1/day	
Throat or pharynx	1. Depress tongue with a tongue depressor. 2. Sample the posterior pharynx, tonsils, and inflamed areas with a sterile swab.	Swab transport (dry swab with or without silica gel is good for *S. pyogenes* and *C. diphtheriae*)	≤2 h, RT	≤24 h, RT	1/day	Throat swab cultures are contraindicated for patients with epiglottitis. Swabs for *N. gonorrhoeae* should be placed in charcoal-containing transport medium and plated ≤12 h after collection. JEMBEC, Bio-Bags, and the GonoPak are better for transport at RT.
Tissue	Collected during surgery or cutaneous biopsy procedure	Anaerobic transport system or sterile, screw-cap container. Add several drops of sterile saline to keep small pieces of tissue moist.	≤15 min, RT	≤24 h, RT	None	Always submit as much tissue as possible. If excess tissue is available, save a portion of surgical tissue at −70°C in case further studies are needed. Never submit a swab that has been rubbed over the surface of a tissue. For quantitative study, a sample of 1 cm^3 is appropriate.

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TABLE 3 Bacteriology collection, transport, and storage guidelines[a] *(Continued)*

Specimen type (reference)	Collection guidelines	Transport device and/or minimum vol	Transport[b] time and temp	Storage time and temp	Replica limits	Comments
Urine						
Female, midstream (29)	1. While holding the labia apart, begin voiding. 2. After several milliliters has passed, collect a midstream portion without stopping the flow of urine. 3. The midstream portion is used for bacterial culture.	Sterile, widemouthed container, ≥1 ml, or urine transport tube with boric acid preservative	Unpreserved: ≤2 h, RT Preserved: ≤24 h, RT	≤24 h, 4°C	1/day	Chlamydial DNA detection in urine from women is less sensitive than in urine from men. Urine is toxic to cell lines and is therefore not the specimen of choice for chlamydial culture. Cleansing before voiding does not improve urine specimen quality; i.e., midstream urine samples are equivalent to clean-catch midstream urine samples.
Male, midstream (29)	1. While holding the foreskin retracted, begin voiding. 2. After several milliliters has passed, collect a midstream portion without stopping the flow of urine. 3. The midstream portion is used for culture.	Sterile, widemouthed container, ≥1 ml, or urine transport tube with boric acid preservative	Unpreserved: ≤2 h, RT	≤24 h, 4°C	1/day	First part of urine stream is used for probe and DNA amplification tests for *Chlamydia*. Collect urine for probe and DNA amplification tests at least 2 h after previous urination.
Straight catheter (29)	1. Thoroughly cleanse the urethral opening with soap and water. 2. Rinse area with wet gauze pads. 3. Aseptically, insert catheter into the bladder. 4. After allowing approx 15 ml to pass, collect urine to be submitted in a sterile container.	Sterile, leakproof container or urine transport tube with boric acid preservative	Unpreserved: ≤2 h, RT Preserved: ≤24 h, RT	≤24 h, 4°C	1/day	Catheterization may introduce urethral flora into the bladder and increase the risk of iatrogenic infection.
Indwelling catheter	1. Disinfect the catheter collection port with 70% alcohol. Clamp catheter below port and allow urine to collect in tubing for 10–20 min. 2. Use needle and syringe to aseptically collect 5 to 10 ml of urine. 3. Transfer to a sterile tube or container.	Sterile leakproof container or urine transport tube with boric acid preservative	Unpreserved: ≤2 h, RT Preserved: ≤24 h, RT	≤24 h, 4°C	1/day	Patients with indwelling catheters always have bacteria in their bladders. Do not collect urine from these patients unless they are symptomatic.
Wound	See Abscess					

[a] Abbreviations: AFB, acid-fast bacilli; BAP, blood agar plate; BHI, brain heart infusion; CHOC, chocolate agar; CVP, central venous pressure; i.v., intravenous; PVA, polyvinyl alcohol fixative; RT, room temperature.
[b] All specimen containers are to be transported in leakproof plastic bags having a separate compartment for the requisition.
[c] One set refers to one culture with both aerobic and anaerobic broths.

General specimen transport guidelines include the following (11, 122).

1. Specimens must be transported promptly to the laboratory. If transport will require more than 2 h, a special holding medium or refrigeration temperature is required (Table 3).

2. Do not store specimens for bacterial culture for more than 24 h even with appropriate holding medium or refrigeration temperature.

3. Optimal transport times of clinical specimens for bacteriological culture depend on the volume of material obtained. Small volumes of fluid (<1 ml) or tissue (<1 cm^3) should be submitted within 15 to 30 min to avoid evaporation, drying, and exposure to ambient conditions. Larger volumes and those specimens in holding medium may be stored as long as 24 h.

4. Bacteria that are especially sensitive to ambient conditions include *Shigella* spp., *Neisseria gonorrhoeae, Neisseria meningitidis, Haemophilus influenzae, Streptococcus pneumoniae,* and anaerobes. Reliable detection of these species requires immediate processing. Delays of up to 6 h result in minimal loss of CFU when transport media are used. Longer delays, even with the use of transport media, result in significant losses of organisms. For delays beyond 6 h, refrigeration improves recovery; however, specimens containing anaerobes should be stored at ambient temperatures (2, 42).

5. Transport of clinical specimens and infectious substances from one laboratory to another, regardless of the distance, requires strict attention to specimen packaging and labeling instructions. Materials for transport must be labeled properly, packaged, and protected during transport. Refer to the Centers for Disease Control and Prevention (CDC) website (http://www.phppo.cdc.gov/nltn/nphtcs/ps2005.aspx) for a complete description of packaging and shipping regulations mandated by the U.S. Department of Transportation.

Specific specimen transport guidelines are summarized in Table 3.

SPECIMENS FOR INFREQUENTLY ENCOUNTERED BACTERIA

Some bacteria cause infections that are infrequently encountered and require special transport conditions or holding medium. In many instances, these specimens will be shipped to reference laboratories; this necessitates relatively long transport times. Table 4 is a list of specimens and transport conditions for these bacteria (39).

PROCESSING OF SPECIMENS

Specimen processing includes detection of bacteria by staining and culturing, performing immunologic assays for microbial antigen, and the use of molecular techniques that identify specific nucleic acid sequences. The recommendations that follow are neither all-inclusive nor applicable to all laboratory settings (Table 5). Gram-stained smears are recommended whenever (i) rapid stain results are necessary for patient care, (ii) analysis of cellularity is used to determine adequacy of specimen, or (iii) results are needed to help interpret culture findings by the laboratory technologist. Anaerobic culture should be included only when ordered with an appropriate specimen source (Table 2) and when the specimen was transported in an oxygen-free container free of contamination with normal skin and mucosal anaerobic flora. Gram-stained smear and culture interpretation may require the intervention and opinion of a laboratory director trained in medical microbiology or pathology, and capable of reviewing clinical information with the patient's physician prior to report generation.

General Considerations

Safety

Refer to chapters 5, 8, and 9 for a complete description of safety issues.

Processing

Specimens for detection of bacteria by methods which include culture, staining, and antigen or nucleic acid detection must be processed in a timely manner. The lability of microorganisms, and their antigens and nucleic acids, mandates holding conditions and processing time limits. Improper handling prior to processing can result in death of pathogenic bacteria or overgrowth of contaminating bacteria. In addition, correct interpretations of culture results generally require a rough quantitation of bacterial densities in the clinical specimen. Allowing bacteria to multiply out of proportion to their original numbers may result in erroneous, sometimes detrimental, interpretations. General considerations for holding specimens and acceptable processing delays are summarized in Table 3 (122).

Processing at a Remote Site

Consolidation of laboratory services results in centralized microbiology laboratories located miles from the sites of specimen collection. Adhering to transport and processing time limits developed for laboratories located within or adjacent to the specimen collection site (e.g., hospital) is impossible for remote laboratories. The best approach to location of a core laboratory and the amount of local processing that should be performed can be determined on the basis of guidelines proposed by the Infectious Disease Society of America (149).

Specimen Labeling and Test Ordering Requirements

Upon arrival of specimens in the laboratory, the time and date of receipt should be recorded. Subsequently, the time of plating, which may differ substantially from the time of receipt, should be recorded. At the time of receipt, all specimens and requisitions should be carefully inspected. Specimens must be labeled and accompanied by a requisition reflecting the physician's order. Specimens are labeled with the patient's name and a description of the specimen source. The requisition must include the following information: patient name, age, sex, identifying number (such as social security number or unique registration/billing number), and location (hospital room, physician's office address, etc.); ordering physician's name; specimen source; date and time of collection; and test ordered. If the information is incomplete, laboratory personnel must call the collecting location and request the missing information. If a specimen is mislabeled or no patient name is provided, another specimen should be collected. Relabeling of a specimen is allowed only if the specimen cannot be recollected, such as tissue collected during a surgical procedure. Laboratory procedures must clearly state the exceptions that are allowed, the steps needed to verify and document exceptions, and the individuals responsible for relabeling. When relabeling has occurred, the course of events must be outlined in the laboratory report so the physician interpreting the results is aware of potential errors.

TABLE 4 Specimen management for infrequently encountered organisms[a]

Organism	Specimen(s) of choice	Transport issues	Comment
Bartonella sp. (cat scratch fever)	Blood, tissue, lymph node aspirate	1 wk at 4°C; indefinitely at −70°C	May see organisms in or on erythrocytes with Giemsa stain. Use Warthin-Starry silver stain for tissue. SPS is toxic.
Borrelia burgdorferi (Lyme disease)	Skin biopsy sample at lesion periphery, blood, CSF	Keep tissue moist and sterile; hand carry to laboratory if possible.	Consider PCR in addition to culture. Culture yield is low. Warthin-Starry silver stain for tissue. AO and Giemsa for blood and CSF.
Borrelia sp. (relapsing fever)	Blood smear (blood)	Hand carry to laboratory if possible.	Use direct wet mount in saline for dark-field microscopy. Stain with Wright's or Giemsa stain. Blood culture is unreliable.
Coxiella (Q fever),[b] *Rickettsia* (spotted fevers; typhus)	Serum, tissue (blood)	Blood and tissue are frozen at −70°C until shipped.	Refer isolation to reference laboratory. Serologic diagnosis is preferred.
Ehrlichia sp.	Blood smear, skin biopsy sample, blood (with heparin or EDTA anticoagulant), CSF, serum	Material for culture sent on ice; keep tissue moist and sterile; hold at 4–20°C until tested or at −70°C for shipment; transport on ice or frozen for PCR test.	Serologic diagnosis preferred. Fix smear in methanol. Tissue stained with FA or Gimenez stain. Refer isolation to reference laboratory. CSF for direct examination and PCR.
Francisella sp. (tularemia)[b]	Lymph node aspirate, scrapings, lesion biopsy sample, blood, sputum	Rapid transport to laboratory or freeze; ship on dry ice.	Send to reference laboratory. Serology helpful. Gram stain of tissue is not productive. IFA available. Culture effective 10% of the time.
Leptospira sp.	Serum, blood (citrate-containing anticoagulants should not be used), CSF (1st wk), urine (after 1st wk)	Blood, <1 h; urine, <1 h or dilute 1:10 in 1% bovine serum albumin and store at 4–20°C.	Serology most helpful. Acidic urine is detrimental. Dark-field microscopy and direct FA available. Warthin-Starry silver stain for tissue.
Streptobacillus sp. (rat-bite fever, Haverhill fever)	Blood, aspirates of joint fluid	High-vol bottle preferred	Do not refrigerate. Requires blood, serum, or ascitic fluid for growth. SPS is inhibitory. AO staining is helpful.

[a] Abbreviations: AO, acridine-orange; FA, fluorescent antibody; IFA, indirect fluorescent antibody.
[b] Laboratory safety hazard. Class II biological safety cabinet required. Also see chapter 9.

Laws governing specimen labeling can be reviewed at the Centers for Medicare and Medicaid Services website (http://www.hcfa.gov). Specimens from outpatient facilities require additional information for Medicare and Medicaid billing. Patient diagnosis, in the form of an ICD-9 code, is needed to confirm the need for a particular test. If a test is not deemed necessary for a specific diagnosis, the patient must sign an advanced beneficiary notice documenting that the test is not considered necessary and, if performed, the patient will be required to pay the test charge. Medicare and Medicaid compliance rules also can be reviewed at the Centers for Medicare and Medicaid Services website.

Specimen Rejection

In spite of acceptable labeling, some specimen collection sites, transport containers, or transport conditions render the specimen unacceptable for processing (164). Table 3 lists acceptable criteria for specimen management based on collection or transport conditions and times. When specimens fall outside these limits, new specimens should be collected whenever possible. In addition, specimens may be rejected because of the quality of specimen material collected rather than the conditions of transport (Table 6). Specimen quality is evaluated by examining the quantity and cellular composition. Although the quantity of many specimens is limited by the collection method or physical size of the infected area, some specimens, such as urine, stool, and sputum, are available in abundance. If another specimen can be collected easily with a larger volume, it is appropriate and necessary to request new or additional material. If the specimen volume must be limited, small volumes of liquid specimens can be extended by adding 1 to 2 ml of sterile saline or a

TABLE 5 Recommendations for Gram stain and plating media for bacteriology specimens or organisms

Specimen or organism	Gram stain	Aerobic media[a]	Anaerobic media[a,b]	Comments
Body cavity fluids	x (separate fluid specimen needed)			Blood culture bottles should be used to incubate large volumes of specimens for all body cavity fluids.
CSF (routine)	x	B C		
CSF (shunt)	x	B C Th		
Pericardial	x	B C	BBA	
Pleural	x	B C	BBA	
Peritoneal	x	B C Mac CNA	BBA LKV BBE CNA	
CAPD	x	B C Th	BBA	
Synovial	x	B C		
Bone marrow	x	B C	BBA	
Catheter tip		B		
Ear external fluid/swab	x	B C Mac		
Ear internal fluid	x	B C	BBA	
Eye	x	B C		
Gastrointestinal tract				
Feces		B Mac HE Ca EB (Sorbitol Mac/ chromogenic agar/Shiga toxin testing optional)		*C. jejuni/coli* in 5% O_2, 10% CO_2, and 85% N_2 at 42°C for all gastrointestinal tract specimens
Rectal swab		B Mac HE Ca EB		
Genitourinary tract				
Vaginal/cervix		B TM		
Urethra/penis	x	TM		
Other	x	B C Mac TM	BBA LKV BBE CNA	
Group B streptococcal screen (vaginal/anal screen)		Selective broth, subculture to B		
Lower respiratory tract				
Sputum	x	B C Mac (PC OFPBL for cystic fibrosis)		
Tracheal aspirate	x	B C Mac CNA		
Bronchoalveolar lavage fluid	x	B C Mac CNA	BBA LKV CNA	Protected bronchoscope brushing (in anaerobic transport) required for anaerobic culture
Bronchoalveolar brushing, washing	x	B C Mac		
Tissue	x	B C Mac Th	BBA LKV BBE CNA	
Upper respiratory tract				
Nasopharynx		B C		
Nose		B chromogenic agar		
Throat		B or SSA		
Urine		B Mac or chromogenic agar		
Wound or abscess				
Swab	x	B C Mac		
Aspirate	x	B C Mac CNA	BBA LKV BBE CNA	
B. pertussis and *B. parapertussis*		Regan Lowe		
Brucella spp.		B C		

(Continued on next page)

TABLE 5 Recommendations for Gram stain and plating media for bacteriology specimens or organisms *(Continued)*

Specimen or organism	Gram stain	Aerobic media[a]	Anaerobic media[a,b]	Comments
C. diphtheriae		Cystine-tellurite or Loeffler's serum		
C. difficile		CCFA		
E. coli O157:H7 (EHEC)		Sorbitol-Mac or chromogenic agar		Shiga toxin EIA more sensitive
F. tularensis		C or BCYE		
H. ducreyi		C + vancomycin (3 μg/ml)		Gram stain resembling "school of fish"
H. pylori	x	B		*Campylobacter* gaseous atmosphere at 35–37°C
Legionella		BCYE		
Leptospira		Fletcher's medium or EMJH		30°C for up to 13 wk
N. gonorrhoeae		TM		
Vibrio		TCBS		
Yersinia		CIN		

[a] B, blood agar; C, chocolate blood agar; Mac, MacConkey agar; Th, thioglycolate broth; Ca, *Campylobacter* agar; HE, Hektoen enteric; EB, enrichment broth; SSA, group A *Streptococcus* selective agar; TM, Thayer-Martin; BCYE, buffered charcoal yeast extract; TCBS, thiosulfate citrate bile salts sucrose; CIN, cefsulodin-Irgasan-novobiocin; BBA, brucella blood agar; LKV, laked blood with kanamycin and vancomycin; BBE, *Bacteroides* bile-esculin; CNA, anaerobic colistin-nalidixic acid; CCFA, cycloserine-cefoxitin-fructose agar; EMJH, Ellinghausen-McCullough-Johnson-Harris medium; CAPD, fluid from chronic ambulatory peritoneal dialysis; PC, *P. cepacia* agar; OFPBL, oxidative-fermentative-polymyxin B-bacitracin-lactose.

[b] Set up anaerobic culture upon request, if specimen is collected and transported appropriately. Call physician if appropriate specimen does not have request for anaerobic culture.

nutrient broth. It is important to add just enough liquid to provide specimen for all tests requested.

Examining the cellular composition of clinical material first requires a gross examination of the specimen. Infection gives rise to purulence (abundant polymorphonuclear cells), blood, necrosis, and mucus (mucous membrane specimens). In general, gross examination should identify yellow to tan purulence, red to rust-colored blood, clear and tenacious mucus, and brown to black discoloration of tissue denoting necrosis. Portions for smear and inoculation to culture media should be taken from these areas. It may be beneficial to ask the assistance of a surgical pathologist when choosing the best portion of excised tissue for examination (197). Ideally, microscopic examination of smears, using a 10× microscope objective, should demonstrate many polymorphonuclear cells and few or no squamous epithelial cells indicating the absence of cutaneous or mucocutaneous contamination with normal bacterial flora. Specimens in which tissue necrosis is present also may show elastin fibers in stained smears. Lower respiratory tract specimens are likely to show alveolar macrophages and Curschmann's spirals, indicating that secretions have originated from the distal airways. Curschmann's spirals are casts of bronchioles found in patients with chronic lung disease caused most commonly by asthma and cigarette smoking. Figures 1 and 2 illustrate elastin fibers and Curschmann's spirals, respectively. Although elastin fibers are present in noninfected surgical wounds and specimens from areas of tissue damage, they are also found in infected tissue where necrosis has occurred. Specimens determined to have gross bacterial contamination from normal flora, indicated by an abundance of squamous epithelial cells, should be rejected. Specimens are rejected by contacting the patient's caregiver, explaining the reason for rejection, and requesting a replacement specimen of acceptable quality. Timely notification and collection of a replacement are necessary, especially in instances where antimicrobial therapy has been initiated. Specimen rejection criteria should be reviewed by appropriate laboratory and medical staff representatives before becoming policy. Examples of acceptable and unacceptable specimens are listed in Table 6.

Culture Interpretation

Following examination of the stained smear and culture incubation, agar culture plates are interpreted for bacterial growth by attempting to differentiate potential pathogens, requiring identification and antimicrobial testing, from contamination by colonizing members of the normal bacterial flora. This is accomplished by examining the relative quantities of each isolate, correlating culture results with Gram-stained smear results, and recognizing usual contaminants and pathogens from respective specimen sites. In general, in cultures of specimens from sites likely to be contaminated (e.g., sputum, urine, and superficial wounds), potential pathogens should outnumber indigenous flora and should be seen in the direct Gram stain. In cultures of specimens from presumably sterile sites (e.g., cerebrospinal fluid [CSF], joint

TABLE 6 Screening specimens requested for routine bacterial culture to ensure quality[a]

Specimen (reference)	Screening method	Results of screen[b]	
		Acceptable	Unacceptable
Sputum (135)	Microscopic examination of Gram-stained smear	<10 SEC/average 10× field	>10 SEC/average 10× field
Endotracheal aspirate (129)	Microscopic examination of Gram-stained smear	<10 SEC/average 10× field and bacteria detected in at least 1 of 20 fields (100×)	>10 SEC/average 10× field and no bacteria detected in 20 fields (100×)
Bronchoalveolar lavage fluid (200)	Microscopic examination of Gram-stained smear	<1% of cells present are SEC	>1% of cells present are SEC
Urine (213)	Urinalysis, Gram stain of urine sediment	<3+ SEC on urinalysis. Positive LE[b] test result with >10 polymorphonuclear leukocytes/mm^3 from symptomatic patient (patients with asymptomatic bacteriuria may not have increased number of leukocytes)	≥3+ SEC on urinalysis or more than 3 potential pathogens by Gram stain implies gross contamination
Superficial wound (178)	Microscopic examination of Gram-stained smear	<2+ SEC, polymorphonuclear leukocytes present	>2+ SEC and no polymorphonuclear neutrophils
Stool for bacterial pathogens (127)	Location of patient? Duration of hospitalization?	Outpatient or inpatient for ≤3 days	In hospital >3 days, or diarrhea developed while in hospital
Other specimens	Screening methods unavailable or unproven		

[a] Reprinted from reference 195 with permission.
[b] LE, leukocyte esterase; SEC, squamous epithelial cells.

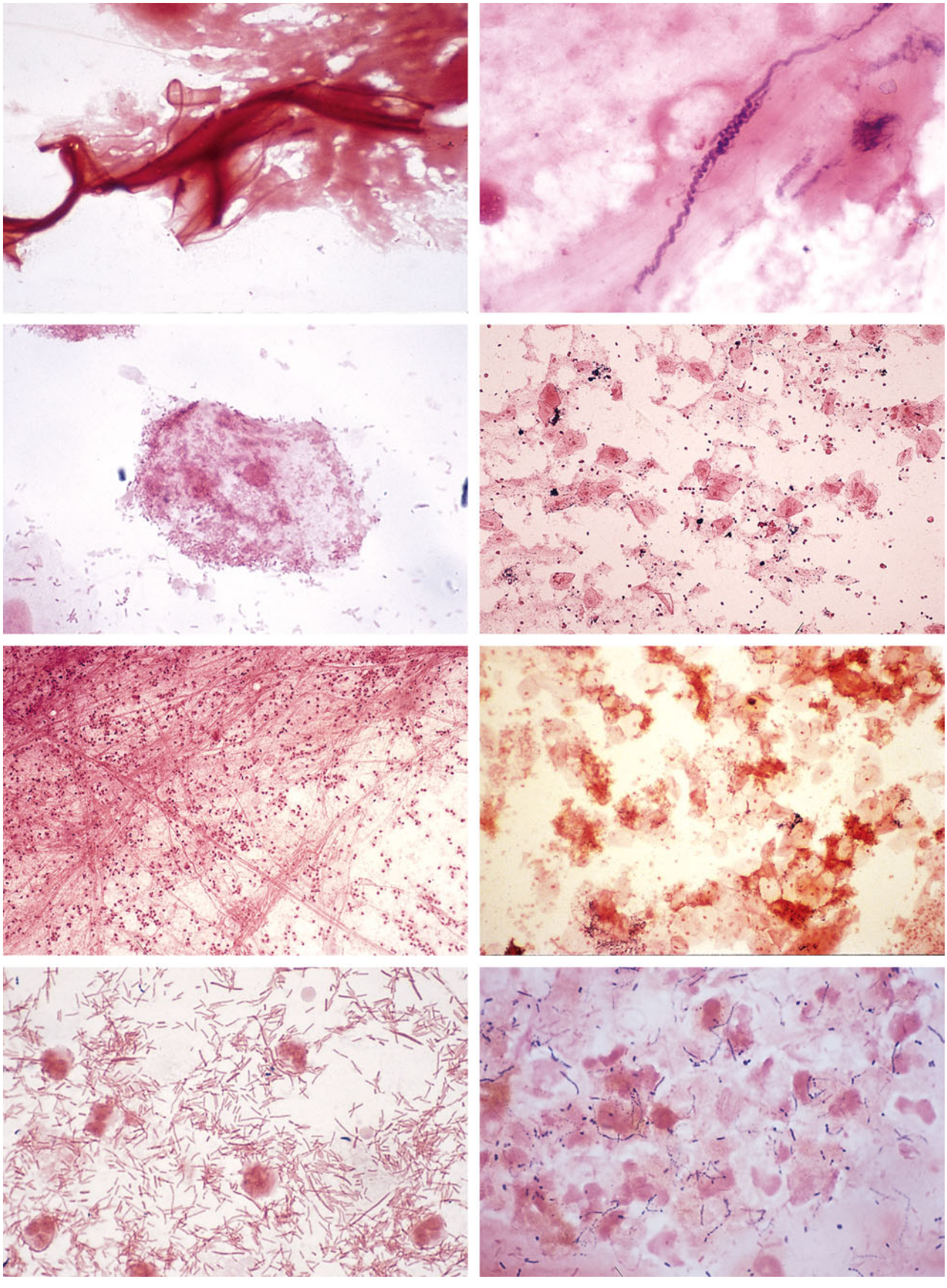
fluids, other body fluids, and deep tissue), potential pathogens occur in any quantity and may or may not be seen in the direct Gram-stained smear. Specific criteria for identifying potentially significant isolates and contaminating members of the normal flora are addressed in the following sections of this chapter. It is a useful policy to save the culture plates for 1 week, allowing physicians the opportunity to call to request further identification or antimicrobial testing when clinically indicated.

Critical Values in Microbiology

Many results in clinical microbiology can have an immediate impact on patient care. Although cultures require days to weeks to become positive, stained-smear results, results of antigen or nucleic acid detection, and culture results from presumably sterile specimen sources may contain information necessitating important changes in therapy or care. Examples of medical emergencies that require immediate notification of the patient's health care provider include a positive blood culture; positive CSF Gram stain or culture; group A streptococcus detected in a surgical wound specimen; Gram stain suggesting gas gangrene, necrotizing fasciitis, or other systemic toxemia; and positive blood smear for malaria. Examples of results that require notification of the physician or health care provider during a day shift, but not so immediate that calls are necessary during evening or overnight hours, include a positive acid-fast stain, new or unusual antimicrobial resistance, and the detection of highly significant or unusual microorganisms such as *Listeria, Legionella,* or *Brucella.* Most critical values are unique to individual medical centers and require laboratories to consult with medical staff representatives before compiling a call list for critical values (95, 108).

Blood and Intravascular Catheter Specimens

Blood is one of the most important specimens received by the laboratory for culture. In most cases, bacteria present in blood (bacteremia) indicate an infection that has spread from a primary site such as the lungs (pneumonia). Such a bacteremia is referred to as a secondary bacteremia. In the absence of an identifiable infected source, the bacteremia is referred to as a primary bacteremia. Primary bacteremia can also result from "silent," subclinical passage of bacteria from contaminated areas of the body, such as mucous membranes, into the blood. Consequences of a clinically apparent primary or secondary bacteremia are sepsis, septic shock, or severe sepsis. Sepsis implies a bacteremia with signs and symptoms, such as fever, chills, and tachycardia. Septic shock indicates the presence of hypotension with sepsis. Severe sepsis is characterized by septic shock with organ system failure and is associated with a 20 to 40% mortality rate (15).



A wide variety of bacteria are involved in bloodstream infections, the majority belonging to groups of bacteria, such as the streptococci/enterococci, staphylococci, or *Enterobacteriaceae,* that grow rapidly in culture (8, 220). It is important to recognize potential contaminants that grow, since treating contaminants as significant isolates is associated with unnecessary expense and dangers of antimicrobial misuse (12). Common contaminants include coagulase-negative staphylococci, corynebacteria, *Bacillus* spp., and propionibacteria. In general, single cultures positive for these bacteria represent contamination. Multiple, separate cultures growing one of these isolates are more likely to indicate a clinically significant bacteremia (90, 125, 219). Contamination is controlled by proper skin antisepsis before venipuncture. Iodine-containing antiseptics, including iodophors (iodine with a detergent) and tincture of iodine (iodine with alcohol, considered more effective than iodophors), or chlorhexidine is needed to reduce the number of viable bacteria at the venipuncture site (106, 124). Contamination rates of less than 3% are desired (172). Higher rates should be investigated and corrected by educational efforts (161).

Bacteremic patients fall into many patterns that determine specimen collection methods. Most patients are intermittently bacteremic, implying that bacteria are present in the blood for periods of time followed by nonbacteremic periods. Other patients who have intravascular sites of primary infection (such as endocarditis) are continuously bacteremic. All patients are likely to have very low quantities of bacteria in the blood, in spite of severe clinical symptoms. For these reasons, multiple blood cultures, each containing large volumes of blood, are required to detect bacteremia. As a rule for adults, two or three separate blood cultures, each inoculated with at least 20 ml of blood, are recommended per 24-h period (8, 219). Pediatric recommendations are similar, except for the volumes of blood recommended (8, 87). Adult and pediatric requirements for blood collection for culture are summarized in Tables 3 and 7.

It is best to collect blood directly into culture bottles during the venipuncture procedure, rather than into transport tubes that are sent to the laboratory for subsequent transfer of blood into the culture bottles. Collection directly into culture bottles enables bacteria to begin growing immediately, decreases the amount of anticoagulant to which bacteria are exposed, and decreases the chances of needlestick accidents for health care personnel. The anticoagulant used in all blood culture systems is sodium polyanethanolsulfonate (SPS) and is known to be inhibitory to meningococci, gonococci, *Peptostreptococcus anaerobius, Streptobacillus moniliformis,* and *Gardnerella vaginalis* (159). Although few practical methods are available to avoid exposure of these bacteria in blood to SPS, it is advisable to diminish the total amount of SPS used by avoiding transport tubes that also include SPS.

Culture of blood is the most sensitive method available for the detection of bacteremia. Semiautomated blood culture systems are present in nearly every clinical laboratory. Refer to chapter 15 for a complete discussion of manual and automated blood culture systems. Occasionally, direct staining of blood collected by venipuncture can provide rapid, nearly immediate, detection of bacteria in blood (157, 163). Gram staining a smear of peripheral blood or buffy coat layer may detect bacterial cells in the blood of patients with meningococcemia, *S. pneumoniae* infection, or overwhelming sepsis caused by other bacteria when the bacterial concentration in blood is very high (approaching 10,000 bacterial cells per ml of blood). In spite of published reports documenting the occasional use of direct staining of blood, the likelihood of results impacting patient management does not warrant use as a routine laboratory procedure.

Some bacteria, such as *Legionella* spp., mycobacteria, some *N. meningitidis* strains, and *N. gonorrhoeae,* fail to grow in routine commercial media and should be sought by using another method, such as lysis centrifugation. Other bacteria may require the addition of adsorbents to bind antimicrobials or inhibitory substances, or more specialized methods such as tissue culture. Prolonged incubation beyond the usual 5-day protocols for automated instruments is not necessary; however, blind subculture may be needed if the patient is receiving antimicrobials at the time of blood collection (7, 150).

Positive blood culture bottles are evaluated initially by examining a Gram-stained smear of the broth. The report should include a description of the bacterial morphology and the Gram reaction. If a presumptive identification of the microorganism can be made, it may be added to the report. For example, a blood culture Gram stain report might state "gram positive in clusters suggesting staphylococci." Specimens in positive blood culture bottles should be subcultured to media based on the organism seen in the Gram-stained smear. In addition, since 5 to 10% of all bacteremias

FIGURE 1 (row 1, left) Gram stain (×100) of surgical wound showing elastin fibers.

FIGURE 2 (row 1, right) Gram stain (×1,000) of sputum showing Curschmann's spirals.

FIGURE 3 (row 2, left) Gram stain (×1,000) of vaginal secretions showing clue cells.

FIGURE 4 (row 2, right) Gram stain (×100) of unacceptable sputum specimen (grossly contaminated with oropharyngeal flora) showing >10 squamous epithelial cells per low-power field.

FIGURE 5 (row 3, left) Gram stain (×100) of acceptable sputum specimen showing <10 squamous epithelial cells per low-power field.

FIGURE 6 (row 3, right) Gram stain (×100) of urine showing 4+ squamous epithelial cells, indicating gross contamination with vaginal or periurethral secretions and bacteria.

FIGURE 7 (row 4, left) Gram stain (×1,000) of urine showing polymorphonuclear leukocytes and 4+ gram-negative bacilli.

FIGURE 8 (row 4, right) Gram stain (×1,000) of a wound showing polymorphonuclear leukocytes, mixed bacterial morphotypes suggesting aerobic and anaerobic bacteria, and both intra- and extracellular bacteria. This appearance suggests a mixed aerobic and anaerobic abscess or closed-space infection.

TABLE 7 Pediatric bacterial blood cultures: recommended blood volume[a]

Patient wt (lb)	Recommended blood vol per culture (ml)	Total blood vol for 2 cultures (ml)	Vol of blood equal to 1% of patient's total blood vol (ml)[b]
<19	1	2	2
18–30	3	6	6–10
30–60	5	10	10–20
60–90	10	20	20–30
90–120	15	30	30–40
>120	20	40	>40

[a] Data from reference 87.

[b] Blood volume calculated by assuming 85 ml/kg in newborns and 73 ml/kg in other patients. Two 20-ml blood specimens collected from an 80-kg adult (40 ml total) represent approximately 0.7% of the patient's total blood volume.

are polymicrobial (contain more than one bacterial type), the addition of a Columbia-colistin-nalidixic acid, or other agar medium inhibitory to gram-negative organisms, to cultures showing gram-negative bacilli in the Gram stain, or the addition of a MacConkey or related selective agar plate to cultures showing gram-positive bacteria, is recommended. Anaerobic media and culture conditions should be used if the morphology of the organism seen in the Gram-stained smear is suggestive of an anaerobic bacterium or if the organism is recovered from an anaerobic culture bottle only.

Special Considerations for Blood Cultures

Anaerobes

Anaerobes have always accounted for a relatively small percentage of organisms recovered from blood. Over the past several years, some investigators have noted a decline in bloodstream infections by anaerobes (134). However, this is not the case in all hospitals (30). Therefore, laboratories should review their own data when deciding whether to include an anaerobic culture bottle as part of the routine setup of blood specimens. Anaerobic media available for use with the automated systems appear to perform adequately. It is important to note that if the anaerobic component of a blood culture is dropped, the inoculum should be added to an additional aerobic bottle to ensure that the full volume of blood is cultured.

Lysis Centrifugation

The Isolator lysis centrifugation system (Wampole Laboratories, Cranbury, N.J.) is a commercially available manual blood culture method (219). The system consists of a tube containing anticoagulant (SPS), EDTA, and saponin. After the tube is filled with blood during phlebotomy, the contents are mixed and centrifuged, and the resulting pellet is inoculated onto agar media. The system effectively recovers aerobic and facultative bacteria and fungi. The Isolator system does not perform as well as other systems when recovering *S. pneumoniae,* other streptococci, *Pseudomonas aeruginosa,* or anaerobes (219). Advantages of the Isolator system are the ability to inoculate the pellet to specific agar media when attempting to detect unusual etiologies of bacteremia, such as those caused by *Legionella pneumophila, Franciscella tularensis,* and *Bartonella* spp., and to provide colony counts, reported as CFU per milliliter of blood (38, 219). Disadvantages of the system are the labor involved with initial processing and the potential for increased contamination that accompanies manipulation during processing (199).

Intravascular Catheter Tips

Culture of intravascular catheter tips is performed to determine the source of a bacteremia and should be performed only when concurrent blood cultures are obtained. Soft tissue infections around a catheter insertion site are cultured as a wound specimen using freshly expressed purulence that can be aspirated. The most common technique used to culture the intravascular portion of a catheter is the semiquantitative method, in which the 5-cm distal portion of the catheter is rolled across a blood agar plate four times (109). The catheter tip is discarded. Growth of more than 15 colonies is considered to be significant; i.e., it implicates the catheter tip as the likely source of a bacteremia if a similar isolate is detected in a blood culture.

Other techniques have been described for the identification of catheter-related infections. A meta-analysis of data was reported concerning the use of three types of catheter segment culture and three blood culture methods to determine the sensitivity and specificity of each method (182). The three catheter segment cultures included qualitative, semiquantitative (e.g., catheter roll method), and quantitative (dilutions to determine bacterial quantities used), while the three blood culture methods included qualitative catheter blood culture (reported as positive or negative), quantitative catheter blood culture, and paired quantitative catheter and peripheral venipuncture blood cultures. Based on this analysis, quantitative catheter segment culture was the most accurate method, with pooled sensitivity and specificity both >90%. The optimal method in all clinical settings for determining that an intravascular catheter is the source of a bacteremia has not been determined. Because the most serious manifestation of catheter infections is bacteremia, ordinary blood cultures may be the best way to determine which patients require therapy (158).

Sterile Body Fluid Specimens

CSF

CSF is collected for the diagnosis of meningitis (67). Bacterial meningitis can be divided into acute and chronic clinical presentations (196). Acute meningitis with onset of symptoms within the previous 24 h is usually caused by pyogenic bacteria. Specific etiologies are related to the age of the patient and whether the disease is community or nosocomially acquired. Chronic meningitis, with symptoms lasting at least 4 weeks, can have a wide variety of causes (Table 8). As with all clinical specimens, even those collected from a presumably sterile site, growth of contaminants occasionally does occur.

CSF is usually obtained by lumbar spinal puncture. Although bacterial staining and culture can be performed with as little as 0.5 ml of fluid, larger volumes are preferred since culture methods are more sensitive when low numbers of bacteria are concentrated by centrifugation before culture. A minimum of 5.0 ml and high-speed (3,000 × *g*) centrifugation are recommended for recovery of *Mycobacterium tuberculosis,* although the yield may still be low. Filtration through a 0.45-μm-pore-size filter results in better concentration of *M. tuberculosis* in CSF. Specimens should be transported to the laboratory immediately in a sterile container maintained at room temperature. All smears should be prepared by cytocentrifugation (176), and cultures should be inoculated with

TABLE 8 Usual etiologies of infectious disease syndromes

Disease	Etiologies
Central nervous system infection	
Acute meningitis, neutrophilic pleocytosis	*S. pneumoniae*
	N. meningitidis
	Listeria monocytogenes
	Streptococcus agalactiae
	H. influenzae
	S. aureus
	Gram-negative rods[a]
	Bacillus anthracis
Acute meningitis, CSF shunt related	Coagulase-negative staphylococci
	S. aureus
	Propionibacterium spp.
	Gram-negative enteric rods (e.g., *E. coli* and *Klebsiella* spp.)
	Gram-negative nonfermenting rods (e.g., *P. aeruginosa* and *Acinetobacter* spp.)
Chronic meningitis, predominantly lymphocytic pleocytosis	*Nocardia* species
	Brucella species
	L. interrogans
	M. tuberculosis
	T. pallidum
	Borrelia burgdorferi
Gastrointestinal tract infection	
Infectious diarrhea[b]	*Salmonella* serotypes
	Shigella spp.
	C. jejuni/coli
	Campylobacter spp. (other)
	EHEC serotypes
	C. difficile
	Vibrio spp.
	Aeromonas spp.
	P. shigelloides
	Y. enterocolitica
	E. coli toxigenic, invasive, and effacing strains
	Listeria monocytogenes (rare)
	Clostridium perfringens
	B. cereus
Ingestion of preformed toxin[c]	S. aureus
	B. cereus
	C. botulinum
Gastritis/gastric and duodenal ulcers	*H. pylori*
Genital tract infection	
Ulcers	*T. pallidum*
	H. ducreyi
	C. trachomatis (LGV)
	Klebsiella granulomatis
Urethritis	*N. gonorrhoeae*
	C. trachomatis
Vulvovaginitis	*N. gonorrhoeae* and *C. trachomatis* in prepubescent girls
BV	Overgrowth of vaginal flora with anaerobic bacteria
Cervicitis	*N. gonorrhoeae*
	C. trachomatis
Endometritis	*Enterobacteriaceae*
	Streptococci (groups A and B)
	Enterococci
	Mixed anaerobic genera
Salpingitis/oophoritis	*N. gonorrhoeae*
	C. trachomatis
	Mixed aerobic and anaerobic flora
Pelvic abscess	Mixed aerobic and anaerobic flora

(Continued on next page)

TABLE 8 Usual etiologies of infectious disease syndromes *(Continued)*

Disease	Etiologies
Epididymitis	*N. gonorrhoeae* *C. trachomatis* *Enterobacteriaceae* *P. aeruginosa* Various gram-positive cocci
Ocular infections	
Conjunctivitis	*S. pneumoniae* *S. aureus* *H. influenzae* *N. meningitidis* *N. gonorrhoeae* *C. trachomatis* (inclusion conjunctivitis) *C. trachomatis* (trachoma) Others[d]
Keratitis	*S. aureus* *S. pneumoniae* *P. aeruginosa* Enterococci *S. pyogenes* (group A) *Enterobacteriaceae* *Pasteurella multocida* Others[e]
Endophthalmitis	*S. aureus* *P. aeruginosa* *Propionibacterium acnes* *S. pneumoniae* *N. meningitidis*
Periorbital cellulitis	*S. aureus* *S. pyogenes* (group A) *S. pneumoniae* *H. influenzae* *Clostridium* spp.
Otitis	
Otitis externa	*S. aureus* *S. pyogenes* (group A) *P. aeruginosa* *Vibrio alginolyticus*
Otitis media	*S. pneumoniae* *H. influenzae* *Moraxella catarrhalis* *S. aureus* Rare pathogens Gram-negative rods Anaerobes
Respiratory tract infection	
Tracheitis, intubated patient	*Enterobacteriaceae* *S. aureus* *P. aeruginosa* Other nonfermenting gram-negative rods
Bronchitis, community acquired	*S. pneumoniae* *H. influenzae* (rarely, other *Haemophilus* species) *M. catarrhalis* *S. aureus* *C. pneumoniae* *M. pneumoniae* *B. pertussis* *S. pyogenes* (group A) Less commonly, same as hospital acquired

(Continued on next page)

TABLE 8 *(Continued)*

Disease	Etiologies
Bronchitis, hospital acquired	*Enterobacteriaceae*
	S. aureus
	P. aeruginosa
	Other nonfermenting gram-negative rods
	Less commonly, same as community acquired
Pneumonia, community acquired	*S. pneumoniae*
	H. influenzae
	M. catarrhalis
	C. pneumoniae
	M. pneumoniae
	L. pneumophila
	Nocardia species
	P. multocida
	Aspiration (anaerobes)
	Less commonly, same as hospital acquired
Pneumonia, hospital acquired	*Enterobacteriaceae*
	S. aureus
	P. aeruginosa
	L. pneumophila
	Other nonfermenting gram-negative rods
	Aspiration (anaerobes)
	Less commonly, same as community acquired
Lung abscess	*S. aureus*
	Klebsiella pneumoniae
	P. aeruginosa
	S. pyogenes (group A)
	Anaerobes (aspiration pneumonia)
	Nocardia species
Empyema	*S. pneumoniae*
	Anaerobes
	Viridans group streptococci, especially *Streptococcus anginosus* group
	S. aureus
	S. pyogenes (group A)
	Gram-negative rods
Urinary tract infection	
Prostatitis	*Enterobacteriaceae*
	P. aeruginosa
	Enterococci
Urethral syndrome	Same us cystitis, but in lower numbers
	C. trachomatis/N. gonorrhoeae
	Unknown—negative culture (about 15% of this disease group)
Cystitis	*Enterobacteriaceae*, especially *E. coli*
	Enterococci
	Staphylococcus saprophyticus (women of childbearing age)
	Nonfermenting gram-negative rods
	Corynebacterium urealyticum (patients with underlying urinary tract pathology)
Pyelonephritis	*Enterobacteriaceae*
	Enterococci
	Agents of bacteremia (descending infection), e.g., *S. aureus*

[a] Gram-negative rods, including *Enterobacteriaceae*, *P. aeruginosa,* and other nonfermenting gram-negative rods.
[b] Disease caused by ingestion of bacterium followed by tissue invasion, toxin production, or other pathogenic mechanism.
[c] Disease caused by ingestion of preformed toxin.
[d] *C. diphtheriae, M. tuberculosis, F. tularensis, T. pallidum, B. henselae* (cat scratch disease), *P. multocida, Bacillus thuringiensis,* and *M. lacunata*.
[e] *T. pallidum, N. gonorrhoeae, Moraxella* sp., *C. diphtheriae, Bacillus* spp., anaerobes, and nontuberculous mycobacteria.

0.5 ml of CSF or sediment resuspended in 0.5 ml of CSF following centrifugation (1,500 × *g* for 15 min) when >1 ml of fluid is received. Recently, PCR has emerged as a potentially useful test for the diagnosis of tuberculous meningitis (27).

Smears should be Gram stained, and the results should be reported to the physician immediately. Results should include a description and semiquantitative enumeration of polymorphonuclear inflammatory cells and bacterial morphology. If the results are suggestive of a bacterial group, this too can be communicated. Because of their low sensitivity and lack of effect on the care and management of patients, and because the cost of antigen testing is much higher than the cost of the Gram stain, direct antigen tests should be considered only when direct communication with the clinical service documents a specific need, such as prior antimicrobial therapy (67, 91, 118, 145, 187). Media are inoculated in accordance with recommendations in Table 5. Broth culture media are not necessary unless CSF is cultured from patients with a CSF shunt or external reservoir (119, 194).

Special Considerations for Sterile Body Fluid Specimens

Anaerobic Bacteria

Anaerobic culture of CSF is very rarely necessary and should be performed only when consultation with the clinical service provides evidence of risk factors for anaerobic etiology such as chronic otitis media with mastoiditis, chronic sinusitis, mixed aerobic-anaerobic soft tissue infection overlying the spine, or the possibility of anaerobic brain abscess, subdural empyema, or epidural abscess (74, 76). Even when a parameningeal abscess contains anaerobes, detection of anaerobes by culture of CSF is unlikely to be helpful, since diagnosis and management are based on microbiological evaluation of the abscess.

Leptospira spp.

Leptospires can be detected in CSF during the first 10 days of acute illness. CSF should be collected before initiation of antimicrobial therapy and while the patient is febrile. Direct detection of leptospires by dark-field examination of CSF has been used in the past but is no longer recommended. Culture should be performed using 1 or 2 drops of CSF inoculated into Ellinghausen-McCullough-Johnson-Harris medium. Cultures should be incubated at room temperature for up to 13 weeks (39) (see chapter 61).

Other Body Fluids

Specimens include pericardial, pleural, peritoneal, peritoneal dialysis, and synovial fluids. A volume of 1 to 5 ml is adequate for the isolation of most bacteria. Fluid submitted on a swab is not optimal. Educational efforts should address the habits of physicians and caregivers who use swabs rather than adding aspirated fluid to an appropriate transport tube. Specimens for anaerobic culture should be transported in an oxygen-free tube. Specimens for the diagnosis of peritonitis associated with chronic ambulatory peritoneal dialysis should include at least 10 ml of fluid (212). All body fluids should be inoculated directly into blood culture bottles in the laboratory or at bedside. For the latter, 0.5 ml should be left in a separate sterile tube for preparation of a smear (14). Body fluids that may clot during transport should be transported in tubes containing anticoagulant. Because heparin, sodium citrate, and EDTA are inhibitory to some bacteria, SPS-containing tubes are recommended. Physicians should be made aware that SPS is inhibitory also and affects the growth of *N. meningitidis, N. gonorrhoeae, P. anaerobius, S. moniliformis,* and *G. vaginalis* (160).

In the laboratory, volumes greater than 1.0 ml for culture should be centrifuged at 1,500 × *g* for 15 min. The supernatant is removed, leaving about 0.5 ml in which to resuspend the sediment. The resuspended sediment is used to inoculate culture media. Cytocentrifugation of 0.5 ml or less (depending on the turbidity of the specimen) should be used to prepare smears for Gram staining before centrifugation to concentrate for culture. Alternatively, smears can be prepared from the same sediment as used to inoculate media; however, this method is less sensitive than cytocentrifugation (176).

Solid media used for culture of body fluid specimens should include chocolate and blood agar, and selective agars for gram-positive and gram-negative organisms if mixed infections are expected (e.g., abdominal fluids from patients with appendicitis) (Table 5). The inoculation of aerobic blood culture bottles with excess fluid is suggested for pericardial, pleural, and synovial fluids, in addition to peritoneal fluids from chronic ambulatory peritoneal dialysis patients, to enhance detection of low numbers of bacteria that may be intracellular or inhibited by prior antimicrobial therapy (16). Cultures should be incubated at 35 to 37°C in the presence of 3 to 5% CO_2 for a minimum of 3 days before being discarded as negative. Blood culture bottles should be incubated for the usual 5- or 7-day protocol.

Some experts question the need to culture abdominal fluid or purulence from patients with intestinal tract perforation, since surgical drainage and broad-spectrum antimicrobials are effective in the majority of cases (137, 185). Complications occur in this clinical setting when microorganisms other than the expected *Enterobacteriaceae,* anaerobes, and enterococci are involved. Standard empirical therapy may not adequately treat *Staphylococcus aureus, P. aeruginosa, Candida* spp., or bacteria more resistant to antimicrobial agents than expected (66). A policy to refuse culture requests or limit identification of isolates that do grow to an arbitrary number (such as 3 or fewer) may not apply uniformly to all patients.

Ear Specimens

Two types of ear specimens are received most commonly by the laboratory, swab specimens for the diagnosis of otitis externa and middle ear fluid specimens for the diagnosis of otitis media (Table 5). Potential pathogens at these two sites differ (Table 8) (20, 25, 168). Since anaerobic bacteria may be involved in middle ear infections, anaerobic culture should be performed on properly collected and transported specimens when requested. Direct examination of Gram-stained smears of middle ear fluid is helpful and is recommended with all culture requests.

External ear specimens may be contaminated with normal flora from the skin or ear canal. Isolates of coagulase-negative staphylococci, diphtheroids, and viridans group streptococci may be listed as presumptive identifications without including results of antimicrobial testing. It is a useful policy to save the culture plates for 1 week, allowing physicians the opportunity to call to request further identification or antimicrobial testing when clinically indicated. Middle ear fluid is less likely to be contaminated. All isolates should be reported, and if requested, antimicrobial testing should be performed on strains with unpredictable susceptibility to antimicrobials.

Eye Specimens

Several types of specimens may be collected for the microbioloigcal analysis of eye infections, including conjunctival scrapings obtained with a swab or sterile spatula for the diagnosis of

conjunctivitis, corneal scrapings collected with a sterile spatula for the diagnosis of keratitis, vitreous fluid collected by aspiration for the diagnosis of endophthalmitis, and fluid material collected by aspiration or tissue biopsy for the diagnosis of periorbital cellulitis (223). Pathogenic bacteria potentially present in these anatomic sites are listed in Table 8. Because the volume of specimen collected from corneal scrapings and vitreous fluid aspiration is very small, direct inoculation of agar culture plates and preparation of smears in the clinic or at the bedside are recommended (223). A close working association is needed between the laboratory and ophthalmologist to ensure a supply of appropriate culture media, the correct technique for inoculation of media, and rapid transport of plates and smears to the laboratory. Media should be inoculated by rubbing the specimen onto a small area of the agar plates. Plates are placed directly into the incubator without cross-streaking by laboratory personnel. This allows the plate reader to detect more easily airborne contaminants that settle on the plate during inoculation procedures that occur outside controlled laboratory conditions.

Media needed for the detection of usual pathogens should include chocolate agar for fastidious bacteria (Table 5). Media for other microorganisms (fungi, viruses, mycobacteria, etc.) should be inoculated if deemed appropriate by the ophthalmologist and microbiologist, and specifically ordered. Incubation at 35 to 37°C in 3 to 5% CO_2 is necessary.

Special Considerations for Eye Specimens

Chlamydia trachomatis

Direct examination of conjunctival smears and detection of chamydial antigen in conjunctival scrapings are useful in the diagnosis of inclusion conjunctivitis or trachoma (45). Diagnosis of chlamydial infection is accomplished using direct fluorescent-antibody (DFA) staining with fluorescein-conjugated monoclonal antibodies or enzyme immunoassays (EIAs) (191). A less sensitive but readily available method is examination of Giemsa-stained conjunctival smears for intracytoplasmic, perinuclear inclusions within epithelial cells. Cell culture for the isolation of *C. trachomatis* is sensitive but more time-consuming and technically demanding than DFA or EIA. Conjunctival scrapings and secretions for culture should be transported in 2SP medium (sucrose phosphate or sucrose phosphate glutamate) with bovine serum and antimicrobials (usually gentamicin, vancomycin, and nystatin or amphotericin B). Swabs with wooden shafts should be avoided since constituents of the wood are toxic to chlamydiae. Specimens for culture should be refrigerated during short delays or stored at –70°C for delays longer than 48 h. Molecular probes and PCR methods are available for the detection of *C. trachomatis* but are not FDA approved at this time for eye specimens (71, 96).

Gastrointestinal Tract Specimens

Feces and, in some cases, rectal swab specimens are submitted to the microbiology laboratory to determine the etiologic agent of infectious diarrhea or food poisoning. Feces should be collected in a clean container with a tight lid and should not be contaminated with urine, barium, or toilet paper. Because intestinal pathogens can be killed by the metabolism of members of the fecal flora rapidly acidifying the specimen, specimens should be transferred to Cary-Blair transport medium soon after collection. Rectal swabs should be placed in a transport system containing an all-purpose medium such as Stuart's.

It should be standard practice in all laboratories to evaluate the appropriateness of stool culture requests. It is well established that hospitalized patients who did not enter the hospital with diarrhea are unlikely to develop enterocolitis caused by bacterial agents other than *Clostridium difficile* (68, 127). For this reason, stool for routine bacterial culture from patients who have been hospitalized for more than 3 days should not be processed without consultation and justification by the patient's physician or caregiver. Diarrhea that develops during hospitalization is likely to be caused by *C. difficile* or occur for noninfectious reasons (60). A simple policy of rejecting stool for routine bacterial culture from patients hospitalized for more than 3 days and offering *C. difficile* testing for nosocomial diarrhea is recommended. On the other hand, *C. difficile* colitis does occur as a community-acquired disease following hospital discharge or the use of outpatient antimicrobial therapy, and requests for diagnostic testing should not be rejected when ordered in the outpatient setting.

Fecal leukocyte examinations have been recommended for the differentiation of inflammatory diarrheas (fecal leukocyte positive) from secretory diarrheas (fecal leukocyte negative). Infectious, inflammatory diarrheas are caused by invasive bacteria, while secretory diarrheas result from toxin-producing bacteria, viruses, and protozoan pathogens (68, 170, 192). Unfortunately, fecal leukocyte morphology degrades in feces during transport and processing delays, making accurate recognition and quantitation difficult. This problem can be solved by using lactoferrin as a surrogate marker for fecal leukocytes (Leuko-Test; TechLab, Blacksburg, Va.), since lactoferrin is not degraded during normal transport and processing times. Lactoferrin-positive stool specimens are considered positive for fecal leukocytes. In addition, invasive pathogens may result in fecal leukocytes being intermittently present or unevenly distributed in stool specimens. Algorithms have been proposed depicting schemes for stool processing based on the use of a lactoferrin assay (63), but they are not commonly used or recommended for the diagnosis of acute, infectious diarrhea (68). The lactoferrin assay is used in the evaluation of patients with inflammatory bowel disease (52).

Usual gastrointestinal pathogens are listed in Table 8 (63). Inclusion of less frequently encountered pathogens should be considered when epidemiological factors suggest an increased likelihood. This may require periodic surveys of one's community to establish which pathogens are most common, especially when considering the addition of selective media or toxin assay for the routine detection of enterohemorrhagic *Escherichia coli* (EHEC), non-*jejuni/coli Campylobacter, Vibrio* spp., and *Yersinia enterocolitica.*

Selective and differential media are used to detect *Salmonella* and *Shigella* spp. (Table 5). These should include one that is differential but not selective for these pathogens, such as MacConkey agar, and one that is a mildly selective medium, such as Hektoen enteric or xylose-lysine desoxycholate agar. In some settings, a highly selective medium such as salmonella-shigella agar is also included. In addition, enrichment broth, such as gram-negative broth or Selenite-F broth, may increase detection of *Salmonella* and is recommended for testing sensitive populations such as food handlers. Subculture of gram-negative and Selenite-F broths to a mildly selective and differential medium after 6 to 8 h and 12 to 18 h of incubation, respectively, is necessary to prevent overgrowth of normal flora and decreased usefulness of the broth (116). All agar plates should be incubated in air at 35 to 37°C for 2 days before being reported as negative. The decision of whether to use a highly selective agar medium and an enrichment broth will vary from one laboratory to another.

Optimally, additional media are used for a trial period to determine their value, which is measured by the detection of strains not present on the two standard media. In settings where such a trial is not possible, the use of MacConkey, Hektoen enteric, or xylose-lysine desoxycholate agar and an enrichment broth is recommended (63).

Campylobacter jejuni and *Campylobacter coli* are detected with a medium such as campylobacter agar with 10% sheep blood and selective antimicrobial agents (Table 5). Media are incubated at 42°C in a microaerophilic atmosphere of nitrogen (85%), carbon dioxide (10%), and oxygen (5%) for up to 3 days. Special enrichment broths are available for the recovery of *Campylobacter;* however, their routine use does not increase the number of campylobacter-positive cultures significantly (1). Detection of other *Campylobacter* species may require media without antibiotics and 37°C incubation (33).

Special Considerations for Gastrointestinal Tract Specimens

Other Enteric Pathogens

A physician order for testing for *Vibrio* spp., *Y. enterocolitica, Aeromonas* spp., and *Plesiomonas shigelloides* may be needed in some geographic locations or epidemiological situations, since incidence is so low in most parts of the United States that the routine use of selective media is not justified. Media used for these enteric pathogens include thiosulfate citrate bile salts sucrose agar for vibrios, cefsulodin-Irgasan-novobiocin agar for *Y. enterocolitica,* and blood agar or selective blood agar to demonstrate hemolysis and provide a medium for oxidase testing for *Aeromonas* spp. and *P. shigelloides* (both oxidase positive) (63). All of these enteric pathogens grow on usual media, but detection is enhanced and simplified using specific selective media.

EHEC

The prevalence of EHEC varies in different parts of the United States and the rest of the world. It has been established that in addition to *E. coli* O157:H7, other serotypes are implicated as enterohemorrhagic strains. In fact, in the United States approximately 50% of Shiga toxin-producing strains, those capable of causing hemorrhagic colitis and hemolytic uremic syndrome, are not serotype O157:H7 (5, 50). For this reason, many laboratories have chosen to perform a Shiga toxin assay by EIA to detect all serotypes, rather than culture or antigen detection for O157:H7 strains only. Some Shiga toxin-producing strains may not harbor all the mechanisms needed to be fully pathogenic in humans. Strategies include testing all specimens year-round, testing all specimens only during summer months (when incidence is highest), testing only specimens containing gross blood, testing only when specifically ordered, testing only specimens from pediatric patients, or testing based on a combination of these factors (58). The best approach for a specific laboratory can be determined by a culture survey of all stool specimens during summer months. Alternatively, one can contact neighboring hospital laboratories or local health departments, which may be able to provide prevalence data. Laboratories should check with public health authorities to see if EHEC-containing stools need to be forwarded for serotyping of toxin-producing *E. coli* strains.

C. difficile

Approximately 15% of people who develop diarrhea following antimicrobial use have antibiotic-associated diarrhea caused by *C. difficile.* If use of the offending antimicrobial continues and disease remains undiagnosed and untreated, pathology may progress to more severe colitis, pseudomembranous colitis, and, possibly, death (9, 60). Nearly 100% of cases of pseudomembranous colitis are caused by *C. difficile.* Disease is caused by toxins (toxin A and toxin B) produced by *C. difficile.* The disease is diagnosed by detecting the organism or its toxins in stool, in conjunction with clinical criteria (84). Cell culture cytotoxicity assay for the detection of toxin B, EIA for the detection of toxin A or toxins A and B, latex agglutination or EIA for the detection of glutamate dehydrogenase (an antigen associated with *C. difficile* and occasionally other bacterial species), and culture of *C. difficile* from stool followed by toxin testing of the isolate are all methods used for diagnosis (9, 110). In practice, an EIA for toxin A or one that detects both toxins A and B is most practical since it requires minutes to hours to complete, compared to 24 to 48 h for the cell culture cytotoxicity assay or 2 to 4 days for culture and toxin testing of the isolate. A single specimen tested by EIA identifies 85 to 100% of specimens eventually proven to be toxin positive, whereas a single specimen tested by cell culture identifies nearly 100% of specimens eventually proven to be positive (110, 201). Testing of a second specimen, after a single negative EIA result, may be needed if symptoms persist and an alternative diagnosis has not been made (110), but testing a second specimen following a single cell culture negative result is not recommended (201).

Although *C. difficile*-associated gastrointestinal disease is most common in hospitalized patients, it can occur in any patient treated with antimicrobials, whether institutionalized or in the community. Testing should not be performed on formed stool (no diarrhea), or as a follow-up to therapy to confirm cure. Repeat testing is appropriate only if symptoms persist or recur (110). Symptoms in those successfully treated will resolve over a few days, in spite of the fact that patients may continue to carry *C. difficile* and even remain toxin positive for days or weeks (181). It is a faulty practice to require negative toxin or culture for *C. difficile* before allowing patients admission to long-term care facilities. Patients who do not have diarrhea and are not incontinent of stool are not an increased risk to other patients, even if carrying *C. difficile* and its toxins, when usual infection control measures are followed (9).

Rare and even fatal disease can be caused by toxin A-deficient strains (toxin B only produced), necessitating occasional use of a toxin A-plus-B immunoassay or cell culture. It is estimated that 2.4% of strains causing human disease are toxin A deficient (85). For epidemiological purposes, stool or rectal swabs placed in anaerobic transport medium may be cultured anaerobically to isolate *C. difficile.* The sample is inoculated to a selective medium such as cycloserine-cefoxitin-fructose agar and incubated anaerobically for 48 h (60).

Since 2002, outbreaks of severe *C. difficile* disease in North America and Europe have been reported. The strains involved are reported to be hyperproducers of toxins A and B. The putative cause is mutation of the regulator controlling production of both toxins. Peak average toxin levels were 16 to 32 times higher than normal in strains without regulator mutations (215). In addition, a third toxin, named the binary toxin, which is found in approximately 5% of all *C. difficile* strains, is reported to be present in nearly two-thirds of strains causing severe disease. Further investigation is needed to establish whether this newly detected toxin is an additional factor in the pathogenicity of *C. difficile* (3, 115).

S. aureus, Methicillin-Resistant *S. aureus* (MRSA), and *Bacillus cereus*

Stool specimens or gastric contents collected from persons with short-incubation food poisoning (2 to 6 h) can be

evaluated for *S. aureus* and *B. cereus*. In general, investigation is beneficial for general public health, rather than a sick individual who recovers quickly, and is best performed by public health laboratories rather than hospital clinical microbiology laboratories. Specimens should be examined by Gram stain, and, because both of these organisms may be present normally in food, quantitative cultures must be performed. A series of dilutions (10^{-1} to 10^{-5}) of the specimen are prepared in buffered gelatin diluent, and 0.1-ml samples of the undiluted specimen and each of the dilutions are plated onto colistin-nalidixic acid or phenylethyl alcohol blood agar. The presence of 10^5 CFU or more of *S. aureus* or *B. cereus* per g of specimen is of potential significance (22, 120).

S. aureus is a possible cause of antibiotic-associated diarrhea (18). The incidence is controversial but receiving more attention. Gram stain of smears of nonformed stool showing sheets of staphylococcal clusters in combination with appropriate clinical findings suggests the diagnosis.

MRSA may be a cause of nosocomial antibiotic-associated diarrhea (18). The overall incidence is unknown. Diagnosis consists of the detection of heavy growth of MRSA in combination with the detection of staphylococcal enterotoxin in stool. Greater recognition of this disease should confirm its significance and result in rapid diagnostic methods and appropriate treatment.

Clostridium botulinum

The clinical diagnosis of foodborne and infant botulism may be confirmed by detecting botulinal toxin, *C. botulinum,* or both in feces (121). Optimally, 25 to 50 g of stool, 15 to 20 ml of serum, and a sample of suspect food should be collected. Most clinical laboratories are not properly equipped to process specimens from persons with suspected botulism. In the United States, when a case of botulism is suspected, investigators at the CDC should be notified to ensure appropriate diagnosis, treatment, and investigation of the potential outbreak. Botulinal toxin could be used as a biological weapon (see chapter 9). Unexpected numbers of cases or unusual presentations should be investigated.

Helicobacter pylori

H. pylori is an important cause of gastritis and peptic ulcer disease (206). The organism can be observed in tissue sections by using hematoxylin and eosin, Giemsa, or Warthin-Starry silver staining. In addition, organisms can be visualized in touch preparations of dissected tissue stained with the Gram stain. The presence of *H. pylori* in stomach or small bowel lesions can be confirmed by culture, antigen detection, urease detection, or the detection of exhaled bacterial metabolite (*H. pylori* breath test) (101, 205, 206). Tissue biopsy specimens collected during endoscopy are used for culture and urease detection. Specimens for culture should be placed in transport medium (medium containing 20% glycerol, such as brucella broth, are best for transport and storage) and transported to the laboratory immediately, or refrigerated during delays (72). Lightly minced tissue is inoculated to freshly prepared blood agar and incubated in a humid, microaerophilic atmosphere (5 to 10% carbon dioxide, 80 to 90% nitrogen, and 5 to 10% oxygen) at 37°C for 7 days (Table 5). The addition of 5% hydrogen should improve the yield of *H. pylori.* Tissue for urease detection is placed as soon as possible into the detection system and processed as specified by the manufacturer. Stool for antigen detection should be collected and handled according to instructions included with the Premier Platinum HpSA test (Meridian Biosciences, Inc., Cincinnati, Ohio) (64, 205). In some clinical situations serologic testing for *H. pylori* antibody may be necessary. Serum should be collected and stored at refrigeration temperature for short periods (up to 1 week) or frozen at –70°C for longer periods (101).

Screening for VRE or Beta-Hemolytic Streptococci

Identifying Carriers of Vancomycin-Resistant Enterococci (VRE) for infection control purposes, detecting group B streptococci in pregnant patients, and detecting group A streptococci during investigations of outbreaks of necrotizing fasciitis or streptococcal toxic shock require collection of a rectal swab specimen. Carriers of VRE can be identified by culturing rectal swab or perirectal swab material (218). Specimens are inoculated to selective enrichment broth, such as Enterococcosel broth with 6 μg of vancomycin per ml, or agar media, such as colistin-nalidixic acid blood agar containing 6 μg of vancomycin per ml (see chapter 30). Carriers of group A streptococci can be identified by culturing rectal swab specimens on sheep blood agar or selective streptococcal agars used to identify patients with streptococcal pharyngitis. Carriers of group B streptococci are identified by culturing vaginal secretions and rectal swab material as discussed below (28).

Genital Tract Specimens

Genital tract specimens are sent to the microbiology laboratory to determine the etiology of various clinical syndromes, including vulvovaginitis, bacterial vaginosis (BV), genital ulcers, urethritis, cervicitis, endometritis, salpingitis, and ovarian abscess in females and urethritis, epididymitis, prostatitis, and genital ulcers in males. These diseases and their etiologies are listed in Table 8 (6).

Many specimens are contaminated with normal skin or mucous membrane flora. Pathogens such as *Haemophilus ducreyi, N. gonorrhoeae, Trichomonas vaginalis, Treponema pallidum,* and *C. trachomatis* are always significant. Other organisms, such as *S. aureus,* beta-hemolytic streptococci, *Enterobacteriaceae,* and anaerobes, are pathogenic only in certain clinical situations. The specimen source, relative quantity of potential pathogen compared to normal flora, and Gram stain interpretation help the technologist determine which isolates require identification and antimicrobial testing. At a minimum, isolates from presumably sterile specimens and pure or predominant potential pathogens from specimens likely to be contaminated with normal flora and containing polymorphonuclear neutrophils should be identified and reported. Mixtures of anaerobes do not require individual identification and listing in most cases. Laboratories should avoid isolating, identifying, and performing antimicrobial testing on every bacterial isolate from all specimens (6). In addition to the excessive cost of this approach, unnecessary reporting of bacterial species contributes to excessive treatment of patients. Exact protocols for workup and reporting may require discussion and mutual agreement with knowledgeable clinicians in each practice environment.

Special Considerations for Genital Tract Specimens

Detection of *N. gonorrhoeae* and *C. trachomatis*

Nucleic acid probe and amplification methods are commercially available for direct detection of *N. gonorrhoeae* and *C. trachomatis* in endocervical, urethral, and urine specimens. Users must pay close attention to the types of specimens approved for use with each kit. Specimens should be collected using the procedures and collection kits recommended by the manufacturer. In addition, false-positive

reactions have been reported with some kits, necessitating confirmation of positive results (208).

Although not as sensitive as molecular methods, both EIA and DFA tests are commercially available for the direct detection of *C. trachomatis* in endocervical, urethral, and, with some EIA kits, urine specimens (171). If these kits are used, collection and processing must follow instructions provided by the manufacturer. Cell culture for *C. trachomatis* is recommended for use with specimens not approved for molecular or EIA kits. These are likely to include eye, rectal, and abscess specimens collected during surgery. In addition, culture is the only acceptable diagnostic procedure in some jurisdictions for medical-legal cases.

Culture for *N. gonorrhoeae* is optimal when the specimen is directly inoculated to a selective medium, such as modified Thayer-Martin medium, and incubated immediately (48). Transport of inoculated media to the laboratory must be in an increased CO_2-containing environment. Swab specimens (cotton swabs should be avoided because they may be toxic) should be placed in a transport system containing Stuart's or Amies medium and delivered to the laboratory as quickly as possible. Specimens for *N. gonorrhoeae* culture should be held at refrigeration temperature during transport. As transport time increases, recovery by culture decreases. Specimens requiring more than 24 h for transport are unacceptable. Detection by nonculture methods is recommended in most settings where the additional cost of testing by molecular technique or immunoasssay is not prohibitive (204).

BV and Vaginitis

BV occurs when conditions result in overgrowth of usual vaginal flora with various anaerobic genera, including *Mobiluncus* and *Bacteroides* (184). Although not characterized by a polymorphonuclear response, BV results in an increase in vaginal secretions that are relatively alkaline (pH > 4.5) compared to normal, the usual predominant flora of lactobacilli being replaced by anaerobes, and the presence of aromatic amines which are detected by adding 10% potassium hydroxide and noting a pungent, fishy odor. In addition, excessive growth of a facultative bacterium called *G. vaginalis* generally coincides with BV. Although *G. vaginalis* commonly is a member of the normal vaginal flora, the presence of increased concentrations that adhere to vaginal squamous epithelial cells, called clue cells, is pathognomonic of BV. Clue cells are squamous epithelial cells peppered with anaerobic coccobacilli and *G. vaginalis* bacteria, frequently showing heavier adherence toward the periphery of the cell and appearing like a donut (Fig. 3). As a result of these characteristic changes, BV is diagnosed best without culture (140). Wet-mount or Gram-stained smears should be examined and interpreted according to Table 9. In summary, BV should be diagnosed by performing a "bedside" pH and KOH "whiff test" and a laboratory Gram stain.

Candida and *Trichomonas* infections of the vulvovaginal areas are inflammatory conditions referred to as vaginitis rather than vaginosis. *Candida* spp. and *T. vaginalis* can be detected using microscopic examination of a saline wet-mount preparation. Low-power examination (×10 to ×20) will detect budding yeasts and pseudohyphae of *Candida* spp. or motile trophozoites of *Trichomonas*. Cultures are much more sensitive than direct wet preparations. Alternatively, a combination probe assay (Affirm VPIII identification test; Becton Dickinson, Sparks, Md.) is commercially available for the simultaneous detection of *Candida* spp., *G. vaginalis,* and *T. vaginalis* in vaginal secretions (21). Specimens must be collected using procedures recommended by the manufacturer.

Screening for Group B Streptococcus

Group B streptococcus carriers are identified by culturing vaginal secretions and rectal swab material. Swabs of the vaginal introitus and anorectum are collected and inoculated into a single enrichment broth such as LIM broth. This broth is incubated at 35°C overnight and subcultured to a blood agar plate the following day (173). A rapid molecular assay is available for the detection of group B streptococcus colonization in pregnant women (35). The performance of this test allows for rapid intrapartum detection of group B streptococci.

Dark-Field Examination for *T. pallidum*

Dark-field examination of tissues, tissue exudates, and material collected from chancres can be used to confirm the diagnosis of syphilis. For dark-field microscopy, the specimen should be examined within 20 min of collection to ensure motility of treponemes and should not be exposed to temperature extremes during transport to a dark-field microscope. The test requires a microscope equipped with a dark-field condenser and experienced personnel who are able to recognize *T. pallidum* spirochetes based on the tightness and regularity of the spirals and on their characteristic corkscrew movement (98). A DFA stain can be used and is performed on air-dried smears. The stability of the smear during transport and the easily identified, fluorescing treponemes make the DFA an attractive alternative to dark-field microscopy (98). Unfortunately, reagents for the DFA test are not commercially or widely available; it may be performed at some public health laboratories.

H. ducreyi

If infection with *H. ducreyi* is suspected, material from the base of the ulcer is collected and held at room temperature until needed for processing (102). One swab is used to prepare a smear for Gram staining. The presence of many small, pleomorphic, gram-negative bacilli and coccobacilli arranged

TABLE 9 Diagnosis of BV using a Gram-stained smear of vaginal secretions, by the Vaginal Infection and Prematurity Study Group criteria[a]

Morphotype	Score for indicated no. of morphotypes seen per oil power field				
	None	≤1	1–5	5–30	>30
Lactobacillus	4	3	2	1	0
Gardnerella/Bacteroides spp.	0	1	2	3	4
Curved gram-variable rods (*Mobiluncus* spp.)	0	1	2	3	4

[a] Adapted from reference 140. Total score = *Lactobacillus* morphotypes + *Gardnerella/Bacteroides* morphotypes + *Mobiluncus* morphotypes. A score of 0 to 3 is considered normal, 4 to 6 is intermediate, and 7 to 10 indicates BV.

in chains and groups (school of fish) suggests *H. ducreyi* but is rarely seen (see chapter 41). Recovery of the organisms by culturing on an enriched medium such as GC agar containing 3 μg of vancomycin per ml, 1% hemoglobin, 5% fetal bovine serum, and 1% IsoVitaleX, or Mueller-Hinton agar with 5% horse blood, 1% IsoVitaleX, and 3 μg of vancomycin per ml, is necessary to confirm the diagnosis; culturing at 33°C yields better recovery than does culturing at 35°C (see chapter 41).

Actinomyces spp.

Actinomyces spp. may cause pelvic inflammatory disease in women who use intrauterine contraceptive devices (IUD) (104). An IUD submitted for culture should be placed in a sterile liquid medium (preferably reduced, such as thioglycolate) and vortexed, and the liquid medium should be used to inoculate aerobic and anaerobic culture media. Inflammatory debris and tissue attached to the IUD should be removed and cultured aerobically and anaerobically. *Actinomyces* spp. produce small knots of intertwined bacterial filaments called grains or granules, which may be 1 mm or more in diameter. These grains should be crushed on a slide for staining (Gram stain is acceptable) and transferred to media for culture. The presence of branching gram-positive filaments suggests *Actinomyces,* which characteristically occurs in mixed infections with other aerobic and anaerobic bacteria. Culture confirms the diagnosis.

Lower Respiratory Tract Specimens

Specimens from the lower respiratory tract are submitted to determine the etiology of airway disease (tracheitis and bronchitis), pneumonia, lung abscess, and empyema (10, 138, 179). Table 8 gives a list of lower respiratory tract diseases and their common etiologies. Usual specimens submitted consist of lower respiratory tract secretions and inflammation in the form of expectorated sputum, induced sputum, endotracheal tube aspirations (intubated patients), bronchial brushings, washes or alveolar lavage samples collected during bronchoscopy, and pleural fluid (198). Specimens should be delivered to the laboratory promptly and processed without delay (within 1 h of collection). If delays are unavoidable, the specimen should be refrigerated.

Usual pathogens detected in lower respiratory tract secretions are present in specimens containing acute inflammatory cells (polymorphonuclear leukocytes) and in quantities greater than contaminating respiratory flora. Frequently, pathogenic bacteria are present within the polymorphonuclear leukocytes. There are many ways to assess the quality of respiratory tract specimens. A simple screening method involves assessment of squamous epithelial cells only (128, 129, 135). Squamous epithelial cells are found in the oropharynx but not in the lower respiratory tract. Increased numbers (defined as >10 per 10× objective microscopic field) indicate gross contamination with oropharyngeal contents, which include usual oral bacterial flora (Fig. 4 and 5). Most bacterial lower respiratory tract disease is caused by inapparent aspiration of oropharyngeal contents. It follows that oropharyngeal floras include the bacteria that cause lower respiratory tract disease. Detection of a potential pathogen in a grossly contaminated specimen may represent contamination with oropharyngeal flora. The lack of usefulness of data from contaminated specimens has resulted in policies for screening and rejecting grossly contaminated respiratory tract specimens. Table 6 lists respiratory tract specimens and usual screening policies. Respiratory tract specimens for the detection of *Mycoplasma pneumoniae, Legionella* spp., dimorphic fungi, and *M. tuberculosis* should not be screened for adequacy. All specimens are considered acceptable for the detection of these microorganisms (34).

Once respiratory tract specimens have been deemed acceptable, Gram-stained smears should be examined further for inflammatory cells, bacteria, and other indicators of lower respiratory tract pathology, such as mucus, necrosis, intracellular bacteria, alveolar macrophages, and Curschmann's spirals (Fig. 2) (231). Tracheal and bronchial mucopurulence can be trapped behind an airway obstruction or within a lung abscess cavity or may pool in the bronchi or trachea, resulting in the death and disintegration of cells, reflected as necrosis and cell debris in stained smears. Intracellular bacteria can be differentiated from bacteria "lying" on top of polymorphonuclear leukocytes by their greater concentration within the phagocytic cell than in nearby extracellular areas. Phagocytosis is an active uptake process resulting in concentration of bacteria within the cell. Alveolar macrophages are identified by their vacuolated cytoplasm and round eccentric nucleus, which is difficult to see in Gram-stained smears. Alveolar macrophages and Curschmann's spirals indicate areas within the smear that originated within the alveoli and distal airways (Fig. 2). Occasionally elastin fibers are seen in respiratory tract specimens (Fig. 1).

Bacteria should be reported when detected in Gram-stained smears if they are potential pathogens. Bacteria not in sufficient quantity or not representative of morphotypes resembling potential pathogens should be lumped together and reported as normal respiratory flora. It is important to differentiate contaminating respiratory flora from respiratory flora causing aspiration pneumonia. Aspiration of relatively large amounts of oropharyngeal contents following loss of consciousness, paralysis of muscles involved with swallowing and breathing, or medical procedures such as intubation can result in infection of the airways with mixed respiratory flora leading to lung abscess and empyema (111). Gram stain of sputum from patients with aspiration pneumonia can be highly suggestive of the diagnosis. Stained smears show many polymorphonuclear leukocytes and many mixed respiratory flora morphotypes, especially those suggesting streptococci and anaerobes. Much of the flora is intracellular. Aspiration pneumonia can be detected in hospitalized patients and those admitted directly from the community (10, 138).

Cultures of respiratory tract material should include a medium selective for gram-negative organisms, such as MacConkey's agar, sheep blood agar, and chocolate agar for the detection of *Haemophilus* spp. (Table 5). Culture plates should be incubated at 35°C in 3 to 5% CO_2 for 48 h before being reported as negative. Cultures are interpreted by examining the relative numbers and types of bacteria that grow. Table 10 summarizes interpretative criteria used with respiratory tract specimens (200).

Special Considerations for Lower Respiratory Tract Specimens

Specimens Collected During Bronchoscopy

Bronchoalveolar lavage fluid and bronchial brush specimens from patients with suspected pneumonia should be cultured quantitatively to evaluate the significance of potential pathogens recovered (23). Bronchial brush specimens, which contain approximately 0.01 to 0.001 ml of secretions, should be placed in 1 ml of sterile nonbacteriostatic saline after collection. The specimen should be delivered to the laboratory immediately. In the laboratory, the specimen is agitated on a vortex mixer, a smear is prepared by cytocentrifugation for staining with the Gram stain, and 0.01 ml of specimen is

inoculated to appropriate media by using a pipette or calibrated loop. Colony counts of more than 1,000 CFU of potential pathogens per ml (corresponding to 10^6 CFU of original specimen per ml) appear to correlate with disease. Bronchoalveolar lavage results in collection of 50 ml or more of saline from a larger lung volume. In the laboratory, a smear is prepared by cytocentrifugation and Gram stained (155). The Gram stain report should include a comment about the presence of squamous epithelial cells and intracellular bacteria. Grossly contaminated fluid (>1% of all cells are squamous epithelial cells) may have falsely elevated counts of potential pathogens. Intracellular bacteria are more likely to be potential pathogens. A 0.01- or 0.001-ml aliquot of bronchoalveolar lavage fluid should be inoculated to agar media (Table 5). The recovery of <10,000 bacteria/ml suggests contamination. The recovery of >100,000 bacteria/ml suggests that the isolate is a potential pathogen. Detection of 10,000 to 100,000 bacteria per ml represents a "gray" zone (200). Counts of pathogens may be reduced by prior antimicrobial therapy or variations in "return" of lavage fluid during the bronchoscopy procedure (Table 10).

Legionella spp.

Legionella spp., especially *L. pneumophila,* are important causes of community- and hospital-acquired pneumonia (51). Legionellosis can be diagnosed by culture, DFA staining of smears of respiratory secretions, detection of antigens in urine, or serologic testing. Culture is preferred because, unlike other methods, it is not limited to detection of certain species or serotypes. Before culture, respiratory samples should be diluted 10-fold in a bacteriological broth, such as tryptic soy, or sterile water to dilute inhibitory substances that may be present in the specimen. Because legionellae grow slowly, optimal isolation from highly contaminated specimens, such as sputum, is achieved by decontaminating the specimens with acid before plating (214). The specimen is diluted 1:10 in KCl-HCl buffer (pH 2.2) and incubated for 4 min at room temperature. It is important not to incubate the specimen for longer than 4 min because legionellae may themselves be killed by acid exposure. Specimens are inoculated onto buffered charcoal yeast extract agar with and without antimicrobial agents (e.g., vancomycin, polymyxin B, and anisomycin). The cultures are incubated in humidfied air at 35°C for a minimum of 5 days. Using a dissecting microscope, small colonies with a ground-glass appearance, typical of *Legionella* spp., can be detected after 3 days of incubation.

M. pneumoniae

Mycoplasma pneumoniae is a common cause of pneumonia, referred to as primary atypical pneumonia. Because *M. pneumoniae* is fastidious and grows very slowly, a definitive diagnosis is often based on the results of serologic tests. When culture is required, the specimen of choice is a throat swab; however, sputum or other respiratory specimens are also acceptable. The specimen should be placed immediately into a transport medium containing protein, such as albumin, and penicillin to reduce the growth of contaminating bacteria. Specimens may be stored in the transport medium for up to 48 h at 4°C or frozen for longer periods at –70°C. PCR methods have been used successfully to detect *M. pneumoniae* directly in respiratory tract specimens. Molecular detection by PCR or a related technique may be the most sensitive method for the detection of *M. pneumoniae* (156).

Specimens from Patients with Cystic Fibrosis

Burkholderia cepacia is an important respiratory pathogen in persons with cystic fibrosis (31, 62). This organism grows well on routine media; however, selective media such as *B. cepacia* selective agar, *Pseudomonas cepacia* agar, and oxidative-fermentative-polymyxin B-bacitracin-lactose agar are useful for optimal recovery from respiratory secretions (79, 80). Comparative studies show *B. cepacia* selective agar to be superior (79) (also see chapter 49).

Chlamydia and *Chlamydophila* spp.

Chlamydiae are important causes of respiratory illnesses in children and adults (143). *C. trachomatis* can cause serious respiratory disease in newborn infants. *Chlamydophila pneumoniae*

TABLE 10 Interpretation of bacterial lower respiratory tract culture results[a]

Specimen	Likely to be significant	Not likely to be significant	Additional data suggesting that isolate is significant
Sputum—coughed or induced	Predominant potential pathogen in Gram stain and culture. Neutrophils abundant.	Potential pathogen not present in Gram stain and only 1–2+ growth in culture. Neutrophils not abundant in Gram stain.	Potential pathogen within neutrophils (intracellular bacteria)
Endotracheal tube aspirate (112)	Predominant potential pathogen in Gram stain and culture. Neutrophils abundant.	Potential pathogen only 1–2+ growth in culture. Neutrophils not abundant in Gram stain.	Potential pathogen in quantities $>10^6$ CFU/ml. Potential pathogen within neutrophils (intracellular bacteria)
Bronchoalveolar lavage fluid	Predominant potential pathogen seen in every 100× field of Gram stain. Quantitative culture detects $>10^5$ CFU of potential pathogen/ml.	Potential pathogen not seen in Gram stain. Quantitative culture detects $<10^4$ CFU of potential pathogen/ml.	Potential pathogen within neutrophils (intracellular bacteria)

[a] Reprinted from reference 195 with permission.

causes illness in all age groups, but most disease occurs in adolescents and young adults. *Chlamydophila psittaci* is primarily an animal pathogen but occasionally causes disease in humans exposed to sick animals. Lower respiratory tract secretions, in addition to nasopharyngeal washes, for the detection of chlamydiae are collected and transported to the laboratory immediately in a medium containing antimicrobial agents (e.g., gentamicin and nystatin). If delays in transport or processing occur, the specimen should be stored at 4°C for up to 48 h. Longer storage should be at –70°C or colder. Chlamydiae are detected by rapid cell culture techniques (shell vial) using McCoy cells for *C. trachomatis* and *C. psittaci* and HEp-2 cells for *C. pneumoniae*. As with *M. pneumoniae*, PCR may prove to be the most sensitive method for the detection of respiratory chlamydiae (156).

Nocardia Species

Respiratory specimens for the detection of *Nocardia* species should be transported to the laboratory as soon as they are collected. For short delays, storage at 4°C is acceptable. Direct examination of a Gram-stained smear containing a *Nocardia* species shows thin, beaded gram-positive branching filaments. The filaments are also partially acid fast when stained by the modified Kinyoun method. There are no media used routinely for the specific recovery of *Nocardia* spp. since they grow readily on many common media such as sheep blood and chocolate agar plates, Sabouraud agar for fungi, Lowenstein-Jensen medium for mycobacteria, and charcoal yeast extract agar for legionellae. Mycobacterial decontamination procedures reduce the recovery of *Nocardia*. Selective charcoal yeast extract agar is optimal for culture from contaminated specimens (210). Although *Nocardia* spp. are detected commonly following 1 week of incubation, cultures are incubated for a total of 3 weeks at 35°C.

Upper Respiratory Tract Specimens

Upper respiratory tract specimens include the external nares, nasopharynx, throat, oral ulcerations, and inflammatory material from the nasal sinuses. Although few serious diseases involve these areas, many pathogens colonize or persist in these sites while causing symptomatic infection in deeper, less accessible sites (147).

Throat specimens are collected to diagnose pharyngitis caused by *Streptococcus pyogenes*. Swab specimens should be placed in a standard transport carrier containing Amies or modified Stuart's medium. Refrigeration is preferred if transport requires more than a few hours. Many rapid direct tests for group A streptococci are commercially available, including EIA, optical immunoassay, and nucleic acid-based probe assays (59, 190). The reported sensitivities of EIAs and an optical immunoassay vary between 60 and 95% but can be as low as 31% (25, 217). The nucleic acid-based assay has a sensitivity of >90% (75, 78). When a rapid test is requested, two throat swabs should be collected. If only one swab is received, the culture plate should be inoculated first. Material remaining on the swab is used for the direct test. If the rapid test is positive, the second swab can be discarded, but if the rapid test is negative, the second swab must be used for culture to confirm the negative direct test. The nucleic acid-based probe test is considered sensitive and specific enough by many to obviate confirmatory culture (78). A position paper by representatives of the American Academy of Family Physicians, the American College of Physicians-American Society of Internal Medicine, and the CDC states that rapid tests do not require confirmatory culture when used with adult patients (32). This recommendation does not hold for specimens from children.

To culture group A streptococci, either sheep blood agar or selective blood agar may be used. Selective agar makes the organism easier to visualize by inhibiting accompanying flora but may delay the appearance of colonies of *S. pyogenes*. Cultures should be incubated for 48 h at 35°C in an environment of reduced oxygen achieved by incubating them anaerobically, in 5% CO_2, or in air with multiple "stabs" through the agar surface. Stabbing the agar surface with the inoculating loop pushes inoculum containing streptococci below the surface, where the oxygen concentration is reduced compared to ambient (25, 89). These culture conditions allow the recovery of group C and G streptococci, organisms which may cause pharyngitis but do not cause the serious sequelae associated with group A streptococci (203, 232).

Throat specimens also are used to identify patients infected with *N. gonorrhoeae*. For best results, the specimen should be inoculated immediately to a selective medium, such as modified Thayer-Martin agar. Cultures are incubated at 35°C in the presence of 5% CO_2 for 72 h.

The external nares can be cultured to identify carriers of *S. aureus* by using a single swab to collect secretions from both the left and right nares. The usual carrier systems used for swab transport containing Amies or Stuart's medium are acceptable. In the laboratory, the specimen can be inoculated to a sheep blood agar plate; however, use of selective media such as colistin-nalidixic acid agar, or a selective and differential medium such as BBL CHROMagar *Staph aureus* (BD Diagnostics, Sparks, Md.), BBL CHROMagar MRSA (BD Diagnostics), or mannitol salt agar is helpful in differentiating *S. aureus* or MRSA from other floras and useful when interpreting large numbers of specimens (53, 54, 57, 221). Published comparisons show the CHROMagar formulations to be superior (53, 54). Cultures should be incubated at 35°C for 2 days. Molecular testing by PCR for the detection of *S. aureus* in swabs from the external nares has been shown to be as sensitive as culture but much more rapid (142). A commercially available, FDA-approved molecular test (IDI-MRSA; Infectio Diagnostic, Inc., Sainte-Foy, Quebec, Canada) for the detection of MRSA in nasal swabs is also an option for those seeking a rapid test (216).

Nasopharyngeal secretions and cells are used to identify patients infected with *Bordetella* spp. and carriers of *N. meningitidis* (113). Specimens for the recovery of *Bordetella pertussis* and *Bordetella parapertussis* should be collected with a small-tipped Dacron swab. Cotton may be toxic to the organism. Swabs should be transported to the laboratory in special media. For delays of up to 24 h, Amies medium with charcoal can be used. If the transport time will exceed 24 h, Regan-Lowe transport medium should be used (82, 126). Culture, DFA staining, and PCR can be used for detection. PCR is the most sensitive method for detecting *B. pertussis* and *B. parapertussis* (Dacron swabs are preferred for PCR tests) (107). Culture is performed using Regan-Lowe charcoal agar containing 10% horse blood and cephalexin. Because a few strains of *B. pertussis* do not grow in the presence of cephalexin, the use of Regan-Lowe medium with and without cephalexin is recommended for optimal recovery (77, 183). Cultures are incubated at 35°C for 5 to 7 days in a humid atmosphere. The DFA test has a lower sensitivity and specificity than PCR for *Bordetella* but offers rapid results (107). Depending on the reagents used, either *B. pertussis* or *B. parapertussis* is detected.

Nasopharyngeal swab specimens are used to identify carriers of *N. meningitidis*. Transport in a swab container with Amies or Stuart's medium is acceptable. Specimens should be inoculated as quickly as possible to sheep blood or chocolate agar; however, selective agars for pathogenic *Neisseria*

spp., such as modified Thayer-Martin, are necessary if interference by normal flora is expected. Culture plates are incubated for 72 h in a humidified atmosphere at 35°C in the presence of 5% CO_2.

Vincent's angina is an oral infection characterized by pharyngitis, membranous exudate, fetid breath, and oral ulcerations. Sometimes referred to as fusospirochetal disease or necrotizing ulcerative gingivitis, it is caused by *Fusobacterium* spp., *Borrelia* spp., and other anaerobes. Diagnosis is made by direct examination of a smear of a swab specimen collected from the ulcerated lesions and stained with the Gram stain (147). The presence of many spirochetes, fusiform bacilli, and polymorphonuclear leukocytes is presumptive evidence of this disease. Culture is not helpful. It should be noted that canker sores do not have a microbial etiology and should not be cultured.

Inflammatory material from the nasal sinuses should be cultured to detect the etiologies of sinusitis. Nearly all cases of bacterial sinusitis follow a primary, upper respiratory tract viral infection. Bacteria are trapped in the sinus as a result of damage to the epithelial lining cells of the sinus and inflammation and swelling that narrows or closes the nasal ostium, preventing normal drainage (69). Specimens collected during endoscopic procedures by physicians specializing in otorhinolaryngology are optimal, since they are sampled directly from the infected sinus, avoiding contamination by normal flora in the nasal passages. Aspirates, washes, scrapings or debridements, and biopsy material should be kept moist and sent in a sterile container to the laboratory (69). Examination of Gram-stained smears can provide a rapid, presumptive identification of likely pathogens. Aerobic culture is needed in all cases; anaerobic transport and culture may be needed in cases of chronic sinisitis. Ventilator-associated sinusitis occurs in fewer than 10% of patients with nasotracheal intubation. Members of the nosocomial flora are implicated. Endoscopic inspection is needed to obtain acceptable specimen for culture (222).

Special Considerations for Upper Respiratory Tract Specimens

Arcanobacterium haemolyticum

A. haemolyticum can cause pharyngitis and peritonsillar abscess (88). The organism can be recovered on media used to detect *S. pyogenes*. Colonies of *A. haemolyticum* are beta-hemolytic and easily confused with those of beta-hemolytic streptococci. Rapid differentiation can be accomplished with the Gram stain. *A. haemolyticum* is a diphtheroid-shaped gram-positive rod (see chapter 34). Incubation of plates at 35°C for up to 72 h may be required for optimal detection.

Corynebacterium diphtheriae

Cultures of both throat and nasopharyngeal specimens are used in the diagnosis of diphtheria. When specimens are processed for culture without delay, no special transport medium or conditions are required. For transport to a reference laboratory, specimens should be sent dry in a container with desiccant (44). Alternatively, specimens collected on swabs may be placed in Stuart's or Amies medium for transport to the laboratory. Smears of specimens for *C. diphtheriae* can be stained with the Gram stain and examined for pleomorphic (diphtheroid morphology) gram-positive rods. In addition, smears can be stained with Loeffler's methylene blue stain and examined for pleomorphic, beaded rods with swollen (club-shaped) ends and reddish purple metachromatic granules. Bacteria with these characteristics are suggestive of but not specific for *C. diphtheriae*. Specimens should be inoculated to Loeffler's serum and potassium tellurite media for the recovery of *C. diphtheriae*. Cultures are incubated for 2 days at 35°C in 5% CO_2 before being reported as negative.

Epiglottitis

A throat swab specimen may be helpful in determining the etiology of epiglottitis, a rapidly progressing cellulitis of the epiglottis and adjacent structures with the potential for swollen tissues to cause airway obstruction. Epiglottitis is almost always caused by *H. influenzae* serotype b but occasionally by other bacteria such as *S. pneumoniae* and *S. pyogenes* (202). The specimen should be collected by a physician only in a setting where emergency intubation can be performed immediately to secure a patent airway. Specimens should be inoculated onto enriched medium, such as chocolate agar, and incubated at 35°C in an atmosphere of 5% CO_2 for 72 h. Nearly 100% of patients with epiglottitis caused by *H. influenzae* have a blood culture positive for the same bacterium.

Tissue Specimens

Tissue specimens are obtained during surgical procedures at significant risk and expense to the patient. Therefore, it is mandatory for the laboratory to receive sufficient specimen for both histopathologic and microbiological examination, bearing in mind that many microbiological tests and cultures may have been ordered. Histopathologic examination of the lesion serves to differentiate between infection and malignancy, and also to distinguish between acute and chronic infectious processes (227). Swabs provide too little specimen and should be discouraged and eliminated through educational efforts. For complete setup of routine, anaerobic, fungal, and acid-fast bacillus cultures and smears, one needs approximately 1 cm^3 of tissue.

Tissue should be transported in a sterile container that maintains moisture. To avoid drying, small pieces of tissue can be moistened with a few drops of sterile, nonbacteriostatic saline. Alternatively, very small pieces of tissue can be placed on a square of moistened sterile gauze. This serves to maintain moisture and allow easy identification by those receiving and processing the specimen. Tissue should be gently minced or ground during processing to release microorganisms and to provide equal specimen for all media and smears. This can be accomplished by cutting with a sterile scalpel, grinding with a mortar and pestle or tissue grinder, or using a stomacher (Seward Co., London, United Kingdom) or masticator (available from several suppliers) (180). The resulting homogenate is used to prepare smears for staining and to inoculate culture media. The Gram stain should be examined for the presence of polymorphonuclear leukocytes and bacteria. Grinding renders most cell morphology and tissue architecture difficult to recognize and often renders fungal hyphae nonviable. A small piece of intact tissue should be retained for inoculation into fungal media. Smears should be examined closely for intracellular bacteria, especially common with staphylococci and streptococci. For bacterial culture, processed tissue should be inoculated to enriched agar media. The use of a broth medium is controversial (128, 130). Anaerobic culture should be included when ordered and when tissue is transported in an oxygen-free environment. Large pieces of tissue, approximately 1 cm^3 or greater, maintain a reduced atmosphere in spite of brief aerobic transport. Oxygen-free transport may not be necessary in this circumstance, and the absence of anaerobic transport should not disqualify large pieces of tissue from anaerobic culture. Routine, aerobic cultures are incubated at 35°C in the presence of 5% CO_2 for 72 h.

Collecting and processing tissue offers an opportunity for exogenous contamination. Cultures growing small numbers of bacteria not commonly associated with infection, such as coagulase-negative staphylococci, corynebacteria, propionibacteria, and saprophytic *Neisseria* spp., may represent contamination rather than true "pathogens." In general, growth of these bacterial groups in broth culture only represents contamination (128, 130). One or two bacterial colonies growing on a single plate, of multiple plates inoculated, and not growing in broth culture, if used, generally represent contamination. Growth of one or two bacterial colonies on agar media not in the area of specimen inoculation or on streak lines in the second through fourth plate quadrants also is likely to represent contamination. In addition, it is assumed that bacteria considered contaminants were not detected in Gram-stained smears prepared from the original specimen. On the other hand, detection of the bacteria listed above as unlikely pathogens should always be reported when seen in the original Gram-stained smear, when present in quantities above a few colonies, and when detected on or in multiple media (41).

Special Circumstances for Tissue Specimens

Bone Marrow

Bone marrow aspirates can be submitted for culture in lysis centrifugation tubes (Isolator; Wampole Laboratories). The "pediatric" tube holds a maximum of 1.5 ml of specimen. One or more of the 1.5-ml tubes can be used. Aspirates may also be submitted in a sterile container containing anticoagulant. Sterile tubes with anticoagulant are less desirable since they use heparin, sodium citrate, or EDTA as anticoagulant, all of which are more inhibitory than SPS (49, 166). Although SPS inhibits meningococci, gonococci, *G. vaginalis,* anaerobic cocci, and *S. moniliformis,* these bacteria are unlikely to be detected in bone marrow aspirate specimens (159).

Although bone marrow aspirate cultures may be helpful in identifying disseminated fungal and mycobacterial diseases, they are unlikely to assist in the identification of usual bacterial diseases (211). Patients infected with human immunodeficiency virus may benefit from bone marrow culture when other specimen cultures have been unsuccessful (47). It is policy in some laboratories to consult with the ordering physician and suggest that routine bacterial culture is not necessary. In most cases, blood or other organ system culture is preferred for the identification of disseminated bacterial infections. Even if bone marrow culture is performed, direct Gram stain of bone marrow aspirates is not helpful and should not be a routine component of bacterial culture.

Lymph Nodes

Lymph node cultures from immunocompetent patients are positive only when there is a granuloma or acute inflammatory lesion, in which case etiologies detected are limited to mycobacteria and fungi. Bacterial culture of lymph nodes from immunocompetent patients may not be necessary without specific clinical or epidemiological findings, such as suspected tularemia *(F. tularensis)* (56).

Quantitative Tissue Culture

Tissue from traumatic wound or burn injury may be submitted for quantitative culture, with results of $\geq 10^3$ CFU/ml being used to predict the likelihood of the development of wound-related sepsis (117, 228). Limitations include the lack of reproducible results and the low predictive value compared to histologic examination of tissue. To perform a quantitative culture, a portion of the specimen is weighed and homogenized in saline. The saline suspension is used to prepare serial dilutions for culture. Detailed procedures for quantitative tissue culture are given elsewhere (230).

Bartonella

Bartonella henselae is the agent of cat scratch disease in healthy hosts and causes bacteremia, endocarditis, bacillary angiomatosis, and bacillary peliosis primarily in immunocompromised hosts. Optimal detection of the organism requires PCR (186). Culture is very difficult but can be attempted using Isolator tubes (Wampole Laboratories) and freshly prepared rabbit blood agar plates incubated in a moist environment for an extended period (19). Tissue culture may have a better yield than agar (see chapter 54). Lymph node tissue is macerated and inoculated into media directly. Because the detection of *B. henselae* is time-consuming and expensive, the diagnosis of cat scratch disease is most often made by clinical criteria and exclusion of other diseases. *B. henselae* can be observed in sections of fixed tissue stained with Warthin-Starry or Diederle's silver stain (94, 123).

Urinary Tract Specimens

Diseases of the urinary tract include prostatitis, urethral syndrome, cystitis, and pyelonephritis. Etiologies are summarized in Table 8. Urine, prostatic secretion, or urethral cells or secretion specimens are needed to diagnose these diseases. Urine can be collected by midstream collection, catheterization (straight/in-out or indwelling), cystoscopic collection, or suprapubic aspiration. Foley catheter tips should not be submitted or accepted for culture since they are always contaminated with urethral flora and quantitation is not possible. A first-voided morning urine is optimal, since in most cases bacteria have been sitting in the bladder multiplying for a number of hours. Clean-catch urine, implying cleansing of periurethral areas, has not been shown to improve the quality of urine culture and is not recommended (103, 154).

Urine specimens should be transported to the laboratory immediately and processed within 2 h of collection. If a delay occurs, specimens may be refrigerated for up to 24 h. Transport tubes containing boric acid are available to stabilize the bacterial population at room temperature for 24 h, if refrigeration is not available (99). Boric acid-preserved urines are acceptable for dipstick leukocyte esterase testing (229).

Urine culture is the most common test performed by most microbiology laboratories, and most urine cultures are negative; i.e., no specific potential pathogen is detected. Screening methods are available that attempt to rapidly separate those specimens containing significant counts of bacteria from negative specimens. In general, screening methods compare well with specimens containing 10^5 CFU of bacteria per ml or greater but perform poorly when colony counts are lower. Screening urine specimens by staining with the Gram stain is rapid and economical with regard to reagents but is labor-intensive and requires a trained technologist. The presence of 1 or 2 bacteria of similar morphotype, or more, in each oil immersion field (100× objective lens) correlates with a count of 100,000 or greater by culture (162, 226). Commercially available dipstick tests that detect leukocyte esterase (an enzyme produced by neutrophils) and nitrite (the result of bacterial nitrate reductase acting on nitrate in the urine) are rapid, inexpensive, and simple to perform, but their sensitivity is low in some patient populations (144, 153, 175). False-negative dipstick screening occurs because frequent voiding dilutes the concentration of leukocyte esterase and nitrite in urine, enterococci and other less common urinary tract pathogens do not produce nitrate reductase, and many patients with

asymptomatic bacteriuria do not have significant numbers of leukocytes in their urine. In spite of this, outpatient screening algorithms have been proposed that incorporate enzyme screening in a "reflexive" urine test; i.e., urinalysis is performed, and if positive for leukocyte esterase or nitrate reductase, a culture will be set up, and if negative, a culture will not be done (29). Such screening works best in symptomatic patients, diabetics, and women older than 60 years (144, 153, 175).

In addition to the Gram stain and dipstick tests for leukocyte esterase and nitrite, several urine screening systems have been marketed (151). Notable among these are the FiltraCheck-UTI and a semiautomated version called the Bac-T-Screen, the UTI screen Bacterial ATP Assay, and the Cellenium automated urine screening system (133, 141, 151, 152, 162, 188). Currently, all are no longer available or not actively marketed, and they are not discussed further here.

The standard for quantitative bacterial culture of urine is the inoculation of 0.01 or 0.001 ml of specimen using a calibrated plastic or wire loop to appropriate media, usually sheep blood and MacConkey agars. The loop is dipped vertically into the well-mixed urine, just far enough to cover the loop, and the loopful of urine is spread over the surface of the agar plate by streaking from top to bottom in a vertical line and again from top to bottom perpendicular to this line in a back-and-forth fashion. Prior to plate inoculation, it is necessary to ensure that a film of urine fills the loop with no bubbles to alter the calibrated volume. The inoculum of urine is spread over the entire agar surface to simplify counting of colonies after growth. Urine cultures are incubated at 35°C for 24 to 48 h. Although most urinary tract pathogens grow readily on usual agar media, slowly growing pathogens and those inhibited by the presence of antimicrobials in the patient specimen may not appear after overnight incubation (16 h). One approach uses the results of the leukocyte esterase and nitrite tests to determine which cultures get incubated for a full 48 h. Urine cultures that are negative after overnight incubation but had one or both positive enzyme tests are incubated for an additional day. Those that had negative enzyme tests are reported as "no growth" in the final report (26, 132).

Contamination of urine is detected in approximately 5 to 40% of cultures. Contamination is not reduced by the use of central processing areas, refrigeration, urine screening systems, specimen preservatives, or insulated specimen transport (207).

Agar paddles are available for urine culture in settings where inoculation and incubation of conventional agar plates are not convenient or possible (165). A standard film of urine is distributed over the agar-covered paddle, usually by dipping the paddle into a jar of urine. The paddle is then reinserted into its plastic container for incubation. Following incubation, the density of growth is estimated by comparison to photographs or drawings. A preliminary identification of gram positive or gram negative can be determined by colony color and morphology, and, when appropriate, the entire paddle can be forwarded to a reference laboratory for complete identification and antimicrobial testing of the isolate. Agar paddle culture of urine with approximate colony counts compares favorably with standard culture (165).

The urinary tract above the urethra is sterile in healthy humans, but the urethra is colonized normally with many different bacteria. Because of this, urine collected by midstream voiding techniques becomes contaminated during passage. Commensal bacteria are differentiated from potential pathogens by quantitative culture. Bacterial counts indicating "significant" bacteriuria (isolate is a likely pathogen) vary with the host and type of infection. Table 11 summarizes significant counts for common clinical situations (189).

TABLE 11 Interpretation of urine culture results[a]

Urine specimen and patient[b]	Likely to be significant[b]	Not likely to be significant[b]	Additional data suggesting that isolate is significant
Midstream, female with cystitis	$>10^2$ CFU of potential pathogen/ml, urine LE is positive	Quantity of potential pathogen ≤ quantity of contaminating flora.	
Midstream, female with pyelonephritis	$>10^5$ CFU of potential pathogen/ml, urine LE is positive	Quantity of potential pathogen ≤ quantity of contaminating flora.	Gram stain demonstrates potential pathogen in neutrophils and/or casts.
Midstream, asymptomatic bacteriuria	$>10^5$ CFU of potential pathogen/ml, LE is usually negative	$<10^5$ CFU of potential pathogen/ml; quantity of potential pathogen ≤ quantity of contaminating flora.	Confirm by repeating urine when clinically indicated.
Midstream, male with UTI	$>10^3$ CFU of potential pathogen/ml, urine LE is positive	$<10^3$ CFU of potential pathogen/ml; quantity of potential pathogen ≤ quantity of contaminating flora.	Gram stain demonstrates potential pathogen in neutrophils and/or casts.
Straight catheter, all patients	$>10^2$ CFU of potential pathogen/ml, urine LE positive for symptomatic patients	$<10^2$ CFU of potential pathogen/ml; urine LE is negative.	Gram stain demonstrates potential pathogen in neutrophils and/or casts.
Indwelling catheter, all patients	$>10^3$ CFU of potential pathogens/ml (multiple pathogens may be present)	Bacteriuria detected in asymptomatic patients; urine LE positive or negative.	No reason to culture unless patient is symptomatic.

[a] Reprinted from reference 195 with permission.
[b] LE, leukocyte esterase; UTI, urinary tract infection.

Severe urinary tract infection generally involves the kidneys (pyelonephritis) and results in bacteremia. Rapid diagnosis and administration of appropriate antimicrobial therapy are necessary. In this clinical setting, blood cultures are needed and a stat Gram stain of the urine can be useful. The Gram stain provides an immediate indication of the quality of the urine and a preliminary identification of the likely pathogen. Specimens containing high numbers of squamous epithelial cells are likely to be grossly contaminated with periurethral or vaginal flora and new specimens should be collected immediately, before antimicrobials inhibit growth of the true pathogen (Fig. 6) (195). Gram stain identification of a potential pathogen confirms that empirical therapy is correct or may suggest a change based on an unexpected pathogen, such as *S. aureus* (Fig. 7).

Special Considerations for Urinary Tract Specimens

Leptospires

Leptospira interrogans can be recovered from blood and CSF during the acute stages of disease and from urine after the first week of illness and for several months thereafter. Urine should be processed as soon as possible after collection, because the acidity of urine harms the organisms. If a delay in processing is expected, urine should be neutralized with sodium bicarbonate, centrifuged (1,500 × *g* for 30 min), and resuspended in buffered saline before being used to inoculate media (see chapter 61). Alternatively, the urine may be diluted 1:10 in 1% bovine serum albumin and stored at 5 to 20°C. Undiluted urine and urine diluted 1:10, 1:100, and 1:1,000 in sterile buffered saline should be inoculated to Ellinghausen-McCullough-Johnson-Harris or equivalent medium, with and without neomycin (39). Cultures should be incubated at 30°C for at least 13 weeks (Table 5).

Bacterial Antigen Testing

Bacterial antigen testing kits, for the purpose of diagnosing bacterial meningitis, include procedures for use with urine specimens. In general, these kits should not be used with urine specimens for the diagnosis of bacterial meningitis. In addition, the FDA issued a product alert specifically cautioning against the use of the group B streptococcus antigen kits with urine specimens because of the risk of both false-positive and false-negative results (55).

An EIA is commercially available for the detection of *L. pneumophila* serogroup 1 antigen in urine. The antigen may be detectable in urine for months following an infection. The assay has a sensitivity of 80% when performed on unconcentrated urine (70, 73). Sensitivity is increased when urine is concentrated. The drawback of this assay is that only disease caused by *L. pneumophila* serogroup 1 is detected. A new EIA has been evaluated that detects all *L. pneumophila* serogroup antigens in urine (40).

S. pneumoniae antigen can be detected in urine using a commercially available EIA (NOW *S. pneumoniae* urinary antigen test; Binax, Portland, Maine) (83, 131, 167). Although the sensitivity for detecting urine antigen is 80% for patients with positive blood cultures and 52% for patients with positive sputum cultures, the specificity is high. This test may prove to be useful in settings where culture is not available, but at present it should be used in addition to, not in place of, culture.

Wound and Abscess Specimens

Abscess specimens should be collected by aspiration. Small amounts of purulence or wounds with nothing to aspirate can be irrigated with sterile, nonbacteriostatic saline to facilitate aspiration. In addition, wounds can be sampled by dissecting a small portion of infected tissue. Purulent specimens and wound specimens characterized by ulceration or necrosis, but with little moisture, can be collected with a swab, but this is generally inferior to aspiration or biopsy. Swab specimens contain less material, are more likely to be contaminated with adjacent flora, and are not amenable to optimal anaerobic transport. When swabs are used for collection, two swabs should be used, one for culture and one for preparation of a smear. Deep lesions that communicate with the surface are most problematic. The cutaneous portion and sinus tract are contaminated with bacteria found at the surface. Surgical debridement and sampling are recommended. If surgery is not performed, effort should be made to aspirate a "pocket" of infected material that is not open to the surface. As a last option, fresh specimen should be expressed from deep within the wound. A swab used to collect specimen from the surface overlying the draining wound is not acceptable. This is of particular importance when evaluating diabetic foot ulcers or infected pressure sores (169). Only deep specimens collected by aspiration or during debridement offer useful culture information (17, 105).

Gram staining of wound and abscess specimens is very important. The Gram stain result provides rapid presumptive identification of etiology, it can be used to evaluate the quality of the specimen submitted, and it guides the workup of culture results. Examination of a Gram-stained smear reveals bacterial morphotypes, acute inflammatory cells (polymorphonuclear neutrophils), intracellular bacteria, cell necrosis, and elastin fibers resulting from tissue necrosis. The quality of the wound specimen can be evaluated by comparing the number of polymorphonuclear cells and squamous epithelial cells (Table 12) (178). Excess numbers of squamous epithelial cells suggest gross contamination with cutaneous flora. It is acceptable to limit workup of bacterial isolates when the specimen shows gross contamination. An example of a limited workup would be to list by Gram stain morphology the isolates encountered, with a comment explaining that the physician must call if a replacement specimen cannot be collected and further identification and antimicrobial testing are clinically warranted.

Special Circumstances for Wound and Abscess Specimens

Cellulitis

Specimens from patients with cellulitis but without abscess are very difficult to collect. Recommendations have been made to inject a small volume of sterile, nonbacteriostatic saline into the infected tissue. The few drops that one can aspirate back should be sent for Gram stain and culture. Under the best of conditions, these specimens are unlikely to be positive (65, 81). Blood cultures from patients with cellulitis also are unlikely to be positive (146). In spite of the shortcomings of microbiology testing for patients with cellulitis, seriously ill patients may require biopsy and all should have blood collected for culture.

Necrotizing Fasciitis and Gas Gangrene

Necrotizing fasciitis and gas gangrene (myonecrosis) are medical emergencies requiring immediate diagnosis and therapy that may include antimicrobials, surgical debridement, and the use of immune globulin and immune mediators to combat the fatal complications of severe septic shock (177, 193). Necrotizing fasciitis and gas gangrene are caused most commonly by toxin-producing *S. pyogenes,* other beta-hemolytic

TABLE 12 Screening wound specimens to ensure quality[a]

Quantity of cells per 10× (objective lens) microscopic field					
No. of neutrophils	Q-value for neutrophils	Q-value for squamous epithelial cells present in the following no.[b]:			
		0	1–9	10–24	≥25
		0	−1	−2	−3
0	0	(1)	0	0	0
1–9	+1	1	0	−1	−2
10–24	+2	+2	+1	0	−1
≥25	+3	+3	+2	+1	0

[a] Attach Q-values to squamous cell and neutrophil quantities. Add the two Q-values together. Specimens with positive Q-values (+1 to +3) are more likely to contain increased numbers of potential pathogens and decreased numbers of potential contaminants. Specimens with negative Q-values (or a Q-value of zero) are likely to be contaminated with local flora. Specimens with no squamous cells or neutrophils are scored as one (1), allowing samples from neutropenic patients or those with necrotic or serous secretions to be processed as acceptable.

[b] The first row of numbers in the table is the Q-value for squamous epithelial cells only. The following four rows are the Q-values for the sum of the neutrophils and squamous epithelial cells.

streptococci, *S. aureus*, *Clostridium* spp., and mixed aerobic and anaerobic bacteria (46). The diagnosis is made by clinical examination of the patient and is confirmed by Gram stain and culture. Gram-stained smears generally show proteinaceous fluid, necrotic cell debris, rare or few polymorphonuclear leukocytes (because of cell lysis), and the bacterial etiology. Culture should confirm the etiology and provide antimicrobial testing results where appropriate.

Anaerobic Abscess

Anaerobes characteristically produce purulent infections in areas adjacent to mucous membranes containing anaerobes from the normal flora. Specimens must be transported to the laboratory in sterile, oxygen-free containers (148). As reviewed above, aspirated fluid or excised tissue is the recommended specimen. Specimens should be stored at room temperature (not refrigerated) during transport and processing delays. Infections of the mouth and gums (and adjacent areas), aspiration pneumonia, empyema, intra-abdominal infections, deep tissue abscesses, infections of the female genital tract, infected pressure sores, and diabetic foot ulcers are caused generally by a mixture of aerobes and anaerobes. Because of the usual microscopic appearance of mixed aerobic-anaerobic abscesses, the Gram stain can rapidly identify these infectious processes (Fig. 8). The presence of many polymorphonuclear leukocytes, many bacteria with anaerobic morphotypes (thin gram-negative bacilli, thin and poorly staining gram-positive bacilli, and boxcar-shaped gram-positive bacilli suggesting *Clostridium* spp.), and many intracellular bacteria suggests the presence of a mixed aerobic-anaerobic infectious process. Culture can determine the exact etiologies, characteristically a mixture of aerobes and anaerobes. However, the identification of most anaerobic bacteria and their susceptibility results are not necessary for the management of mixed infections. Aerobic and facultative bacteria present need full identification and antimicrobial testing results for proper therapeutic selection.

Specimens for anaerobic culture should be processed as soon as possible after arrival in the laboratory. Usual media include an anaerobic blood agar plate (CDC blood agar or brucella blood agar), a medium that inhibits gram-positive and facultative gram-negative bacilli such as blood agar with kanamycin and vancomycin, a differential or selective medium such as *Bacteroides* bile-esculin, and a gram-positive selective medium such as colistin-nalidixic acid blood agar or phenylethyl alcohol blood agar (Table 5). Media should be incubated in an anaerobic environment immediately after inoculation. Incubation in anaerobic containers, such as GasPak jars (Becton Dickinson Microbiology Systems, Cockeysville, Md.), AnaeroPack (Mitsubishi Gas Chemical America, Inc., New York, N.Y.), or Bio-Bag Anaerobic Culture Set (Becton Dickinson Microbiology Systems), or in an anaerobic chamber is acceptable (36, 37). Anaerobes grow more slowly than aerobic or facultative bacteria, necessitating a full 48 h of incubation before colony size is large enough to interpret accurately. Negative anaerobic cultures should be held for 3 to 5 days before being reported as negative. Longer incubation is necessary for isolation of *Actinomyces* and some other fastidious anaerobes.

Autopsy Specimens

Microbiology testing as a component of the autopsy examination has been and continues to be controversial (114, 174, 225). Postmortem and agonal invasion of sterile tissues confuses the significance of positive culture results, prompting some to argue against microbiology testing. Others have found that postmortem examination continues to uncover a significant number of infectious diagnoses, whether in the community or university hospital setting, which were missed by modern high-technology medicine (97). In addition, an important portion of missed diagnoses represents treatable diseases (136). The value of autopsy microbiology is further enhanced by its use to identify emerging diseases, etiologies of biological warfare, community outbreaks, nosocomial infections, and antimicrobial resistance and to uncover the cause of death in organ transplant patients and others with immunocompromising conditions. Safety precautions designed to protect the pathologist and dissection assistants during autopsy procedures have been thoroughly reviewed (139).

To minimize contamination of postmortem specimens, the body should be moved to a refrigerated locker (4 to 6°C) as soon as possible after death. Limited movement of the body has been shown to decrease the incidence of false-positive postmortem cultures (43). Although it has been shown that cultures collected within 48 h of death from a refrigerated cadaver did not show an increase in false-positive results, tissue and fluid specimens, as a rule, should be taken

from refrigerated bodies within 15 h of death (93). This serves to diminish the likelihood of postmortem overgrowth of contaminants and improve detection of true pathogens.

Specimens should be obtained by sterilizing the surface of the organ with a hot spatula or iron surface until the surface is thoroughly dry (43). Body fluids, including blood, should be collected first. For blood collection, the wall of the heart and large vessel should be seared and a sterile needle (18 to 20 gauge) should be inserted. A 20-ml volume, or as close to 20 ml as possible, should be collected and injected directly into aerobic and anaerobic blood culture bottles. Blood culture results obtained before opening the chest cavity by percutaneous subxyphoid aspiration have been shown to have greater interpretive value (less contamination but detection of relevant organisms). Most conclude that postmortem blood cultures rarely provide information that is not already known. Solid viscera should be sampled by immediately cutting blocks of tissue from the center of the seared area. Samples should be submitted to microbiology with a requisition providing a full explanation of the studies needed. Postmortem cultures can be very useful for detecting pathogens that are not considered normal human flora, such as *M. tuberculosis*, *Brucella* spp., *B. pertussis*, some systemic fungi (*Histoplasma capsulatum*, *Coccidiodes immitis*, etc.), parasitic helminths, and agents of biological warfare. Tissue samples should be transported to the microbiology laboratory immediately in sterile tubes. The use of transport media and laboratory processing methods should follow recommendations for premortem specimens. An efficient way to avoid unnecessary workup of contaminating microorganisms is to issue a preliminary report to the pathologist who performed the autopsy listing organisms detected by colony or Gram stain morphology, such as "lactose-fermenting gram-negative rod" or "gram-positive cocci in clusters." This is accompanied with a notation that further identification and antimicrobial testing will not be performed unless there is consultation with the laboratory director or technologist conducting the culture investigation. Plates can be held for 1 week and discarded if no additional information is requested.

Specimens for the Detection of Agents of Biological Warfare

An excellent review of the potential agents of biological warfare and their management in the clinical microbiology laboratory can be found in Cumitech 33 and chapter 9 of this Manual (61, 92).

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Reagents, Stains, and Media: Bacteriology

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21

REAGENTS

The reagents listed in this chapter include those in common use and a few highly specialized ones. For information on specific reagents not included here, refer to literature cited in the chapter in which the reagent is mentioned or the general references listed at the end of this chapter (11, 49). Reagents are listed in alphabetical order, with brief descriptions of their intended uses and ingredients. The test protocol is included where appropriate. A fresh 18- to 24-h pure broth culture or well-isolated colonies from nonselective medium are most often appropriate to use for testing. Many of these reagents and tests are available commercially either individually or incorporated into commercial identification systems. The reader should be aware that commercial preparations in which two reagents are already combined may not contain the traditional percentage of each reagent and may require adjustment of laboratory protocols. Mycobacterial mucolytic and decontamination preparations from a number of manufacturers are an example. Unless stated otherwise, the reagents listed in this section should be prepared by dissolving the reagent components in the stated liquid with a magnetic stirring bar. The standard sterilization technique of autoclaving at 121°C at 15 lb/in^2 for 15 min followed by a slow exhaust cycle should be used when needed. However, certain solutions, such as those containing antibiotics or carbohydrates, cannot be autoclaved because the supplements will be denatured. These solutions are sterilized by filtration through a 0.22-μm-pore-size filter. Additionally, certain reagents require different heat sterilization times. Instructions for reagents that require special preparation or sterilization protocols are included in the discussion of the reagent.

It is critical that distilled, deionized water be used in the preparation of all components. Removal of contaminating pyrogens and minerals from water used for culture reagents is imperative, especially for the success of cell culture systems.

Storage of prepared reagents in sterile, airtight, screw-cap containers is recommended. Some reagents require storage in dark containers, and some need to be stored refrigerated (2 to 8°C) instead of at room temperature. Special storage instructions are given when appropriate. Standard safety precautions should be taken when preparing the reagents. Follow the safety guidelines for the chemicals being used, in addition to the laboratory safety protocols. For reagents that are prepared in-house, proper quality control measures must be taken with appropriate positive and negative controls.

■ Acetoin (acetyl-methyl-carbinol)

See Voges-Proskauer (VP) test.

■ *N*-Acetyl-L-cysteine-sodium hydroxide (NALC-NaOH)

NALC is a mucolytic agent used for digestion, and NaOH is a decontamination agent used in the processing of specimens for mycobacteriology. Sodium citrate is included in the mixture to exert a stabilizing effect on the acetylcysteine.

4% NaOH, sterile	50 ml
2.9% Sodium citrate, sterile	50 ml
NALC powder	0.5 g

Mix well in a sterile container. Use within 24 h of preparation.

■ L-Alanine-7-amido-4-methycoumarin (Gram-Sure; Remel)

Gram-Sure is a reagent-impregnated disk with the fluorogenic compound L-alanine-7-amido-4-methycoumarin. This is a rapid disk test that is used as an adjunct to the Gram stain to distinguish between gram-negative and gram-positive aerobic rods or coccobacilli and is used most commonly with gram-positive organisms that may appear gram variable or gram negative, such as *Bacillus* or *Lactobacillus*. The mechanism of the test is dependent on the presence of aminopeptidase in the cell walls of gram-negative organisms, which will hydrolyze the reagent L-alanine-7-amido-4-methycoumarin in the disk from a nonfluorescent substrate to a blue fluorescent compound. A pure colony growth is inoculated into demineralized water and then inoculated onto the disk. The disk is incubated at room temperature for 5 to 10 min and then observed under long-wave UV light for blue fluorescence. Blue fluorescence is gram negative, and the absence of blue fluorescence is gram positive. Obligate anaerobes may fail to give expected results (50).

■ Bile solubility (10% sodium deoxycholate)

The bile solubility test is used as a presumptive identification test for *Streptococcus pneumoniae*. Sodium deoxycholate is a surface-active bile salt. It acts upon the cell wall of pneumococci, resulting in cell lysis. The test is performed

with alpha-hemolytic streptococcal colonies. Oxgall is a dehydrated bile that can be used, but sodium deoxycholate is preferred.

Sodium deoxycholate	1.0 g
Sterile distilled water	9.0 ml

The pH should be 7.0. Store refrigerated in a sterile dark bottle.

Tube method
Prepare a heavy suspension of the organism in 2 ml of buffered broth (pH 7.4) or physiological saline (pH 7.0). The pH of the solution should not be below 6.8. Divide the organism suspension into two tubes. To one tube add a few drops of the 10% sodium deoxycholate solution. To the other tube add the same amount of sterile physiological saline. Incubate at 35°C. If the organism is bile soluble, the tube containing the bile salt will lose its turbidity in 5 to 15 min and show an increase in viscosity concomitant with clearing.

Agar colony test
Put a couple of drops of sodium deoxycholate on the suspected colonies. Incubate the plate right side up for 30 min at 35°C. Pneumococcal colonies will be lysed, but viridans group streptococci will not.

■ Bovine albumin fraction V, 0.2%

The 0.2% bovine albumin solution is used to buffer specimens for mycobacterial culture following decontamination with NALC-NaOH.

Bovine albumin solution, 5%	40.0 ml
Sodium chloride	8.5 g
Distilled water	960.0 ml

Adjust to pH 6.8 ± 0.2 with 4% NaOH. Sterilize by filtration. Aliquot into sterile screw-cap tubes. Store refrigerated.

Following decontamination and concentration by centrifugation, the sedimented specimen is resuspended in 1 to 2 ml of sterile 0.2% bovine albumin fraction V. This suspension is then used to inoculate media and prepare microscopic smears.

■ Catalase

Hydrogen peroxide (H_2O_2) is used to determine if bacteria produce the enzyme catalase. H_2O_2 (3%) is commercially available. Superoxol is a 30% H_2O_2 that is used for identification of *Neisseria* spp. (see chapter 39), and a 15% concentration is often used for differentiation and identification of anaerobes.

Slide method
Transfer a test colony to a clean glass slide and add 1 drop of 3% H_2O_2. Development of bubbles is considered a positive result. Extreme care should be taken to avoid picking up any media from a blood-containing agar plate because catalase is present in erythrocytes and any carryover of blood cells can cause a false-positive reaction. Hydrogen peroxide solution for detection of catalase in anaerobes is typically 15%.

Tube method
Add 1.0 ml of 3% H_2O_2 to an overnight pure culture slant. (Do not use blood agar medium.) Observe for immediate bubbling.

■ Cetylpyridinium chloride-sodium chloride (CPC-NaCl)

CPC-NaCl is used for decontamination of transported sputum specimens for mycobacteriology culture.

CPC	1 g
Sodium chloride	2 g
Distilled water	100 ml

Mix and store in a sealed brown bottle at room temperature. If crystals form, the solution should be gently heated before use.

An equal amount of sputum and CPC-NaCl is mixed until the specimen is liquefied, and then the specimen can be shipped to the testing site. Specimens treated with CPC-NaCl must be cultured on egg-based media or else residual CPC will inhibit mycobacterial growth.

■ Coagulase

The coagulase test is used to detect free coagulase or bound coagulase (clumping factor) and differentiate coagulase-producing *Staphylococcus* from other *Staphylococcus* spp. Dehydrated rabbit plasma reagent with EDTA is commercially available. Rehydrate and perform the test according to the manufacturer's directions. While human plasma is preferred for detection of clumping factor with *Staphylococcus lugdunensis* and *Staphylococcus schleiferi*, it is not recommended for routine testing because it may contain antibodies against staphylococci.

Slide test
The slide test detects bound coagulase (clumping factor). Emulsify a heavy suspension of staphylococci in a small drop of water on a clean glass slide. If autoagglutination occurs, do not continue; instead, perform a tube test. Add 1 small drop of rabbit plasma reagent to the suspension. Mix with a continuous circular motion while observing for the formation of visible white clumps. Known positive and negative controls should be set up in parallel. Negative or delayed positive (20 to 60 s) results should be confirmed by the tube test.

Tube coagulase test
The tube coagulase test detects bound and free coagulase. Dispense 0.5 ml of rabbit plasma into a sterile tube. Inoculate a loopful of the test organism into the tube. Incubate the tube at 35°C for 4 h. Observe for clotting at intervals during the first 4 h because some staphylococci produce fibrolysin, which could lyse the clot. Do not shake or agitate the tube while checking for clotting. The formation of a clot is considered positive. The majority of coagulase-positive *Staphylococcus aureus* isolates will form a clot within 4 h. Incubate the tube at room temperature overnight if no visible clot is observed after 4 h. However, a clot may have formed and subsequently dissolved over the 24 h, so overnight incubation is not always specific. Some investigators have recommended incubation at 35°C.

■ Dyes and pH indicators

A variety of dyes and pH indicators are used in media and reagents. The most common are given in Table 1.

■ Efrotomycin

Efrotomycin is used to separate *Enterococcus casseliflavus* and *Enterococcus gallinarum* (resistant) from *Enterococcus faecium* (susceptible). Dissolve 100 mg of efrotomycin (Merck

TABLE 1 Dyes and pH indicators

Indicator	pH and color
Acid fuchsin (Andrade's)	5.0, pink
	8.0, pale yellow
Bromcresol green	3.8, yellow
	5.4, blue
Bromcresol purple	5.2, yellow
	6.8, purple
Bromphenol blue	3.0, yellow
	4.6, blue
Bromthymol blue	6.0, yellow
	7.6, dark blue
Chlorcresol green	4.0, yellow
	5.6, blue
Chlorphenol red	5.0, yellow
	6.6, red
Cresolphthalein	8.2, colorless
	9.8, red
m-Cresol purple	7.4, yellow
	9.0, purple
Cresol red	7.2, yellow
	8.8, red
Methyl red	4.4, red
	6.2, yellow
Neutral red	6.8, red
	8.0, yellow
Phenolphthalein	8.3, colorless
	10.0, red
Phenol red	6.8, yellow
	8.4, red
Resazurin	Oxidized: blue, nonfluorescent
	Reduced: red, fluorescent
Thymol blue	8.0, yellow
	9.6, blue
Triphenyl-tetrazolium chloride	Oxidized: colorless
	Reduced: red

Sharpe & Dohme) in 0.1 ml of dimethyl sulfoxide and dilute in 9.9 ml of sterile distilled water. Dispense 10 μl of this solution onto filter paper disks and dry in the dark at room temperature for 5 to 6 h. A heavy inoculum of bacteria is spread with a loop or swab over half of a Trypticase soy blood agar plate, the efrotomycin disk is then placed on the heavy inoculum, and the plate is incubated for 18 to 24 h at 35°C. Organisms with any growth inhibition are considered efrotomycin susceptible. The availability of this antibiotic may be limited. An alternative test is the 1-*O*-methyl-α-D-glucopyranoside test (see below).

■ Ehrlich reagent

See Indole test.

■ Ferric ammonium citrate, 1%

Hydrolysis of esculin to esculetin is detected when the product reacts with ferric ammonium citrate to form a brown or black complex.

Dissolve 1.0 g of ferric ammonium citrate in 100 ml of distilled water. Store in a dark bottle, refrigerated, for up to 1 year.

After esculin broth is inoculated with the test organism and incubated for 1 to 2 days, a few drops of ferric ammonium citrate are added. A brown-black color develops immediately in positive tests. This test can also be performed by incorporating an iron salt into esculin agar medium.

■ Ferric chloride reagent

Ferric chloride reagent is used in both the phenylalanine deaminase test and the sodium hippurate hydrolysis test.

Ferric chloride ($FeCl_3 \cdot 6H_2O$)	12 g
Hydrochloric acid, 2%	100 ml

Hydrochloric acid (2%) is prepared by adding 5.4 ml of concentrated hydrochloric acid (37%) to 94.6 ml of distilled water.

Test procedure. Adding 4 or 5 drops of ferric chloride reagent onto overnight growth on phenylalanine agar or broth performs the phenylalanine deaminase test. If phenylpyruvic acid has formed, a brown color develops in the medium (positive reaction). Ferric chloride reagent can also be added to inoculated broths (e.g., heart infusion broth or Todd-Hewitt broth) supplemented with hippurate. Hydrolysis of hippurate produces benzoic acid and glycine. An insoluble brown ferric benzoate precipitate will form in a positive hydrolysis reaction.

■ Fildes enrichment

Fildes enrichment is a source of growth factors used to supplement media for the isolation of fastidious organisms. Commercial preparations are available and include the following ingredients.

Pepsin	4.0 g
Sodium chloride	5.4 g
Sodium hydroxide	70.0 ml
Hydrochloride acid, concentrated	24.0 ml
Sheep blood	200 ml
Deionized water	600 ml

The pH should be 7.0 ± 0.2. Media should be supplemented to a final concentration of 5.0%.

■ Formate-fumarate

Supplementation of media with formate and fumarate has been used to characterize selected anaerobes (e.g., *Bacteroides ureolyticus*).

Sodium formate	3.0 g
Fumaric acid	3.0 g
Distilled water	50.0 ml

To adjust the pH, add 20 pellets of NaOH, stirring until the pellets are dissolved and the fumaric acid is in solution. Bring the final pH to 7.0 with 4 N NaOH. Sterilize by filtration. Store refrigerated for up to 6 months. Add 0.5 ml of this solution to 10 ml of thioglycolate broth. Anaerobic growth in supplemented broth is then compared with the growth in unsupplemented broth.

■ β-Galactosidase

See *o*-Nitrophenyl-β-D-galactopyranoside (ONPG).

■ β-Glucuronidase (see also β-Methylumbelliferyl-β-D-glucuronidase [MUG] test)

Detection of β-glucuronidase activity is useful for the rapid identification of *Escherichia coli,* members of the *Streptococcus*

anginosus group, and other bacteria. A solution of 0.1% (wt/vol) *p*-nitrophenyl-β-D-glucopyranoside (colorimetric substrate) in 0.067 M Sorensen phosphate buffer (pH 8.0) is prepared. Tubes containing 0.5 ml of the substrate solution are inoculated with a loopful of bacteria from an overnight culture. The tubes are incubated at 35°C and examined after 4 h for the appearance of a yellow color (liberated *p*-nitrophenol). The fluorometric substrate 4-methylumbelliferyl-β-D-glucuronide is commercially available and yields a fluorescent product when hydrolyzed by β-glucuronidase.

■ Glycine-buffered saline

Glycine-buffered saline (0.043 M glycine, 0.15 M NaCl [pH 9.0]) is used in some serological procedures and is also used as a transport medium for enteric organisms.

Glycine	3.23 g
NaCl	8.77 g
Distilled water	1,000 ml

■ Hemin solution, 5-mg/ml stock

Hemin solution is one of the additives in thioglycolate and *Brucella* base medium that makes them enriched for fastidious organisms. Dissolve 0.5 g of hemin in 10 ml of 1 N NaOH. Bring the volume up to 100 ml with distilled water (final concentration of stock solution, 5 mg/ml). Sterilize by autoclaving. Store refrigerated for up to 1 month. It is used at a final concentration of 5μg/ml of medium.

■ Hippurate test

The hippurate test measures the hydrolysis of sodium hippurate. Hippurate is hydrolyzed to benzoic acid and glycine by the enzyme hippurate hydrolase (hippuricase), which is produced by some bacteria, including group B streptococci (GBS), some *Listeria* spp., *Gardnerella vaginalis, Campylobacter jejuni,* and *Legionella pneumophila*. The procedure described here detects the presence of glycine with the ninhydrin reagent. Ferric chloride reagents can also be used (see above).

Test procedure. A 1% (wt/vol) solution of sodium hippurate is prepared in 0.067 M Sorensen phosphate buffer (pH 6.4). Tubes containing 0.5 ml of this solution are inoculated and incubated at 35°C for 2 h, after which 0.2 ml of the ninhydrin reagent is added. Development of a deep blue-purple color within 5 min is a positive reaction.

For *L. pneumophila,* inoculate a 0.5-ml aliquot of 1% sodium hippurate solution with a loopful of organism and incubate at 35°C in ambient air for 18 to 20 h. Add 0.2 ml of ninhydrin reagent, mix well, and incubate for an additional 10 min at 35°C. Observe for 20 min for blue-purple color development.

Ninhydrin reagent, 3.5%

Ninhydrin	3.5 g
Acetone	50 ml
1-Butanol	50 ml

Mix acetone and butanol in a sterile dark container. Add ninhydrin, mix, and store at room temperature.

■ Indole test

The indole test is used for the determination of the organism's ability to produce indole from deamination of tryptophan by tryptophanase. Both the Ehrlich and Kovàcs reagents should be stored refrigerated away from light. For the Ehrlich and Kovàcs reagents, dissolve the aldehyde in alcohol and then slowly add acid to the mixture.

Ehrlich reagent

Ethyl alcohol, 95%	95 ml
p-Dimethylaminobenzaldehyde	1 g
Hydrochloric acid, concentrated	20 ml

Test procedure. Indole is first extracted with xylene. Add 1 ml of xylene to a 48-h tryptone broth or other tryptophan-containing broth medium. Shake the tube vigorously for 20 s and let stand for 1 to 2 min to allow the xylene extract to come to the top of the broth. Gently add 0.5 ml of the Ehrlich reagent down the side of the tube. Do not shake the tube. A red ring at the interface of the medium and the reagent phase within 5 min represents a positive test. Ehrlich's reagent is preferred for organisms that produce small amounts of indole such as nonfermenters and anaerobes.

Kovàcs indole reagent

Pure amyl or isoamyl alcohol	150 ml
p-Dimethylaminobenzaldehyde	10 g
Hydrochloric acid, concentrated	50 ml

Test procedure. Add 5 drops of Kovàcs reagent to either 48-h-old 2% tryptone broth or an 18- to 24-h-old tryptophan broth culture. Do not shake the tube after the addition of reagent. A red color at the surface of the medium is a positive test.

Spot indole test

p-Dimethylaminocinnamaldehyde (DMACA)	200 mg
Hydrochloric acid, concentrated	2 ml
Distilled water	18 ml

Add the acid to the water, and let it cool before adding DMACA.

Test procedure. Moisten a piece of Whatman no. 3 paper with a couple drops of the reagent. Remove a well-isolated colony from an 18- to 24-h-old culture onto a blood agar plate with a sterile inoculating loop or a wooden stick and smear it onto the moistened filter paper. Observe for a blue to blue-green color within 2 min, which is a positive reaction. No color change or a pinkish tinge is considered negative. This test should be used only on colonies from media containing sufficient tryptophan and no glucose. Colonies from media containing dyes (e.g., MacConkey or eosin-methylene blue [EMB] agar) may cause misleading results and should not be used. Colonies from mixed cultures should not be used, as indole-positive colonies can cause indole-negative colonies to appear weakly positive.

■ LAP (leucine aminopeptidase or leucine arylamidase) test

The LAP test detects the presence of leucine aminopeptidase (LAP). The substrate leucine-α-naphthylamide is hydrolyzed by LAP to leucine and free α-naphthylamine. a-Naphthylamine reacts with DMACA to form a red color. The LAP test, along with pyrrolidonyl-α-naphthylamide (PYR) hydrolysis, is helpful in the presumptive characterization of catalase-negative, gram-positive cocci (streptococci, enterococci, and streptococcus-like organisms). Some commercial identification kits include an assay for this enzyme, and commercial rapid disk tests are also available.

■ Lysozyme solution

Lysozyme	50 mg
Hydrochloric acid, 0.01 N	50 ml

Mix and sterilize by filtration. Store refrigerated. It may be stored for only 1 week.

Test procedure. Add 5 ml of lysozyme solution to 95 ml of basal glycerol broth, dispense in 5-ml aliquots, and keep refrigerated. Growth of the test organism in the lysozyme-supplemented glycerol broth is compared with the growth in the unsupplemented glycerol broth.

■ Lysozyme test

The lysozyme test measures the ability of organisms, such as *Nocardia*, to grow in the presence of lysozyme.

Basal glycerol broth

Peptone	1.0 g
Beef extract	0.6 g
Glycerol	14.0 ml
Distilled water	200 ml

Mix well and autoclave to sterilize. Store refrigerated. It may be stored for up to 3 months.

■ McFarland standard

For different McFarland standards, mix the designated amounts of 1% anhydrous barium chloride ($BaCl_2$) and 1% (vol/vol) cold pure sulfuric acid (H_2SO_4) as shown in Table 2 in screw-cap tubes. Tightly seal the tubes. When the barium sulfate is shaken up well, the density in each tube corresponds approximately to the bacterial suspension listed in Table 2. Store the prepared standard tubes in the dark at room temperature. The absorbance of the 0.5 McFarland standard should be 0.08 to 0.10 at 625 nm using a spectrophotometer with a 1-cm light path. The standard should be checked regularly to make sure the density is still accurate.

■ 1-*o*-Methyl-α-D-glucopyranoside (MGP) (α-methyl-D-glucoside)

The MGP test is used to separate *E. casseliflavus* and *E. gallinarum* (positive) from *Enterococcus faecalis* and *E. faecium* (negative). Heart infusion broth is prepared with 1% MGP and 0.006% bromcresol purple indicator, distributed into 2-ml aliquots, and autoclaved for 10 min. The broth is inoculated with a drop of an overnight broth culture or several colonies from a blood agar plate and incubated for 1 day at 35°C. Prolonged incubation (for up to 7 days) may be necessary. Development of a yellow color indicates a positive reaction.

TABLE 2 McFarland standards protocol

Standard	Vol (ml)		Corresponding bacterial suspension (10^8 CFU/ml)
	1% $BaCl_2$	1% H_2SO_4	
0.5	0.05	9.95	1.5
1	0.1	9.9	3
2	0.2	9.8	6
3	0.3	9.7	9
4	0.4	9.6	12
5	0.5	9.5	15
6	0.6	9.4	18
7	0.7	9.3	21
8	0.8	9.2	24
9	0.9	9.1	27
10	1.0	9.0	30

■ Methylumbelliferyl-β-D-glucuronidase (MUG) test (see also β-Glucuronidase)

The MUG test is the fluorogenic assay for β-glucuronidase. The enzyme hydrolyzes the substrate 4-methylumbelliferyl-β-D-glucuronide to yield 4-methylumbelliferyl, which fluoresces blue under long-wave UV light. The test is normally used for the presumptive identification of *E. coli* and more recently for streptococcal strains. The colorimetric test method is described under β-Glucuronidase.

Dissolve 50 mg of 4-methylumbelliferyl-β-D-glucuronide in 10 ml of 0.05 M Sorensen phosphate buffer, pH 7.5. Dilute 1:16 of the stock 4-methylumbelliferyl-β-D-glucuronide and add 1.25 ml to a vial containing 50 sterile paper disks. Allow the disks to be thoroughly saturated until no liquid remains in the vial. Spread the saturated disks out and allow to dry completely. The disks can be stored in a dark bottle at −20°C for 1 year or at 4°C for 1 month.

Test procedure. Wet disk with 1 drop of sterile water. Apply the organism to the disk using a wooden stick or loop and then incubate the disk for up to 2 h at 35°C. Shine a long-wave UV light on the disk. A positive reaction is indicated by blue fluorescence. A negative reaction is indicated by the lack of fluorescence.

■ Middlebrook enrichment (oleic acid-albumin-dextrose-catalase [OADC] and albumin-dextrose-catalase [ADC])

The Middlebrook enrichment is added to various Middlebrook media. The OADC and enrichment contain oleic acid as a carbon source, and both supplements contain dextrose as a carbon source and bovine albumin fraction V and catalase as growth factors. WR 1339 Triton encourages cording in *Mycobacterium tuberculosis*. All the enrichments described below are prepared in 100 ml and added to 900 ml of preautoclaved medium that has been cooled to 50 to 55°C.

OADC enrichment

Bovine albumin fraction V	5.0 g
Dextrose	2.0 g
Sodium chloride	0.85 g
Oleic acid	0.05 g
Catalase	4.0 mg (0.004 g)

Add all components to distilled and deionized water and bring the volume to 100 ml. Mix thoroughly and filter sterilize.

OADC enrichment with WR 1339

Add 0.25 g of WR 1339 Triton to the above OADC ingredients. Prepare as described above.

ADC enrichment

Bovine albumin fraction V	5.0 g
Dextrose	2.0 g
Catalase	4.0 mg (0.004 g)

Add all components to distilled and deionized water and bring the volume to 100 ml. Mix thoroughly and filter sterilize.

■ Modified oxidase

The test is used for differentiation of *Micrococcus* and related organisms from most other aerobic gram-positive cocci. Six percent tetramethyl-*p*-phenylenediamine dihydrochloride (the same chemical used in Kovàcs oxidase reagent) dissolved in dimethyl sulfoxide is used as the reagent. Keep the reagent away from light. A loopful of colonies from blood agar plates is smeared onto filter paper, and the reagent is dropped onto the bacterial growth. Development of a blue to purple-blue color in 2 min indicates a positive reaction. Commercially prepared disks are available (26).

■ Nessler reagent

The Nessler reagent is used in the determination of acetamide hydrolysis by some gram-negative bacteria.

Nessler reagent

Solution A
Dissolve 1 g of mercuric chloride in 6 ml of distilled water. Add 2 or 3 drops of concentrated hydrochloric acid (HCl) to dissolve the sediment.

Solution B
Dissolve 2.5 g of potassium iodide in 6 ml of distilled water completely. Add to solution A.

Solution C
Dissolve 6 g of potassium hydroxide in 6 ml of distilled water completely. Add to the mixture of solutions A and B. Add 13 ml of distilled water. Mix well. Filter using a sintered-glass funnel before use and store in a dark bottle. Note: do not use a Nalgene filter. The Nessler reagent solution may decompose at room temperature after several weeks and should therefore be checked with each use.

Test procedure. Inoculate 1 ml of mineral-based broth medium (carbon assimilation medium) supplemented with 0.1% acetamide. After incubation for 24 h at 30°C, 1 drop of Nessler reagent is added. A positive reaction is indicated by a red-brown sediment due to the presence of ammonia from the action of acylamidase. Acetamide agar is available commercially.

■ Nitrate reduction

The nitrate reduction test is used to determine the ability of an organism to reduce nitrate to nitrite or free nitrogen gas.

Reagent A

N,*N*-Dimethyl-naphthylamine	0.6 ml
Acetic acid (5 N), 30%	100 ml

Reagent B

Sulfanilic acid	0.8 g
Acetic acid (5 N), 30%	100 ml

Store each reagent in a brown glass bottle in the refrigerator. Store away from light.

Test procedure. At the time of testing, mix equal portions of the reagents and then add 10 drops to the overnight growth from the nitrate broth culture. A positive reaction is indicated by the development of a red color within 1 to 2 min, which means that nitrate has been reduced to nitrite. Negative reactions are confirmed by adding a pinch (approximately 20 mg) of zinc dust with development of red color within 5 to 10 min, which indicates that nitrate has not been reduced by the organism. If the tube remains clear, nitrate has been reduced to free nitrogen gas, and a clear tube is considered a positive reaction.

■ *o*-Nitrophenyl-β-D-galactopyranoside (ONPG)

The ONPG test is used to determine the ability of an organism to ferment lactose. It is especially useful for identification of members of the family *Enterobacteriaceae*. ONPG-impregnated tablets can be purchased commercially. Commercially prepared reagents are recommended because it is tedious and difficult to prepare the reagent in-house.

■ Oxalic acid, 5%

Oxalic acid is used as a decontamination agent for specimens that contain *Pseudomonas* spp. when culturing for mycobacteria. The reagent is especially helpful when processing respiratory specimens from cystic fibrosis patients.

Oxalic acid	50 g
Distilled water	1,000 ml

Autoclave to sterilize and store at room temperature. The solution has an expiration date of 1 year.

■ Oxidase test

The oxidase test detects the presence of a cytochrome oxidase system. Production of a dark blue-purple color on either a filter paper strip or disk indicates a positive test. A number of reagents can be used for this test.

Kovàcs oxidase reagent:
1% tetramethyl-*p*-phenylenediamine dihydrochloride (in water)
Gordon and McLeod's reagent:
1% dimethyl-*p*-phenylenediamine dihydrochloride (in water)
Gaby and Hadley (indolphenol oxidase) reagents:
1% α-naphthol in 95% ethanol
1% *p*-aminodimethylaniline HCl

Kovàcs reagent is less toxic and more sensitive than the other reagents. Add a few drops of the reagent to a strip of filter paper (Whatman no. 2 or equivalent) and then smear a loopful of the organism on the paper using a platinum loop or wooden stick. A wire loop containing iron may give a false-positive reaction. The oxidase test should not be performed on colonies growing on medium containing a high concentration of glucose because the fermentation of glucose may inhibit oxidase activity. Only colonies from nonselective, nondifferential media should be used to detect oxidase. A positive reaction with the Kovàcs reagent develops within 10 to 15 s and is characterized by a dark purple-black color. If the Kovàcs solution becomes blue due to autoxidation, it should be discarded. The dimethyl compound in Gordon and McLeod's reagent is more stable than the tetramethyl compound (Kovàcs reagent). A positive reaction is characterized by a blue color and develops within 10 to 30 min.

■ Oxidase, modified.

See Modified oxidase test.

■ Phosphate-buffered saline (PBS)

10× stock solutions

1. 0.1 M NaH_2PO_4 (sodium phosphate, monobasic). Dissolve 13.9 g of NaH_2PO_4 in 1,000 ml of deionized water.
2. 0.1 M Na_2HPO_4 (sodium phosphate, dibasic). Dissolve 26.8 g of $Na_2HPO_4 \cdot 7H_2O$ in 1,000 ml of deionized water.
3. 8.5% NaCl (sodium chloride). Dissolve 85.0 g of NaCl in 1,000 ml of deionized water. Sterilize by autoclaving for 20 min or by filtration. Store refrigerated.

Working PBS

Prepare a solution of the desired pH by combining the 10× stocks.

0.1 M NaH_2PO_4	See Table 3
0.1 M Na_2HPO_4	See Table 3
8.5% NaCl	100 ml
Deionized water	to 1,000 ml

■ Polysorbate 80

See Tween 80.

■ Potassium chloride solution (0.2 M, pH 2.2)

Potassium chloride is used to treat respiratory specimens for the recovery of *Legionella.*

For 100 ml of potassium chloride, 0.2 M (pH 2.2), solution

Potassium chloride, 0.2 M (solution A): place 1.49 g of potassium chloride into a volumetric flask and dilute to 100 ml with deionized water

Hydrochloric acid, 0.2 M (solution B): add 1.67 ml of concentrated hydrochloric acid to 75 ml of deionized water in a volumetric flask and QS to 100 ml. Add 13.5 ml of solution B to 86.5 ml of solution A and mix.

Potassium hydroxide (0.1 N), used for neutralizing the specimen after mixing with potassium chloride solution: add 0.56 g of potassium hydroxide to a volumetric flask and dilute to 100 ml with deionized water.

TABLE 3 Preparation of pH-specific 0.1 M sodium phosphate buffer[a]

pH	Vol (ml)		pH	Vol (ml)	
	A	B		A	B
5.7	93.5	6.5	6.9	45.0	55.0
5.8	92.0	8.0	7.0	39.0	61.0
5.9	90.0	10.0	7.1	33.0	67.0
6.0	87.7	12.3	7.2	28.0	72.0
6.1	85.0	15.0	7.3	23.0	77.0
6.2	81.5	18.5	7.4	19.0	81.0
6.3	77.5	22.5	7.5	16.0	84.0
6.4	73.5	26.5	7.6	13.0	87.0
6.5	68.5	31.5	7.7	10.5	89.5
6.6	62.5	37.5	7.8	8.5	91.5
6.7	56.5	43.5	7.9	7.0	93.0
6.8	51.0	49.0	8.0	5.3	94.7

[a] A, 0.1 M NaH_2PO_4; B, 0.1 M Na_2HPO_4.

Test procedure. Approximately 0.5 ml of specimen is mixed thoroughly with 4.5 ml of the 0.2 M potassium chloride (pH 2.2) solution, and the mixture is allowed to stand for 15 min at room temperature. The mixture is then neutralized to pH 7.0 with 0.1 N KOH and is inoculated onto isolation media.

■ Pyrrolidonyl-α-naphthylamide (PYR) hydrolysis

PYR is hydrolyzed by organisms that possess the enzyme pyrrolidonyl arylamidase (56). The PYR test is used for rapid presumptive identification of group A alpha-hemolytic streptococci *(Streptococcus pyogenes)*, *Enterococcus* spp., and other gram-positive cocci that grow aerobically and form cells arranged in pairs and chains. A pure culture or isolated colony for testing is critical given the number of PYR-positive organisms with similar Gram stain morphology and hemolytic characteristics.

PYR substrate (L-pyrrolidonyl-α-naphthylamide)

Dissolve L-pyrrolidonyl-α-naphthylamide in methyl alcohol first and then dilute with sterile distilled water. Adjust pH to 5.7 to 6.0. It is used at 0.01% in broth or agar media and at 0.02% in filter paper strips.

PYR reagent (DMACA)

DMACA	200 mg
Hydrochloric acid, concentrated	2 ml
Distilled water	18 ml

Add the acid to the water, and let it cool before adding DMACA.

Tube method. Prepare PYR broth (Todd-Hewitt broth containing 0.01% PYR substrate). Autoclave to sterilize. Dispense 0.15 ml per tube. Emulsify colonies from a blood agar plate in the PYR broth to a turbidity of McFarland no. 2 standard (milky suspension). Incubate at 35°C for 2 h. Add 1 drop of the PYR reagent to each tube, with gentle shaking. Observe for development of a cherry red color after 2 min. Yellow, orange, or orange-pink color is considered negative.

Spot paper strip method. Cut Whatman no. 3 filter paper into strips. Saturate the strips with 0.02% PYR substrate. Dry at room temperature and store desiccated at 2 to 6°C. Prior to testing, moisten the strip with sterile distilled water. Using an inoculating loop or wooden stick, rub colonies onto strip. Incubate for 10 min at 35°C. Then add PYR reagent to the strip and observe for development of a red color change. Yellow, orange, or pink is considered negative. For both methods, a positive reaction is indicated by cherry red to dark purple-red color. A negative reaction is indicated by orange or yellow (no change) color. PYR media, disks, and strips are available commercially.

■ Saline

Saline is used as a diluent in a variety of procedures. Normal or physiological saline is 0.85%.

Sodium chloride	8.5 g
Distilled water	1,000 ml

Other concentrations (e.g., 0.45%) are also used.

■ Skim milk, 20%

Skim milk is used to stabilize bacterial suspensions, particularly those containing anaerobes, for freezing.

Skim milk powder	20 g
Distilled water	100 ml

After the skim milk is dissolved in the water, dispense 0.25 to 0.5 ml into 2-ml vials. Autoclave at 110°C for 10 min. The vials can be refrigerated for up to 6 months.

■ Sodium bicarbonate ($NaHCO_3$), 20 mg/ml

Sodium bicarbonate is added to thioglycolate broth to enrich it for the recovery of anaerobes. Dissolve 2 g of $NaHCO_3$ in 100 ml of distilled water. Filter sterilize and store refrigerated for up to 6 months. Add 0.5 ml to 10 ml of thioglycolate broth.

■ Sodium citrate (0.1 M), 2.9%

Sodium citrate, dihydrate	29.4 g
Distilled water	1,000 ml

Dissolve and autoclave. Store at room temperature. If a precipitate forms, discard and prepare a fresh solution.

■ Sodium hydroxide (1 N), 4%

Sodium hydroxide	40 g
Distilled water	1,000 ml

Dissolve and autoclave. Store at room temperature. If a precipitate forms, discard and prepare a fresh solution.

■ Sodium polyanetholesulfonate (SPS) disks

SPS disks are used to differentiate *Peptostreptococcus anaerobius* (which is inhibited by SPS) from other anaerobic cocci. Dissolve 5 g of SPS in 100 ml of distilled water, filter sterilize, and then dispense 2 μl onto 6-mm-diameter sterile filter paper disks. Allow the disks to dry at room temperature for 72 h. The dried disks are stable at room temperature for up to 6 months. A zone of inhibition of 12 mm indicates that the organism is susceptible.

■ Sorensen pH buffer solutions (M/15 phosphate buffer solutions)

Solution A
M/15 (0.067 M) sodium phosphate, dibasic. Dissolve 9.464 g of anhydrous Na_2HPO_4 in 1 liter of distilled water.

Solution B
M/15 (0.067 M) potassium phosphate, monobasic. Dissolve 9.073 g of anhydrous KH_2PO_4 in 1 liter of distilled water. Mix *x* ml of solution A and solution B as indicated in Table 4 for a buffer of the desired pH.

■ Tween 80 (polysorbate 80), 10%

Tween 80 (polysorbate 80)	10 ml
Distilled water	90 ml

Mix Tween 80 with water until dissolved. Autoclave at 121°C at 15 lb/in^2 for 10 min. Swirl the solution immediately after autoclaving and during cooling to resolubilize the Tween 80.

TABLE 4 Sorensen pH buffer solutions

pH	Vol (ml)	
	Solution A	Solution B
5.29	0.25	9.75
5.59	0.5	9.5
5.91	1.0	9.0
6.24	2.0	8.0
6.47	3.0	7.0
6.64	4.0	6.0
6.81	5.0	5.0
6.98	6.0	4.0
7.17	7.0	3.0
7.38	8.0	2.0
7.73	9.0	1.0
8.04	9.5	0.5

Store refrigerated. The solution can be used for 6 months. Add 0.5 ml of 10% Tween 80 to 10 ml of broth medium when it is used as a medium supplement.

■ Urease test

Rapid enzymatic test used for identification purposes for a number of organisms. The procedure for identifying *Haemophilus* spp. is described.

KH_2PO_4	0.1 g
K_2HPO_4	0.1 g
NaCl	0.5 g
Phenol red, 1:500	0.5 ml

Add all ingredients into 100 ml of distilled water. Adjust the pH to 7.0 with NaOH, and add 10.4 ml of a 20% (wt/vol) aqueous solution of urea. To make 1:500 phenol red, dissolve 0.2 g of phenol red in NaOH and add distilled water to 100 ml. Red color developing within 4 h after inoculation indicates urease activity.

■ Vitamin K_1, 10-mg/ml stock

Vitamin K_1 (3-phytylmenadione) is added to enrich media for the recovery of anaerobes. Mix 0.2 g of vitamin K_1 in 20 ml of 95% ethanol by aseptic technique. Vitamin K_1 is a viscous liquid, and it may be hard to measure the exact amount. Adjust the amount of 95% ethanol accordingly to obtain a 10-mg/ml stock. Store refrigerated in a sterile dark bottle. The stock solution can be further diluted in sterile distilled water to obtain a 1-mg/ml working solution, which can be stored refrigerated in a dark bottle for up to 30 days.

■ Voges-Proskauer (VP) test

The VP test is used to detect acetoin (acetyl-methylcarbinol), which is produced by certain microorganisms during growth in a buffered peptone-glucose broth (MR-VP broth). The VP test is commonly used to aid in the differentiation between genera (such as *E. coli* from the *Klebsiella* and *Enterobacter* groups) and other species of the *Enterobacteriaceae* family. The test can be used as a differential test for other organism groups (viridans group streptococci).

Reagent A: 5%-α-naphthol
Dissolve 5 g of α-naphthol in 100 ml of absolute ethanol. Store refrigerated in a brown glass bottle away from light.

Reagent B: 40% KOH
Dissolve 40 g of potassium hydroxide in 100 ml of distilled water.

Test procedure. Inoculate MR-VP broth and incubate until good growth is obtained. Add 0.6 ml of the α-naphthol solution and 0.2 ml of the 40% KOH to 2.5 ml of culture broth. Shake well after the addition of each reagent. A positive reaction, indicated by the formation of a pink-red product, occurs within 5 min. However, allow 15 min for color development before considering the test negative.

STAINS

Direct Examination of Specimens

The first step in the processing of most clinical material is microscopic examination of the specimen. Direct examination is a rapid, cost-effective diagnostic aid. Methods for direct examination are designed to reveal and enumerate microorganisms and eukaryotic cells. Visible microorganisms may denote the presumptive etiologic agent, guiding the laboratory in the selection of the appropriate isolation media and the physician in the selection of the appropriate empirical antibiotic therapy. The quality of the specimen and the measure of the inflammatory response can also be evaluated. In addition, the direct smear serves as a quality control indicator for attempts to isolate observed organisms (1, 27, 28, 33, 54, 55).

Smear Preparation

Smears may be made from clinical material, culture broths, or isolated colonies. These smears should be prepared on clean glass slides, since dirt and grease may interfere with adhesion of the sample to the slide and with the staining process. The best smears are prepared after thoughtful selection of those portions of the sample most likely to reveal the etiologic agent (e.g., a purulent portion of sputum). Smears should contain enough material for an adequate survey of the specimen but should not be overly thick because thick smears may peel or flake off the slide during staining procedures. Thick smears also make the timing of decolorization harder to judge. Smears from swabs should be prepared by rolling the swab over the slide. This method of application helps to preserve host cell morphology and microorganism cell arrangements. Tissue smears may be prepared either by touching freshly exposed cut surfaces directly onto a slide or by first using a tissue grinder or stomacher to homogenize the sample. However, tissue grinding may distort cellular morphology. Smears from aspirates or body fluids may be prepared in several different ways, depending upon the amount of material and equipment available. When the quantity of a liquid sample is limited, a single drop placed on a slide will suffice. If more sample is available, the material should be centrifuged (1,500 × *g* for 15 min) to concentrate any cells, and the sediment can then be used to prepare the smear. An additional option for the preparation of smears from liquid samples is the use of cytocentrifugation. The cytocentrifuge method uses specimen funnels that are mounted with a slide and filter card and placed in the centrifuge. During centrifugation, the filter card absorbs the supernatant, while cells and microorganisms are centrifuged through a hole in the filter paper strip and are deposited in a continuous layering fashion onto a 6-mm-diameter circular area of the slide. The method is sensitive for the detection of pathogens from sterile body fluids, particularly cerebrospinal fluid (CSF) and peritoneal fluids (10, 62). The method has also been used for detection of acid-fast organisms and *Pneumocystis jiroveci* from respiratory specimens (8, 29). The deposition of specimen in a discrete area and the ability to lyse erythrocytes during centrifugation are particularly advantageous characteristics that allow more rapid and enhanced resolution in a smear examination.

Samples are fixed to the slides with either heat or methanol. Methanol fixation is preferred since heating may produce artifacts, may create aerosols, and may not adhere the specimen adequately to the slide (51). Once dry, the fixed smear is ready for staining.

Smear Preparations and Staining Methods

The following staining methods and techniques are individual methods used most often in the clinical bacteriology laboratory. They are presented in the following categories: wet mounts and single-stain methods, differential, acid-fast, and fluorescent. The stain method and significant characteristics are described. The principles of stains used for all microorganisms are described in chapter 14. Readers are referred to chapters 81, 117, and 134 for those staining procedures most commonly performed in the specialties of virology, mycology, and parasitology, respectively, and to other specific references (6, 23, 39, 47, 51, 58, 66).

Wet Mounts and Single-Stain Methods

■ **Colloidal carbon wet mounts (India ink, nigrosin)**

See chapter 117.

■ **KOH with and without lactophenol cotton blue**

See chapter 117.

■ **Lugol's iodine**

See chapter 134.

■ **Methylene blue stain**

Methylene blue is a simple direct stain used for a variety of purposes. The stain reveals the morphology of fusiform bacteria and spirochetes from oral infections (Vincent's angina). It may also establish the intracellular location of microorganisms such as *Neisseria.* Methylene blue is the stain of choice for identification of the metachromatic granules of diphtheria; however, one should be careful about overstaining, because this will lessen the contrast between the bacteria and the granules. Methylene blue stains organisms or leukocytes a deep blue in a light gray background. *Corynebacterium diphtheriae* appears as a blue bacillus with prominent darker blue metachromatic granules.

Basic procedure
Fix the prepared slide in absolute methanol for 1 to 3 min or heat fix. Air dry the slide and then stain with 0.5 to 1.0% aqueous methylene blue for 30 to 60 s and up to 10 min for possible *C. diphtheriae* granules. Rinse in water, blot dry, and examine at ×100 to ×1,000 magnification.

■ **M'Fadyean stain**

The M'Fadyean stain is a modification of the methylene blue stain developed originally for detecting *Bacillus anthracis* in clinical specimens. The rectangular bacteria stain black-deep

blue in chains of two to a few cells. Virulent *B. anthracis* rods will be surrounded by a clearly demarcated zone giving the appearance of a reddish-pink capsule ("M'Fadyean reaction").

Basic procedure
As *B. anthracis* is suspected, safety precautions must be taken throughout the procedure. All materials, including spent staining washes, should be discarded into disinfectant effective against endospores or autoclaved. See chapter 7. The staining reagent is prepared by dissolving 0.05 mg of methylene blue solution per ml in 20 mM potassium phosphate adjusted to pH 7.3. After the prepared slide is air dried, it is fixed in absolute methanol for 2 to 3 min. The slide is then dried and a large drop of the methylene blue solution is placed on the slide for 1 min. Rinse the slide under water into a 10% hypochlorite solution, blot, and allow to dry. Examine at ×100 to ×1,000 magnification.

Differential Staining Methods

■ Gram staining

Gram staining is the single most useful test in the clinical microbiology laboratory. It is the differential staining procedure most commonly used for direct microscopic examination of specimens and bacterial colonies because it has a broad staining spectrum. First devised by Hans Christian Joachim Gram late in the 19th century, it has remained basically the same procedure and serves in dividing bacteria into two main groups: gram-positive organisms, which retain the primary crystal violet dye and which appear deep blue or purple, and gram-negative organisms, which can be decolorized, thereby losing the primary stain and subsequently taking up the counterstain safranin and appearing red or pink. The staining spectrum includes almost all bacteria, many fungi, and parasites such as *Trichomonas, Strongyloides,* and miscellaneous protozoan cysts. The significant exceptions include organisms such as *Treponema, Mycoplasma, Chlamydia,* and *Rickettsia,* which are too small to visualize by light microscopy or which lack a cell wall. Mycobacteria are generally not seen by Gram staining; however, in smears illustrating heavy infections, the organisms may give a beaded appearance that is somewhat similar to that of *Nocardia* spp. or may exhibit organism "ghosts" (27). Gram staining can also be used to differentiate epithelial and inflammatory cells, thus providing information about the state of infection and the quality of the specimen (34, 54, 55, 63).

The Gram reaction, morphology, and arrangement of the organisms give the physician clues to the preliminary identification and significance of the organisms. Problems with analysis of the Gram staining generally result from errors in preparation of the slide, such as a smear that is too thick, excessive heat fixing (which can distort organisms), improper decolorization, and inexperience. Overdecolorization results in an abundance of bacteria that appear to be gram negative, while underdecolorizing results in too many bacteria that appear to be gram positive. If a chain of cocci resembling streptococci (normally gram positive) and epithelial cells appears to be gram negative, the slide is overdecolorized. Slides stained by Atkins' Gram staining method are less sensitive to decolorization because the mordant is more effective in retaining crystal violet. This allows better visualization of gram-positive organisms, especially those very sensitive to decolorization such as *S. pneumoniae* and *Bacillus* spp. Atkins' method does not offer a significant advantage over the conventional Gram staining procedure in visualizing gram-negative organisms, and in fact, such visualization may be more difficult with some specimens, such as blood cultures.

Other variations of the Gram stain exist in which the counterstain components may be different, specifically for the purpose of enhancing the appearance of gram-negative organisms. An example is the use of basic carbol fuchsin instead of safranin for easier identification of gram-negative anaerobic organisms (Table 2).

Laboratorians should note that organisms may not always stain true to their cell wall. Anaerobic organisms, older cultures, and organisms that are exhibiting the effects of antibiotics may be especially difficult to interpret. Two methods that may help in the cell wall identification include a string test noted to help distinguish gram-negative anaerobes. Another test is a simple fluorogenic disk test, Gram-Sure (Remel), that helps to differentiate aerobic gram-negative organisms (fluoresce blue with long-wavelength UV light) and gram-positive organisms (no fluorescence). See the reagent section for a full description (50).

Laboratories should evaluate various Gram staining procedure and then institute one as the routine procedure. Two methods of Gram staining are presented here. The first method is the conventional Gram staining method used by most laboratories. The second is an altered Gram staining method devised by Atkins (3) in 1920 that uses gentian violet, a different mordant, and acetone as the decolorizing agent. Each of the staining components is readily available commercially from a number of manufacturers (see Appendix 1).

Basic procedure—conventional Gram staining
The prepared slide is fixed in 95% methanol for 2 min. After air drying, the slide is flooded with crystal violet (10 g of 90% dye in 500 ml of absolute methanol). After at least 15 s, the slide is washed with water and flooded with iodine (6 g of I_2 and 12 g of KI in 1,800 ml of H_2O). The slide is washed with water after 15 s, decolorized with acetone-alcohol (400 ml of acetone in 1,200 ml of 95% ethanol), washed immediately, and counterstained for at least 15 s with safranin (10 g of dye in 1,000 ml of H_2O). This slide is then washed, blotted dry, and examined at ×100 to ×1,000 magnification.

Basic procedure—Atkins' Gram staining
The primary stain is gentian violet (20 g of crystal violet is dissolved in 200 ml of 95% methanol, 8 g of ammonium oxalate is dissolved in 800 ml of distilled water, and the solutions are mixed together and filtered after 24 h). The mordant is Atkins' iodine (20 g of crystals is dissolved in 100 ml of 1 N NaOH; the mixture is then combined with 900 ml of distilled water and stored in a brown bottle at room temperature). Acetone is the decolorizer, and safranin is the counterstain. The staining procedure is the same as that used for conventional Gram staining. The two dyes and the mordant should each be allowed to remain on the slide for at least 15 s. More time has little effect. The most critical aspect of this stain is the amount of time that the decolorizer is used. Unfortunately, the amount of decolorizer is directly related to the thickness of the specimen on the slide. The old benchmark that the slide should continue to receive decolorizer until no more crystal violet is seen to be washing away is still true but is difficult to attain in practice.

■ Spore stain (Wirtz-Conklin)

The Wirtz-Conklin spore stain is a differential stain for detection of spores. Spore-forming bacteria and cell debris will appear pink-red with green-staining spores.

Basic procedure
After the prepared slide is air dried and heat fixed, it is flooded with 5 to 10% aqueous malachite green. Stain is left

on the slide for 45 min. Alternatively, the slide can be heated gently to steaming for 3 to 6 min. Heating to steaming enhances the uptake of the stain into the spores. The slide is then rinsed with tap water. Aqueous safranin (0.5%) is used as a counterstain for 30 s. Rinse in water, blot dry, and examine at ×400 to ×1,000 magnification.

■ Wayson stain

The Wayson stain is a modification of the methylene blue stain and is actually a mix of two stains. It has been used for screening CSF for bacteria and amoebae and for demonstrating bipolar staining and examining tissue specimens for *Yersinia pestis* (15). The advantage of this stain is that the contrast between organisms and proteinaceous background is good. Organisms stain dark blue, leukocytes stain light blue and purple, and the background is light blue. However, slides stained by this method cannot be restained with the Gram stain, and the tinctorial qualities of the stain deteriorate over time.

Basic procedure

The staining reagents are prepared by dissolving 0.2 g of basic fuchsin in 10 ml of 95% ethyl alcohol and 0.75 g of methylene blue in 10 ml of 95% ethyl alcohol. The two solutions are added together slowly into 200 ml of 5% phenol in distilled water. The stain is then filtered and stored in an opaque bottle at room temperature. After the prepared slide is air dried, it is fixed in methanol for 2 min and stained for 1 min. Rinse in water, blot dry, and examine at ×100 to ×1,000 magnification.

■ Gimenez stain

The Gimenez stain formula and full description are in chapter 68 and Table 2 in chapter 14. This differential stain is used for the visualization of *Rickettsia* and *Coxiella* from cell cultures and *L. pneumophila*. Carbol fuchsin is the primary stain and fast green and Malachite green are the counterstains, allowing greater contrast with the organisms and background for easier visualization of the organisms. The stain must be heated 48 h prior to use and filtered.

Acid-Fast Staining Methods

The cells of certain organisms contain long-chain (30- to 90-carbon) fatty acids (mycolic acids) that give them a coat impervious to crystal violet and other basic dyes. Heat or detergent must be used to allow penetration of the primary dye into the bacterium. Once the dye has been forced into the cell, it cannot be decolorized by the usual solvent process. The acid-fast stains are useful for identification of a specific group of bacteria (e.g., *Mycobacterium, Nocardia, Rhodococcus, Tsukamurella, Gordonia,* and *Legionella micdadei*) and the oocysts of *Cryptosporidium, Isospora, Sarcocystis*, and *Cyclospora*. The Ziehl-Neelsen (Z-N) stain is one of the original acid-fast stains described. A number of modifications of the Z-N staining procedure have been made to differentiate various acid-fast organisms as well as to simplify the staining process (7, 41, 48, 66).

■ Ziehl-Neelsen (Z-N)

Basic procedure

The prepared slide is heat fixed for 2 h at 70°C. The slide is then flooded with carbol fuchsin (0.3 g of basic fuchsin is dissolved in 10 ml of 95% ethanol, 5 ml of phenol, and 95 ml of water; the solution is filtered before use). Heat the slide slowly to steaming and maintain for 3 to 5 min at 60°C. After cooling, wash with water and decolorize the slide with acid-alcohol (97 ml of 95% ethanol in 3 ml of HCl). Wash and counterstain for 20 to 30 s with methylene blue (0.3 g of dye in 100 ml of H_2O). Wash, blot dry, and examine at ×400 to ×1,000 magnification. An acid-fast organism will stain red, and the background of cellular elements and other bacteria will be blue, the color of the counterstain.

■ Kinyoun stain (Kinyoun modification of Z-N stain)

The basic difference between the Z-N and Kinyoun stains is the replacement of heating with the use of a higher concentration of phenol in the primary stain. The primary stain consists of 4 g of basic fuchsin in 20 ml of 95% alcohol, 8 g of phenol, and 100 ml of distilled water. The Z-N and Kinyoun stains have the same sensitivity and specificity; however, the Kinyoun (cold) staining procedure is less time-consuming and is easier to perform.

■ Modified Kinyoun stain (modified acid-fast stain)

Another modification of the acid-fast staining procedure has been the use of a weaker decolorizing agent (0.5 to 1.0% sulfuric acid) in place of the 3% acid-alcohol. This particular stain helps differentiate those organisms known to be partially or weakly acid-fast, particularly *Nocardia, Rhodococcus, Tsukamurella, Gordonia,* and *Diezia.* These organisms do not stain well with the Z-N or Kinyoun stain.

The acid-fast stains are important clinically and are relatively simple to use. Definitive identification of an acid-fast organism from a clinical specimen cannot be made by staining alone, but certain clues may be helpful. Mycobacteria often appear as slender, slightly curved rods and may show darker granules that give the impression of beading. *M. tuberculosis* can appear as beaded rods arranged in parallel strands or "cords"; *Mycobacterium kansasii* may form long, often broad and banded cells; and *Mycobacterium avium* complex cells appear as short, uniformly staining coccobacilli. *Nocardia* spp. often branch and almost always show a speckled appearance.

Difficulty in interpretation can result from smears that are too thick or insufficiently decolorized, yielding an acid-fast artifact. As a quality control measure, a known acid-fast organism such as nonpathogenic *M. tuberculosis* HRV 37 and a non-acid-fast organism such as *Streptomyces* can be stained in parallel with the clinical specimen.

Factors such as age, exposure to drugs, and a particular acid-fast organism itself may vary the acid-fast presentation. For example, while *M. tuberculosis* is consistently acid fast (with the Z-N or Kinyoun stain), rapidly growing mycobacteria and *Nocardia* are not. Therefore, use of the modified Kinyoun stain may be necessary for these organisms (41, 48, 66). Other modifications used in tissue preparations, such as the Fite-Faraco stain and Pottz stain, may be preferred for unusual isolates such as *Mycobacterium leprae* (66).

Detection of small numbers of acid-fast organisms in clinical specimens is generally significant. However, the use of acid-fast stains for gastric aspirates in the interpretation of pulmonary disease in adults or for stool specimens from human immunodeficiency virus-positive patients in diagnosing *Mycobacterium avium-Mycobacterium intracellulare* infection yields very poor specificity (false-positive smears with saprophytic organisms) as well as poor sensitivity (65). In addition, patients receiving adequate therapy may still have positive smears without positive cultures for a number of weeks. Rarely, small numbers of acid-fast organisms in a smear

may represent transferred contamination or the use of reagents contaminated with nonviable saprophytic mycobacteria (e.g., *Mycobacterium gordonae*). All smear-positive but culture-negative specimens should be investigated carefully.

Fluorescent Staining Procedures

See chapter 13 for fluorochrome filter recommendations.

■ Acridine orange

Acridine orange is a fluorochrome that can be intercalated into nucleic acid in both the native and the denatured states (61). The staining procedure is rapid and is more sensitive than the Gram staining procedure in the detection of organisms in blood culture broths, CSF, and buffy coat preparations (34, 44, 45, 52). Acridine orange is also useful in a number of miscellaneous infections, such as *Acanthamoeba* infections, infectious keratitis, and *Helicobacter pylori* gastritis (31). Bacterial and fungal DNAs fluoresce orange under UV light, and mammalian DNA fluoresces green. Results for cellular specimens or heavily laden bacterial specimens may be difficult to interpret owing to excessive fluorescence, and some interobserver variability may be noted.

Basic procedure

After the prepared slide is air dried and fixed in methanol, it is flooded with the acridine orange solution (stock solution, 1 g of dye in 100 ml of H_2O; working solution, 0.5 ml of stock added to 5 ml of 0.2 M acetate buffer [pH 4.0]). After 2 min, rinse the slide with tap water, air dry, and examine with UV light at ×100 to ×1,000 magnification.

■ Auramine-rhodamine

Auramine and rhodamine are nonspecific fluorochromes that bind to mycolic acids and that are resistant to decolorization with acid-alcohol (28). Staining procedures with these fluorochromes are thus equivalent to the fuchsin-based acid-fast procedures. The stain has become commonplace in laboratories that routinely perform acid-fast examinations because it allows rapid screening of specimens and because the procedure is more sensitive than the traditional acid-fast procedures. Acid-fast organisms fluoresce orange-yellow in a black background. If the secondary stain is not used, the organisms will fluoresce a yellow-green color. Smears with suspicious organisms may be confirmed directly with a Kinyoun stain. However, a single organism or a small number of organisms may be difficult to confirm.

Basic procedure

The prepared slide is fixed at 65°C for at least 2 h. It is then stained for 15 min with the auramine-rhodamine solution (1.5 g of auramine O, 0.75 g of rhodamine B, 75 ml of glycerol, 10 ml of phenol, and 50 ml of H_2O) and rinsed with water, followed by decolorization for 2 to 3 min with 0.5% HCl in 70% ethanol. After being rinsed, the slide is counterstained with 0.5% potassium permanganate for 2 to 4 min. The slide is rinsed, dried, and examined under UV light at ×100 to ×400 magnification.

Antibody Staining Methods

See chapter 18 on immunoassays.

MEDIA

This section reviews the basic components necessary in media for the growth and identification of organisms isolated in the clinical microbiology laboratory, specifically bacteriology. Media for the major groups of microorganisms are listed alphabetically. Refer to chapter 14 for discussion of principles of different media. The specific intended use and significant components are provided for each medium. Because media may be purchased from a number of suppliers and minor formulation variations exist for each medium, formulas as well as inoculation and incubation conditions, quality control, and limitations to the use of the media are not specifically mentioned except in rare instances. Readers are referred to comprehensive references on microbiological media (4, 7, 12, 20, 24, 25, 38, 40, 60, 70), including the *Handbook of Media for Clinical Microbiology*, 2nd edition (5), by R. M. Atlas and J. W. Snyder. In addition, the package inserts or websites from each company with purchased and/or specialized media offer excellent and specific descriptions. Recently, the first edition of a combined manual of both Difco and BBL products has been published as *The Difco and BBL Manual* (9). This manual includes numerous color photographs that depict colony morphology and color reactions as well as extensive descriptions of media and relevant references. *The Difco Manual*, 10th (18) and 11th (19) editions, from BD Biosciences, and other media supplier manuals continue to be good references because of historical media, as are other supplier manuals that may contain their own unique products. The formulations of a given medium from different manufacturers do vary slightly and may have been modified from the original description of the medium. A typical comment from the manufacturer is that the "classical" formula has been adjusted to meet performance standards. Again, the package insert or the formula being prepared from a reference should be followed closely. Chapters in this Manual are noted when appropriate to describe a referenced method cited by the authors for a specific organism. These referenced methods and media are found in the Isolation Procedures section within the chapter cited. Appendix 1 describes additives commonly added to media. Refer to Appendix 2 for suppliers of media and reagents as mentioned throughout this Manual. Media are available as prepared plates, tubes, bottles, or dehydrated products.

Preparation of Media

When preparing media from dehydrated materials, the manufacturers' instructions should be followed closely. Chemically cleaned glassware and distilled and/or demineralized water should always be used unless specified otherwise. Care in terms of accuracy should be taken when measuring liquid and dry ingredients. Mixing and solubilization of ingredients are typically done on hot plates, with magnetic stir bars placed in the bottom of the flask or beaker. Excessive heating should be avoided. Autoclaving or filtration sterilizes the media. Autoclaving of volumes of up to 500 ml at 121°C for 15 min is adequate. Larger volumes may require up to 20 to 30 min. The stir bars should be removed before sterilization. For quality control of autoclaving, specialized tape or paper is placed on the medium flask at the time of autoclaving. Enrichments such as blood and other labile additives such as filter-sterilized antibiotics should be added aseptically after the base medium has cooled.

While many dehydrated media are available, most clinical laboratories rely on prepared media from a commercial manufacturer because of convenience and lack of appropriate medium preparation facilities. In addition, overnight shipping even for small-volume purchases is often available for specialized media from the laboratory's routine medium distributor.

Quality Control

The Clinical and Laboratory Standards Institute (CLSI) has specific requirements for quality assurance of commercially prepared media, as documented in standard M22-A3 (13). However, these recommendations do not apply to all media. In addition, any medium that is prepared by the user requires its own specific quality control. Storage of media should be in the dark at 2 to 8°C. Storage in the dark is preferred because additives, such as dyes, will deteriorate faster in the light. The date that the medium was received in the laboratory and the medium expiration date should be marked and easily visible when stored. Media should be in use only up to the expiration date. Prolonged or incorrect storage of media, including transport media, can lead to desiccation of the media, changing the composition of nutrients and selective agents.

Plating of Specimens on Media and Incubation

Media should be warmed to room temperature before inoculation of specimens. In addition, a medium that has obvious contamination, such as colony growth or turbidity in broth medium, or that looks damaged in any way should not be used. Damage may include such things as a cracked petri dish and agar that has changed color, demonstrates precipitates, or is dehydrated.

Finally, it is important that the appropriate atmospheric incubation and temperature of media be used to optimize those pathogens likely to be in specimens submitted. Examples would include those conditions optimal for *Enterobacteriaceae* and *Campylobacter*. Isolation of *Enterobacteriaceae* from MacConkey agar may not be optimal if the agar is placed in CO_2, since the acidity with increased CO_2 may inhibit some of these organisms (S. M. Kircher, R. J. Cote, N. J. Dick, and W. F. Seip, *Abstr. 2000 Gen. Meet. Am. Soc. Microbiol.*, abstr. C-242, 2000), and *Campylobacter* should always be in a microaerophilic environment and at 42°C.

Bacteriology Media

■ A7 and A8 agars

A7 and A8 agars are selective and differential media used for the cultivation, identification, and differentiation of *Ureaplasma* spp. and *Mycoplasma hominis*. Both media contain soy and casein digests in an agar base and a supplement solution that contains yeast extract, horse serum, cysteine enrichment solution, and penicillin. Incorporation of urea aids in the identification of urease production to differentiate the organisms on the basis of the appearance of golden to dark brown colonies. There are two main differences between the agars: the use of manganous sulfate in A7 agar and putrescine dihydrochloride and calcium chloride in A8 agar for the detection and enhancement of growth of urease-positive colonies. *Ureaplasma* colonies are small golden brown colonies that are usually identified at 72 h. *Mycoplasma* colonies have a fried egg appearance and may have a golden or amber color. See also Mycotrim GU and Mycotrim RS.

■ Alkaline peptone water

Alkaline peptone water is an enrichment broth used for the isolation of small numbers of *Vibrio* and *Aeromonas* organisms from stool specimens. Adjustment of the broth to pH 8.4 and inclusion of sodium chloride at a concentration of 0.5 to 1.0% make it selective for *Vibrio* species.

■ American Trudeau Society medium

American Trudeau Society medium is a nonselective enriched medium used for the isolation and cultivation of mycobacteria. It is an egg-based medium. The coagulated egg provides fatty acids essential for support of mycobacterial growth. Glycerol and potato flour provide other nutrients. Malachite green is a partially selective agent that inhibits bacteria. The concentration of malachite green is low, and no other antibiotics are present in this medium; thus, it is very susceptible to proteolytic damage caused by contaminating organisms. This medium is best for specimens not usually contaminated with other microorganisms, e.g., tissue biopsy specimens or CSF. See also Lowenstein-Jensen and Middlebrook media.

■ Amies transport medium with and without charcoal

Amies transport medium is a modification of Stuart's medium. The glycerol phosphate used to maintain the pH in Stuart's medium has been found to enhance the growth of certain organisms that utilize this as a nutrient and to allow overgrowth of potential contaminants. In Amies medium, phosphate buffer replaced the glycerol phosphate ingredient and other salts were added to control the permeability of bacterial cells. Amies medium with charcoal is preferred for the isolation of *Neisseria* spp. because the charcoal neutralizes metabolic products toxic to the organisms.

■ Anaerobic blood agar (CDC)

The Centers for Disease Control and Prevention (CDC) formulation of anaerobic blood agar is a general-purpose medium used for the isolation and cultivation of anaerobic bacteria. The nutritive base is tryptic soy agar supplemented with yeast extract, vitamin K, hemin, and sheep blood. This medium is best for the isolation of anaerobic gram-positive cocci. See chapter 55 for other selective anaerobic medium recommendations for gram-positive bacteria.

■ 10B arginine broth; Shepard's broth

10B arginine broth is a medium used for the transport and growth of *M. hominis* and *Ureaplasma urealyticum*. The medium contains nitrogenous components, amino acids, and other components necessary for growth, and penicillin G or a semisynthetic penicillin is added to reduce bacterial contamination. Shepard's broth contains cefoperazone. Two primary compounds, namely, urea and arginine, as well as the phenol red indicator, aid in the identification of the organisms. *Ureaplasma* hydrolyzes urea and releases ammonia; *Mycoplasma* deaminates the arginine. Both reactions result in an alkaline pH shift and change the color of the medium from yellow to pink. *Ureaplasma* depletes urea in the medium quickly (<12 h), with subsequent death of the culture. Thus, after the color changes, the cultures need to be subcultured quickly to a medium such as A8 or A7 agar that supports the organisms. Limitations of the medium are that other species of *Mycoplasma* and other bacteria may also change the color of the medium.

■ Ashdown agar

Ashdown agar is a selective and differential medium consisting of Trypticase soy agar with 4% glycerol, 0.005 mg of crystal violet per ml, 0.05 mg of neutral red per ml, and 0.004 mg of gentamicin per ml, specifically designed for the isolation

of *Pseudomonas pseudomallei,* the causative agent of melioidosis. Glycerol and neutral red allow differentiation from other pseudomonads since *P. pseudomallei* appears flat, with rough wrinkled colonies due to the glycerol, and absorbs the neutral red dye, whereas other pseudomonads do not. The medium is made selective by the addition of crystal violet, which inhibits gram-positive organisms, and gentamicin, which inhibits gram-negative organisms. Ashdown broth with 50 mg of colistin per liter enhances recovery over direct plating by 25% (2, 68).

■ *Bacillus cereus* medium

B. cereus medium is an enriched medium used for the isolation of *B. cereus*. The base includes agar, yeast extract, and buffers. Mannitol combined with bromcresol purple as the indicator dye makes the medium differential. An egg yolk emulsion is added for the detection of the lecithinase activity seen with *B. cereus*.

■ BACTEC 12B radiometric medium

BACTEC 12B medium (BD Biosciences) is a liquid nonselective medium used for the isolation and identification of *Mycobacterium* species in conjunction with the BACTEC system. The medium consists of a 7H9 broth base. The radiometric BACTEC system incorporates ^{14}C-labeled palmitic acid and detects radioactive carbon dioxide; the medium is referred to as 7H12 or BACTEC 12B medium. An antibiotic enrichment supplement is added to the medium to make it selective for mycobacteria. This supplement includes the antibiotics polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin, with polyoxylene stearate as a *Mycobacterium* growth enhancer. A total of 0.5 ml of processed specimen may be accommodated in the vial. The BACTEC 12B medium is most commonly used for susceptibility testing. Newer nonradiometric systems are available for monitoring for mycobacterial growth and/or used for susceptibility testing. These include the 9200 series (BD Biosciences), MGIT (BD Biosciences), ESPII (TREK Diagnostic Systems), and MB/BacT ALERT 3D (bioMérieux, Inc.). Manual medium systems also exist for the detection of mycobacteria, such as the Isolator (Wampole Laboratories) and Septi-Chek AFB (BD Biosciences). The reader is referred to chapters 36 and 77 for specifics on medium formulations and antibiotic supplements for each system.

■ *Bacteroides* bile esculin agar

Bacteroides bile esculin agar is an enriched, selective, and differential medium used for the isolation and presumptive identification of members of the *Bacteroides fragilis* group and *Bilophila wadsworthia*. The nutritive base includes casein and soybean peptones, hemin, and vitamin K. The differential characteristic of esculin hydrolysis is identified by the product, esculetin, which reacts with the ferric ammonium citrate to form a complex and produce brown-black coloration around the colony. The selective agents include bile, which inhibits most gram-positive bacteria and anaerobic organisms other than members of the *B. fragilis* group, and gentamicin, which inhibits facultative anaerobes. Bile esculin agar with kanamycin and enriched with vitamin K and hemin is a formulation that is more enriched and selective than *Bacteroides* bile esculin agar for isolation of the *B. fragilis* group. This medium includes beef extract and pancreatic digest of gelatin, vitamin K, and hemin as the nutritive base. Bile inhibits the same organisms as described above, and kanamycin is inhibitory for facultatively anaerobic and aerobic gram-negative bacilli.

■ Baird-Parker agar base

Baird-Parker agar base is a beef extract, peptone, and yeast extract base used to prepare egg-tellurite-glycine-pyruvate agar (ETGPA). ETGPA is an enriched, selective, and differential agar used for the detection of coagulase-positive staphylococci *(S. aureus)* from food and other nonclinical sources. Glycerol and lithium make the medium selective by inhibiting many bacteria. Tellurite is also inhibitory to bacteria. The egg yolk emulsion is an enrichment. Tellurite and the egg yolk emulsion also act as differential determinants. When *S. aureus* reduces tellurite, it imparts a black color to the colony, and lecithinase activity is demonstrated by a clearing around the colony. *S. aureus* appears as black-brown colonies with clear zones around them.

■ Barbour-Stoenner-Kelly medium (Sigma-Aldrich Co., St. Louis, Mo.)

Barbour-Stoenner-Kelly medium is a complicated medium for the isolation of *Borrelia burgdorferi*. Bovine serum albumin, rabbit serum, and sodium bicarbonate are significant components. While a defined medium has been described, variability in components makes for differences in the ability to isolate the organism (59).

■ Bile esculin agar

Bile esculin agar is a selective and differential medium used for the isolation and differentiation of *Enterococcus* and *Streptococcus bovis* (group D *Streptococcus*) from non-group D *Streptococcus*. The nutritive base includes peptone and beef extract. The selective agent is bile (oxgall), which inhibits gram-positive organisms and most strains of streptococci except *S. bovis* and *Enterococcus*. Esculin hydrolysis is a characteristic to differentiate enterococci and *S. bovis* from other organisms. Esculin in the medium is hydrolyzed to esculetin and dextrose. A black-brown pigment forms when the iron salt (ferric citrate) is used as the color indicator of esculin hydrolysis and subsequent esculetin formation. *S. bovis* and *E. faecalis* grow on the medium and exhibit blackening around the colony.

■ Bile esculin agar plus vancomycin at 6 µg/ml

Bile esculin agar plus vancomycin is a selective and differential medium used to identify vancomycin-resistant streptococci and enterococci. Colonies appear the same as they do on bile esculin agar. Growth of group D streptococci and enterococci occurs with esculin hydrolysis in the presence of bile, appearing as blackening of the medium.

■ Bile esculin azide agar and broth (Enterococcosel)

Bile esculin azide agar or broth is a selective and differential medium for *S. bovis* (group D streptococcus) and enterococci. As with bile esculin agar, esculin is incorporated into the medium, and precipitation with ferric ions forms a brown-black pigment, which identifies these species. Bile esculin azide medium has a reduced percentage of bile and makes the medium less inhibitory to non-group D streptococci.

Sodium azide is incorporated to inhibit gram-negative organisms. The addition of 6 μg of vancomycin per ml makes the medium selective for vancomycin-resistant enterococci. Incorporation of aztreonam subsequently increases the selectivity by inhibiting other organsims, especially gram-negative organisms, contaminating specimens.

■ Bismuth sulfite agar

Bismuth sulfite agar is a highly selective and differential medium used for the isolation of *Salmonella enterica* serovar Typhi and other enteric bacilli. Beef extract, peptones, and dextrose are the nutritive base. Bismuth sulfite, a heavy metal, and brilliant green are selective agents which inhibit most commensal gram-positive and gram-negative organisms. Ferrous sulfate is an indicator for hydrogen sulfide production, which occurs when the hydrogen sulfide produced by *Salmonella* reacts with the iron salt. This reaction causes a black or green metallic colony and a black or brown precipitate. Colony morphology and color help differentiate *Salmonella* species. This medium may be inhibitory for some species of *Shigella*. Readers are referred to chapter 42 for other selective media for enteric organisms.

■ Blood agar

See Columbia agar with 5% sheep blood.

■ Blood culture media

All blood culture medium formulations are based on a nutrient peptone broth with variations due to hydrolysis or digestion of the source protein. Additives for neutralization of serum components and/or inactivation of antibiotics, such as SPS, chelators, or resins, vary with the manufacturer. The reader is referred to chapter 15 for formulations for specific blood culture media and various commercial systems.

■ Bordet-Gengou medium

Bordet-Gengou medium is an enriched medium used for the isolation and cultivation of *Bordetella pertussis* from clinical specimens. The medium contains potato infusion, glycerol, and peptones as the nutritive base. Sheep blood allows detection of hemolytic reactions and provides other nutrients for *Bordetella*. The medium can be supplemented with methicillin, which inhibits some of the normal oral flora that is obtained upon collection of the specimen. Culture plates should be held for at least 7 days. Incubation up to 12 days identifies *Bordetella parapertussis*. Bordet-Gengou medium is not as effective for the recovery of *Bordetella* as Regan-Lowe medium, and freshly prepared medium must be used for optimum recovery.

■ Brain heart infusion agar

Brain heart infusion agar is a general-purpose medium used for the isolation of a wide variety of pathogens, including yeasts, molds, and bacteria. The basic formula includes brain heart infusion from solids as well as meat peptones, yeast extract, and dextrose. One variation that exists is brain heart infusion agar with vitamin K and hemin for the enrichment of anaerobes. The anaerobic formulation may be optimal for the isolation of *Eubacterium* spp. but inferior for the isolation of anaerobic gram-negative organisms, especially those that produce pigment.

■ Brain heart infusion agar with 7% horse blood and brain heart infusion agar with 1% serum (see Brain heart infusion agar)

Brain heart infusion agar with horse blood or serum enriches the medium for isolation of *Helicobacter* spp.

■ Brain heart infusion broth

Brain heart infusion broth is a general-purpose clear liquid medium that is used to cultivate a wide variety of organisms. It is also used for the preparation of inocula for susceptibility tests and identification. The medium is especially useful as a blood culture medium. The main nutritive base includes infusion from brains and beef heart. Peptones, glucose, sodium chloride, and buffers are other additives. Sodium chloride acts as an osmotic agent, and disodium phosphate acts as a buffer. Formulations with 6.5% NaCl are used for the isolation of salt-tolerant streptococci, formulations with 0.1% agar that reduce O_2 tension favor anaerobes, and formulations with Fildes enrichment are used for the isolation of fastidious organisms such as *Haemophilus* and *Neisseria*. Brain heart infusion broth is also used for the preparation of inocula for antimicrobial susceptibility testing and broth dilution MIC testing procedures. The medium contains infusions of brain, casein, and meat peptones.

■ Brain heart infusion-vancomycin agar (see Brain heart infusion agar)

Brain heart infusion-vancomycin agar is a selective medium used for the isolation of vancomycin-resistant enterococci. The base is brain heart infusion agar. Vancomycin (6 μg/ml) is added to select for vancomycin-resistant enterococci.

■ Brilliant green agar

Brilliant green agar is a highly selective and differential medium used for the isolation of *Salmonella* species except for serovar Typhi. The nutritive base contains meat and casein peptones. Brilliant green dye at a high concentration is the selective agent and inhibits most gram-positive and gram-negative bacteria, including *Shigella* species and serovar Typhi. Phenol red is the pH indicator. Yeast extract provides additional nutrients. Sugars included in the medium are sucrose and lactose. Acid production in the fermentation of these sugars produces yellow-green colonies with a yellow-green zone. Nonfermenters of sucrose and lactose may range in color from white to reddish pink with a red zone around the colony *(Salmonella)*.

■ Brucella agar

Brucella agar is a medium designed originally for the purpose of isolating *Brucella* spp. from dairy products. Brucella agar with 5% horse blood can be used as a general-purpose medium for the isolation of both fastidious aerobic and anaerobic organisms. The nutritive base includes a peptone mix, including meat peptones, dextrose, and yeast extract.

■ Brucella agar with cefoxitin and cycloserine

Brucella agar with cefoxitin and cycloserine is a selective and differential sheep blood medium used for the isolation of *Clostridium difficile*. Brucella agar is the nutritive base. Vitamin K and sheep blood provide other growth enhancers. Cefoxitin and cycloserine inhibit most gram-positive and

gram-negative organisms, respectively. *Enterococcus* is not inhibited. Another differential characteristic is that *C. difficile* colonies fluoresce yellow-green under UV light.

■ Brucella agar with hemin and vitamin K

Brucella agar with hemin and vitamin K is a general-purpose nonselective and enriched medium used for the isolation and cultivation of anaerobic bacteria. Casein peptones, dextrose, and yeast extract are the nutritive base. Hemin and vitamin K provide further enrichments. Defibrinated sheep blood allows determination of hemolytic reactions. Because of the high carbohydrate content, colonies with beta-hemolytic reactions may have a greenish hue. The medium is better for gram-negative organisms.

■ Brucella agar with 5% horse blood

Horse blood enriches brucella agar for fastidious organisms, such as *H. pylori*, by providing both hemin (factor X) and NAD (factor V) factors. The use of horse blood also allows determination of hemolytic reactions. However, hemolytic reactions for *Streptococcus* and *Haemophilus* on horse blood differ from those on media with sheep blood.

■ Brucella broth

Brucella broth is a liquid medium that is used to cultivate *Campylobacter* species and to identify the organisms to the species level. Brucella base that contains peptones, dextrose, and yeast extract is the nutritive base.

■ Buffered charcoal yeast extract (BCYE) (selective and nonselective)

BCYE is a specialized enriched agar medium used for the isolation and cultivation of *Legionella* species from environmental and clinical specimens. *Legionella* species, especially *L. pneumophila*, require specific nutrients for growth. One is iron and the other is the amino acid L-cysteine. These are provided in BCYE by ferric pyrophosphate and L-cysteine hydrochloride. The nutritive base is yeast extract and α-ketoglutarate. *N*-(2-Acetamido)-2-aminoethanesulfonic acid buffer maintains the pH of the medium. Charcoal acts as a detoxifying agent and surface tension modifier. Antibiotics may also be added to the medium. Typically, manufacturers provide the medium in two combinations of three antibiotics. One is polymyxin B, anisomycin, and vancomycin, and the other is polymyxin B, anisomycin, and cefamandole (PAC). In these combinations polymyxin B inhibits gram-negative rods, anisomycin inhibits yeasts, vancomycin inhibits gram-positive organisms, and cefamandole inhibits both gram-positive and gram-negative organisms. BCYE with PAC may be inhibitory to some strains of *L. micdadei*. Nonselective BCYE can support the growth of other fastidious organisms such as *Nocardia* and *Francisella*. BCYE can also be a differential medium with the addition of the dyes bromcresol purple and bromthymol blue. *L. pneumophila* produces light blue colonies with a pale green tint. This differential medium is also called Wadowsky-Lee medium and can be used for isolation of actinomycetes.

■ Buffered glycerol saline

Buffered glycerol saline is a multipurpose transport medium. The transport medium has been used for the isolation of bacteria, such as *Aeromonas* spp., as well as viruses. In addition, glycerol-containing media may also be used for long-term storage of isolates and for transport and storage of biopsy specimens.

■ *Burkholderia cepacia* selective agar

B. cepacia selective agar is an enriched and selective medium used for the isolation of *B. cepacia*. Trypticase peptone, yeast extract, sodium chloride, sucrose, and lactose are the nutritive base. The medium is made selective by the addition of polymyxin B, gentamicin, vancomycin, and crystal violet. The medium supports the growth of *B. cepacia* and inhibits >90% of other isolates. This agar is more sensitive and more selective than *Pseudomonas cepacia* (PC) agar, oxidative-fermentative polymyxin B-bacitracin-lactose medium, and other selective media for isolation of *B. cepacia* from patients with cystic fibrosis (35). See chapter 49.

■ *Campylobacter* blood agar

Campylobacter blood agar is an enriched selective blood agar medium used for the isolation of *Campylobacter* species. The nutritive base is brucella agar. Sheep blood provides heme and other growth factors. The selectivity of the medium comes from the incorporation of five antimicrobial agents: trimethoprim, vancomycin, amphotericin B, polymyxin B, and cephalothin. These agents inhibit normal stool flora such as members of the family *Enterobacteriaceae*, staphylococci, and yeasts. Plates should be incubated in a microaerophilic environment. Due to the dextrose content of the brucella base, weak oxidase reactions may be exhibited. Some species of *Campylobacter* (e.g., *Campylobacter fetus* subsp. *fetus* and *Campylobacter upsaliensis*) are inhibited by cephalosporins. Media with cephalothin have been shown to be more inhibitory to *C. jejuni* and *Campylobacter coli* than those with cefoperazone. See chapter 59 for additional selective media.

■ *Campylobacter* charcoal differential (CCD) agar

CCD agar is a blood-free selective medium used for the isolation of *Campylobacter* from stool specimens. Cefoperazone replaces cefazolin in other selective agars. The nutritive base is Preston agar, which consists of beef extract and peptones. This agar has been shown to be less inhibitory than other *Campylobacter* agars for all *Campylobacter* species as well as more inhibitory to contaminating organisms (36).

■ *Campylobacter* thioglycolate medium

Campylobacter thioglycolate medium is a selective holding medium used for the isolation of *Campylobacter* species. The low concentration of agar in thioglycolate broth provides a reduced oxygen content. The selective agents include the same as those in *Campylobacter* agar: trimethoprim, vancomycin, polymyxin B, cephalothin, and amphotericin B.

■ Cary-Blair transport medium

Cary-Blair transport medium was specifically designed to enhance the survival of enteric bacterial pathogens. The medium has a low nutrient content, which allows organism survival without replication; sodium thioglycolate, which allows a low oxidation-reduction potential; and a high pH, which minimizes the destruction of bacteria when acid is produced.

■ Cefoperazone-vancomycin-amphotericin B (CVA) medium

CVA medium is a selective and enriched blood agar medium used for the isolation of *Campylobacter* species. The nutritive agar base is brucella agar. Sheep blood provides hemin and other growth nutrients. The antibiotics in this medium are vancomycin, amphotericin B, and cefoperazone, which inhibit gram-positive organisms, fungi, and anaerobic gram-negative organisms. The limitations of this medium are that some campylobacters (e.g., *C. fetus* subsp. *fetus*) are inhibited and that weak oxidase reactions may occur due to the dextrose in the brucella agar base. CVA is a good medium to use if only a single selective medium for *Campylobacter* can be used.

■ Cefsulodin-Irgasan-novobiocin (CIN) medium (*Yersinia* selective agar)

CIN, or *Yersinia* selective agar, is a selective and differential medium used for the isolation and differentiation of *Yersinia enterocolitica* from clinical specimens and food sources. The nutritive base includes peptones and beef and yeast extracts. The selective agents are sodium desoxycholate, crystal violet, cefsulodin, Irgasan (triclosan), and novobiocin. The medium is available with various concentrations of cefsulodin (4, 8, or 15 μg/ml). The lower concentration is recommended for better growth of clinically significant *Yersinia* spp. as well as growth of *Aeromonas* spp. Mannitol is the sugar, and neutral red is the indicator. Organisms that ferment mannitol in the presence of the neutral red dye cause a pH drop around the colony. The colony becomes transparent, with the absorption of the red dye to form a red bulls-eye appearance in the center with *Yersinia*, and *Aeromonas* will have a pink center with an uneven clear apron. Most other bacteria, including other enteric mannitol fermenters, are inhibited. Some yersiniae may require cold enrichment at 4°C and subsequent subculture to CIN medium. See also chapters 44 and 46.

■ Cetrimide agar

Cetrimide agar is a selective and differential medium used for the identification of *Pseudomonas aeruginosa*. Cetrimide is the selective agent and inhibits most bacteria by acting as a detergent. *Pseudomonas* produces a number of pigments. Two pigments can be detected with this medium. The magnesium chloride and potassium sulfate stimulate the blue-green pyocyanin pigment, and the fluorescent yellow-green pigment can be seen with a UV light. The low iron content of the medium also stimulates pigment production.

■ Charcoal selective medium

Charcoal selective medium is an enriched selective medium used for the isolation of *Campylobacter* species. In this medium the nutritive base is a Columbia agar base, and charcoal is used to effectively replace blood components. The selective agents used in this medium include vancomycin, cefoperazone, and cycloheximide. Vancomycin and cefoperazone effectively inhibit gram-positive and gram-negative organisms, including *Pseudomonas*. Fungi are inhibited by cycloheximide. The limitation of this medium is that some *Campylobacter* species, e.g., *C. fetus* subsp. *fetus*, are inhibited by cephalosporins.

■ Chocolate agar

Chocolate agar is a general-purpose medium used for the isolation and detection of a wide variety of microorganisms, including fastidious species such as *Neisseria* and *Haemophilus*. Chocolate agar originated as a GC agar base which includes meat and casein peptones, phosphate buffer to maintain pH, and cornstarch to detoxify fatty acids in the medium. Hemoglobin is added to the medium to provide hemin (X factor). The appearance of hemin in dry powdered form is reddish brown. When hemoglobin is hydrated and added to the medium, it gives the agar base the "chocolate" appearance. Other enrichments added include defined supplements, such as IsoVitaleX or Vitox, which provides NAD (V factor). Both of these components added to the GC agar base make the enriched chocolate agar. Levinthal agar is a variation on chocolate agar. The medium is a selective and differential medium used for the isolation and identification of *Haemophilus influenzae* type b. The medium is a chocolate agar base that is made transparent by the removal of particulate matter by either centrifugation or filtration through sterile filter paper. The medium contains bacitracin to inhibit respiratory flora and *H. influenzae* type b antiserum, allowing detection of an immunoprecipitation reaction. Colonies of encapsulated strains show a bright iridescence (red-blue-green-yellow) when light is transmitted from behind the clear medium.

■ Chopped meat glucose broth

Chopped meat glucose broth is an enriched medium that supports the growth of most anaerobes. It is most commonly used to isolate *Clostridium botulinum* from mixed bacterial growth. Beef heart, peptones, and dextrose supply the essential nutrients. The −SH groups from cooked and denatured muscle protein are the reducing agents in the medium. Vitamin K and hemin are additives used to maximize the growth of specific anaerobes. The medium helps to induce sporulation of *Clostridium* spp.

■ CHROMagar (Rambach agar)

CHROMagar is a microorganism-specific, chromogenic culture medium used for isolation and identification for a variety of organisms. CHROMagar was developed by Alain Rambach, who first formulated agars that were monochromogenic for the detection of *E. coli* and *Salmonella* spp. Second-generation agars are multicolor. Both types are differential and selective. The nutritive agar base includes peptone and glucose. Different additives, proprietary chromogenic mixtures, and antibiotics have resulted in a series of media specific for such organisms as *Listeria, S. aureus,* methicillin-resistant *S. aureus* (MRSA), *E. coli* O157, yeasts, and other organisms. BD Biosciences has a licensing agreement for development of the CHROMagar media. Many other chromogenic media with proprietary components are also now available through a number of manufacturers. Most commonly evaluated are chromogenic media for the detection of MRSA. These include MRSA ID (bioMérieux), MSA-CFOX (Oxoid), and MRSA*Select* (Bio-Rad) (17, 64). Greater recovery of organisms, increased specificity, and elimination of nonselective media are benefits of chromogenic agar use. Cost per plate compared to those of standard media and limited shelf life need to be considered before implementation.

■ Columbia agar with 5% sheep blood

Columbia agar with 5% sheep blood is a general-purpose medium used for the isolation of a variety of microorganisms, including fastidious organisms. This medium contains meat

and casein peptones and beef extract, yeast extract, and cornstarch as the nutritive base. Sheep blood allows determination of hemolytic reactions and provides X factor. However, the substantial carbohydrate content may make beta-hemolytic streptococci appear to be alpha-hemolytic or take on a greenish hue. NADase enzyme in sheep blood destroys the V factor (NAD); thus, organisms that require this factor do not grow. Incorporation of horse or rabbit blood allows beta-hemolysis to be seen better. Addition of 20 μg of ampicillin per ml is helpful in isolation of *Aeromonas*.

■ Columbia broth

Columbia broth is a general-purpose clear liquid medium used especially for blood culture medium. The broth supports the growth of a wide range of microorganisms. The base is similar to Columbia agar, with meat peptones, casein, and yeast extract. Salt and Tris buffers have been added to enhance the growth of microorganisms and increase the buffering capacity, respectively. For the purpose of blood culture medium, additional ingredients include carbon dioxide, which is stimulatory for many organisms; cysteine, which improves isolation of anaerobic and aerobic organisms from blood; SPS, a polyanionic anticoagulant which inactivates aminoglycosides and which interferes with the complement, lysozyme activity, and the phagocytic activity inherent in a blood specimen; and glucose, which provides a hypertonic medium for the isolation of cell wall-deficient forms.

■ Columbia-colistin-nalidixic acid agar with 5% sheep blood

Columbia-colistin-nalidixic acid agar with 5% sheep blood is a selective and differential medium commonly used in the isolation of gram-positive aerobic and anaerobic organisms from mixed clinical specimens. The base is Columbia agar with 5% sheep blood, which allows the detection of hemolytic reactions and provides additional enrichment and X factor (heme). The medium is made selective by the inclusion of the antibiotics colistin and nalidixic acid, which inhibit gram-negative organisms. Swarming *Proteus* spp. are inhibited. Supplementation with glutathione and lead acetate allows a selective and differential medium for anaerobic gram-positive cocci.

■ Cycloserine-cefoxitin-fructose agar

Cycloserine-cefoxitin-fructose agar is a selective and differential agar medium used for the isolation of *C. difficile*. The nutritive base includes animal peptones and fructose. The selective agents include cycloserine and cefoxitin. Cycloserine inhibits gram-negative organisms, especially *E. coli*, and cefoxitin is a broad-spectrum antibiotic that is active against both gram-positive and gram-negative organisms. *Enterococcus* is not inhibited. The medium is made differential by the addition of neutral red. *Clostridium* raises the pH of the medium and allows the neutral red indicator to change to yellow. Both the colony and the surrounding medium turn yellow. In addition, *C. difficile* colonies yield a gold-yellow fluorescence when viewed under long-wave UV light.

■ Cysteine-albumin broth with 20% glycerol

Cysteine-albumin broth with 20% glycerol is used for transport and storage of gastric biopsy specimens for the recovery of *H. pylori* (37).

■ Cystine glucose blood agar

Cystine glucose blood agar is an enriched medium used for the isolation of *Francisella* spp. The nutritive base is beef heart infusion, peptones, and glucose. *Francisella* requires cystine for growth. Rabbit blood provides hemoglobin enrichment.

■ Cystine-tellurite blood agar

Cystine-tellurite blood agar is a modification of Tinsdale agar and is both a selective and differential medium used for the detection of *C. diphtheriae*. Casein peptones, beef infusion, and yeast extract are the nutritive base. Potassium tellurite is both the selective and differential agent. Gram-negative organisms and most organisms of the upper respiratory flora are inhibited; *Corynebacterium* spp. are the exception. The potassium tellurite also allows differentiation of *C. diphtheriae* from other biotypes by the dull metal gray or black colony appearance indicative of tellurite reductase activity and the brown halo around the colony consistent with cystinase activity. Other organisms, such as staphylococci, may reduce tellurite and produce black colonies. These are easily differentiated by Gram staining.

■ Cystine tryptic agar

Cystine tryptic agar is a pancreatic digest of casein-enriched medium that is most commonly used for cultivation and maintenance of fastidious organisms. Addition of 1% carbohydrates and phenol red for determination of fermentation reactions is especially helpful for differentiating *Neisseria* spp.

■ Diagnostic sensitivity agar

Diagnostic sensitivity agar is a medium used in the cultivation of organisms for susceptibility testing. The base is proteose peptone, veal infusion solids, agar, and glucose, with other additives. The medium is available as a premixed powder from Oxoid.

■ DNA-toluidine blue agar

DNA-toluidine blue agar is a differential medium used most commonly for the detection and differentiation of *Staphylococcus* spp. The nutritive base is tryptic soy agar. Supplementation with DNA permits detection of DNase activity (or heat-stable staphylococcal nuclease) that has endo- and exonucleolytic properties and can cleave DNA or RNA produced in most coagulase-positive staphylococci. The medium is blue due to the toluidine blue O, and DNase activity is detected by a pink zone around the colony secondary to the metachromatic property of the dye.

■ Dubos Tween-albumin broth

Dubos Tween-albumin broth is a nonselective medium used for the isolation and cultivation of mycobacteria. Polysorbate 80 (Tween 80) is an oleic acid ester and acts as an essential fatty acid necessary for the growth of mycobacteria. In addition, Tween 80 acts as a dispersal agent and allows a small inoculum to grow more homogeneously. Casein peptone and asparagine provide other nutrients. Phosphates provide a buffering system, and albumin, a source of protein, provides protection from toxic substances in the medium. Cultures of *M. tuberculosis* may form cords in the medium, and other mycobacteria grow more diffusely. See also Middlebrook 7H9 broth.

■ Egg yolk agar (modified McClung-Toabe agar)

Egg yolk agar medium (modified McClung-Toabe agar) is a selective and differential medium used for the isolation and differentiation of *Clostridium* spp. McClung and Toabe (51a) reported on the use of egg yolk medium for the identification of species of clostridia by the detection of lecithinase and lipase activities. Degradation of lecithin results in an opaque precipitate around the colony, and lipase destroys fats in the egg yolk, which results in an iridescent sheen on the colony surface. Proteolysis can also be determined with egg yolk agar, as indicated by a translucent clearing of the medium around the colony. The medium should be incubated anaerobically for a minimum of 48 h. It should be held for up to 7 days for the detection of lipase activity. Addition of neomycin makes the egg yolk agar moderately selective by inhibiting some facultative anaerobic gram-negative rods. Lecithinase positivity is also seen with some *Bacillus* spp. Lipase and proteolytic activity can also be demonstated with some anaerobes.

■ Ellinghausen-McCullough/Johnson-Harris medium

Ellinghausen-McCullough/Johnson-Harris medium is an enriched semisolid medium used for the isolation and cultivation of *Leptospira*. Stuart's medium is the base to which multiple modifications for optimization of the recovery of *Leptospira* have been made since the original description. Bovine albumin and Tween provide lipids and long-chain fatty acids. B vitamins and ammonium ion provide essential vitamins and a nitrogen source. Lysed erythrocytes provide other essential supplements, such as iron. The medium is made selective by adding 5-fluorouracil either alone or with fosfomycin and nalidixic acid (21). This medium is available as a dehydrated medium base and supplement from BD Biosciences.

■ Enterococcosel agar

See Bile esculin azide agar and broth.

■ Eosin-methylene blue (EMB) agar

EMB agar is a selective and differential medium used for the isolation and differentiation of enteric pathogens from contaminated clinical specimens. Pancreatic digest makes up the nutritive base. Eosin and methylene blue are the selective agents and inhibit gram-positive organisms. The sugars are lactose and, in certain modifications, sucrose. Organisms that ferment lactose bind to the dyes under acidic conditions and appear as blue-black colonies with a metallic sheen. Under less acidic conditions, other coliforms appear as mucoid and brown-pink colonies. Nonfermenters such as *Salmonella, Shigella,* and *Proteus* will appear amber or transparent (colorless). EMB agar should be stored in the dark because of the loss of support of growth when it is exposed to visible light.

■ ESP Culture System II

ESP Myco medium is used with ESP Culture System II (TREK Diagnostic Systems) for the detection of mycobacterial growth. The medium is a Middlebrook 7H9 broth enriched with glycerol, Casitone, and cellulose sponge disks. OADC enrichment is added prior to use.

■ ETGPA

See Baird-Parker agar base.

■ Fastidious anaerobic agar (*Fusobacterium* selective agar)

Fastidious anaerobic agar is an enriched sheep blood medium used for the isolation and cultivation of anaerobic organisms. Peptone, glucose, agar, and starch make up the solid base. Sheep blood, vitamin K, and hemin are enrichments for anaerobes. A greater amount of hemin is used in fastidious anaerobic agar than in other anaerobic media. This medium is used for the isolation of *Fusobacterium* spp. and formate-fumarate-requiring species.

■ Fletcher's medium

Fletcher's medium is an enriched semisolid medium used for the isolation and growth of *Leptospira*. The medium is a peptone and beef extract base with a 1.5% agar concentration. Rabbit serum supplies long-chain fatty acids and albumin and has been found to be superior to other serum sources for the isolation of *Leptospira*. 5-Fluorouracil may be added to select for *Leptospira*. Cultures should be incubated in air and at 28 to 30°C. See Ellinghausen-McCullough/Johnson-Harris medium.

■ FlexTrans viral and chlamydia transport medium

FlexTrans viral and chlamydia transport medium (Trinity Biotech) is intended to be used as a transport medium for viruses and/or chlamydiae. The medium consists of minimal essential medium, bovine serum albumin, glutamine, and sucrose, with a phenol red indicator. Microbial growth is inhibited by the incorporation of the antibiotics amphotericin B, gentamicin, and streptomycin.

■ GC agar base

GC agar base is a chocolate agar base used with various additives for the purpose of isolating *Neisseria gonorrhoeae* and other fastidious organisms, such as *Haemophilus* spp., including *Haemophilus ducreyi*, and for susceptibility testing of *N. gonorrhoeae*. GC agar base includes digest of casein, animal peptones, cornstarch, NaCl, and buffers. A 1% defined growth supplement with yeast and X (hemin) and V (NAD) factors is added. The supplement contains a low concentration of cysteine to avoid activation of various beta-lactam antibiotics such as penems, carbapenems, and clavulanic acid. GC agar base with 5% fetal bovine serum and 10% CVA enrichment allows for isolation of *H. ducreyi* (53).

■ GN broth

GN broth is an enriched selective broth medium used for the isolation of gram-negative rods. Specifically, *Salmonella* and *Shigella* are isolated more effectively in GN broth than on solid medium alone. The nutritive base includes casein and meat peptones as well as mannitol and dextrose. The concentration of mannitol limits the growth of some other contaminating enteric organisms. Sodium deoxycholate and sodium citrate inhibit gram-positive and some gram-negative organisms. Enteric organisms do not overgrow the pathogens in the first 6 h of incubation, at which time the broth should be subcultured.

■ Haemophilus test medium (HTM) and broth

HTM is an enriched medium used for susceptibility testing of *Haemophilus* species. The medium contains beef and casein

extracts. Yeast extract, hematin, and nicotinamide (NAD) provide necessary growth factors and enrichments. Antagonists to sulfonamides and trimethoprim are removed by thymidine phosphorylase. The calcium and magnesium concentrations are adjusted to the concentrations recommended by the CLSI. Although the agar medium is a clear agar base and should exhibit clear endpoint interpretations, some investigators have reported difficulties with interpreting zone sizes and poor growth with some strains. Broth microdilution methods also use HTM, which consists of cation-adjusted Mueller-Hinton broth and the supplements described above. Broth dilution methods with HTM provide clearer endpoints. See chapter 75 for specific formulations and references.

■ Heart infusion agar and broth

Heart infusion agar and broth are general-purpose media used for the isolation of a variety of microorganisms. Heart muscle infusion, casein peptones, and yeast extract are the nutritive base. Fastidious organisms do not grow well on or in this medium because no additional enrichments or sheep blood is incorporated. Incorporation with 5% rabbit blood allows detection of the more fastidious *Actinomyces.*

■ Hektoen enteric agar

Hektoen enteric agar is a selective and differential medium used for the isolation and differentiation of enteric pathogens from contaminated clinical specimens. Animal peptones and yeast extract provide the nutritive base. Bile salts and the indicator dyes (bromthymol blue and acid fuchsin) in the medium are the selective agents and inhibit gram-positive organisms. Lactose, sucrose, and salicin are the carbohydrates incorporated to differentiate fermenters from nonfermenters. In addition, differentiation of species occurs with the use of sodium thiosulfate and ferric ammonium citrate, which allow detection of hydrogen sulfide production. Organisms that produce hydrogen sulfide appear with the formation of a black precipitate on the colony. Fermenters such as *E. coli* produce colonies which are yellow-pink, *Shigella* spp. are green or transparent, and *Salmonella* spp. are green or transparent with black centers.

■ Hemin-supplemented egg yolk agar

See Neomycin egg yolk agar.

■ Isolator or lysis-centrifugation tube (Wampole Laboratories)

The Isolator is a unique system for the purpose of recovering organisms from blood through a simultaneous process of lysis and centrifugation. The tubes contain saponin, a lysing agent, and EDTA, an anticoagulant as well as a fluorocarbon that acts as a cushioning agent during centrifugation. The system creates a layer that is subsequently plated onto media appropriate for organism recovery. The system is especially good for the recovery of dimorphic fungi, yeasts, mycobacteria, and *Bartonella* spp. Recovery of anaerobes, *Haemophilus* spp., and pneumococci may be reduced.

■ Iso-Sensitest agar and broth

Iso-Sensitest agar and broth are media used for susceptibility testing in countries outside the United States. The base includes hydrolyzed casein, peptones, and glucose, with other additives. The medium is available as a premixed powder from Oxoid Unipath. See chapter 70 for specific uses.

■ John E. Martin Biological Enrichment Chamber (JEMBEC) (BBL) and InTray GC System transport medium (Biotest)

The JEMBEC and InTray GC devices are transport/inoculation media for direct plating of specimens for the detection of *N. gonorrhoeae.* JEMBEC contains GC-Lect agar, and InTray GC contains modified Thayer-Martin medium, both of which are a chocolate agar base. Each system is self-contained with a tablet that allows production of a CO_2 atmosphere and enhances recovery of the pathogen. The InTray GC system can be stored and transported at room temperature. GC-Lect helps to specifically inhibit *Capnocytophaga* spp. See chapter 39 for specific antibiotic components for various media used for the purpose of isolating *Neisseria* spp.

■ Kanamycin-vancomycin laked sheep blood agar

Kanamycin-vancomycin laked sheep blood agar is an enriched, selective, and differential medium used for the isolation and cultivation of anaerobic bacteria, especially slowly growing and fastidious anaerobes from clinical specimens, such as *Bacteroides* spp. and *Prevotella* spp. The base is CDC anaerobic blood agar. The selective agents are kanamycin and vancomycin (7.5 μg/ml) to prevent growth of obligate facultative gram-negative and gram-positive bacteria and facultative anaerobic bacteria, respectively. Use of a medium with 2 μg of vancomycin per ml allows better growth of *Porphyromonas* spp. Laked blood is used to allow optimal pigmentation of anaerobes such as the pigmented *Prevotella* spp.

■ Lactobacillus MRS broth

Lactobacillus MRS (deMan, Rogosa, and Sharpe) broth is a nonselective liquid medium used for the isolation and cultivation of lactobacilli from clinical specimens and dairy and food products. The nutritive base includes peptones, yeast extract with buffers, and glucose. Polysorbate 80 (Tween 80) supplies fatty acids and magnesium for additional growth requirements. Sodium acetate and ammonium citrate may inhibit normal flora, including gram-negative bacteria, oral flora, and fungi, and improve the growth of lactobacilli. The growth of lactobacilli is favored when the pH is adjusted to 6.1 to 6.6. Gas production can help identify *Leuconostoc* from *Pediococcus* in conjunction with arginine degradation to differentiate these organisms from *Lactobacillus* (16). See chapter 31.

■ Levinthal agar with bacitracin and *H. influenzae* antiserum

See Chocolate agar.

■ Lim broth

Lim broth is a modification of Todd-Hewitt broth and is an enriched selective liquid medium used for the isolation and cultivation of *Streptococcus agalactiae*. Peptones, salts, and dextrose provide the nutritive base. Yeast extract provides B vitamins and additional enrichment. The antibiotics colistin and nalidixic acid inhibit gram-negative organisms. Lim broth has shown better recovery of GBS than Todd-Hewitt broth with gentamicin and nalidixic acid (22). See also StrepB carrot broth and GBS broth.

■ Lithium chloride-phenylethanol-moxalactam agar

Lithium chloride-phenylethanol-moxalactam agar is an enriched and selective agar used for the isolation and cultivation of *Listeria monocytogenes*. Peptones and beef extract are the nutritive base. Phenylethyl alcohol, glycine anhydride, and lithium chloride suppress the growth of gram-positive and gram-negative organisms. Moxalactam makes the agar more selective by inhibiting gram-negative organisms such as *Pseudomonas* and additional gram-positive organisms. Enrichment of *Listeria* in a broth for 24 h is subsequently subcultured onto the selective medium for isolation when trying to isolate the organisms from contaminated sites. The medium is not differential, but use of oblique lighting may show *Listeria* colonies to be blue, while other colonies appear yellow or orange (14, 46, 67).

■ Loeffler's medium

Loeffler's medium is an enriched nonselective medium used for the cultivation of corynebacteria, especially *C. diphtheriae*. The nutritive base is heart infusion and peptones with dextrose. Horse serum and egg cause the medium to coagulate during sterilization and provide other nutritive proteins. The medium enhances the production of metachromatic granules within the cells of the organisms. These granules are seen when smears of the organism are viewed with the methylene blue stain.

■ Lombard-Dowell egg yolk agar

See Neomycin egg yolk agar.

■ Lowenstein-Jensen medium

Lowenstein-Jensen medium is an enriched nonselective medium used for the isolation and cultivation of mycobacteria. It is similar to American Trudeau Society medium in its content and its ability to grow mycobacteria. Lowenstein-Jensen medium is an egg-based medium with glycerol and potato flour and, as with all egg-based media, is susceptible to liquefying when specimens are contaminated with other bacteria. The concentration of malachite green is twice that in American Trudeau Society medium, and thus, the malachite green is somewhat more inhibitory for contaminating organisms. Inorganic salts may make the medium more enriched for mycobacteria.

■ Lowenstein-Jensen medium (Gruft modification)

The Gruft modification of Lowenstein-Jensen medium is an enriched selective medium used for the isolation of mycobacteria. Penicillin and nalidixic acid are added to the medium and inhibit gram-positive and gram-negative organisms, respectively. RNA is added as a growth stimulant.

■ Lowenstein-Jensen medium (Mycobactosel modification)

The Mycobactosel modification of Lowenstein-Jensen medium is an enriched selective medium for the isolation of mycobacteria. Different antibiotics from the Gruft modification are added to make the medium more selective against bacteria. Cycloheximide, lincomycin, and nalidixic acid inhibit saprophytic fungi, gram-positive organisms, and gram-negative organisms, respectively. No RNA is added.

■ Lowenstein-Jensen medium with 1% ferric ammonium citrate

Lowenstein-Jensen medium with 1% ferric ammonium citrate is an enriched and selective egg-based medium used for the recovery of *Mycobacterium haemophilum*. Ferric ammonium citrate is the additive which allows this organism to grow.

■ Lowenstein-Jensen medium with 5% NaCl

Lowenstein-Jensen medium with 5% NaCl is an enriched selective medium used to differentiate sodium chloride-tolerant strains of *Mycobacterium*. Most rapid growers, e.g., the *Mycobacterium fortuitum* complex, as well as the more slowly growing organism *Mycobacterium triviale*, will grow on this medium. The exception is the more resistant organism *Mycobacterium chelonae*, which will not grow on this medium.

■ Lysis-centrifugation tube

See Isolator.

■ MacConkey agar

MacConkey agar is a selective and differential medium used for the isolation of gram-negative organisms. The nutritive base includes a variety of peptones. The medium is made selective by the incorporation of bile (although at levels less than those used in other enteric media) and crystal violet, which inhibit gram-positive organisms, especially enterococci and staphylococci. An agar concentration greater than that described in the original reference helps to inhibit swarming *Proteus*. The medium is made differential by use of the combination of neutral red and lactose. When an organism ferments lactose, the drop in pH causes the colony to take on a pink-red appearance.

■ MacConkey agar with sorbitol (SMAC)

SMAC is a selective and differential medium used for the isolation and differentiation of sorbitol-negative *E. coli.* Shiga toxin-producing strains of *E. coli,* such as *E. coli* O157:H7, which may cause hemorrhagic colitis, are indistinguishable from other *E. coli* serotypes on routine stool isolation media such as MacConkey agar because they all ferment lactose. SMAC has D-sorbitol instead of the lactose in the MacConkey agar formulation. Shiga toxin-producing strains of *E. coli* do not ferment sorbitol and appear as colorless colonies. Sorbitol-fermenting strains are pink. The medium inhibits enterococci with crystal violet and other gram-positive organisms with bile salts. SMAC with cefixime and tellurite may be more selective for detection of *E. coli*. However, some strains may be inhibited. SMAC does not identify all Shiga toxin-producing strains of *E. coli.*

■ MacConkey broth

MacConkey broth is a differential medium containing the indicator bromcresol purple used for the detection of coliform organisms from contaminated food, water, or stools. The broth contains peptone, lactose, bile salts, and sodium chloride. Bromcresol purple is less inhibitory than neutral red for coliforms. The color change from purple to yellow is a more sensitive and definitive indication of acid formation.

■ Mannitol-egg yolk-polymyxin B agar

Mannitol-egg yolk-polymyxin B agar is an enriched, selective, and differential medium used for the isolation of *B. cereus* from mixed clinical specimens. The nutritive base includes peptone and beef extract. Egg yolk emulsion is added for the detection of lecithinase activity, which is usually limited to *B. cereus*. Phenol red and mannitol are combined to make the medium differential. Contaminating gram-negative organisms are inhibited by the polymyxin B.

■ Mannitol salt agar

Mannitol salt agar is a selective and differential medium used for the isolation of *S. aureus*. The nutritive base includes peptones, beef extract, and mannitol. Phenol red is the indicator. The selective nature of the medium is the high salt content (7.5% NaCl), which inhibits most organisms except staphylococci. The differential component for identification of *S. aureus* is the combination of mannitol and phenol red. The color change around the colony from red to yellow upon the fermentation of mannitol and the subsequent drop in the pH of the medium identify staphylococci.

■ Martin-Lewis agar

Martin-Lewis agar is an enriched and selective medium for the isolation of *N. gonorrhoeae.* Martin-Lewis agar is a modification of the modified Thayer-Martin formulation. The nutritive base is chocolate agar. The specific differences from the modified Thayer-Martin formulation are the use of a greater concentration of vancomycin (4.0 versus 3.0 μg/ml), which inhibits more gram-positive organisms, and the replacement of nystatin with anisomycin, which improves the inhibition of *Candida* species. Trimethoprim and colistin are incorporated as well for inhibition of other commensal organisms. Some strains of pathogenic *Neisseria* have been reported to be inhibited by vancomycin and trimethoprim. See also JEMBEC and Thayer-Martin agar. Chapter 39, Table 3, lists antimicrobial agent amounts for each *Neisseria* selective medium.

■ MB/BacT ALERT (bioMèrieux)

MB/BacT ALERT contains a modified Middlebrook 7H9 medium supplemented with casein, bovine serum albumin, and catalase. It is used with MB/BacT ALERT 3D (bioMérieux, Inc.) for the cultivation and detection of mycobacterial growth.

■ MGIT (mycobacteria growth indicator tube) (BD Diagnostic Systems)

MGIT is a Middlebrook 7H9-based broth system that contains a fluorescence indicator, which is used for the detection of mycobacterial growth. The 7H9 broth is supplemented with multiple growth enrichments prior to use. Chapter 36, on *Mycobacterium,* describes and compares this system with the BACTEC system in more detail.

■ Middlebrook 7H10 agar

Middlebrook 7H10 agar is an enriched nonselective agar-based medium used for the isolation and cultivation of mycobacterial species. Essential ingredients include inorganic salts, glycerol, and OADC enrichment. The OADC enrichment includes oleic acid, which is a fatty acid used in the metabolism of mycobacteria; albumin, which protects against toxic agents and which is a source of protein; dextrose, which is used as a source of energy; and catalase, which destroys toxic peroxides in the medium. This is the recommended medium for mycobacterial susceptibility testing. However, because standard formulations of the agar and especially the OADC supplement may vary, quality control is critical. Middlebrook medium allows visualization of mycobacterial colonies 1 to 2 weeks earlier than Lowenstein-Jensen formulations.

■ Middlebrook 7H11 agar

Middlebrook 7H11 agar is a nonselective agar-based medium used for the isolation and cultivation of *Mycobacterium* species. The formulation is identical to that of Middlebrook 7H10 medium except for the addition of casein hydrolysate. Casein hydrolysate is added as a growth stimulant for drug-resistant strains of *M. tuberculosis*. The formulation of Middlebrook 7H11 thin-pour agar is identical to that of Middlebrook 7H11 agar except that the agar plate has a reduced volume. The plates are sealed and every 2 days are examined along the isolation streak lines for evidence of microcolonies. This technique allows for faster detection on solid medium than in standard tube media or on thick media on plates.

■ Middlebrook 7H9 broth with glycerol

Middlebrook 7H9 broth with glycerol is an enriched nonselective broth for the isolation of *Mycobacterium* species. Glycerol, inorganic compounds, and cations supply essential nutrients and stimulate growth. ADC enrichment is added to the broth. ADC enrichment includes albumin, which binds to free fatty acids that are toxic to *Mycobacterium* species; dextrose, which supplies energy; and catalase, which destroys toxic peroxides that may be present in the medium.

■ Mitchison 7H11 selective agar

Mitchison 7H11 selective agar is an enriched selective agar-based medium used for the isolation of *Mycobacterium* species. The basic formulation is Middlebrook 7H11 agar: glycerol, inorganic salts, casein hydrolysate, malachite green, and OADC enrichment. Antibiotics are added to make the medium very selective for mycobacteria. Carbenicillin and polymyxin B, amphotericin B, and trimethoprim are active against most members of the family *Enterobacteriaceae*, yeasts, and *Proteus* species, respectively.

■ Modified Irgasan-ticarcillin-potassium chromate broth

Modified Irgasan-ticarcillin-potassium chromate broth is a selective broth used for the isolation of *Y. enterocolitica*. The base is the modified Rappaport-Vassiliadis enrichment broth with minor alterations. Irgasan and ticarcillin replace the carbenicillin. The chromate makes the medium more selective by inhibiting members of the family *Enterobacteriaceae. Enterobacteriaceae* have A nitrase activity, which splits chlorate to toxic by-products. *Yersinia* spp. have B nitrase activity, which cannot split the chlorate.

■ Modified Thayer-Martin agar

Modified Thayer-Martin agar is an enriched and selective agar for the isolation of pathogenic *Neisseria* species from

clinical specimens with mixed flora. The nutritive base is chocolate agar. Modified Thayer-Martin agar has three significant changes from the original Thayer-Martin medium. The medium has less agar and less dextrose, and these characteristics improve the growth of *Neisseria*. The third change is the addition of trimethoprim, which inhibits swarming *Proteus* spp. Vancomycin and colistin inhibit gram-positive and gram-negative bacteria, respectively. This medium is recommended over the original formulation for the isolation of pathogenic *Neisseria*. Some strains of pathogenic *Neisseria* have been reported to be inhibited by vancomycin and trimethoprim. See also Martin-Lewis agar.

■ Mueller-Hinton agar with and without 5% sheep blood

Mueller-Hinton agar is the agar recommended by the CLSI for routine susceptibility testing of nonfastidious microorganisms by the Kirby-Bauer disk diffusion susceptibility method. Mueller-Hinton agar with 5% sheep blood is used for susceptibility testing of *S. pneumoniae*. Beef and casein extracts and soluble starch in an agar base make up the nutritive base of the medium. Starch protects the organism from toxic materials that may be in the medium. Calcium and magnesium concentrations are controlled. Mueller-Hinton agar with 5% chocolatized blood plus 1% IsoVitaleX and 3 μg of vancomycin per ml is used for the isolation of *H. ducreyi.* See GC agar and chapter 41.

■ Mueller-Hinton agar with 2% NaCl

Mueller-Hinton agar with 2% NaCl is a selective medium used for testing the susceptibility of *Staphylococcus* to the penicillinase-resistant penicillins methicillin, nafcillin, and oxacillin by agar dilution or with the gradient-based system (E test). The sodium chloride added to the medium enhances the growth of staphylococci. Heteroresistant methicillin-resistant strains are more easily detected with this medium by increasing the incubation time to 24 h and by incubation at cooler temperatures (30 to 35°C).

■ Mueller-Hinton agar with 4% NaCl and 6 μg of oxacillin per ml

Mueller-Hinton agar with 4% NaCl and 6 μg of oxacillin per ml is the selective, differential medium used to screen *S. aureus* (not coagulase-negative staphylococci) for resistance to penicillinase-resistant penicillins (e.g., nafcillin, methicillin, and oxacillin). A sample from overnight growth on nonselective medium is used. Incubation for a full 24 h at 35°C in ambient air is recommended before interpretation of growth.

■ Mueller-Hinton broth

Mueller-Hinton broth is a magnesium and calcium cation-adjusted liquid medium used in procedures for susceptibility testing of aerobic gram-positive and gram-negative organisms by both macrodilution and microdilution methods. The nutritive base includes beef extract and peptones. Starch is a detoxifying agent.

■ Multiprobe media (M4-3, M5, and M4-RT) (Remel)

M4-3 contains vancomycin, amphotericin B, and colistin and is suitable for transport of viruses, chlamydiae, *Mycoplasma,* and *Ureaplasma*. M5 is similar to M4-3, but it does not contain gelatin. M5 is suitable for transport of viruses, chlamydiae, *Mycoplasma,* and *Ureaplasma*. M4-RT contains gentamicin and amphotericin B and is suitable only for transport of viruses and chlamydiae. All the media are supplemented Hanks' balanced salt solution buffered with HEPES buffer, with phenol red as the pH indicator. The antibiotics are added to inhibit bacterial organisms, and therefore, the medium cannot be used for bacterial culture.

■ Mycobactosel agar

Mycobactosel is a BBL trade name for an enriched selective agar-based medium used for isolation of *Mycobacterium* species. The medium is called by other names, depending on the manufacturer. The basic formulation is a Middlebrook 7H11 base, glycerol, inorganic salts, casein hydrolysate, malachite green, and OADC enrichment. Antibiotics are added to make the medium selective. The principle used for the Middlebrook 7H11 formulation to which antibiotics are added is the same as that used for Mitchison 7H11 medium. The antibiotics differ between Mycobactosel agar and Mitchison 7H11 medium. The antibiotics in Mycobactosel agar are cycloheximide, lincomycin, and nalidixic acid, which inhibit saprophytic fungi, gram-positive organisms, and gram-negative organisms, respectively.

■ MycoTrim GU and MycoTrim RS (Irvine Scientific)

The MycoTrim GU and MycoTrim RS culture systems are unique triphasic flask systems specifically designed for the isolation and identification of *M. hominis, U. urealyticum* (GU), and *Mycoplasma pneumoniae* (RS). The flask systems contain both agar and an enriched broth formulated for growth of these organisms that contain PPLO broth, containing meat digests, peptones, beef extracts, glucose, arginine, urea, horse serum, yeast extract, phenol red indicator, calcium, phosphate, and agar at a concentration that allows spreading growth of the colonies. The specimen is inoculated into the flask, and antibiotic disks (cefoperazone and nystatin) are added to prevent bacterial contamination. The system is incubated agar side up. Growth of the organism results in a pH change and is indicated by a color change in the media from red to orange and/or yellow. If no color change occurs in the first 24 h, the agar surface is reinoculated with the growth media. Because mycoplasmas do not cause turbidity, the color change is crucial. Colonial growth is confirmed by visualizing colonies using a light microscope and the 10× objective. The color change and colony morphology, including size and appearance, distinguish between *M. hominis, U. urealyticum,* and *M. pneumoniae*.

■ NAG medium

NAG medium is an enriched and selective medium used for the isolation and cultivation of *Haemophilus* species from clinical specimens with mixed flora. The agar base is blood agar with *N*-acetyl-D-glucosamine (NAG), hemin, and NAD. NAG medium allows spheroblastic *H. influenzae* to revert morphologically. Spheroblastic forms may be seen in specimens from patients receiving beta-lactam antibiotics. Bacitracin makes the medium selective by inhibiting gram-positive organisms that occur as normal respiratory flora. This medium has been found to be especially helpful in isolating *H. influenzae* from respiratory specimens from cystic fibrosis patients. Placement of cefsulodin disks on the primary streak helps to inhibit *Pseudomonas* spp. to make the medium more selective.

■ Neomycin egg yolk agar

Neomycin egg yolk agar is a selective and differential medium used for the differentiation of anaerobic organisms that are lipase positive, including *Clostridium* spp., *Prevotella intermedia, Fusobacterium necrophorum*, and some strains of *Prevotella loescheii*. The nutritive base includes peptones and yeast extract. Vitamin K and L-cystine make the medium optimal for the isolation of anaerobes. Egg emulsion adds enrichment and makes the medium differential by detecting lipase activity. Neomycin makes the medium selective by inhibiting both gram-positive and gram-negative organisms and differential by the fermentation of lactose.

■ Neomycin-vancomycin agar

Neomycin-vancomycin agar is an enriched and selective medium that is particularly good for the isolation and cultivation of *Fusobacterium* from clinical specimens. The nutritive base is fastidious anaerobe agar with 5% sheep blood. The selective agents include neomycin and vancomycin, which inhibit gram-negative and gram-positive organisms, respectively.

■ New York City medium

New York City medium is an enriched and selective medium for the isolation of pathogenic *Neisseria* from clinical specimens. It also supports the growth of large-colony mycoplasmas and *U. urealyticum*. The medium is a clear peptone-cornstarch agar base with lysed horse erythrocytes, horse plasma, and yeast dialysate, which are used instead of the hemoglobin and the supplements used for the other enriched and selective media for *Neisseria*. The antibiotics that make the medium selective include vancomycin, colistin, and amphotericin B, which inhibit gram-positive bacteria, gram-negative bacteria, and fungi, respectively. While human blood products can replace the horse blood products, sheep blood cannot be used.

■ Nucleic acid transport (NAT) (Medical Packaging Corporation)

NAT is a nucleic acid transport device that is FDA cleared for use with multiple amplification and hybridization testing formats.

■ Oxford agar

Oxford agar is an enriched and selective medium used for the isolation of *L. monocytogenes.* Columbia agar is the base, and it is supplemented with esculin and ferric ammonium citrate for the detection of esculin hydrolysis by listeriae. Suppression of contaminants is accomplished by the addition of lithium, cycloheximide, colistin, acriflavine, cefotetan, and fosfomycin. A modified Oxford agar replaces cycloheximide, acriflavine, cefotetan, and fosfomycin with moxalactam. *Listeria* spp. appear black with a black halo.

■ Oxidative-fermentative polymyxin B-bacitracin-lactose agar

Oxidative-fermentative polymyxin B-bacitracin-lactose medium is a selective and differential medium used for the isolation of *B. cepacia* from respiratory specimens from patients with cystic fibrosis. The nutritive base is an oxidative-fermentative medium with peptones. When acid is produced from the utilization of the lactose sugar, as occurs with *B. cepacia*, the bromthymol blue indicator changes the colony from green to yellow. Polymyxin B and bacitracin are the selective agents and inhibit some gram-negative and gram-positive organisms, respectively. Other organisms seen in cystic fibrosis patients may grow on this medium and are differentiated by the inability to produce acid from lactose. See also *Burkholderia cepacia* selective agar and *Pseudomonas cepacia* (PC) agar.

■ P agar

P agar is an enriched medium used for cultivation and isolation of staphylococci. The agar base includes peptone, yeast extract, NaCl, and glucose.

■ Peptone yeast extract broth

Peptone yeast extract broth is used in the analysis of metabolic products by gas-liquid chromatography because there is negligible acid volatility within the medium.

■ Petragnani medium

Petragnani medium is an egg-based medium. It contains more than twice the concentration of malachite green in Lowenstein-Jensen medium and is most commonly used for the isolation and cultivation of mycobacteria from heavily contaminated specimens. It is also used for the cultivation and maintenance of *Mycobacterium smegmatis*.

■ Phenylethyl alcohol agar

Phenylethyl alcohol agar is an enriched and selective blood agar medium used for the detection and isolation of anaerobic organisms, particularly fastidious and slowly growing bacteria, from clinical specimens with mixed flora. The base is Trypticase soy agar with yeast extract, vitamin K, cystine, and hemin. The medium is selective as a result of the incorporation of phenylethyl alcohol, which reversibly inhibits DNA synthesis and thus inhibits facultative anaerobic gram-negative bacteria, such as members of the family *Enterobacteriaceae.* Phenylethyl alcohol agar inhibits swarming by *Proteus* spp. and *Clostridium septicum.*

■ PLM-5 TM

PLM-5 TM is a proprietary medium formulation (Intergen Co., Purchase, N.Y.) similar to Ellinghausen-McCullough/Johnson-Harris medium that is used for the isolation and cultivation of *Leptospira*. It has less agar and 5-fluorouracil.

■ Polymyxin B-acriflavine-lithium chloride-ceftazidime-esculin-mannitol (PALCAM) agar

PALCAM agar is an enriched, differential, and selective agar medium used for the isolation of *L. monocytogenes*. Columbia agar supplemented with glucose, mannitol, and yeast extract is the nutritive base. Esculin and ferric ammonium citrate are added to detect esculin hydrolysis by listeriae. Fermentation of mannitol is detected with the indicator dye phenol red. Lithium, acriflavine, ceftazidime, and polymyxin B are added as selective agents. For contaminated specimens an enriched broth is inoculated and incubated for 24 h. Subsequently 0.1 ml is subcultured onto PALCAM agar. Listeria colonies appear gray-green with a sunken black center.

■ Polymyxin B-lysozyme-EDTA-thallous acetate agar

Polymyxin B-lysozyme-EDTA-thallous acetate agar is a selective agar used for the isolation of *B. anthracis* from environmental specimens. Heart infusion agar is the base. Thallous acetate and EDTA are additional additives speculated to have advantages for the recovery of *B. anthracis*. Lysozyme is an additive which inhibits *Bacillus* spp. other than *B. cereus* and *B. anthracis*. The addition of thallous acetate, EDTA, and lysozyme together has an additive effect which results in the inhibition of most non-*B. anthracis* species. Polymyxin B inhibits gram-negative organisms. Colonies of *B. anthracis* grown on polymyxin B-lysozyme-EDTA-thallous acetate agar are smaller and smoother than those grown on plain heart infusion agar and are creamy white with a ground-glass texture. *B. cereus* is usually inhibited (43).

■ Polymyxin B-pyruvate-egg yolk-mannitol-bromthymol blue agar

Polymyxin B-pyruvate-egg yolk-mannitol-bromthymol blue agar is an enriched, selective, and differential medium used for the isolation of *B. cereus*. The nutritive agar base includes peptones, agar, and buffers. Egg yolk emulsion allows detection of lecithinase activity, which is unique to *B. cereus*. Sodium pyruvate is added to reduce the size of the colonies, which may be important when performing plate counts. Bromthymol blue and mannitol combine to make the medium differential. *B. cereus* does not produce acid from mannitol and has a distinctive bright blue color. Polymyxin B inhibits contaminating gram-negative organisms from clinical specimens with mixed flora, such as stool specimens. Mannitol-egg yolk-polymyxin B agar and *B. cereus* medium are similarly used for the isolation of *B. cereus*.

■ Polysorbate 80 medium

See Ellinghausen-McCullough/Johnson-Harris medium.

■ PRAS media

Prereduced anaerobically sterilized (PRAS) media are specifically manufactured (Anaerobe Systems) and packaged to eliminate oxygen to enhance growth of anaerobic organisms. Systems come with and without swabs and are available in various collection formats: tubes, widemouthed collection containers for surgical specimens, and prepackaged plates.

■ *Pseudomonas cepacia* (PC) agar

PC agar is a selective medium used for the isolation of *B. cepacia* (formerly *Pseudomonas cepacia*) from respiratory specimens from cystic fibrosis patients. The medium was originally derived from a holding medium containing salts, phenol red, and agar in a phosphate buffer. Selective agents include crystal violet, ticarcillin, and polymyxin B, which inhibit many gram-positive and gram-negative organisms. PC agar may inhibit *B. cepacia* as well (30, 35, 69). See also *Burkholderia cepacia* selective agar and oxidative-fermentative polymyxin B-bacitracin-lactose agar.

■ Rambach agar

See CHROMagar.

■ Rappaport-Vassiliadis enrichment broth

Rappaport-Vassiliadis enrichment broth is a selective and enriched broth used for the isolation and cultivation of *Salmonella* spp. from food and environmental specimens. A modified Rappaport-Vassiliadis broth is a more selective broth used for the isolation and cultivation of *Y. enterocolitica* from foods. Basic Rappaport-Vassiliadis medium contains soybean peptone digest with salts and malachite green. Malachite green suppresses the growth of contaminating bacteria. The modified Rappaport-Vassiliadis broth uses pancreatic digest of casein with salts, malachite green, and carbenicillin.

■ Regan-Lowe medium

Regan-Lowe medium is an enriched and selective medium used for the isolation of *B. pertussis*. Beef extract pancreatic digest, horse blood, and niacin are the nutritional base. Starch and charcoal neutralize substances, such as fatty acids and peroxides, that are toxic to *Bordetella*. Cephalexin is added to inhibit the normal flora in the nasopharynx. Regan-Lowe transport medium contains half-strength charcoal and horse blood, provides better isolation of *Bordetella* than Bordet-Gengou medium, and has a longer shelf life.

■ Salmonella-shigella agar

Salmonella-shigella agar is a selective and differential medium used for the isolation and differentiation of *Salmonella* and *Shigella* from clinical specimens and other sources. The nutritive base contains animal and casein peptones and beef extract. The selective agents are bile salts, citrates, and brilliant green dye, which inhibit gram-positive organisms. The high degree of selectivity of the medium inhibits some strains of *Shigella*, and the medium is not recommended as a primary medium for isolation of this species. The medium contains only lactose and thus differentiates organisms on the basis of lactose fermentation. The formation of acid on fermentation of lactose causes the neutral red indicator to make red colonies. Non-lactose-fermenting organisms are clear on the medium. As with Hektoen enteric agar, sodium thiosulfate and ferric ammonium citrate allow the differentiation of organisms that produce hydrogen sulfide. Lactose fermenters, such as *E. coli*, have colonies which are pink with a precipitate, *Shigella* appears transparent or amber, and *Salmonella* appears transparent or amber with black centers. Some strains of *Shigella dysenteriae* are inhibited.

■ Schaedler's agar

Schaedler's agar is a general-purpose medium used for the isolation and cultivation of anaerobic bacteria. The nutritive base includes vegetable and meat peptones, dextrose, and yeast extract. Sheep blood, vitamin K, and hemin provide other additives that stimulate the growth of fastidious anaerobes. Because of the high carbohydrate content, colonies with beta-hemolytic reactions may have a greenish hue. This medium may be better than other nonselective anaerobic media for the isolation of fastidious anaerobic organisms.

■ Schleifer-Kramer agar

Schleifer-Kramer agar is a selective medium used for the isolation of *Staphylococcus* from heavily contaminated specimens such as feces. The nutritional base includes casein

peptones with beef and yeast extracts, glycine, and sodium pyruvate. Sodium azide at 0.45% makes the medium selective for staphylococci and some other gram-positive organisms by inhibiting gram-negative organisms.

■ Selenite broth

Selenite broth is an enrichment broth medium used for the isolation of *Salmonella* and *Shigella* species. Casein and meat peptones provide nutrients. Selenite inhibits enterococci and coliforms that are part of the normal flora if the broth medium is subcultured within 12 to 18 h. However, reduction of selenite produces an alkaline condition that may also inhibit the recovery of *Salmonella*. Lactose and phosphate buffers are added to allow stability of the pH. When fermenting organisms produce acid, the acid neutralizes the effect of the selenite reduction and subsequent alkalinization. Cystine added to selenite broth enhances the recovery of *Salmonella*.

■ Sensitest agar

Sensitest agar is a medium used in susceptibility testing outside of the United States. The base is pancreatic digest of casein, peptones, and glucose with other additives. The medium is available as a premixed powder from Oxoid Unipath. See chapter 70 for specific uses.

■ Septic-Chek biphasic mycobacterial media

The Septic-Chek system (Becton Dickinson Microbiology Systems) is a mycobacterial culture system which contains modified 7H9 broth and three types of solid media, modified Lowenstein-Jensen, Middlebrook 7H11, and chocolate agars, with various supplements.

■ Skirrow medium

Skirrow medium is an enriched selective blood agar medium used for the isolation of *Campylobacter* spp. from specimens with mixed flora. The nutritive base is a blood-based agar. Hematin is provided by sheep blood. The selective agents are trimethoprim, vancomycin, and polymyxin B, which inhibit the normal flora found in fecal specimens.

■ STGG

STGG medium (skim milk, tryptone, glucose, and glycerin) is a transport medium that has been used for collection of nasopharyngeal swabs for the purposes of isolation and preservation of *S. pneumoniae*. Collection and storage of nasopharyngeal swabs on STGG at −70 or −20°C were shown to be equal to direct plating of nasopharyngeal swabs onto selective medium (57).

■ Storage media

See chapter 6.

■ StrepB carrot broth kit (Hardy Diagnostics) and GBS broth (Northeast Laboratory Services)

Carrot broth and GBS broth are media that allow for the detection of red, red-orange, or orange pigment production by beta-hemolytic *S. agalactiae* (GBS) due to the hemolytic reaction with substrates such as starch, protease, peptone, and serum. There is a direct genetic linkage identified with the hemolytic activity and pigment production, with about 95% of GBS strains demonstrating hemolysis. The medium is supplemented with growth-promoting components that are added in the form of a disk (antibiotics). Generation of a bright orange or red color occurs within 6 to 24 h. These media have shown to be very specific, with more rapid turnaround time to detection of GBS than subculture to Lim broth (32). Also see Lim broth.

■ Streptococcus selective agar

Streptococcus selective agar is a selective medium for detection of streptococci. The agar base is Columbia agar. Various antibiotic supplements have been used to make the medium selective for streptococci and reduce the numbers of gram-negative organisms. Colistin and oxolinic acid are one combination less detrimental to *Streptococcus* spp. (42).

■ Stuart's transport media with and without charcoal

Stuart's transport medium is an early transport medium first described in 1948. This medium uses glycerol phosphate to maintain the specimen as well as maintain the pH, agar, methylene blue as a redox indicator, and sodium thioglycolate to allow the survival of anaerobes. The glycerol phosphate has also been found to be used as an energy source by certain contaminants which may overgrow the desired pathogen. Charcoal may be added as a detoxifying agent.

■ Sucrose-phosphate-glutamate transport medium

Sucrose-phosphate-glutamate transport medium is used for the maintenance and transport of *Chlamydia* species and viruses. Sucrose and two buffer solutions are the base. Bovine serum and glutamic acid are additives. Glutamic acid is a stabilizing agent that is especially useful for enveloped viruses. The antibiotic combination may be the same as or slightly different from that in 2-sucrose-phosphate. Most commonly the antibiotic combination is vancomycin, streptomycin, and nystatin, which inhibit both gram-positive and gram-negative organisms, as well as yeasts.

■ 2-Sucrose-phosphate transport media

2-Sucrose-phosphate medium is used for transport of specimens for the purpose of culturing *Chlamydia trachomatis* and *Mycoplasma* spp. Sucrose (0.2 M) and two potassium phosphate buffers are the base. Fetal bovine serum allows *Chlamydia* to maintain infectivity, and nystatin and gentamicin are added to inhibit yeasts and bacteria. 4-Sucrose-phosphate agar and broth (Remel) with a higher concentration of sucrose are also used for the isolation of *Mycoplasma* spp.

■ Tetrathionate broth base

Tetrathionate broth base is an enriched liquid medium used for the isolation of *Salmonella* species in contaminated clinical specimens and other products. The nutritive base includes pancreatic digest of casein and peptic digest of animal tissue with sodium thiosulfate. Bile salts inhibit gram-positive organisms and tetrathionate, which is formed when an iodine-potassium iodide solution is added and which is inhibitory to other normal intestinal flora. Addition of brilliant green inhibits gram-positive and gram-negative organisms, including some *Salmonella* spp.

■ Thayer-Martin agar

Thayer-Martin agar is an enriched and selective medium used for the isolation of *Neisseria* from clinical specimens with mixed flora. The nutritive base is chocolate agar, which is a GC agar base with casein and meat peptones, cornstarch for the neutralization of fatty acids, and phosphate buffer for control of the pH. The chocolate agar occurs with the addition of hemoglobin, which provides hemin or X factor, and IsoVitaleX enrichment, which provides NAD, vitamins, and other nutrients, to improve the growth of pathogenic *Neisseria*. The medium is made selective by the addition of vancomycin, colistin, and nystatin, which inhibit the normal flora of gram-positive bacteria, gram-negative bacteria, and fungi, respectively. Some strains of pathogenic *Neisseria* have been reported to be inhibited by vancomycin and trimethoprim. See also Modified Thayer-Martin agar and Martin-Lewis agar.

■ Thioglycolate with hemin and vitamin K

Thioglycolate broth with hemin and vitamin K is an enriched liquid medium used to support the growth of microaerophilic and anaerobic organisms, including fastidious organisms. Casein and soy peptones supply the basic nutrients. Sodium thioglycolate and L-cystine are the reducing agents in the medium, while hemin and vitamin K are additional additives that allow more fastidious anaerobes to thrive. A small amount of agar helps to slow the diffusion of oxygen and is more suitable for anaerobic organisms.

■ Thiosulfate citrate bile salt sucrose (TCBS)

TCBS is a highly selective and differential medium for the recovery of *Vibrio* spp. except *Vibrio hollisae* and *Vibrio cincinnatiensis.* The medium is inhibitory to gram-positive organisms by incorporation of oxgall, a naturally occurring substance containing a mixture of bile salts and sodium cholate, a pure bile salt. Peptic and casein digests are the nutritive base, and sucrose is a fermentable carbohydrate for the metabolism of *Vibrio*. Sodium thiosulfate provides a sulfur source, and ferric citrate detects hydrogen sulfide production. Bromthymol blue and thymol blue are the pH indicators. Alkaline pH enhances the recovery of *Vibrio cholerae*. Color appearance on the agar is dependent on the species. See chapter 47.

■ Tinsdale agar

See Cystine-tellurite blood agar.

■ Todd-Hewitt broth with gentamicin and nalidixic acid

Todd-Hewitt broth is used for the isolation of beta-hemolytic streptococci from mixed flora, especially vaginal specimens for GBS. Beef heart infusion and peptone are the nutritive base, with dextrose as the energy source and sodium-based buffers to protect the hemolysin from inactivation. The antibiotics make the medium selective by inhibiting gram-negative rods.

■ Tryptic or Trypticase soy agar base with 5% sheep blood

Tryptic or Trypticase soy agar base with 5% sheep blood is a general-purpose medium used for the isolation of a wide variety of organisms. The medium contains soybean and casein peptones as the nutritive base. The addition of sheep blood enriches the medium, and the sheep blood allows the growth of more fastidious organisms by providing hemin (X factor). V factor (NAD) is inactivated by enzymes in the sheep blood and thus does not allow the growth of organisms that require the NAD additive, such as *H. influenzae*. The use of sheep blood provides an excellent means of interpretation of hemolytic reactions, especially those of *Streptococcus* spp.

■ Tryptic or Trypticase soy broth

Tryptic or Trypticase soy broth is a general-purpose clear liquid medium used for the cultivation of a wide variety of organisms. It is also recommended by the CLSI for preparation of an inoculum for Kirby-Bauer disk diffusion susceptibility testing and is the CLSI's choice as a sterility testing medium. The base includes digests of casein and soybean, with additional additives of glucose, sodium chloride to maintain osmotic equilibrium, and buffers. For the purpose of a blood culture medium, additional additives include carbon dioxide to enhance the growth of microorganisms and SPS, an anticoagulant, to inactivate blood components and aminoglycosides. Formulations with 6.5% NaCl exist for the purposes of differentiating enterococcal species or salt-tolerant streptococci. Fildes enrichment is added to cultivate fastidious organisms such as *Haemophilus* spp.

■ Trypticase soy agar with horse or rabbit blood

Trypticase soy agar with horse or rabbit blood medium is used for the isolation of *Haemophilus* species. The nutritive base is a combination of soy and casein peptones. The medium provides smaller but adequate amounts of X (hemin) and V (NAD) factors compared to the amounts in sheep blood and is used for the isolation of *Haemophilus* species. In addition, the medium with horse or rabbit blood allows determination of hemolytic reactions.

■ University of Vermont modified listeria enrichment broth

University of Vermont modified listeria enrichment broth is an enriched and selective liquid medium used for the isolation of *L. monocytogenes*. The nutritive base contains pancreatic digest of casein and animal tissue and beef and yeast extract and is supplemented with esculin, acriflavine, and nalidixic acid.

■ V agar

V agar is an enriched and selective medium used for the isolation of *G. vaginalis* from clinical specimens. The nutritive base is GC agar. The addition of 2% hemoglobin, 5% fetal bovine serum, and a supplement containing NAD enhances the recovery of the organism. Vancomycin (3 μg/ml) is added to the medium to inhibit contaminating bacteria. Many formulations with the GC agar base, the enrichments, and vancomycin exist.

■ VMGA III medium

VMGA III (viability medium, Göteborg, anaerobic) is a transport and collection medium specifically designed to maintain viability of mixed anaerobes from peridontal and endodontal sites. The medium is particularly useful for transport of paper

points that are placed in gingival crevices to soak up secretions. A total of 0.5 to 2.0 ml of prereduced, buffered salt suspension is placed into a tube of similar total volume (2 ml) with or without six to eight glass beads (0.1 to 1.5 mm in diameter). The beads aid in dispersing polysaccharide matrices that occur within gingival plaques and granular aggregates (39).

■ Wadowsky-Yee medium

See Buffered charcoal yeast extract (BCYE).

■ Wilkins-Chalgren broth and agar

Wilkins-Chalgren medium is recommended for susceptibility testing with anaerobic organisms. The medium contains specific nutrients that support the growth of anaerobes such as yeast extract, vitamin K, hemin, and arginine. The use of peptones allows a more standardized medium.

■ Xylose-lysine-desoxycholate agar

Xylose-lysine-desoxycholate agar is a selective and differential medium used for the isolation and differentiation of enteric pathogens from clinical specimens. The nutritive base includes carbohydrates and yeast extract. This medium is more supportive of fastidious enteric organisms such as *Shigella*. The selective agent is desoxycholate, which inhibits gram-positive organisms. Phenol red is the color indicator. As with Hektoen enteric and salmonella-shigella agars, ferric ammonium citrate (indicator) and sodium thiosulfate (sulfur source) allow identification of organisms that produce H_2S with the appearance of colonies with a black center. The medium contains xylose, which most enteric organisms ferment. The most important exception is *Shigella*, the colonies of which appear to be transparent or the color of the red media. The enteric organisms that contain the lysine decarboxylase enzyme utilize the lysine in the medium. For *Salmonella*, which contains the lysine enzyme, this reaction reverts the pH to an alkaline state and the colony appears to be transparent or red with a black center. The lactose and sucrose in the medium help to differentiate other enteric organisms. When other enteric organisms ferment these sugars, they maintain the pH at an acidic condition and the colonies appear yellow or yellow-red. A number of other similar media for isolation of enteric pathogens exist, including xylose-galactosidase medium, which is more specific for *Aeromonas* spp. See chapters 43 and 46, which give specific references for performance of the various xylose-containing media.

■ Yersinia selective agar

See Cefsulodin-Irgasan-novobiocin medium.

APPENDIX 1
Medium Additives

N-(2-Acetamido)-2-aminoethanesulfonic acid (ACES): allows optimal pH buffering capacity without inhibition of bacteria as seen with other inorganic buffers

Acriflavine: selective agent; suppresses gram-positive organisms

ADC enrichment: a supplement added to mycobacteriology media that includes albumin, dextrose, catalase, and sodium chloride; catalase destroys peroxides that may be in the medium

Agar used in broth medium (0.05 to 0.1%): used to reduce O_2 tension

Albumin: protects against toxic by-products in medium; binds free fatty acids

Antibiotics: one or many may be added to make a medium selective; inhibitory capacity may vary depending on the concentration used

Bicarbonate-citric acid pellet: used to generate CO_2 gas within closed environment after exposure to moistures; used in transport devices for isolating *Neisseria gonorrhoeae*

Bismuth sulfite: heavy metal that is inhibitory to commensal organisms

Carbohydrates: energy source; used to make medium differential when combined with an indicator

Cetrimide: acts as a quaternary ammonium cationic detergent that causes nitrogen and phosphorus to be released from bacterial cells other than *Pseudomonas aeruginosa*

Charcoal: detoxifying agent, surface tension modifier, scavenger of radicals and peroxides

Cornstarch: works as a detoxifying agent; may provide additional nutrients as an energy source

Dent's supplement (Oxoid): vancomycin, trimethoprim, cefsulodin, and amphotericin B added to Columbia blood agar and laked horse blood for isolation of *Helicobacter*

Dextrose: makes the medium hypertonic; energy source

Egg yolk: used to demonstrate lecithinase, lipase, and proteolytic activities and fatty acids

Ferric ammonium citrate: iron salt used in combination with other agents (esculin, sodium thiosulfate) to make medium differential by producing a black precipitate

Fildes enrichment: peptic digest of sheep blood that provides a rich source of nutrients, including X (hemin) and V (NAD) factors; X originally stood for unknown and V originally stood for vitamin

Glycerol: a purified alcohol and an abundant source of carbon; used in culture, transport, and storage medium and reagent preparation

Glycine: a selective agent that is inhibitory to organisms

Horse serum: an enrichment used in growth media for such organisms as *Mycoplasma* and *Ureaplasma*

IsoVitaleX (BBL): provides V factor (NAD) and additional nutritive ingredients, such as vitamins, amino acids, ferric ion, and dextrose, to stimulate growth of fastidious organisms

Laked blood or laked horse blood: created by freeze-thaw cycles of blood; enhances pigment production of anaerobes and used in susceptibility testing of fastidious organisms

Lithium chloride: a selective agent that inhibits organisms

Malachite green: a dye that partially inhibits bacteria

NAD (V factor): necessary for growth of some fastidious organisms

OADC enrichment: a supplement added to mycobacteriology media that includes oleic acid, albumin, dextrose, catalase, and sodium chloride; the oleic acid provides fatty acids utilized by mycobacteria, and the catalase destroys peroxides that may be in the medium

Oxgall (bile): inhibits specific organisms; allows medium to be selective

Peptones: carbohydrate-free source of nutrients

Phenylethyl alcohol: reversibly inhibits DNA synthesis; results in inhibition of facultative anaerobic gram-negative organisms

Pyridoxal: liquid supplement added to media for isolation of fastidious organisms; also comes in the form of a disk to be used in satelliting tests

Rabbit blood: enhances pigment production of anaerobes; hemolytic reactions of streptococci are "correct"; additive to heart infusion agar for isolation of *Bartonella* spp.

Serum: albumin, fatty acids

Sheep blood and human blood: provide hemin and other nutrients; allow true hemolytic reactions of streptococci; NADase enzyme inactivates the NAD in the sheep blood and is not available for organisms

Skirrow's supplement: vancomycin, trimethoprim, and polymyxin B added to Columbia agar and laked horse blood for isolation of *Helicobacter*

Sodium azide: a selective agent that inhibits gram-negative organisms

Sodium bicarbonate: neutralization agent used with gastric wash or lavage specimens for recovery of acid-fast organisms
Sodium bisulfite: disinfectant, antioxidant, or reducing agent
Sodium chloride: maintains osmotic equilibrium; when added at a high concentration it may be a selective agent
Sodium citrate: a selective agent inhibitory to organisms
Sodium desoxycholate: a salt of bile acid and a selective agent that inhibits gram-positive and spore-forming organisms
Sodium polyanetholesulfonate (SPS): a polyanionic anticoagulant that inactivates aminoglycosides and interferes with the complement cascade, lysozyme activity, and phagocytic activity inherent in blood. May be inhibitory to *Neisseria, Gardnerella, Streptobacillus, Peptostreptococcus, Francisella,* and *Moraxella* spp.
Sodium pyruvate: growth stimulant
Sodium selenite: a selective agent that inhibits coliforms
Sodium thioglycolate: a reducing agent
Starch: a polysaccharide and detoxifying agent incorporated into some media as a differential agent
Tellurite: is toxic to egg-clearing strains of bacteria; imparts black color to colony
Tween 80 (polysorbate 80): an oleic acid ester that stimulates growth and provides fatty acids as well as acts as a dispersal agent
Vitamin K: ingredient required for optimal growth of certain obligate anaerobes, such as the *Bacteroides* group
Vitox (Oxoid): provides V factor (NAD) and other essential growth factors to stimulate growth of fastidious organisms; see IsoVitaleX.
Yeast extract: water-soluble product that provides B vitamins and protein

APPENDIX 2
Product Suppliers and Manufacturers of Reagents, Stains, Microscopes, and Media

1. American Type Culture Collection
   P.O. Box 1549
   Manassas, VA 20108
   http://www.atcc.org
2. AdvanDx, Incorporated
   10A Roessler Road
   Woburn, MA 01801
   http://www.advandx.com
3. Anaerobe Systems
   15906 Concord Circle
   Morgan Hill, CA 95037
   http://www.anaerobesystems.com
4. Applied Biosystems
   850 Lincoln Centre Drive
   Foster City, CA 94404
   http://www.appliedbiosystems.com
5. BBL/Difco (see BD Biosciences)
6. BD Diagnostic Systems
   7 Loveton Circle
   Sparks, MD 21152
   http://www.bd.com/ds
7. BD Biosciences
   1 Becton Drive
   Franklin Lakes, NJ 07417
   and
   2350 Qume Drive
   San Jose, CA 95131
   http://www.bdbiosciences.com
8. BioMed Diagnostics Inc.
   1388 Antelope Road
   White City, OR 97503-1619
   http://www.biomed1.com
9. Carl Zeiss, Microimaging, Incorporated
   1 Zeiss Drive
   Thorwood, NY 10594
   http://www.zeiss.com/micro
10. Chromager
    4, place du 18 Juin 1940
    Paris, F-75006
    France
    http://www.chromager.com
11. Copan Diagnostics, Incorporated
    2175 Sampson Avenue, Suite 124
    Corona, CA 92879
    http://www.copanusa.com
12. Fisher Scientific International Inc.
    Liberty Lane
    Hampton, NH 03842
    http://www.fisherscientific.com
13. Hardy Diagnostics
    1430 West McCoy Lane
    Santa Monica, CA 93455
    http://www.hardydiagnostics.com
14. Heipha Diagnostica Biotest
    Lilienthalstrasse 16
    D-69214 Eppelheim
    Germany
    http://www.heipha.de
15. Intergen Company
    2 Manhattanville Road
    Purchase, NY 10577
    http://www.intergenco.com
16. Invitrogen (Gibco)
    1600 Faraday Avenue
    P.O. Box 6482
    Carlsbad, CA 92008
    http://www.invitrogen.com
17. Irvine Scientific
    2511 Daimler Street
    Santa Ana, CA 92705-5588
    http://www.nucleus@irvinesci.com
18. Key Scientific Products
    1402 Chisholm Trail
    Round Rock, TX 78681
    http://www.keyscientific.com
19. Marcor Development Corporation
    341 Michele Place
    Carlstadt, NJ 07072-2304
    http://www.marcordev.com
20. Medical Chemical Corporation
    19430 Van Ness Avenue
    Torrance, CA 90501
    http://www.med-chem.com
21. Medical Packaging Corporation
    941 Avenido Acaso
    Camarillo, CA 93012
    http://www.devicelink.com
22. Medical Wire & Equipment-MWE
    29 Leafield Way
    Corsham
    Wiltshire
    SN13 9RT
    United Kingdom
    http://www.mwe.co.uk
23. Nikon Instruments, Incorporated
    1300 Walt Whitman Road
    Melville, NY 11747
    http://www.nikonusa.com
24. Northeast Laboratory Services
    Rt. 137 China Rd.
    Winslow, ME 04901
    http://www.nelabservices.com
25. Olympus America, Incorporated
    2 Corporate Center Drive
    Melville, NY 11747
    http://www.olympusamerica.com
26. Oxoid Limited (Remel distributor in the United States)
    Wade Road
    Basingstoke
    Hampshire

RG24 8PW
United Kingdom
http://www.oxoid.com

27. PML Microbiologicals
27120 SW 95th Avenue
Wilsonville, OR 97070
http://www.pmlmicro.com
28. Remel, Incorporated
12076 Santa Fe Drive
Lenexa, KS 66215
http://www.remelinc.com
29. Sigma-Aldrich Company
P.O. Box 14508
St. Louis, MO 63178
http://www.sigma-aldrich.com
30. Starplex Scientific, Incorporated
50A Steinway Boulevard
Etobicoke, Ontario M9W 6Y3
Canada
http://www.starplexscientific.com
31. Wampole Laboratories
Half Acre Road
Cranbury, NJ 0851

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Algorithm for Identification of Aerobic Gram-Positive Cocci

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Most gram-positive cocci recovered from aerobic cultures can be differentiated with the tests shown in Tables 1 and 2. These organisms include "aerotolerant anaerobes," facultative anaerobes, microaerophiles, and obligate aerobes. The genera display various colony morphologies and hemolytic and catalase reactions (Tables 1 and 2). Their cellular morphologies as revealed by Gram staining of broth cultures are generally either streptococcal, in which gram-positive cocci or coccobacilli are arranged in pairs and/or chains, or staphylococcal, in which cells appear as cocci arranged in pairs, tetrads, clusters, and irregular groups. No taxonomic kinship is implied by division of these bacteria into two groups based on cellular morphology. As new genera and species are described and characterized, it becomes increasingly difficult to identify some of the less frequently isolated organisms solely on the basis of phenotypic traits. The reader is referred to the chapters noted in the tables for more detailed descriptions of these organisms.

TABLE 1 Characteristics of catalase-negative gram-positive cocci that grow aerobically and form cells arranged in pairs and chains[a]

PYR	LAP	6.5% NaCl	BE	Motility	45°C	Probe	HIP	Satellitism	10°C	Vancomycin resistance	Organism(s) (chapter)
+	+	+	+	+	+						*Enterococcus* (30)
					−						*Vagococcus* (31)
				−		+					*Enterococcus* (30)
						−					*Lactococcus* (31)
			−				+	−			*Facklamia* spp.[b] (31)
							−	V			*Ignavigranum* (31)
		−	+	+							*Vagococcus* (31)
				−							*Lactococcus* (31)
			−					+			*Abiotrophia, Granulicatella* (31)
								−			*Gemella* spp.[c] (31)
	−	+									*Globicatella* (31)
		−									*Dolosicoccus* (31)
−	−									+	*Leuconostoc*[d] (31)
										−	*Globicatella* (31)
	+								+		*Lactococcus* (31)
									−		*Streptococcus*[e] (29)

[a] See chapters 21 and 29 to 31 for descriptions of the methods for performing the tests referred to in this table. Reactions shown are typical, but exceptions may occur. Abbreviations, terms, and symbols: PYR, production of pyrrolidonyl arylamidase; LAP, production of leucine aminopeptidase; 6.5% NaCl, growth in 6.5% NaCl; BE, hydrolysis of esculin in the presence of 40% bile; 45°C, growth at 45°C; probe, reaction with commercially available nucleic acid probe for the genus *Enterococcus;* HIP, hydrolysis of hippurate; satellitism, satelliting growth behavior; 10°C, growth at 10°C; +, most strains positive; −, most strains negative; V, variable reactions are observed.

[b] The reactions listed in this table are typical for *F. hominis, F. sourekii,* and *F. ignava. F. languida* cells tend to be arranged in clusters, and isolates are hippurate hydrolysis negative (Table 2).

[c] G. *morbillorum,* G. *bergeri,* and G. *sanguinis* cells tend to be arranged in pairs and chains, in contrast to the cells of G. *haemolysans,* which are arranged in pairs, tetrads, and clusters (Table 2).

[d] *Leuconostoc* is distinguished from the other catalase-negative organisms in Table 1 by its ability to produce gas as an end product of glucose metabolism and by its intrinsic resistance to vancomycin. The phenotypically similar genus *Weissella* contains organisms formerly classified as leuconostocs and the species formerly named *Lactobacillus confusus* (see chapter 31).

[e] Most streptococci are PYR negative, with the exception of *S. pyogenes* isolates and some strains of *S. pneumoniae,* which are PYR positive.

TABLE 2 Differentiating features of gram-positive cocci that grow aerobically and form cells arranged in clusters or irregular groups[a]

Catalase	Obligate aerobe	Oxidase	PYR	LAP	NaCl	ESC	Hemolysis	Vancomycin resistance	BGUR	Organism (chapter)
+	+	+			+[b]					*Micrococcus* (28)
		−			+[c]					*Alloiococcus* (28)
	−	−			+[b]					*Staphylococcus* (28)
					−[b]					*Rothia mucilaginosa*[d] (28, 34)
−	−		+	+	+[c]	+			−	*Dolosigranulum* (31)
									+	*Aerococcus sanguinicola* (31)
						−			−	*Facklamia languida*[e] (31)
					−[c]	+				*Rothia mucilaginosa*[d] (28, 34)
						−				*Gemella haemolysans*[f] (31)
				−			α			*Aerococcus viridans*[g] (31)
							γ			*Helcococcus kunzii*[g] (31)
			−	+				R		*Pediococcus*[h] (31)
								S	+	*Aerococcus urinae* (31)
				−					+	*Aerococcus urinaehominis* (31)
									−	*Aerococcus christensenii* (31)

[a] See chapters 21, 28, and 31 for descriptions of methods for performing the phenotypic tests referred to in this table. Reactions shown are typical; exceptions may occur. Abbreviations and symbols: PYR, production of pyrrolidonyl arylamidase; LAP, production of leucine aminopeptidase; NaCl, growth in the presence of either 5 or 6.5% NaCl (see footnotes *b* and *c*); ESC, esculin hydrolysis; BGUR, production of β-glucuronidase; +, most strains positive; −, most strains negative; V, variable reactions are observed; α, alpha-hemolysis on sheep blood agar; γ, nonhemolytic reaction on sheep blood agar; S, susceptible; R, resistant.

[b] Growth in the presence of 5% sodium chloride.

[c] Growth in the presence of 6.5% sodium chloride.

[d] *R. mucilaginosa* isolates are usually catalase negative or weakly positive but may be strongly catalase positive.

[e] *Ignavigranum ruoffiae* (Table 1) exhibits reactions identical to those of *F. languida* in the PYR, ESC, and NaCl tests. However, *I. ruoffiae* cells are arranged primarily in chains and *F. languida* cells usually form clusters. Other *Facklamia* species form cells arranged in pairs and chains (Table 1).

[f] *G. haemolysans* cells tend to be arranged in pairs, tetrads, and groups, in contrast to the cells of other *Gemella* species, which usually occur in pairs and short chains (Table 1).

[g] *H. kunzii* strains form tiny, pinpoint, nonhemolytic colonies on blood agar after 24 h of aerobic incubation at 35°C, and *A. viridans* isolates form larger, alpha-hemolytic colonies under similar incubation conditions. In contrast to *H. kunzii, A. viridans* prefers aerobic incubation atmospheres. Two additional species of *Helcococcus* isolated from human sources have been described, each based on a single isolate. In contrast to *H. kunzii,* the new species *H. sueciensis* and the proposed species "*H. pyogenes*" are PYR negative (see chapter 31).

[h] The genera *Pediococcus* and *Tetragenococcus* have similar phenotypic characteristics, except that tetragenococci are vancomycin susceptible. The bile esculin test can differentiate between tetragenococci (positive) and *Aerococcus urinae* (negative); see chapter 31.

Algorithm for Identification of Aerobic Gram-Positive Rods

GUIDO FUNKE

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The aim of the algorithm for the identification of aerobic gram-positive rods described in this chapter is simply to guide the reader to the appropriate chapter of this Manual for further information. The algorithm emphasizes that Gram stain (performed on 24- to 48-h-old colonies from rich media) and macroscopic morphologies are the initial key features for the differentiation of aerobic gram-positive rods. All strains of aerobic gram-positive rods (except the non-rapidly growing mycobacteria) are initially grown on blood agar plates.

Regular rods are organisms with cells whose longitudinal edges are usually not curved but are parallel. If spore formation is not observed initially, it can be tested for on a nutritionally depleted medium. Catalase activity should be tested with colonies from media lacking heme groups. The type of metabolism can be checked in oxidative-fermentative media or in cystine Trypticase agar medium. Irregular rods are organisms with cells whose longitudinal edges are curved and not parallel. Diagnostic end products of glucose metabolism are detected by chromatographic methods. Slight beta-hemolysis is best observed when cells are incubated in a CO_2-enriched atmosphere. Yellow- or orange-pigmented colonies are always composed of irregular rods. Some genera that stain partially acid-fast (e.g., *Gordonia* and *Rhodococcus*) may also show a yellow-orange pigment. Rods exhibiting vegetative substrate filaments may show branched hyphae, which either form spores or reproduce by fragmentation. It is obvious that vegetative substrate filaments might not be present initially (i.e., within 48 h), and so these organisms are prone to initial misidentification.

For the yellow-orange genera (e.g., *Microbacterium, Curtobacterium,* and *Leifsonia*), as well as the rods exhibiting vegetative substrate filaments, chemotaxonomic methods must very often be used for definitive identification to the genus level; for example, partially acid-fast bacteria can be identified to the genus level by analysis of mycolic acids.

Genera which contain strict anaerobic gram-positive rods may also contain aerobically growing species. This is particularly true for the genus *Actinomyces* (as it is presently defined). *Clostridium tertium* (a strong gas producer) may also grow aerobically. Furthermore, some aerobic gram-positive cocci (e.g., *Leuconostoc* spp. or *Streptococcus mutans*) might initially be misidentified as gram-positive rods because of their initial Gram stain appearance. Likewise, but less frequently, some gram-positive rods (e.g., *Rhodococcus* spp.) might be initially misidentified as gram-positive cocci because of their initial Gram stain appearance.

This algorithm should serve only as the basis of a preliminary identification of an unknown aerobic gram-positive rod, and the reader is referred to the chapters given in Table 1 for further information.

TABLE 1 Algorithm for identification of aerobic gram-positive rods

Cellular morphology	Yellow-orange pigment	Vegetative substrate filaments	Spore formation	Catalase	Metabolism[a]	H_2S in TSI	Other unusual Gram stain feature	Diagnostic end product of glucose metabolism[b]	Slight beta-hemolysis	Slow acid production	Acid-fast stain	Partially acid-fast stain	Aerial vegetative filaments	Motility	Growth at 50°C	Organism (chapter)
Regular			+													*Bacillus,* including *Paeni-*, *Brevi-*, *Aneurini-*, and *Virgibacillus* (32)
			−	+	O											*Kurthia*
					F											*Listeria* (33)
				−		+										*Erysipelothrix* (33)
						−										*Lactobacillus* (56)
Irregular	−			+			Club-shaped rods									*Corynebacterium* (34)
							Slim, long rods									*Turicella* (34)
							Very coccoid rods									*Dermabacter* (34)
							May show jointed rods									*Arthrobacter* (34)
							May show short rods									*Brevibacterium* (34)
							May show branching									*Actinomyces* (56), *Propionibacterium* (56), *Rothia* (28, 34)
				−			Coccoid rods, Gram variable									*Gardnerella* (34)
								S	+ −							*Arcanobacterium* (34) *Actinomyces* (56)
								A								*Bifidobacterium* (56)
								L								*Rothia* (28, 34)
	+	+														*Oerskovia*, *Cellulosimicrobium* (both 34)

(Continued on next page)

TABLE 1 Algorithm for identification of aerobic gram-positive rods *(Continued)*

Cellular morphology	Yellow-orange pigment	Vegetative substrate filaments	Spore formation	Catalase	Metabolism[a]	H_2S in TSI	Other unusual Gram stain feature	Diagnostic end product of glucose metabolism[b]	Slight beta-hemolysis	Slow acid production	Acid-fast stain	Partially acid-fast stain	Aerial vegetative filaments	Motility	Growth at 50°C	Organism (chapter)
		−		+	O					+						*Curtobacterium* (34)
										−						*Microbacterium*, *Leifsonia* (both 34)
					F											*Microbacterium*, *Cellulomonas*, *Exiguobacterium* (all 34)
				−												*Microbacterium* (34)
	−	+									+					*Mycobacterium* (36, 38)
											−	+	+			*Nocardia* (35)
													−			*Tsukamurella*, *Gordonia*, *Rhodococcus*, *Dietzia* (all 35)
												−		+		*Dermatophilus* (35)
														−		*Actinomadura* (both 35)
													+		+	*Saccharomonospora*, *Saccharopolyspora*, *Thermoactinomyces* (all 35)
															−	*Actinomadura*, *Amycolatopsis*, *Nocardiopsis*, *Pseudonocardia*, *Streptomyces* (all 35)

[a] O, oxidative; F, fermentative.
[b] S, succinic acid; A, acetic acid; L, lactic acid.

Algorithms for Identification of Aerobic Gram-Negative Bacteria*

PAUL C. SCHRECKENBERGER AND DAVID LINDQUIST

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These algorithms are meant to assist in the identification of organisms that are not readily identified by methods in place in most clinical laboratories. Microbiologists planning to identify an unknown gram-negative rod begin with colonies on an agar plate. Our definition of "good growth on blood agar plate (BAP)" is the presence of distinct colonies (approximately 1 mm) on Trypticase soy agar with 5% sheep blood after 24 h of incubation at 35°C in room atmosphere. Poor growth indicates that more than 24 h of incubation is necessary for the development of distinct colonies. If an organism fails to grow on BAP after 72 h, it is considered to show "no growth." Morphological and phenotypic criteria were chosen not only for their discriminatory value but also because the methods are available in most laboratories. Cellular morphology is determined by using a Gram stain from a young colony on a BAP. The description of "tiny coccobacilli" used for *Brucella* and *Francisella* in Table 2 implies almost indiscernible cells resembling grains of sand. For many organisms with pleomorphic morphologies, we chose to represent the dominant shape.

The urea test refers to conventional Christensen's urea reaction after 24 h of incubation, whereas the rapid urea result is read after 4 h. Glucose fermentation refers to an acid reaction in the butt of a Kligler iron agar (KIA) or triple sugar iron agar (TSI) tube. "Glucose oxidized" refers to acid production in the upper portion of oxidative-fermentative (OF) media. "BHI+serum" refers to brain heart infusion agar with 10% (vol/vol) serum added. The oxidase test refers to results obtained with the *N,N,N,N*-tetramethyl-*p*-phenylenediamine dihydrochloride reagent. Motility is best observed by preparing a wet preparation from a young colony on a BAP. Decarboxylase reactions are determined by using an extremely turbid inoculum in Moeller's media (heavier than usual inoculum). Polymyxin B sensitivity is indicated by any zone of inhibition surrounding a 300-U disk on a BAP. For glucose-nonfermenting rods and other fastidious organisms, the indole test is performed using the Ehrlich's extraction method (see chapter 50). "Esculin" refers to hydrolysis of esculin in media without bile.

These algorithms are dichotomous, since many organisms may fall into more than one group due to phenotypic variability of a given trait. The presence of two or more atypical traits or a major variation from the ideal phenotype depicted in an algorithm, due to antibiotic use, auxotrophy, or other reasons, may limit the algorithm's utility. These algorithms are intended as a guide to presumptive identification of an unknown isolate. The reference chapter describing the organism should be consulted to determine the definitive identification. To use the algorithms, start with Table 1 for gram-negative bacteria that grow well on blood agar in 24 h at room atmosphere and Table 2 for fastidious gram-negative bacteria. Note that Table 1 consists of three parts, which we have designated Table 1a, Table 1b, and Table 1c. In each case begin in the upper left-hand column of the table; if the test organism matches the given characteristic, then continue horizontally to the right to the next reaction. If the reaction in the box matches your test organism, continue moving horizontally until you reach the organisms listed in the right-hand column. When the reaction in the box does not match your test organism, then move down the column vertically to find the reaction that matches. Repeat the process until you reach the right-hand column. Be sure to check all your reactions with the organism characteristics given in the referenced chapter.

*This chapter contains information presented in chapter 23 by Paul C. Schreckenberger and Jane D. Wong in the eighth edition of this Manual.

TABLE 1a Identification algorithm for gram-negative bacteria that grow well on blood agar (part 1)

Cell morphology	Glucose fermented	Pigmented colonies	Oxidase	6% NaCl	Motility	Sucrose fermented	H_2S in TSI	Indole	Glucose oxidized	Fluorescent pigment	Yellow-pigmented colonies	Polymyxin B	ONPG[a]	Lysine decarboxylase	Arginine decarboxylase	Urea	Esculin	OF mannitol	OF maltose	Growth on MacConkey	Lactose, trehalose, or xylose fermented	Phenylalanine deaminase	H_2S in TSI	ONPG	Nitrate to gas	Organism group (chapter)
Rod	+	Purple																								*Chromobacterium* (40)
		Other	+	+																						*Vibrio* (47)
				−	+																					*Aeromonas* (46), *Plesiomonas* (45)
					−	+																				*Pasteurella* (40), *Actinobacillus* (40)
						−	+																			*Pasteurella bettyae* (40)
							−	+																		EF4a (40)
								−																		*Pasteurella avium* (40), *Actinobacillus actinomycetemcomitans* (40)
			−	+																						*Vibrio metschnikovii* (47)
				−																+	+					*Enterobacteriaceae* (42–45)
																					−	+				*Providencia, Morganella* (42, 45)
																						−	+			*Edwardsiella* (42, 45)
																							−			*Pasteurella bettyae* (40)
																				−						*P. bettyae* (40)
	−	Pink																								*Asaia, Azospirillum, Methylobacterium, Roseomonas* (50)
		Not pink	+		+		+																			*Shewanella* spp. (50)

		−	+						*Balneatrix alpica* (50)
			−	+	+				*Pseudomonas aeruginosa, P. fluorescens, P. putida* (48)
				−	+				*Agrobacterium* Yellow Grp, O-1, O-2, *Sphingomonas* spp. (50)
					−	R	+		*Pseudomonas*-like group 2 (50)
								+	*Burkholderia cepacia* complex (49)
								−	*B. stabilis* (49)
							−	+	*B. pseudomallei* (49)
								−	*Ralstonia mannolilytica* (49)
									Pandoraea spp., *Ralstonia* spp. (49)
						S		+	*Ochrobactrum anthropi*, *Achromobacter* groups B and E (50)
									CDC Vb-3 (48), OFBA-1, *Ochrobactrum* spp. (50)
									Ochrobactrum spp. (50), *Acidovorax* spp. (49), *Pseudomonas*-like group 2 (50)
									P. mendocina (48), CDC Ic , *Ochrobactrum* spp. (50)
								−	*Rhizobium radiobacter* (50)
									Ochrobactrum anthropi, Achromobacter Group F, *Halomonas venusta, Inquilinus limosus* (50)
									P. stutzeri (48), *Ochrobactrum anthropi* (50)
									Pseudomonas-like group 2, *Herbaspirillum*, CDC halophilic nonfermenter group 1 (50)
									CDC group O-3, *Inquilinus limosus*, *Massilia timonae* (50), *Brevundimonas vesicularis* (49)
									P. stutzeri (48), *Achromobacter xylosoxidans* (50)
									A. xylosoxidans (50), *Brevundimonas diminuta* (49)

[a]ONPG, *O*-nitrophenyl-beta-D-galactopyranoside.

TABLE 1b Identification algorithm for gram-negative bacteria that grow well on blood agar (part 2)

Cell morphology	Glucose fermented	Pigmented colonies	Oxidase	Motility	H_2S in TSI	Indole	Glucose oxidized	Yellow-pigmented colonies	Polymyxin B	Arginine decarboxylase	Rapid urea	NO_2 reduction	Acetamide	Esculin	OF mannitol	Urea	Growth in 6.5% NaCl	Gelatin	NO_3 to NO_2	Curved rods	Organism group (chapter)
Rod	−	Not pink	+	+	−	−	−		R												*Pandoraea* spp. (49)
									S	+										+	*Laribacter hongkongensis* (50)
																				−	*P. pseudoalcaligenes, P. alcaligenes, Pseudomonas* species group 1 (48)
										−	+										*Bordetella bronchiseptica, Oligella ureolytica, Cupriavidus pauculus* (50)
											−	+							+		*Pseudomonas* species group 1 (48), *Achromobacter denitrificans* (50)
																			−		*Alcaligenes faecalis* (50)
												−	+		+						*Delftia acidovorans* (49)
															−				+		*Achromobacter piechaudii* (50)
																			−		*Bordetella avium* (50, 51)
													−				+				CDC halophilic group 1 (50)
																	−		+		*P. pseudoalcaligenes, P. alcaligenes* (48), *Comamonas terrigena, C. testosteroni* (49), *Achromobacter piechaudii, Cupriavidus taiwanensis* (50)
																			−		*Brevundimonas diminuta, Brevundimonas vesicularis* (49), *Bordetella hinzii* (50, 51), *P. alcaligenes* (48), *Cupriavidus gilardii, Cupriavidus respiraculi* (50)
				−		+		+													*Chryseobacterium indologenes/gleum, Empedobacter brevis*, CDC group IIi (50)
								−			+										*Bergeyella zoohelcum* (50)
											−		+		+						*Elizabethkingia meningoseptica* (50)
															−						CDC group IIc, IIh, IIi (50)
														−				+			*Weeksella virosa, Empedobacter brevis* (50)
																		−			CDC group IIe, IIg (50)
						−	+							+	+						*Sphingobacterium spiritivorum, Inquilinus limosus* (50)
															−	+			+		*Sphingobacterium thalpophilum* (50)
																			−		*Sphingobacterium multivorum* (50)
																−					*Sphingomonas paucimobilis, Flavobacterium mizutaii* (50)
														−					+		EF-4b, *Paracoccus yeei, Psychrobacter immobilis* (50)
																			−		EO-3, EO-4 (50)
							−				+							+			*Myroides* spp. (50)
																		−			*Oligella ureolytica* (50)
											−			+							*Alishewanella fetalis* (50)
														−							*Neisseria weaveri, N. elongata*, Gilardi rod group 1 (39, 50)

TABLE 1c Identification algorithm for gram-negative bacteria that grow well on blood agar (part 3)

Cell morphology	Glucose fermented	Pigmented colonies	Oxidase	Motility	Glucose oxidized	DNase	Urea	Mannitol	Lysine decarboxylase or OF lactose	Lysine decarboxylase	OF maltose	Esculin	Gelatin	NO_2 reduced	NO_3 reduced	Brown diffusible pigment	Growth at 42°C	Organism group (chapter)
Rod	−	Not pink	−	+	+			+	+									*Burkholderia cepacia* complex (48)
									Both negative		+	+						*Pseudomonas luteola* (47)
												−						*P. oryzihabitans* (47)
											−							*Burkholderia gladioli* (48)
								−		+								*Stenotrophomonas maltophilia* (48)
										−								*Sphingomonas paucimobilis* (49)
					−								+					*Bordetella ansorpii* (50, 51)
													−				+	*Kerstersia gyiorum* (50)
																	−	*Bordetella trematum* (50, 51)
				−	+													*Acinetobacter baumannii*, EO-5 (50)
					−		+											*Bordetella parapertussis* (50, 51)
							−								+			CDC NO-1 (50)
															−	+		*Bordetella holmesii* (50, 51)
																−		*Acinetobacter lwoffii* (50)
Diplococci			+															*Neisseria* (39), *Moraxella catarrhalis* (50)
			−															*Acinetobacter* (50)
Coccobacilli			+	+														*Oligella ureolytica* (50)
				−	+										+			EF-4b, *Paracoccus yeei, Psychrobacter immobilis* (saccharolytic) (50)
															−			EO-3, EO-4 (50)
					−	+												*Moraxella canis, M. catarrhalis* (50)
						−	+											*Psychrobacter phenylpyruvica* (50), *Brucella* spp. (52)
							−							+				*Oligella urethralis* (50)
														−	+			*Moraxella lacunata, M. nonliquefaciens, M. osloensis, P. immobilis* (asaccharolytic) (50)
															−			*M. atlantae, M. lincolnii, M. osloensis, P. immobilis* (asaccharolytic) (50)
			−	−	+													*Acinetobacter baumannii*, EO-5 (50)
					−		+											*Bordetella parapertussis* (50, 51)
							−								+			CDC NO-1 (50)
															−	+		*Bordetella holmesii* (50, 51)
																−		*Acinetobacter lwoffii* (50)

TABLE 2 Identification algorithm for gram-negative bacteria with poor or no growth on blood agar[a]

Growth on BAP	Growth only on:	Cellular morphology	Urea	Pigmented colonies	Oxidase	6% NaCl	H_2S in TSI	Cauliflower-like colonies	O-shaped cells	Require X ± V	Organism group (chapter)
Poor		Tiny coccobacilli	+								*Brucella* (52)
			−		+		+				*Francisella philomiragia* (52)
							−				*Bordetella* (50)
					−						*Francisella* (52)
		Rods		Pink							*Asaia, Azospirillum, Methylobacterium, Roseomonas* (50)
				Other				+			*Bartonella* (54)
								−			*Haemophilus aphrophilus* (41), *Cardiobacterium, Dysgonomonas, Eikenella, Kingella, Simonsiella, Suttonella* (40)
		Diplococci or coccobacilli							+		*Paracoccus yeei* (50)
									−		*Neisseria* (39), *Moraxella* (50)
None	Chocolate	Diplococci or coccobacilli									*Neisseria* (39)
		Fusiform rod									*Capnocytophaga* (40)
		Rod						+			*Bartonella, Afipia* (54)
								−		+	*Haemophilus* (41)
										−	*Francisella* (52)
	BCYE	Long gram-negative rods									*Legionella* (53)
		Regular rods			+						*Bordetella* (51)
					−						*Francisella* (52)
	BHI+ serum	Pleomorphic, beaded filamentous rods									*Streptobacillus* (40)
		Small rods									*Bartonella, Afipia* (54)

[a] Abbreviations: BCYE, buffered charcoal yeast extract agar; X, hemin; V, nicotinamide adenine dinucleotide.

Algorithm for Identification of Anaerobic Bacteria

DIANE M. CITRON

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Anaerobic bacteria are defined for the purposes of this algorithm as organisms displaying better growth when incubated in an anaerobic environment than in the presence of oxygen. The use of selective and differential agars for the primary setup of clinical specimens and prompt incubation in an anaerobic environment can provide rapid presumptive identification of important groups of anaerobes based on distinctive characteristics, such as bile resistance, pigmented colonies, a double zone of beta-hemolysis, or the presence of fusiform cells on Gram stain. Examples of such agars include Bacteroides-bile-esculin (BBE) for isolation and presumptive identification of the *Bacteroides fragilis* group and *Bilophila wadsworthia*. Brucella agar supplemented with laked blood, kanamycin, and vancomycin inhibits enteric and gram-positive organisms and is thus useful for isolation and characterization of *Bacteroides, Prevotella,* and some strains of fusobacteria. Pigmented *Prevotella* spp. produce pigment more rapidly and intensely on laked blood agar. Phenylethyl alcohol blood agar (PEA) and colistin nalidixic acid blood agar inhibit enteric organisms and swarming *Proteus* spp. but allow for growth and isolation of many gram-positive and gram-negative anaerobes. PEA also inhibits the swarming by *Clostridium septicum,* which can completely overgrow and contaminate other anaerobic organisms in a mixed culture. All media for culture of anaerobes should be supplemented with vitamin K_1 and hemin.

Simple tests, such as tests for susceptibility (inhibition zone diameters, $\geq$10 mm) to the special potency disks with 1,000 μg of kanamycin, 5 μg of vancomycin, and 10 μg of colistin; tests for growth in the presence of 20% bile; the spot indole test; and tests for nitrate reduction, catalase and urease production, and lecithinase and lipase production on egg yolk medium are rapid and useful for initial grouping of many anaerobes (1).

The gram-positive non-spore-forming rods are difficult to group by simple tests. Nitrate-reducing strains include *Actinomyces, Propionibacterium,* and some of the *Eubacterium*-like group members. Most *Propionibacterium* spp. and some species of *Actinomyces* are catalase positive. Lactobacilli and bifidobacteria do not reduce nitrate and are catalase, urease, and indole negative.

The genus *Clostridium* includes the gram-positive spore-forming rods; however, many members of this group appear to be gram negative, and spores can be difficult to see under routine conditions. While many species require extensive testing for complete identification, some very clinically important species can be recognized by easily observable characteristics.

Anaerobic bacteria are a diverse group of organisms and include members that often exhibit seemingly contradictory characteristics, such as gram-negative rods sensitive to vancomycin (certain clostridia, *Porphyromonas* spp.), gram-positive rods resistant to vancomycin (some lactobacilli), or rods that appear as cocci (some *Prevotella* and *Porphyromonas* spp.). The algorithm in Table 1 includes characteristics that should be helpful for suggesting the correct category for anaerobes encountered in clinical specimens.

REFERENCE

1. **Jousimies-Somer, H., P. Summanen, D. M. Citron, E. J. Baron, H. M. Wexler, and S. M. Finegold.** 2002. *Wadsworth-KTL Anaerobic Bacteriology Manual,* 6th ed. Star Publishing Co., Belmont, Calif.

TABLE 1 Algorithm for identification of bacteria that grow better anaerobically than aerobically

Cellular morphology	Gram reaction	Growth on BBE	Pattern on kanamycin and vancomycin disk[a]	Pigment or red fluorescence	Nitrate	Catalase	Spores	Spreading, irregular, peaked, or large colony	Large boxcar-shaped cells, beta-hemolysis	Organism (chapter)
Rod or coccobacillus	−	+	R/R							*B. fragilis* group (58)
			S/R		−	−				*Fusobacterium mortiferum/varium* group (58)
					+	+				*Bilophila wadsworthia* (58)
		−	R/S	+/−	−	−/+				*Porphyromonas* (58)
			R/R	+/−	−	−				*Prevotella* (58)
				−			rare	+		*Clostridium innocuum* (57)
			S/S	+			rare	−		*Clostridium ramosum* (57)
				−			rare	−		*Clostridium clostridioforme* group (57)
			S/R		+	−				*Bacteroides ureolyticus*, *B. ureolyticus*-like, or *Campylobacter*-like spp. (58, 59)
					−					*Fusobacterium* (58)
	+/variable		S/R							*Lactobacillus* (56)
			S/S	−	−	−				*Lactobacillus*, *Bifidobacterium*, or "*Eubacterium*-like" group (56)
				+/−	+	+/−				*Actinomyces*, *Propionibacterium*, "*Eubacterium*-like" group (56)
				+	−	−	rare			*Clostridium ramosum* (57)
				−	+/−	−	+	+		*Clostridium* spp. (57)
							−	+	+	*Clostridium perfringens* (57)
Coccus	+		S/S							Anaerobic gram-positive cocci (55)
	−		S/R							*Veillonella*, *Acidaminococcus*, or *Megasphaera* (55)

[a] R, resistant; S, susceptible.

Algorithms for Identification of Curved and Spiral-Shaped Gram-Negative Rods*

IRVING NACHAMKIN

26

Curved and spiral-shaped bacteria have a common microscopic morphology but represent diverse bacterial pathogens (Table 1). These organisms are curved, helical, or spiral-shaped gram-negative rods. Specific detection of these organisms may require a combination of tests including microscopy, histologic staining of tissue, biochemical tests, antigen tests, serologic tests, bacteriologic culture, and molecular approaches (Fig. 1).

Most bacteria in this group of organisms are isolated from patients with gastrointestinal and related infections. *Campylobacter jejuni* subsp. *jejuni* is the most frequently isolated curved gram-negative rod associated with diarrheal illness, but under proper culture conditions, other *Campylobacter* spp., *Helicobacter* spp., and *Vibrio* species may be detected in routine stool cultures. *Helicobacter pylori* is the most common curved gram-negative rod isolated from gastric tissue, but other *Helicobacter* species have also been reported in this site.

Other less commonly isolated curved gram-negative rods include the anaerobes *Desulfovibrio* spp., *Sutterella wadsworthensis, Wolinella succinogenes,* and *Anaerobiospirillum succiniciproducens,* which may be isolated from blood, abscess material, or other clinical samples. Several oxidase-positive nonfermenters may also have a curved appearance, including *Laribacter hongkongensis, Herbaspirillum* species 3, and CDC O-3 (see chapter 50).

The spirochetes *Borrelia* spp. and *Leptospira* spp. cause systemic infections and are infrequently isolated in clinical laboratories, usually only with specialized media. These bacteria are strictly aerobic and have optimal growth temperatures at 28 to 30°C (*Leptospira* spp.) and 30 to 33°C (*Borrelia* spp.). *Treponema* spp. of clinical importance are identified based on clinical and epidemiologic findings, as well as microscopic, serologic, and molecular test procedures.

* This chapter contains information presented in chapter 25 by James Versalovic in the eighth edition of this Manual.

TABLE 1 Curved gram-negative bacilli that may be encountered in clinical specimens[a]

Clinical finding	Specimen type(s)	Curved gram-negative organisms encountered		Microscopic appearance in specimen	Culture conditions and media	Chapter
		Genus or group	Species			
Gastroenteritis	Stool; intestinal biopsy specimen	*Arcobacter*	*A. butzleri*, *A. cryaerophilus*, *A. skirrowii*	Slightly curved, curved, S-shaped, or helical	Microaerobic, may grow aerobically or anaerobically, nonhemolytic, grows on nonselective blood agar (with filtration method), may grow on Campy-CVA	59
		Brachyspira	*B. aalborgi*, *B. pilosicoli*	Spirochete	Anaerobic, prolonged incubation (1–2 weeks) on anaerobic media, selective media may be required	58
		Campylobacter	*C. jejuni* subsp. *jejuni*, *C. jejuni* subsp. *doylei*, *C. coli*, *C. upsaliensis*, *C. fetus* subsp. *fetus*, *C. lari*, *C. curvus*, *C. rectus*, *C. hominis*, *C. lanienae*, *C. hyointestinalis*, *C. sputorum*	Curved, spiral, gull, S-shaped GNR	Microaerobic, 37 or 42°C, increased H_2 required for some non-*C. jejuni*/*C. coli* species, selective media required such as Campy-CVA, charcoal-based media such as CCDA; filtration method for less common species and H_2-requiring species	59
		Helicobacter (Gastric) (Intestinal)	*H. pylori*, *H. bizzozeronii*, *H. canis*, *H. canadensis*, *H. cinaedi*, *H. fennelliae*, *H. pullorum*, *Helicobacter* spp. and flexispira taxon 8	Curved, spiral, gull, S-shaped GNR	Microaerobic, 37°C, increased H_2 required for intestinal species, nonselective blood agar for *H. pylori*, selective supplements (Skirrows, Dents) may be needed for contaminated gastric samples	60
		Laribacter	*L. hongkongensis*	Gull or spiral-shaped	Asaccharolytic nonfermenter, aerobic growth on blood agar, MacConkey agar	50
		Vibrio	*V. cholerae*, *V. parahaemolyticus*, *V. fluvialis*, *V. alginolyticus*, *V. cincinnatiensis*, *V. damsela*, *V. furnissii*, *V. hollisae*, *V. metschnikovii*, *V. mimicus*	Comma-shaped or plain rods, larger than *Campylobacter*	Aerobic conditions, 37°C, grows on routine laboratory media, blood agar, MacConkey agar; use selective medium such as TCBS for primary isolation from stool samples	47
Bacteremia	Blood	*Borrelia* Lyme group	*B. afzelii*, *B. burgdorferi*, *B. garinii*	Not seen in routine BC bottles	Difficult to isolate, special media required for isolation; BSK, MKP	62
		Relapsing fever group	*B. recurrentis*, *B. miyamotoi*, *B. lonestari*, *B. hermsii*			

(Continued on next page)

TABLE 1 *(Continued)*

Clinical finding	Specimen type(s)	Curved gram-negative organisms encountered		Microscopic appearance in specimen	Culture conditions and media	Chapter
		Genus or group	Species			
		Campylobacter	*C. jejuni* subsp. *jejuni*, *C. fetus* subsp. *fetus*, *C. upsaliensis*, *C. lari*, *C. concisus*	Curved, spiral, gull, S-shaped GNR	Microaerobic, incubate subcultures at 37°C, increased H_2 required for some non-*C. jejuni*/*C. coli* species	59
		Herbaspirillum	*Herbaspirillum* species 3 (formerly EF-1)	Curved or helical GNR	Growth properties not described	50
		Helicobacter	*H. cinaedi*, *H. fennelliae*	Curved, spiral, gull, S-shaped GNR	Microaerobic, incubate subcultures at 37°C, increased H_2 required	60
		Leptospira	*L. biflexa*, *L. interrogans*	Not seen in routine BC bottles but may be seen in direct wet preparations of anticoagulated blood	Aerobic growth, 28–30°C, specialized media required for isolation such as EMJH, PLM-5	61
		Vibrio	*V. vulnificus*, *V. damsela*, *V. metschnikovii*, *V. cincinnatiensis*	Comma-shaped or straight rods	Aerobic growth, 37°C, grows on routine blood agar, MacConkey agar	47
		CDC O-3		Thin, medium to slightly long curved rods with tapered ends	Saccharolytic nonfermenter, no growth or poor growth on MacConkey agar, grows on Campy-CVA medium	50
		Spirillum	"Spirillum minus"	Not seen in routine BC bottles	Cannot be cultured in vitro	
Tissue infection, oral	Tissue biopsy specimen, abscess fluid	*Campylobacter*	*C. concisus*, *C. curvus*, *C. rectus*, *C. gracilis*, *C. showae*	Curved, spiral, gull, S-shaped GNR	Microaerobic, incubate cultures at 37°C, increased H_2 required for oral species, use nonselective blood agar or CCDA with filtration method	59
Tissue infection, skin, wound, other	Skin biopsy specimen, lesion fluid	*Borrelia*	Lyme group spp.	Histologic stains required	Difficult to isolate, special media required for isolation; BSK, MKP	62
		Treponema	*T. pallidum* subsp. *pallidum* (syphilis), *T. carateum* (pinta), *T. pallidum* subsp. *pertenue* (yaws), *T. pallidum* subsp. *endemicum* (endemic)	Spirochetes; silver stain; dark-field; DFA	Has not been isolated in vitro	63
		Vibrio	*V. vulnificus*, *V. damsela*, *V. alginolyticus*, *V. harveyi*	Comma-shaped or straight rods, larger than *Campylobacter* spp.	Aerobic, grows on routine laboratory media, blood agar, MacConkey agar	47

(Continued on next page)

TABLE 1 Curved gram-negative bacilli that may be encountered in clinical specimens[a] *(Continued)*

Clinical finding	Specimen type(s)	Curved gram-negative organisms encountered		Microscopic appearance in specimen	Culture conditions and media	Chapter
		Genus or group	Species			
		Anaerobes	*Desulfovibrio, Sutterella wadsworthensis, Wolinella succinogenes, Anaerobiospirillum, succiniciproducens*	Curved rods	Growth under anaerobic conditions, use anaerobe media	58
		CDC O-3		Thin, medium to slightly long curved rods with tapered ends	Saccharolytic nonfermenter, no growth or poor growth on MacConkey agar, grows on Campy-CVA medium	50
		Herbaspirillum	*Herbaspirillum* species 3	Curved or helical GNR	Growth properties not described	50
		Spirillum	"Spirillum minus"	Short, tightly coiled GNR	Unable to isolate in vitro	
	Urine	*Leptospira*	*L. biflexa, L. interrogans*	Spirochete with curved ends observed by darkfield	Aerobic growth, 28–30°C, specialized media required for isolation such as EMJH, PLM-5	61

[a] Not all species listed have been shown to cause human diseases; they are listed if they have been isolated from human clinical specimens. BC, blood culture; GNR, gram-negative rod; DFA, direct fluorescent antibody; Campy CVA, Campy-cefoperazone, vancomycin, amphotericin; CCDA, charcoal cefoperazone deoxycholate agar; TCBS, thiosulfate-citrate-bile salts-sucrose agar; BSK, Barbour-Stoenner-Kelly medium; MKP, modified Kelley medium; EMJH, Ellinghausen-McCullough-Johnson-Harris medium.

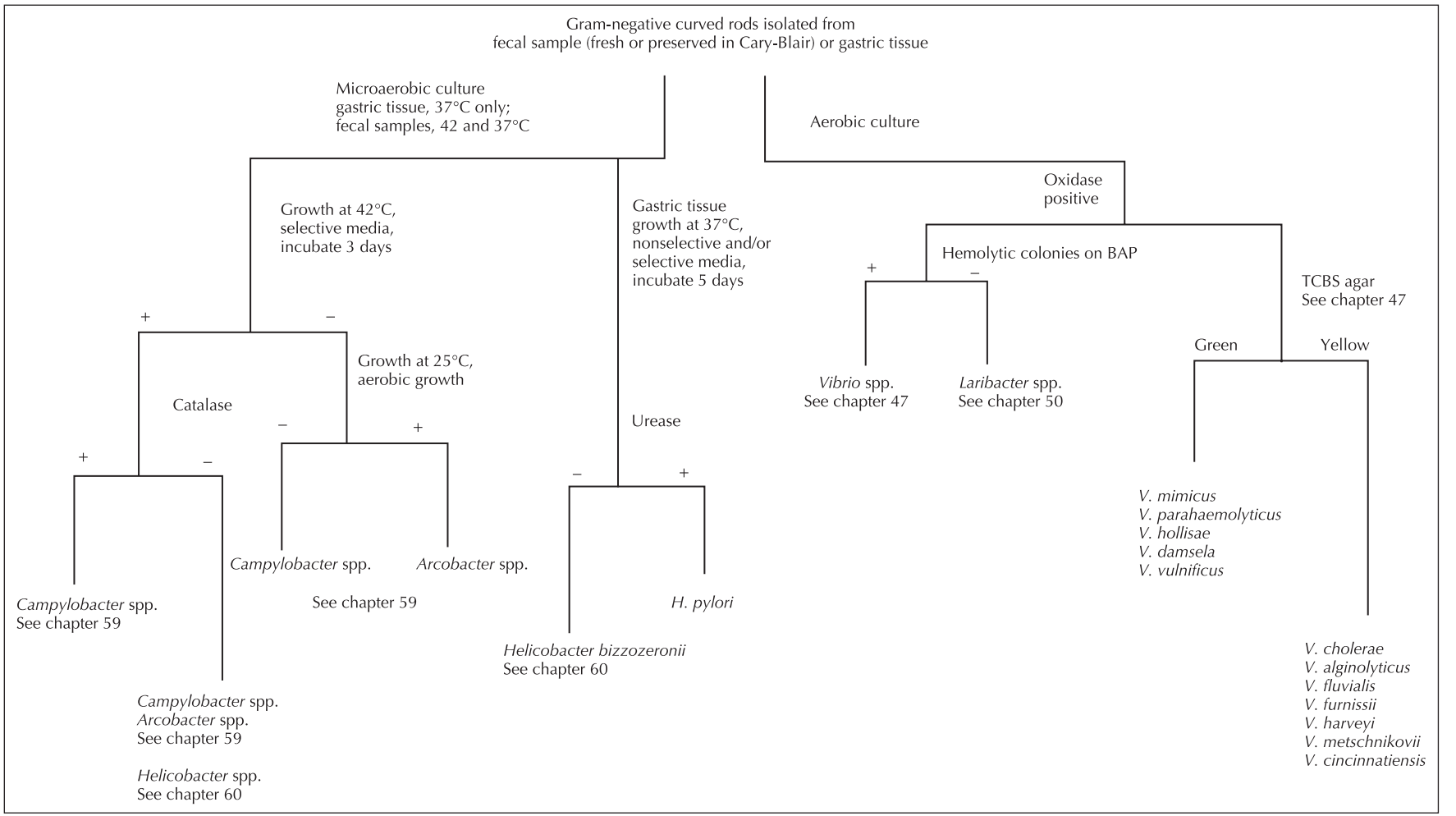


FIGURE 1 Algorithm for identification of curved gram-negative bacilli from fecal and gastric clinical samples. BAP, blood agar plate; TCBS, thiosulfate-citrate-bile salts-sucrose.

Algorithms for Identification of *Mycoplasma, Ureaplasma,* and Obligate Intracellular Bacteria

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The bacteria discussed in chapters 64 to 69 differ from bacteria described in other parts of this Manual by several characteristics, including lack of efficient characterization with the Gram stain method and except for *Mycoplasma* and *Ureaplasma* species, the requirement for intracellular growth. Thus, the most frequently used tests in clinical microbiology laboratories, the Gram stain and culture on artificial media, are unable to detect these organisms in clinical samples. Diagnosis of infections caused by these bacteria has traditionally been accomplished by Romanowsky staining (Giemsa and Wright stains) of clinical samples, by detection of antibody responses to infection using a variety of serological tests, or by histopathologic analysis of biopsy samples. Molecular diagnostic tools and better culture methods have significantly improved the ability to detect these agents and to diagnose the diseases that they cause. For some of these infections, molecular tools are becoming standard practice. The following tables summarize the epidemiology of these infections (Table 1) and the diagnostic tests most often used for the detection of the causative bacteria (Table 2).

TABLE 1 Epidemiology and clinical disease associated with *Anaplasma, Chlamydia, Chlamydophila, Coxiella, Ehrlichia, Mycoplasma, Rickettsia, Tropheryma,* and *Ureaplasma* infections

Organism	Disease	Reservoir	Vector and mode of transmission
Anaplasma phagocytophilum	Human granulocytotropic anaplasmosis (HGA); fever, headache, myalgia, systemic involvement except for central nervous system	White-footed mouse, other small mammals, ruminants, deer	*Ixodes scapularis* (deer or black-legged tick), *I. pacificus* (western black-legged tick), *I. ricinus* (rabbit tick), and *I. persulcatus* tick bites
Chlamydia trachomatis	Endemic trachoma, inclusion keratoconjunctivitis, urethritis, epididymitis, endometritis, salpingitis, perihepatitis, pneumonia, lymphogranuloma venereum	Humans	Sexual contact, hand-eye contact, insect fomites, infected birth canal
Chlamydophila pneumoniae	Pneumonia, bronchitis, sinusitis, pharyngitis	Humans	Inhalation of infected aerosols
Chlamydophila psittaci	Psittacosis (pneumonia), systemic infections	Birds, domestic animals	Inhalation of infected aerosols
Coxiella burnetii	Acute Q fever (self-limited febrile illness ± pneumonia, hepatitis); chronic Q fever (endocarditis, endovascular infections)	Cattle, sheep, goats, cats, rabbits, dogs, ticks	Inhalation of infected aerosols; ingestion of nonpasteurized dairy products
Ehrlichia chaffeensis	Human monocytotropic ehrlichiosis (HME): fever, headache, myalgia, systemic involvement including central nervous system	White-tailed deer, dogs and other canids, raccoons	*Amblyomma americanum* (Lone Star tick) and potentially *Dermacentor variabilis* (American dog tick) tick bites
Ehrlichia ewingii	"Ewingii" ehrlichiosis: fever, headache, myalgia, predominantly in immunocompromised individuals	Dogs and other canids	*Amblyomma americanum* (Lone Star tick) tick bites
Mycoplasma genitalium	Urethritis, cervicitis, endometritis, conjunctivitis	Humans	Sexual contact, vertical transmission in utero or intrapartum
Mycoplasma hominis	Acute pyelonephritis, bacterial vaginosis, pelvic inflammatory disease, postabortion bacteremia	Humans	Sexual contact, vertical transmission in utero or intrapartum
Mycoplasma pneumoniae	Tracheobronchitis, pneumonia, pharyngitis, extrapulmonary complications (meningoencephalitis, arthritis, etc.)	Humans	Contact with infectious aerosols or fomites
Orientia tsutsugamushi	Scrub typhus	Chiggers (larval mites)	*Leptotrombidium* spp. (chigger) bites
Rickettsia africae	African tick-bite fever	Not established	*Amblyomma* spp. tick bites
Rickettsia akari	Rickettsialpox	Mice and other small mammals	*Allodermanyssus sanguineus* (mouse mite) bites
Rickettsia conorii	Boutonneuse fever or Mediterranean spotted fever	Small mammals and ticks	*Rhipicephalus sanguineus* (Brown dog) tick bites
Rickettsia felis	Murine typhus-like illness	Fleas, opossums, cats, dogs	*Ctenocephalides felis* (cat fleas); contamination of infected flea feces into flea bite
Rickettsia prowazekii	Epidemic typhus	Humans, lice, flying squirrels	*Pediculus humanus* subsp. *corporis* (body louse); contamination of infected louse feces into louse bite
Rickettsia rickettsii	Rocky Mountain spotted fever	Ticks, small and medium-size mammals	*Dermacentor variabilis* (American dog tick), *Dermacentor andersoni* (wood tick), *Rhipicephalus sanguineus* (North and Central America), *Amblyomma cajennense* (Central and South America) tick bites
Rickettsia typhi	Murine typhus	Rats and other rodents, opossums	*Xenopsylla cheopis* (rat fleas) and *Ctenocephalides felis* (cat fleas); contamination of flea bite wound with infected flea feces
Tropheryma whipplei	Malabsorption, arthralgias, lymphadenopathy, culture-negative endocarditis, encephalitis	Humans	Environmental contacts (sewage, human stool, saliva); genetic predispositions
Ureaplasma urealyticum	Urethritis, epididymo-orchitis, urinary calculi, abortion, chorioamnionitis	Humans	Sexual contact, in utero or peripartum vertical transmission

TABLE 2 Diagnostic tests for *Anaplasma, Chlamydia, Chlamydophila, Coxiella, Ehrlichia, Mycoplasma, Rickettsia,* and *Ureaplasma*

Organism	Diagnostic test[a]
Anaplasma phagocytophilum	**Microscopy:** Giemsa or Wright stain of peripheral blood or buffy coat smears is positive in approximately 60% of infected persons. **Antigen tests:** None available. **Molecular tests:** EDTA-anticoagulated blood collected during the pretreatment acute phase of illness is used for PCR amplification. Species- and genus-specific tests are available. Current test of choice for diagnosis during active infection. **Culture:** EDTA-anticoagulated peripheral blood is inoculated onto HL-60, THP1, or other myelocytic cell lines. Positive cultures may be obtained between 3 and 30 days from many samples if inoculated within 24 h and if obtained before antimicrobial therapy. Lack of timely results precludes frequent use. **Serologic tests:** IFA is the most frequently used test. A fourfold or greater rise in titer or a single peak titer of ≥80 in a patient with typical clinical features of HGA confirms infection. Test sensitivity is between 90 and 100%; specificity is approximately 95%.
Chlamydia trachomatis	**Microscopy:** Organisms may be detected by Giemsa stain or DFA test. DFA is more sensitive, but neither test should be used alone. **Antigen tests:** Commercial EIAs vary in sensitivity but may cross-react with other bacterial LPS and used alone are not suitable for screening. EIA using blocking antibodies may increase specificity to 99.5%. Point-of-care tests are only 62–72% sensitive compared to culture and less sensitive compared to molecular tests. **Molecular tests:** Commercial NAATs (PCR, transcription-mediated amplification, strand-displacement amplification) are available and are tests of choice for confirmation of *C. trachomatis* infections. **Culture:** Recovered in many different cell cultures commonly including McCoy and HeLa cells. Test sensitivity is dependent on the quality of the submitted specimen. **Serologic tests:** MIF test is most sensitive and specific (test of choice). Diagnosis for acute *C. trachomatis* infection is confirmed by fourfold or greater rise in titer. Rising antibody titers may not be observed with chronic, repeated, or systemic infections. A single IgM titer of ≥32 supports a diagnosis of neonatal pneumonia.
Chlamydophila pneumoniae	**Microscopy:** Organisms may be detected by Giemsa stain or DFA test directed against LPS, but both tests are relatively insensitive. **Antigen tests:** Available EIA tests directed against LPS detect all *Chlamydia* and *Chlamydophila* species but are licensed only for *C. trachomatis.* **Molecular tests:** None commercially available. **Culture:** Recovered best if inoculated onto HL cells or Hep-2 cells. **Serologic tests:** MIF test is the test of choice. Diagnosis is confirmed by fourfold or greater rise in titer or single samples with IgM titer of ≥16 and/or IgG titer of ≥512.
Chlamydophila psittacii	**Microscopy:** Organisms may be detected by Giemsa stain or DFA test directed against LPS, but both tests are relatively insensitive. **Antigen tests:** Available EIA tests directed against LPS detect all *Chlamydia* and *Chlamydophila* species but are licensed only for *C. trachomatis*. **Molecular tests:** None commercially available. **Culture:** Recovered in many different cell cultures commonly including McCoy and HeLa cells. **Serologic tests:** MIF test is most sensitive and specific (test of choice).
Coxiella burnetii	**Microscopy:** DFA or immunohistochemistry tests may be performed but are insensitive and not widely available. **Antigen tests:** None commercially available. **Molecular tests:** PCR available only through reference laboratories; more sensitive than culture from frozen tissue, blood, or for chronic disease; less useful for serum, especially if stored frozen. **Culture:** May be cultivated in a variety of cell lines, especially Vero cells and HEL cells, or by inoculation into embryonated chicken yolk sacs or laboratory animals. **Serologic tests:** Most frequently used diagnostic test. The IFA test is recommended. Acute Q fever is confirmed by a fourfold or greater rise in titer to phase II antigens, or a single IgM titer of ≥50 and IgG titer of ≥200. Chronic Q fever is confirmed in a single serum sample with a ≥800 IgG titer to phase I antigen. A decreasing antibody titer suggests successful therapy.
Ehrlichia chaffeensis	**Microscopy:** Giemsa or Wright stain of peripheral blood or buffy coat smears is positive in up to 29% of infected persons. **Antigen tests:** None available. **Molecular tests:** EDTA-anticoagulated blood collected during the pretreatment acute phase of illness is used for PCR amplification. Species- and genus-specific tests are available. Current test of choice for diagnosis during active infection. Sensitivity ranges from 56 to 100%. **Culture:** EDTA-anticoagulated peripheral blood or CSF is inoculated onto DH82, THP1, HEL-22, Vero, HL-60, or other cell lines. Positive cultures may be obtained between 5 and >30 days from most samples if inoculated within 12 h and if obtained before antimicrobial therapy. Lack of timely results precludes frequent use.

(Continued on next page)

TABLE 2 *(Continued)*

Organism	Diagnostic test[a]
	Serologic tests: IFA is the most frequently used test. A fourfold or greater rise in titer or a single peak titer of ≥64 in a patient with a clinically compatible illness is considered evidence of infection. Test sensitivity is believed to be high.
Ehrlichia ewingii	**Microscopy:** Giemsa or Wright stain of peripheral blood or buffy coat smears occasionally reveals bacterial clusters (morulae) in neutrophils of infected persons.
	Antigen tests: None available.
	Molecular tests: EDTA-anticoagulated blood collected during the pretreatment acute phase of illness is used for PCR amplification. Species- and genus-specific tests are available in some reference and public health laboratories. Current test of choice for diagnosis during active infection. Sensitivity is not currently known but is suspected to be high.
	Culture: No method of in vitro culture has been developed.
	Serologic tests: No specific antibody test is available. Antibody tests are based largely on cross-reactivity with *E. chaffeensis* and alone are not diagnostic.
Mycoplasma genitalium	**Microscopy:** May be detected in genital fluids using DNA fluorochrome stains (Hoechst 33258 or acridine orange), but these are not specific.
	Antigen tests: Not recommended for diagnostic purposes.
	Molecular tests: PCR amplification tests are highly sensitive and have unknown specificity, but clinical studies have yielded variable results when compared with culture and serology. May be the only practical means for detection of the pathogen. Commercial kits are not currently available in the United States.
	Culture: Organisms are isolated from a variety of genital fluids. Wood-shafted cotton swabs should be avoided. Mycoplasmas are extremely labile, and appropriate transport medium should be used. Can be recovered on SP4 glucose broth supplemented with arginine. Growth conditions are not well established. Widely considered insensitive for diagnosis confirmation.
	Serologic tests: MIF and Western immunoblot methods have been described, although none are commercially available.
Mycoplasma hominis	**Microscopy:** May be detected in body fluids using DNA fluorochrome stains (Hoechst 33258 or acridine orange), but these are not specific.
	Antigen tests: None commercially available.
	Molecular tests: PCR tests have been developed but are less useful than culture.
	Culture: Organisms are isolated from a variety of clinical samples. Wood-shafted cotton swabs should be avoided. Mycoplasmas are extremely labile, and appropriate transport medium should be used. Can be recovered on SP4 glucose broth supplemented with arginine, on Shepard's 10B broth, or A8 agar. Growth occurs within 2 to 4 days.
	Serologic tests: Not recommended for routine use.
Mycoplasma pneumoniae	**Microscopy:** May be detected in body fluids by using DNA fluorochrome stains (Hoechst 33258 or acridine orange), but these are not specific.
	Antigen tests: Not recommended for diagnostic purposes.
	Molecular tests: PCR amplification tests are highly sensitive and have unknown specificity, but clinical studies have yielded variable results when compared with culture and serology. Commercial kits are not currently available in the United States.
	Culture: Organisms are isolated from a variety of clinical samples. Wood-shafted cotton swabs should be avoided. Mycoplasmas are extremely labile, and appropriate transport medium should be used. Can be recovered on SP4 glucose broth supplemented with arginine. Growth occurs after 21 days. Widely considered insensitive for diagnosis confirmation.
	Serologic tests: EIA tests are more sensitive and specific than CF and IFA; detection of seroconversion by demonstration of a fourfold increase in antibody titer is preferred, but detection of IgM antibodies in single serum samples may be useful. The cold agglutinin test is not recommended for diagnosis of *M. pneumoniae* infection.
Orientia tsutsugamushi	**Microscopy:** DFA or immunohistochemistry on skin or other tissues may be performed, but tests are not widely available.
	Antigen tests: Not available.
	Molecular tests: PCR amplification performed on EDTA-anticoagulated blood, buffy coat leukocytes, plasma, or tissue samples obtained during the acute phase of illness; available only through reference laboratories.
	Culture: Isolation is performed by intraperitoneal inoculation of mice. Performed only in reference and research laboratories.
	Serologic tests: With the IFA test in a region of endemicity, a titer of ≥400 is 98% specific and 48% sensitive. Lower cutoffs are used for populations in which the infection is not endemic. Indirect immunoperoxidase is also sensitive and specific with diagnostic cutoffs of ≥128 for IgG and ≥32 for IgM. Dot EIA kits are available and have lower sensitivity and specificity than IFA. Weil-Felix (*Proteus*) febrile agglutinins test is insensitive and nonspecific.

(Continued on next page)

TABLE 2 Diagnostic tests for *Anaplasma, Chlamydia, Chlamydophila, Coxiella, Ehrlichia, Mycoplasma, Rickettsia,* and *Ureaplasma (Continued)*

Organism	Diagnostic test[a]
Rickettsia africae and Rickettsia conorii	**Microscopy:** DFA or immunohistochemistry on skin biopsy of rash or eschar is sensitive and specific for spotted fever group rickettsiae. Antibodies are not commercially available. **Antigen tests:** Not available. **Molecular tests:** PCR amplification performed on EDTA-anticoagulated blood, buffy coat leukocytes, plasma, skin biopsy, or tissue samples obtained during acute phase of illness; available only through reference laboratories. **Culture:** Heparin-anticoagulated plasma or buffy coat cells or triturated skin biopsy specimens are inoculated into shell vials seeded with cell lines such as Vero, L-929, HEL, or MRC5. Infected cells are detected by immunofluorescence or PCR after 48 to 72 h; sensitivity is up to 59%. **Serologic tests:** IFA is sensitive using *R. africae, R. conorii,* or other spotted fever group rickettsial antigens (e.g., *R. rickettsii*) but is low during the acute phase of illness. A fourfold increase in titer is generally considered most specific, but single titers of ≥128 for IgG and ≥32 for IgM are considered diagnostically significant. A Dot EIA test that is modestly less sensitive and specific is available for *R. conorii*.
Rickettsia akari	**Microscopy:** DFA or immunohistochemistry on skin biopsy of rash or eschar is sensitive and specific for spotted fever group rickettsiae. Antibodies are not commercially available. **Antigen tests:** Not available. **Molecular tests:** PCR amplification performed on skin biopsy specimens of eschars obtained during acute phase of illness; available only through reference laboratories. **Culture:** Heparin-anticoagulated blood plasma or buffy coat cells are inoculated into shell vials seeded with cell lines such as Vero, L-929, HEL, or MRC-5. Infected cells are detected by Giemsa, Gimenez, or fluorescent antibody staining after 48 to 72 h. Sensitivity is not known. **Serologic tests:** IFA is sensitive using either *R. akari* or other spotted fever group rickettsial antigens (e.g., *R. rickettsii*) but is low during the acute phase of illness. A fourfold increase in titer is generally considered most specific, but single titers of ≥128 for IgG and ≥32 for IgM are considered diagnostically significant. *R. akari*-specific testing can be obtained in reference or public health laboratories.
Rickettsia felis	**Microscopy:** Not available. **Antigen tests:** Not available. **Molecular tests:** PCR amplification performed on EDTA-anticoagulated blood, buffy coat leukocytes, plasma, or tissue samples obtained during the acute phase of illness; available only through reference laboratories. **Culture:** Not available except through research laboratories. **Serologic tests:** IFA is sensitive using either *R. felis* or other spotted fever or typhus group rickettsial antigens but is low during the acute phase of illness. A fourfold increase in titer is generally considered most specific; diagnostic titers have not been established. *R. felis*-specific testing can be obtained in reference or public health laboratories.
Rickettsia prowazekii	**Microscopy:** Not available. **Antigen tests:** Not available. **Molecular tests:** PCR amplification performed on EDTA-anticoagulated blood, buffy coat leukocytes, plasma, or tissue samples obtained during the acute phase of illness; available only through reference laboratories. **Culture:** Heparin-anticoagulated blood plasma or buffy coat cells are inoculated into shell vials seeded with cell lines such as Vero, L-929, HEL, or MRC-5. Infected cells are detected by Giemsa, Gimenez, or fluorescent-antibody staining after 48 to 72 h. Sensitivity is not known. **Serologic tests:** IFA is sensitive using either *R. prowazekii* or *R. typhi* as antigen. A fourfold increase in titer is generally considered most specific, but single titers of ≥128 for IgG and ≥32 for IgM are considered diagnostically significant.
Rickettsia rickettsii	**Microscopy:** DFA or immunohistochemistry on skin biopsy of rash is 70% sensitive and 100% specific. Antibodies are not commercially available. **Antigen tests:** Not available. **Molecular tests:** PCR amplification performed on EDTA-anticoagulated blood, buffy coat leukocytes, plasma, or tissue samples obtained during the acute phase of illness; available only through reference laboratories. **Culture:** Heparin-anticoagulated blood plasma or buffy coat cells are inoculated into shell vials seeded with cell lines such as Vero, L-929, HEL, or MRC-5. Infected cells are detected by Giemsa, Gimenez, or fluorescent-antibody staining after 48 to 72 h. Sensitivity is not known. **Serologic tests:** IFA is sensitive using *R. rickettsii* or other spotted fever group rickettsial antigens (for example) but is low during the acute phase of illness. A fourfold increase in titer is generally considered most specific, but single titers of ≥128 for IgG and ≥32 for IgM are considered diagnostically significant.
Rickettsia typhi	**Microscopy:** DFA or immunohistochemistry on skin biopsy of rash is sensitive and specific. Antibodies are not commercially available.

(Continued on next page)

TABLE 2 *(Continued)*

Organism	Diagnostic test[a]
	Antigen tests: Not available. **Molecular tests:** PCR amplification performed on EDTA-anticoagulated blood, buffy coat leukocytes, plasma, or tissue samples obtained during the acute phase of illness; available only through reference laboratories. **Culture:** Heparin-anticoagulated blood plasma or buffy coat cells are inoculated into shell vials seeded with cell lines such as Vero, L-929, HEL, or MRC-5. Infected cells are detected by Giemsa, Gimenez, or fluorescent-antibody staining after 48 to 72 h. Sensitivity is not known. **Serologic tests:** IFA is sensitive using either *R. prowazekii* or *R. typhi* as antigen. A fourfold increase in titer is generally considered most specific, but single titers of ≥128 for IgG and ≥32 for IgM are considered diagnostically significant. A Dot EIA test that is modestly less sensitive and specific is available for *R. typhi*.
Tropheryma whipplei	**Microscopy:** Tissue biopsy with periodic acid-Schiff stain or immunohistochemistry. **Antigen tests:** None available. **Molecular tests:** PCR tests are currently the preferred method for specific diagnosis; performed with small intestinal biopsy specimens, blood, cardiac valve tissues; available through reference laboratories. **Culture:** Blood, small intestine tissue, cardiac valves, CSF, aqueous humor, and synovial fluid can be inoculated onto human fibroblast cell culture. Sensitivity of culture is not known, and at least 30 days are required before detection. **Serologic tests:** Not currently available.
Ureaplasma urealyticum	**Microscopy:** May be detected in body fluids using DNA fluorochrome stains (Hoechst 33258 or acridine orange), but these are not specific. **Antigen tests:** None commercially available. **Molecular tests:** PCR tests have been developed but are less useful than culture. **Culture:** Organisms are isolated from a variety of clinical samples. Wood-shafted cotton swabs should be avoided. Ureaplasmas are extremely labile, and appropriate transport medium should be used. Can be recovered on Shepard's 10B urea broth and A8 urea agar. Growth occurs within 2 to 4 days. **Serologic tests:** Not recommended for routine use.

[a]Abbreviations: CF, complement fixation; CIE, counterimmunoelectrophoresis; CSF, cerebrospinal fluid; DFA, direct fluorescent antibody; EIA, enzyme immunoassay; IFA, indirect fluorescent antibody; Ig, immunoglobulin; MIF, microimmunofluorescence; NAAT, nucleic acid amplification tests.

Staphylococcus, Micrococcus, and Other Catalase-Positive Cocci

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TAXONOMY

Members of the genera *Staphylococcus* and *Micrococcus* are gram-positive, catalase-positive cocci that were placed together with *Stomatococcus* and *Planococcus* in the family *Micrococcaceae* (162). However, the results of DNA base composition testing (155a), DNA-rRNA hybridization (86), and comparative oligonucleotide cataloguing of 16S rRNA (117, 179) indicate that the genera *Staphylococcus* and *Micrococcus* are not closely related. The genus *Staphylococcus* is most closely related to the newly described genus *Macrococcus* (100), but it also has a relatively close relationship to the genera *Bacillus, Brochothrix, Gemella, Listeria,* and *Planococcus*. These genera have been tentatively arranged together with staphylococci and several other genera in the family *Bacillaceae* (22) of the broad *Bacillus-Lactobacillus-Streptococcus* cluster (118, 178) or the order *Bacillales* (22). The genus *Micrococcus* is most closely related to the genus *Arthrobacter* of the coryneform or actinomycete group (177, 179). The genus *Micrococcus* has been dissected into the six genera, *Micrococcus* (comprising the species *Micrococcus luteus*, *Micrococcus lylae,* and *Micrococcus antarcticus*), *Kocuria* (comprising the species formerly known as *Micrococcus roseus, Micrococcus varians,* and *Micrococcus kristinae*), *Kytococcus* (formerly *Micrococcus sedentarius*), *Nesterenkonia* (formerly *Micrococcus halobius*), *Dermacoccus* (formerly *Micrococcus nishinomiyaensis*), and *Arthrobacter* (formerly *Micrococcus agilis,* a member of the "*Arthrobacter globiformis-Arthrobacter citreus* group") (95, 110, 176). The genus *Kocuria* is more closely related to the genus *Rothia* than to other actinomycetes and the genus *Kytococcus* is most closely related to the genus *Dermacoccus* (176). Additional gram-positive, catalase-positive cocci include *Alloiococcus otitis* (1, 49), *Rothia mucilaginosa* (formerly *Stomatococcus mucilaginosus*) (8, 37), and on occasion *Aerococcus* species. *Aerococcus* will be discussed in chapter 31.

DESCRIPTION OF THE GENERA

Members of the genus *Staphylococcus* are gram-positive cocci (0.5 to 1.5 μm in diameter) that occur in irregular grape-like clusters and, less often, singly and in pairs, tetrads, and short chains (three or four cells). They are nonmotile, non-spore forming, and usually catalase positive and are typically unencapsulated or have limited capsule formation under laboratory conditions. The species do not form gas from carbohydrates and are facultative anaerobes with the exception of *Staphylococcus saccharolyticus* and *S. aureus* subsp. *anaerobius,* which initially grow anaerobically but may become more aerotolerant on subculture (Table 1). The genus *Staphylococcus* is currently composed of 37 species (readers are referred to http://www.bacterio.cict.fr/, which provides a list of validly published names of species). Some uncommon strains of staphylococci require the presence of CO_2 or factors such as hemin or menadione for growth. Small-colony variants (SCVs) of *S. aureus* have been described which grow as small, nonpigmented, and nonhemolytic colonies on routine media such as blood agar. SCVs are often auxotrophic for menadione or hemin, have a reduced range of carbohydrate utilization, may fail to express several putative virulence factors, and are resistant to gentamicin owing to poor drug uptake. SCVs are proposed to have a defect in the electron transport chain.

The genome of *S. aureus* has a guanine-plus-cytosine (G+C) content of approximately 32% and is composed of a single chromosome of around 2.8 Mb predicted to carry approximately 2,500 genes. Several sequenced strains also contain a plasmid. Whole genome sequencing is in progress or has been completed for eight *S. aureus* isolates. Published sequences are available for strains N315, Mu50, MW2, COL, MRSA252, and MSSA476 (6, 59, 76, 99); sequencing of strains NCTC 8325 and Michigan vancomycin-resistant *S. aureus* (VRSA) is ongoing. Whole genome sequences have been published for *S. epidermidis* ATCC 12228 and RP62A (59, 206). The whole genome sequence of *S. epidermidis* consists of a single chromosome plus various numbers of plasmids (*S. epidermidis* ATCC 12228 contains six plasmids and strain RP62A contains one) totalling approximately 2.5 Mb. This species has a G+C content of 32% and contains around 2,400 to 2,500 coding sequences. Both *S. epidermidis* genomes contain fewer putative virulence determinants than that of *S. aureus*.

Members of the genus *Micrococcus* are gram-positive cocci (1 to 1.8 μm in diameter) occurring mostly in irregular clusters, tetrads, and pairs. Micrococci and staphylococci have been confused with one another for more than a century on the basis of similar cellular morphologies and Gram-stained appearances and positive catalase activities. Both genera are commonly found on mammalian skin and may be present in various human and veterinary clinical specimens, although micrococci are found less frequently

TABLE 1 Differentiation of members of the genus *Staphylococcus* from other gram-positive cocci[a]

Genus and exceptional species	G+C content (molecular %) of DNA	Strict aerobe	Facultative anaerobe or microaerophile	Strict anaerobe	Tetrad cell arrangement	Strong adherence on agar	Motility	Growth on:					Catalase reaction result[d]	Benzidine test result[e]	Modified oxidase test result[f]	Anaerobic acid production from glucose[g]	Aerobic acid production from glycerol	Resistance to:			
								5% NaCl agar	6.5% NaCl agar	12% NaCl agar	P agar in 18 h[b]	Schleifer-Kramer agar[c]						Lysostaphin (200 μg/ml)	Erythromycin (0.4 μg/ml)	Bacitracin (0.04 U)[h]	Furazolidone (100 μg)[i]
Staphylococcus spp.	30–39	−	*d*	−	*d*	−[j]	−	+	+	*d*	+	+	+	+	−	*d*	+	−	+	+	−
S. aureus subsp. *anaerobius*		−	±	±	−	−	−	+	+	*d*	−	ND	−	−	−	+	+	−	+	ND	−
S. saccharolyticus		−	±	±	+	−	−	+	+	±	−	ND	−	±	−	+	+	−	+	ND	−
S. hominis		±[k]	±	−	+	−	−	+	+	±	+	+	+	+	−	+	+	−	+	+	−
S. auricularis		−	+	−	+	−	−	+	+	±	−	ND	+	+	−	+	+	−	+	+	−
S. saprophyticus, *S. cohnii*, *S. xylosus*		*d*	*d*	−	−	−	−	+	+	±	+	+	+	+	−	−	+	−	+	+	−
S. kloosii, *S. equorum*, *S. arlettae*		±	±	−	−	−	−	+	+	±	*d*	+	+	+	−	−	+	−	+	+	−
S. intermedius		−	+	−	−	−	−	+	+	+	+	±	+	+	−	+	+	−	+	+	−
S. sciuri, *S. lentus*, *S. vitulinus*		±	±	−	*d*	−	−	+	+	*d*	*d*	+	+	+	+	−	+	−	+	+	−
Macrococcus[l]	38–45	±	±	−	*d*	−	−	+	+	±	*d*	ND	+	+	+	−	*d*	−	+	+	−
Enterococcus	34–42	−	+	−	−	−	*d*	+	+	(±)	±	(±)	−	−	−	+	*d*	+	+	+	−
Streptococcus	34–46	−	+	*d*	−	−	−	*d*	*d*	−	−	−	−	−	−	+	*d*	+	−	*d*	−
Aerococcus	35–40	−	+	−	+	−	−	+	+	+	−	ND	−	−	−	(+)	ND	+	ND	−	−
Planococcus	39–52	+	−	−	*d*	−	+	+	+	+	−	ND	+	+	ND	−	−	+	ND	ND	−
Alloiococcus	44–45	+	−	−	−	−	−	+	+	ND	ND	ND	±	−	−	ND	−	ND	ND	ND	ND
R. mucilaginosa	56–60	−	+	−	*d*	+	−	−	−	−	−	ND	±	+	−	+	*d*	+	ND	−	*d*
Micrococcus	66–75	+	−	−	+	−	−	+	+	*d*	−	−	+	+	+	−	−	+	−[m]	−	+
Kocuria kristinae	67	±	±	−	+	−	−	+	+	±	−	(±)	+	+	+	(+)	+	+	−	−	+

[a] Symbols and abbreviations: +, 90% or more species or strains positive; ±, 90% or more species or strains weakly positive; − , 90% or more species or strains negative; *d*, 11 to 89% of species or strains positive; ND, not determined. Parentheses indicate a delayed reaction.

[b] Growth on P agar is under aerobic conditions at 35 to 37°C. Positive growth is indicated for detectable formation of colonies of at least 1 mm in diameter; ± indicates detectable formation of colonies of between 0.5 and 1 mm in diameter. Growth on sheep or bovine blood agar is slightly greater but less discriminative between staphylococci and other genera.

[c] Growth is under aerobic conditions at 35 to 37°C for 24 to 48 h. Positive growth is indicated for a number of CFU on selective medium comparable to that on plate count agar and a colony of 0.5 mm in diameter; ± indicates a significant reduction in the number of CFU on the selective medium compared to that on plate count agar, and parentheses indicate a colony of pinpoint size to 0.5 mm in diameter.

[d] Sometimes a weak catalase or pseudocatalase reaction can be observed with certain strains of species designated as catalase negative. In some species, catalase activity may be activated by hemin supplementation.

[e] Detects the presence of cytochromes. Some strains of benzidine test-negative species can synthesize cytochromes on aerobic media supplemented with hemin (50).

[f] See reference 163.

[g] Standard oxidation/fermentation test (163).

[h] A disk is used. Positive indicates resistance and no zone of inhibition. *Micrococcus*, *Kocuria*, *Kytococcus*, *Stomatococcus*, and *Aerococcus* spp. are susceptible and have an inhibition zone of 10 to 25 mm in diameter.

[i] A disk is used. Positive indicates resistance and no zone of inhibition or a zone of up to 9 mm in diameter. Susceptible species have an inhibition zone of 15 to 35 mm in diameter.

[j] Some strains of *S. epidermidis* adhere tenaciously to the surface of agar, and this property is correlated with heavy slime production.

[k] *S. hominis* does not demonstrate growth in the anaerobic portion of a thioglycolate medium within 24 h and may produce only very poor growth in this portion following 3 to 5 days of incubation. However, it will grow and ferment glucose anaerobically (standard oxidation-fermentation test). Failure to grow anaerobically in thioglycolate may be due in part to inhibition by the ingredients.

[l] *Macrococcus* species can also be differentiated from *Staphylococcus* species on the basis of their generally larger Gram-stained cell size (≥2 μm) and larger number of chromosome fragments produced by digestion with NotI (12 to 36 fragments).

[m] A few *Micrococcus* strains demonstrate high-level (MIC, ≥50 μg/ml) erythromycin resistance.

than staphylococci and are generally regarded as saprophytic rather than as opportunistic pathogens. By the mid-1960s, a clear distinction could be made between staphylococci and micrococci on the basis of DNA base composition (155a). Members of the genus *Staphylococcus* have a G+C content of 30 to 39%, whereas members of the *Micrococcus* and related genera have G+C contents within the range of 66 to 75%. The genus *Micrococcus* is currently composed of three species (110, 176). *Micrococcus luteus* is the most common micrococcal species found in nature and in clinical specimens. In the diagnostic laboratory, staphylococci can be distinguished from micrococci on the basis of the former's resistance to bacitracin and susceptibility to furazolidone. Micrococci are oxidase positive, and staphylococci are oxidase negative, with the exception of *S. lentus*, *S. sciuri*, *S. vitulus,* and *S. fleurettii*. The more distantly related species *Kocuria varians*, *Kocuria kristinae*, and *Kytococcus sedentarius* are occasionally found in clinical specimens and can be distinguished from micrococci on the basis of their cellular fatty acid compositions and results of several simple tests listed below in the section on the identification of *Micrococcus* species.

Alloiococci are aerobic, gram-positive cocci, weakly catalase positive, occurring mostly in clusters and pairs. The G+C content is 44 to 45%. These organisms can be easily distinguished from the aerobic micrococci by their negative oxidase reaction.

R. mucilaginosa is a gram-positive coccus occurring mostly in clusters, is a facultative anaerobe, and is catalase variable. The G+C content is 56 to 60.4%. *R. mucilaginosa* can easily be distinguished from staphylococci and micrococci by its inability to grow on 5% NaCl agar.

Members of the genus *Macrococcus* (including the species *Macrococcus caseolyticus* [formerly *Staphylococcus caseolyticus*], *Macrococcus equipercicus, Macrococcus bovicus,* and *Macrococcus carouselicus*) can be distinguished from staphylococci on the basis of their higher G+C contents (38 to 45%), absence of cell wall teichoic acid (with the possible exception of *Macrococcus caseolyticus*), smaller genome size of approximately 1.5 to 1.8 Mb, and larger Gram-stained cell size of 1.3 to 2.5 μm in diameter. They can also be distinguished from most species of staphylococci by their oxidase activity. They are susceptible to a wide range of antibiotics and do not exhibit the antibiotic resistance profiles characteristic of many staphylococcal species. However, like staphylococci, macrococci have a cell wall peptidoglycan that has L-lysine as the diamino acid with an interpeptide bridge that is susceptible to the action of lysostaphin. Since the clinical significance of macrococci has yet to be established and the genus has a rather restricted host range including only whales and related aquatic mammals and hoofed animals, they will not be described further in this chapter.

NATURAL HABITATS

Staphylococci are widespread in nature, their major habitat being the skin and mucous membranes of mammals and birds. They may be found in the mouth, mammary glands, and intestinal, genitourinary, and upper respiratory tracts of these hosts. Staphylococci generally have a benign or symbiotic relationship with their hosts but can become pathogenic after gaining entry into the host tissue through trauma of the cutaneous barrier, inoculation by needles, or direct implantation of medical devices. Staphylococci found on humans and other primates include *S. aureus, S. epidermidis, S. capitis, S. caprae, S. saccharolyticus, S. warneri, S. pasteuri, S. haemolyticus, S. hominis, S. lugdunensis, S. schleiferi, S. auricularis, S. saprophyticus, S. cohnii, S. xylosus,* and *S. simulans* (88, 141). Most of these species produce resident populations on humans, although *S. xylosus* and *S. simulans* are usually transient residents on humans and are acquired primarily from domestic animals and their products. Some of the human staphylococcal species are transient or temporary residents on domestic animals. *S. aureus* is a major (resident and transient) species for primates, while specific ecovars or biotypes can be found occasionally living on different domestic animals or birds (41, 87). *S. schleiferi, S. intermedius,* and *S. felis* are commonly found living on carnivora (141). *S. lutrae* has been isolated from the European otter (53). *S. xylosus, S. kloosii,* and *S. sciuri* are common residents on a variety of rodents (141). *S. hyicus, S. chromogenes, S. sciuri, S. lentus,* and *S. vitulus* are common residents of ungulates and, in addition, may be isolated from their food products (42). The last three species are also common residents of whales and related aquatic mammals. Other staphylococci associated with food products include *S. fleurettii, S. condimenti, S. carnosus,* and *S. piscifermentans* (150, 183, 193). *S. nepalensis* has been isolated from goats of the Himalayas (173), and *S. pseudointermedius* has been isolated from a range of mammals (43).

Some *Staphylococcus* species demonstrate habitat or niche preferences on their particular hosts (88). For example, *S. capitis* subsp. *capitis* is found in large populations on the adult human head, especially the scalp and forehead, where sebaceous glands are numerous and well developed. *S. capitis* subsp. *ureolyticus* is also found on the head but may produce relatively large populations in the axillae of some individuals. *S. auricularis* has a strong preference for the external auditory meatus. *S. hominis* and *S. haemolyticus* generally produce larger populations on areas of the skin where apocrine glands are numerous, such as the axillae and pubic areas (88). *S. aureus* prefers the anterior nares as a habitat (93). The novobiocin-resistant staphylococci, particularly *S. cohnii*, are found in large populations on the human feet. *S. saprophyticus* appears in high numbers in the female genitourinary tract (154).

Micrococci are widespread in nature and are commonly found on the skin of humans and other mammals (91). They are generally believed to be temporary residents and are most frequently found on the exposed skin of the face, arms, hands, and legs. Alloiococci have been isolated from human middle ear fluid (1, 49), and *R. mucilaginosa* is probably a normal inhabitant of the mouth and upper respiratory tract (8).

CLINICAL SIGNIFICANCE

Staphylococcus

S. aureus is an important cause of community-acquired sepsis and is a leading nosocomial pathogen. Disease manifestations can be broadly divided into toxin-mediated diseases (such as food poisoning, scalded skin syndrome, and toxic shock syndrome [TSS]), infection of the skin and soft tissues (furuncles or boils, cellulitis, and impetigo), infection of deep sites (such as bone and joints and the heart valve, spleen, and liver [almost any organ can be involved]), and infection of the lung and urinary tract. An important complication of *S. aureus* bacteremia is dissemination of the organism to one or more distant sites. Additional manifestations of hospital-acquired infection include surgical wound infection, ventilator-associated pneumonia, bacteremia associated with intravenous devices, and infection associated with other types of prosthetic

material such as cerebrospinal fluid shunts, prosthetic joints, and vascular grafts.

S. aureus toxin-mediated diseases range in severity from self-limiting to life threatening. TSS is associated with colonization by or infection with a strain of *S. aureus* that is positive for one of the superantigens, most commonly TSS toxin-1 (TSST-1; around 75% of all TSS cases). TSST-1 is a member of a superantigen family that has the ability to stimulate T cells and induce tumor necrosis factor and the cytokine interleukin-1. Gaining notoriety in the 1980s in association with the use of high-absorbancy tampons (45), TSS is now also recognized to occur in nonmenstrual settings including invasive disease and *S. aureus* colonization of sites such as postoperative wounds. Those affected present with severe disease with high fever, hypotension, an erythematous rash that becomes desquamating 1 to 2 weeks later, and involvement of three or more organs (45). Diagnosis is made on clinical grounds. A clinical case definition devised by the Centers for Disease Control and Prevention is useful (26), although not all cases of suspected TSS have sufficient criteria to fulfill this definition. In such cases, circumstantial evidence for the diagnosis includes the isolation of an *S. aureus* strain that is capable of producing TSST-1 or another superantigen (the remainder being positive for staphylococcal enterotoxin B or C). Methods for detecting TSST-1 and enterotoxin production include enzyme-linked immunosorbent assay (128), reverse passive latex agglutination (Oxoid, Ogdensburg, N.Y.), and PCR (7, 125, 129, 164).

Staphylococcal scalded skin syndrome affects primarily neonates and young children and results from the action of *S. aureus* exfoliative toxins on skin epidermidis. Fragile blisters form which rupture and lead to skin loss associated with poor temperature control, fluid loss, and secondary infection (102). Diagnosis is made on the basis of clinical features, including Nikolsky's sign, in which the skin wrinkles on gentle pressure. *S. aureus* may be isolated from a site of localized infection, such as the umbilical stump, but blister fluid is usually culture negative for *S. aureus*.

Staphylococcal food poisoning results from ingestion of preformed staphylococcal enterotoxins (45). Nausea and vomiting occur after an incubation period of 2 and 6 h. Abdominal pain and diarrhea are also common features. Diagnosis is made on clinical grounds; suspected food can be cultured for the presence of *S. aureus*.

S. aureus bacteremia is a major scourge of modern medical care. Around two-thirds of all cases are related to nosocomial infection, much of which is associated with the use of intravenous devices. One of the greatest challenges faced in caring for individuals with *S. aureus* bacteremia is to determine whether the disease is uncomplicated or complicated (that is, associated with bacterial spread to one or more distant sites). A study of clinical identifiers for complicated disease represents significant progress (55). The most common deep-site infections involve bones, joints, and heart valves. Rates of *S. aureus* endocarditis have increased, with the use of intravenous catheters potentially contributing to this rise. A rare but important syndrome of rapidly progressive *S. aureus* necrotizing pneumonia and other invasive diseases affecting previously healthy children and young adults have recently been described (17, 60, 160). The strains responsible were positive for Panton-Valentine leukocidin, and pneumonia was often preceded by influenza-like symptoms. The increase in community-associated methicillin-resistant *S. aureus* (MRSA) infections is further discussed later in "Antimicrobial Susceptibilities."

The role of coagulase-negative staphylococci (CoNS) as nosocomial pathogens has been recognized and well documented over the last 2 decades, especially for the species *S. epidermidis* (147). An increase in the number of infections involving CoNS has paralleled the increasing use of prosthetic and in-dwelling devices and the growing number of immunocompromised patients in hospitals. *S. epidermidis* has been documented as a pathogen in numerous cases of bacteremia; native and prosthetic valve endocarditis; surgical (particularly sternal) wounds, urinary tract, and ophthalmologic infections; and prosthetic joint-, ventricular shunt-, peritoneal dialysis-, and intravascular catheter-related infections (158, 197). *S. saprophyticus* is an important opportunistic pathogen in human urinary tract infections, especially in young, sexually active females. It has been proposed as an agent of nongonococcal urethritis in males and as a cause of other sexually transmitted diseases, prostatitis, wound infections, and septicemia (158). *S. haemolyticus*, the second most frequently encountered CoNS species associated with human infections, has been implicated in native valve endocarditis, septicemia, peritonitis, urinary tract infections, and wound, bone, and joint infections (89, 158). *S. lugdunensis* has been reported as a cause of endocarditis (189). The aggressive nature of *S. lugdunensis* endocarditis, as reflected by the frequent need for valve replacement and the high mortality rate, indicates that rapid recognition of *S. lugdunensis* is needed. *S. lugdunensis* has also been implicated in arthritis, bacteremia, urinary tract infection, and prosthetic material-associated infection (89).

Other CoNS have been implicated in a variety of infections. For example, *S. capitis, S. caprae, S. saccharolyticus, S. simulans,* and *S. warneri* have been implicated in endocarditis; *S. capitis, S. cohnii, S. hominis, S. schleiferi, S. simulans,* and *S. warneri* have been implicated in bacteremia; *S. epidermidis* and *S. haemolyticus* have been implicated in meningitis (79); *S. warneri* and *S. simulans* have been implicated in osteomyelitis; *S. cohnii* has been implicated in native valve endocarditis and pneumonia; *S. cohnii, S. xylosus, S. schleiferi, S. hominis,* and *S. caprae* have been implicated in urinary tract infections; *S. caprae* and *S. cohnii* have been implicated in arthritis; *S. schleiferi* and *S. caprae* have been implicated in wound and joint infections and osteomyelitis (20); *S. warneri* has been implicated in multifocal discitis; and *S. capitis, S. schleiferi,* and *S. warneri* have been implicated in catheter-related infections (89, 158). *S. schleiferi* may cause wound infections in humans and has been implicated in infections related to pacemaker insertion (25, 75). *S. hominis* subsp. *novobiosepticus* has been isolated from human blood cultures and has been associated with clinically significant septicemia (90). *S. sciuri* has been isolated from wounds and from individuals with skin and soft tissue infections (123).

The coagulase-positive species *S. intermedius* and the coagulase-variable species *S. hyicus* are of particular importance in veterinary infections. *S. aureus* and these two coagulase-positive species are serious opportunistic pathogens of animals. *S. intermedius* has been associated with a variety of canine infections including cellulitis, otitis externa, pyoderma, abscesses, reproductive tract infections, mastitis, and wound infections (61). *S. intermedius* infections in humans are usually associated with animal bites. This species has been implicated in a food-poisoning outbreak involving butter blend products (85). *S. hyicus* has been implicated in infectious exudative epidermitis and septic polyarthritis in pigs and mastitis in cows. *S. schleiferi* subsp. *schleiferi* and *S. schleiferi* subsp. *coagulans* have been isolated

from dogs with pyoderma, and *S. schleiferi* subsp. *coagulans* has been isolated from dogs with external otitis (81).

Micrococcus, Alloiococcus, and *R. mucilaginosa*

Members of the genus *Micrococcus* and the related coccal genera *Kocuria* and *Kytococcus* are generally harmless saprophytes that inhabit or contaminate the skin, mucosa, and perhaps also the oropharynx; however, they can be opportunistic pathogens for the immunocompromised. *Micrococcus luteus* has been implicated as the causative agent in cases of intracranial abscesses, pneumonia, septic arthritis, endocarditis, and meningitis (165). *Kytococcus sedentarius* has been associated with prosthetic valve endocarditis. Other infections associated with micrococci and their relatives include bacteremia, continuous ambulatory peritoneal dialysis peritonitis, and infection associated with ventricular shunts and central venous catheters (3, 139).

Significant levels of inflammatory cells along with the observation of intracellular *Alloiococcus* led Faden and Dryja to suggest that this organism plays a pathogenic role in persistent otitis media (49). Additional studies employing either culture (65) or PCR (11, 74) support the role of alloiococci in chronic otitis media, but its pathogenic potential should be the subject of further investigation since it has also been found as an inhabitant of the external outer ear in healthy individuals (57).

R. mucilaginosa has been implicated in numerous reports of infection since the mid to late 1980s. This organism has been isolated in cases of bacteremia, endocarditis, endophthalmitis, intravascular catheter-related and central nervous system infections, pneumonia, peritonitis, and septicemia (64, 66, 103) and from a patient with cervical necrotizing fasciitis arising from an infected parotid cyst (116).

PREVENTION OF STAPHYLOCOCCAL INFECTION

There are no licensed vaccines available for the prevention of staphylococcal disease. Data on the efficacy of passive vaccination using hyperimmune antistaphylococcal immunoglobulin for the prevention of staphylococcal disease in high-risk groups have not been published to date. Hospital infection control measures are central to the prevention of nosocomial infection. Guidelines for the prevention of nosocomial transmission of MRSA are available (132). The recommendations continue to include the use of contact precautions for patients colonized by or infected with MRSA but also suggest that hospitals implement a program of active surveillance cultures to identify possible reservoirs in patients at high risk for carriage of MRSA at the time of hospital admission. A full discourse is outside the scope of this chapter; readers are referred to standard texts. Nasal carriage of *S. aureus* has been suggested as a risk factor for the development of infection (196). Infection rates are higher in carriers than in noncarriers, and patients with *S. aureus* sepsis are often infected with their own strain. Multiple studies have been conducted to determine the effect of temporary eradication of *S. aureus* carriage. This is usually achieved by the topical application of mupirocin to the anterior nose. Most studies have been conducted with the dialysis population. Several studies have shown a reduction in the rate of *S. aureus* infection in those receiving hemodialysis (14, 77, 94). Topical mupirocin applied to the peritoneal dialysis catheter exit site reduces *S. aureus* exit site infection and peritonitis (9, 185, 187). The effect of routine decolonization using mupirocin on the rate at which drug resistance will emerge is unclear. A double-blind, placebo-controlled trial of intranasal mupirocin treatment of patients undergoing surgery did not result in a significantly reduced rate of *S. aureus* surgical site infections overall, but the treatment did decrease the rate of all nosocomial *S. aureus* infections among patients who were *S. aureus* carriers (144). An evidence-based review of intranasal mupirocin treatment concluded that this was highly effective at eradication of nasal carriage in the short term, but this did not convert into clinical benefit overall (104). However, this review also reported that subgroup analyses and several small studies revealed lower rates of *S. aureus* infection among selected populations (such as the dialysis population) (104). A systematic review of the effect of mupirocin on rates of *S. aureus* infection in the peritoneal dialysis and hemodialysis populations provided further support for nasal eradication in these patients (182). A Cochrane review of the effects of topical and systemic antimicrobial agents on nasal and extranasal MRSA carriage, adverse events, and the incidence of subsequent MRSA infections concluded that there is insufficient evidence to support the use of topical or systemic antimicrobial therapy for eradicating nasal or extranasal MRSA (112). In summary, the strongest evidence for the efficacy of interrupting nasal *S. aureus* carriage rests with the renal dialysis population; the optimal use of nasal decolonization in other patient groups is less well defined. Despite the use of mupirocin for several decades, little resistance has been observed to date (16, 24, 111, 204). Rectal carriage may represent a reservoir for *S. aureus* for patients in the intensive care unit and liver transplant recipients (174); it is unclear whether nasal decolonization would interrupt gut or rectal carriage.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

The general principles of collection, transport, and storage of specimens as described in chapters 5, 6, and 20 of this Manual are applicable to the organisms listed in this chapter. No special methods or precautions are usually required for these organisms because they are easily obtained from clinical material of most infection sites and are relatively resistant to drying and to moderate temperature changes. Some strains of staphylococci may require anaerobic conditions or CO_2 supplementation for satisfactory growth, but these survive transport and limited storage in air.

DIRECT EXAMINATION

The direct microscopic examination of normally sterile fluids such as cerebrospinal fluid and joint aspirates may be useful. Direct examination of certain nonsterile fluids may also be useful if the microscopist evaluates the specimen by noting the presence of inflammatory cells versus epithelial cells. Even if large numbers of gram-positive cocci are present, only a presumptive report of "gram-positive cocci resembling staphylococci (or micrococci)" should be made. Culture and appropriate identification techniques must confirm this report; microscopy alone cannot adequately differentiate various species of staphylococci or micrococci from one another or from planococci, some streptococci, aerococci, various anaerobic cocci, and other cocci related to micrococci.

ISOLATION PROCEDURES

Considering the widespread distribution of staphylococci and micrococci over the body surface, careful procedures should be used to isolate organisms from the focus or foci of

infection without collecting surrounding normal flora (89). Distinguishing contaminants from the infecting staphylococci and micrococci continues to be an important clinical challenge.

The basic procedures for culture and isolation described in chapter 20 of this Manual should be followed. Every specimen should be plated onto blood agar (preferably sheep blood agar) and other media as indicated. On blood agar, abundant growth of most staphylococcal species occurs within 18 to 24 h, and abundant growth of micrococci occurs within 36 to 48 h. Although most CoNS colony morphologies cannot be distinguished from one another, preliminary identification testing should begin after overnight incubation. Further incubation of the primary isolation plate for 2 to 3 days allows the colony morphology to develop and helps to determine if the culture is mixed or pure. Failure to hold plates for 72 h can result in (i) selection of more than one species or strain if two or more colonies are sampled to produce an inoculum, (ii) selection of an organism(s) not producing the infection if the specimen contains two or more different species or strains, and (iii) incorrect labeling of a mixed culture as a pure culture. Colonies should be Gram stained, subcultured, and tested for genus, species, and when applicable, strain properties. The degree to which staphylococci are identified is discussed in more detail below. Most staphylococci of major medical interest produce growth in the upper as well as the lower anaerobic portions of the thioglycolate broth or semisolid agar.

Specimens from heavily contaminated sources such as feces should be streaked onto a selective medium such as mannitol-salt agar, Columbia colistin-nalidixic acid agar, lipase-salt-mannitol agar (Remel, Lenexa, Kans.), or phenylethyl alcohol agar. These media inhibit the growth of gram-negative organisms but allow staphylococci and certain other gram-positive cocci to grow. In addition, two chromogenic agars, CHROMagar Staph aureus (BD, Sparks, Md.) and *S. aureus* ID (bioMérieux, Hazelwood, Mo.), have been evaluated for their ability to isolate *S. aureus* from clinical specimens (46, 52, 58, 127, 146, 161). CHROMagar Staph aureus medium has been shown to inhibit *Pseudomonas* strains from cystic fibrosis patients as well as provide a better means to recover SCVs.

PCR methodology has been described that can detect MRSA directly from clinical samples. Most reports describe the use of a selective preenrichment step in overnight broth prior to PCR (51, 70, 82). One report described PCR using nasal swab material following an enrichment step for *S. aureus* with the use of immunomagnetic separation (56). Direct PCR has also been applied to a small number of nasal swabs without an enrichment step (80) and to clinical blood culture specimens (140). Detection of *S. aureus* directly from blood cultures using peptide nucleic acid fluorescence in situ hybridization (AdvanDx, Woburn, Mass.) has also been described (33).

IDENTIFICATION

Staphylococcus Species

Laboratory tests that differentiate members of the genus *Staphylococcus* from other gram-positive cocci are listed in Table 1. *Staphylococcus* species can be identified on the basis of a variety of conventional phenotypic characteristics, as shown in Table 2. The most clinically significant species can be identified on the basis of several key characteristics; these are shown in Table 3. In more specialized settings, species can be identified on the basis of molecular constituents such as cellular fatty acids (97) and further identified or characterized using genotypic tests such as analysis of macrorestriction patterns, ribotyping (21), amplification of DNA regions (62, 119, 126, 203), gene sequencing of loci including the 16s RNA gene, *hsp, sodA,* and *tuf* (73, 101, 170), and use of a PCR-enzyme-linked immunosorbent assay system (201). Most molecular methods pertaining to species-level identification are confined to the reference or research laboratory. Further discussion of the role of bacterial typing in clinical microbiology and the methods available is provided later in this chapter.

Colony Morphological Appearance

On nonselective blood agar, nutrient agar, tryptic soy agar, or brain heart infusion agar, isolated colonies of most staphylococci are 1 to 3 mm in diameter within 24 h and 3 to 8 mm in diameter after 3 days of incubation in air at 34 to 37°C, depending on the species. Exceptions are *S. aureus* subsp. *anaerobius, S. saccharolyticus, S. auricularis, S. equorum, S. vitulus,* and *S. lentus*, which grow more slowly and usually require 24 to 36 h for detectable colony development. Colony morphology can be a useful supplementary characteristic in the identification of species. In order for morphology differences among species to be observed, isolated colonies need to develop for several days at 34 to 37°C (and should be further augmented by 2 days of growth at room temperature).

The typical 24-h *S. aureus* colony is pigmented (cream yellow to orange), smooth, entire, slightly raised, and hemolytic on routine blood agar. Rare strains that produce abundant capsule material may have a glistening, wet appearance. Colonies reach 6 to 8 mm in diameter by 3 days of incubation. SCVs of *S. aureus* may require at least 48 h of incubation to become visible. Colony size is usually about 1/10 that of the wild type, and the colony lacks pigment. SCVs are often auxotrophic for menadione or hemin, have a reduced range of carbohydrate utilization, and are resistant to gentamicin (152). They may be isolated in purity or mixed with colonies with normal morphotypes, giving the appearance of a mixed culture, and may remain stable upon subculture or may rapidly revert to the wild type. They are most commonly isolated from patient populations with unusually persistent infections such as cystic fibrosis or chronic osteomyelitis and/or from patients who have prolonged exposure to aminoglycosides and trimethoprim-sulfamethoxazole (113, 152). Normal growth may be restored if the isolate is grown in the presence of menadione, hemin, and/or CO_2 supplementation (153).

The typical 24-h CoNS colony is nonpigmented, smooth, entire, glistening, slightly raised to convex, and opaque. Rare strong slime producers develop a mucoid colony morphology. Colony diameter reaches 2.5 to 6 mm by 3 days of incubation. Colonies of *S. haemolyticus* are smooth, butyrous, and opaque and are usually larger than those of *S. epidermidis* and *S. hominis*. *S. haemolyticus* and *S. hominis* may be nonpigmented or cream to yellow-orange. Colonies of *S. lugdunensis* are usually 4 to 7 mm in diameter by 3 days of incubation, smooth and glossy and may be nonpigmented or cream to yellow-orange. The edges are entire and rather flat, and the centers are slightly domed. *S. schleiferi* colonies are usually 3 to 5 mm in diameter by 3 days of incubation and are nonpigmented. They are smooth and glossy and are slightly convex, with entire edges. Colonies of *S. saprophyticus* are large (5 to 8 mm in diameter by day 3), entire, very glossy, opaque, smooth, butyrous, and more convex than the colonies of the aforementioned species. Approximately one-half of the

strains are pigmented, ranging from cream to yellow-orange. Colonies of *S. intermedius* and *S. hyicus* are relatively large, usually 5 to 8 mm in diameter. They are slightly convex, entire, smooth, glossy, and usually nonpigmented. Colonies of *S. intermedius* are translucent. Those of *S. hyicus* are more opaque, becoming translucent with prolonged incubation.

Colonies of the same strain generally exhibit similar features of size, consistency, edge, profile, luster, and color on nonselective media commonly used for the culture of staphylococci or micrococci. Certain strains may exhibit variant morphotypes, and in these situations, chromosomal analyses can be used to clarify the relationship of each morphotype. At least one colony of each morphotype should be selected from the primary isolation plate for subsequent analyses. Members of the same strain usually have the same biotype profile. However, further differentiation may be necessary if the strain has a common biotype profile.

Coagulase Production

The ability to clot plasma continues to be widely used as a criterion for the identification of pathogenic staphylococci associated with acute infections, i.e., *S. aureus* in humans and animals and *S. intermedius* and *S. hyicus* in animals. This activity is due to the action of coagulase which is secreted during bacterial growth. The test is performed by mixing 0.1 ml of an overnight culture in brain heart infusion broth with 0.5 ml of reconstituted plasma, incubating the mixture at 37°C in a water bath or heat block for 4 h, and observing the tube for clot formation by slowly tilting the tube 90° from the vertical. Alternatively, a large, well-isolated colony on a noninhibitory agar can be transferred into 0.5 ml of reconstituted plasma and incubated as described above. Any degree of clotting constitutes a positive test result. A flocculent or fibrous precipitate is not a true clot and should be recorded as negative. If no clot is formed by 4 h, the tube should be reincubated overnight at 35 to 37°C. Some strains of *S. intermedius* and most coagulase-producing strains of *S. hyicus* require more than 4 h for a positive coagulase test. If incubation exceeds 4 h, the following points must be considered: (i) staphylokinase produced by some strains may lyse the clot after prolonged incubation, yielding false-negative results; (ii) false-positive or false-negative results may occur if the plasma used is not sterile; and (iii) an inoculum from an agar-grown colony may not be pure and a contaminant may produce false results after prolonged incubation. For uncommon *S. aureus* strains requiring a longer clotting period, other characteristics (Table 3) should also be tested to confirm identity. Additional characteristics are required to identify rare coagulase-negative *S. aureus* strains.

Slide Agglutination Test

The slide agglutination test detects bacterial aggregation of *S. aureus* in the presence of plasma through the action of a cell wall-associated protein termed clumping factor A, which is an adhesin for fibrinogen. A positive slide test may also aid in the identification of *S. lugdunensis* and *S. schleiferi* (Table 3). It is performed by making a heavy uniform suspension of growth in distilled water, stirring the mixture to a homogeneous composition so as not to confuse clumping with autoagglutination, adding 1 drop of plasma, and observing for clumping within 10 s. Slide tests must be read quickly because false-positive results may appear with reaction times of longer than 10 s. In addition, colonies for testing must not be picked from media containing high concentrations of salt (e.g., mannitol-salt agar) because autoagglutination and false-positive results may occur. Some uncommon strains of *S. intermedius* may give a positive slide test result. Alternative methods include commercial kits that detect one or more of the following: clumping factor, protein A, and other surface antigens. Latex agglutination tests often have a higher specificity and sensitivity than the conventional slide test for the identification of *S. aureus*, although they are generally less reliable for the identification of *S. lugdunensis*. Some members of the *S. saprophyticus* and *S. sciuri* species groups and *Macrococcus* species may produce positive results with latex agglutination tests, but they are usually negative in the slide test. Due to the low levels of bound coagulase and protein A in MRSA strains, the detection of MRSA by rapid agglutination tests requires the incorporation of antibodies against staphylococcal capsular polysaccharide. Latex agglutination tests that detect both serotype 5 and serotype 8 capsular polysaccharides of methicillin-susceptible *S. aureus* and MRSA strains are available (54); however, false-positive reactions can occur (13, 68, 191). When the test organism is suspected to be *S. aureus*, negative slide tests should be confirmed by the coagulase test. Various manufacturers provide commercial latex agglutination kits, including the Slidex Staph (bioMérieux), BBL Staphyloslide (BD), Staphaurex (Murex Diagnostics Inc., Norcross, Ga.), Staphaurex and Staphaurex Plus (Remel), Mastastaph (Mast, Merseyside, United Kingdom), and Staphytect Plus and Dry Spot Staphytect Plus (Oxoid). A variety of plasmas may be used for the coagulase or slide agglutination tests. Dehydrated rabbit plasma containing EDTA is commercially available. Human plasma should not be used unless it has been tested for clotting capability and lack of infectious agents.

Heat-Stable Nuclease Production

A heat-stable staphylococcal nuclease (thermonuclease [TNase]) that has endo- and exonucleolytic properties and can cleave DNA or RNA is produced by most strains of *S. aureus, S. schleiferi, S. intermedius,* and *S. hyicus*. Some strains of *S. epidermidis, S. simulans,* and *S. carnosus* demonstrate weak TNase activity. TNase can be detected by a metachromatic-agar diffusion procedure and DNA-toluidine blue agar. A commercial TNase test with toluidine blue agar is available (Remel), and results can be interpreted in 4 h. Regular DNase agar such as that used for differentiation of *Enterobacteriaceae* should not be used for TNase detection.

Phosphatase Activity

Detection of phosphatase activity is based on the hydrolysis of *p*-nitrophenylphosphate into phosphate and *p*-nitrophenol by alkaline phosphatase. This process has been incorporated into several commercial biochemical test systems for staphylococcal species identification. Phosphatase activity is indicated by the release of yellow *p*-nitrophenol from the colorless substrate. Strains of *S. aureus, S. schleiferi, S. intermedius,* and *S. hyicus* and most strains of *S. epidermidis* are alkaline phosphatase positive. Phosphatase-negative strains of *S. epidermidis* can be distinguished from the related species *S. hominis* on the basis of their strong anaerobic growth in thioglycolate within 18 to 24 h or resistance to polymyxin B on a 300-U disk (BD).

Pyrrolidonyl Arylamidase Activity

Pyrrolidonyl arylamidase (pyrrolidonase) activity can be determined by the hydrolysis of pyroglutamyl-ß-naphthylamide (L-pyrrolidonyl-ß-naphthylamide [PYR]) into L-pyrrolidone and ß-naphthylamine, which combines with a PYR reagent (*p*-dimethylaminocinnamaldehyde) to produce a red color.

TABLE 2 Differentiation of *Staphylococcus* species

| Species | Characteristic[a] | | |

TABLE 2 Differentiation of *Staphylococcus* species *(Continued)*

Species	Characteristic[a]																																						
						Expression of:																				Acid production (aerobically) from:														
	Large colonies[b]	Colony pigmentation[c]	Anaerobic growth[d]	Aerobic growth[e]	Coagulase test result	Clumping factor[f]	Heat-stable nuclease	Hemolysins[g]	Catalase[h]	Oxidase[i]	Alkaline phosphatase	Arginine arylamidase	Pyrrolidonyl arylamidase[j]	Ornithine decarboxylase	Urease[j]	β-Glucosidase[j]	β-Glucuronidase[j]	β-Galactosidase[j]	Arginine utilization[j]	Acetoin production	Nitrate reduction	Esculin hydrolysis	Novobiocin resistance[k]	Polymyxin B resistance[l]	D-Trehalose	D-Mannitol	D-Mannose	D-Turanose	D-Xylose	D-Cellobiose	L-Arabinose	Maltose	α-Lactose	Sucrose	*N*-Acetylglucosamine	Raffinose
S. cohnii subsp. *urealyticus*	+	*d*	(+)	+	−	−	−	(*d*)	+	−	+	−	*d*	−	+	−	+	+	−	*d*	−	−	+	−	+	+	+	−	−	−	(+)	+	−	*d*	−
S. xylosus	+	*d*	*d*	+	−	−	−	−	+	−	*d*	−	*d*	−	+	+	+	+	−	*d*	*d*	*d*	+	−	+	+	+	*d*	+	−	*d*	+	*d*	+	+	−
S. kloosii	*d*	*d*	−	+	−	−	−	(*d*)	+	−	*d*	−	*d*	−	*d*	*d*	*d*	*d*	−	*d*	−	d	+	−	+	+	−	−	(*d*)	−	*d*	*d*	(*d*)	(±)	−	−
S. equorum	−	−	−	(+)	−	−	−	(*d*)	+	−	(+)	−	−	−	+	ND	+	*d*	−	−	+	*d*	+	ND	+	+	+	*d*	+	(*d*)	+	*d*	*d*	+	*d*	−
S. arlettae	*d*	+	−	+	−	−	−	−	+	−	(+)	−	−	−	−	ND	+	*d*	−	ND	−	−	+	ND	+	+	+	+	+	+	+	+	+	+	−	+
S. gallinarum	+	*d*	(+)	+	−	−	−	(*d*)	+	−	(+)	−	−	−	+	+	*d*	*d*	−	−	+	+	+	−	+	+	+	+	+	+	+	+	*d*	+	+	+
S. succinus	ND	−	−	+	ND	ND	ND	ND	+	−	+	ND	−	ND	+	ND	ND	ND	−	−	−	ND	ND	ND	+	ND	+	ND	ND	ND	ND	ND	+	ND	ND	(*d*)
S. simulans	+	−	+	+	−	−	−	(*d*)	+	−	(*d*)	−	+	−	+	−	*d*	+	+	*d*	+	−	−	−	*d*	+	*d*	−	−	−	−	(±)	+	+	+	−
S. carnosus subsp. *carnosus*	+	−	+	+	−	−	−	−	+	−	+	−	+	−	−	−	−	+	+	+	+	−	−	−	*d*	+	+	−	−	−	−	−	*d*	−	ND	−
S. carnosus subsp. *utilis*	−	−	+	+	−	ND	ND	ND	+	ND	−	ND	ND	ND	−	ND	−	−	+	ND	*d*	−	ND	ND	*d*	−	−	−	ND	−	−	−	−	−	ND	−
S. piscifermentans	ND	*d*	+	+	−	ND	−	−	+	−	+	ND	ND	ND	+	+	−	−	+	−	+	+	−	ND	+	*d*	−	−	−	−	−	*d*	*d*	*d*	ND	−
S. condimenti	ND	*d*	+	+	−	ND	ND	ND	+	ND	+	ND	ND	ND	+	ND	−	+	+	ND	+	−	ND	ND	+	+	+	−	ND	−	−	−	+	±	ND	−
S. felis	+	−	+	+	−	−	−	(*d*)	+	−	+	ND	ND	ND	+	−	−	+	+	−	+	ND	−	ND	+	+	+	ND	−	−	−	−	+	*d*	+	−
S. lutrae	−	−	+	+	+	−	(±)	+	+	−	+	ND	ND	ND	+	ND	ND	+	−	−	+	ND	−	ND	+	*d*	+	ND	+	ND	ND	+	+	ND	ND	ND
S. intermedius	+	−	(+)	+	+	*d*	+	*d*	+	−	+	−	+	−	+	*d*	−	+	*d*	−	+	−	−	−	+	(*d*)	+	*d*	−	−	−	(±)	*d*	+	+	−
S. delphini	+	−	(+)	+	+	−	−	+	+	−	+	ND	ND	ND	+	ND	ND	ND	+	−	+	ND	−	ND	−	(+)	+	ND	−	ND	−	+	+	+	ND	ND
S. hyicus	+	−	+	+	*d*	−	+	−	+	−	+	−	−	−	*d*	d	+	−	+	−	+	−	−	+	+	−	+	−	−	−	−	+	+	+	−
S. chromogenes	+	+	+	+	−	−	−	−	+	−	+	−	*d*	−	+	*d*	−	−	+	−	+	−	−	+	+	*d*	+	*d*	−	−	−	*d*	+	+	*d*	−
S. sciuri subsp. *sciuri*	+	*d*	(+)	+	−	−	−	(±)	+	+	+	−	−	−	−	+	−	−	−	−	+	+	+	−	+	+	(*d*)	(±)	(*d*)	+	*d*	(*d*)	(*d*)	+	−	−
S. sciuri subsp. *carnaticus*	−	*d*	(*d*)	+	−	*d*[p]	−	(±)	+	+	*d*	−	−	−	−	+	−	−	−	−	+	+	+	−	+	+	(*d*)	ND	+	(*d*)	*d*	(*d*)	(*d*)	+	−	−
S. sciuri subsp. *rodentium*	*d*	*d*	(*d*)	+	−	+[p]	−	(±)	+	+	*d*	−	−	−	−	+	−	−	−	−	+	+	+	−	(+)	+	(+)	ND	(*d*)	*d*	(*d*)	(*d*)	−	+	−	−
S. lentus	−	*d*	(±)	(+)	−	−	−	−	+	+	(±)	−	−	−	−	+	−	−	−	−	+	+	+	−	+	+	(+)	(±)	(±)	+	*d*	*d*	*d*	+	*d*	+
S. fleurettii	−	−	+	+	−	−	−	ND	+	+	*d*	−	−	−	−	ND	−	−	−	*d*	+	*d*	+	ND	+	ND	+	+	*d*	−	*d*	+	−	+	ND	−
S. nepalensis	−	−	+	+	−	−	−	−	+	−	+	−	+	−	+	ND	+	+	−	−	+	+	+	−	+	+	+	*d*	+	−	+	+	+	+	+	−
S. pseudointermedius	+	−	ND	+	+	−	+	+	+	ND	+[q]	ND	+	ND	+	+	−	+	ND	+	+	ND	−	ND	+	(+)	+	(+)	−	−	−	+	+	+	+	−

S. vitulinus	−	+	−	(+)	−	−	−	+	+	−	ND	−	−	*d*	−	−	−	−	*d*	+	ND	(*d*)	+	−	−	*d*	*d*	−	−	−	+	−	−

[a] Symbols and abbreviations (unless otherwise indicated): +, 90% or more strains positive; ±, 90% or more strains weakly positive; −, 90% or more strains negative; *d*, 11 to 89% of strains positive; ND, not determined. Parentheses indicate a delayed reaction.

[b] Positive is defined as a colony diameter of ≥ 6 mm after incubation on P agar at 34 to 35°C for 3 days and at room temperature (ca. 25°C) for an additional 2 days; exceptions are *S. succinus* (4 to 6 mm on tryptic soy agar) and *S. fleuretti* (8 to 12 mm on tryptic soy agar).

[c] Positive is defined by the visual detection of carotenoid pigments (e.g., yellow, yellow-orange, or orange) during colony development at normal incubation or room temperatures. Pigments may be enhanced by the addition of milk, fat, glycerol monoacetate, or soaps to P agar.

[d] Growth is in a semisolid thioglycolate medium. Symbols: +, moderate or heavy growth down the tube within 18 to 24 h; ±, heavier growth in the upper portion of the tube and weaker growth in the lower, anaerobic portion of tube; −, no visible growth within 48 h but very weak diffuse growth or a few scattered, small colonies may be observed in the lower portion of the tube by 72 to 96 h. Parentheses indicate delayed growth appearing within 24 to 72 h, sometimes noted as large, discrete colonies in the lower portion of the tube.

[e] Growth is on P agar or bovine, sheep, or human blood agar at 34 to 37°C. *S. equorum* grows slowly at 35 to 37°C; its optimum growth temperature is 30°C. Anaerobic species *S. saccharolyticus* and *S. aureus* subsp. *anaerobius* grow very slowly in the presence of air. *S. aureus* subsp. *anaerobius* requires the addition of blood, serum, or egg yolk for growth on primary isolation medium. *S. auricularis, S. lentus,* and *S. vitulus* produce just-detectable colonies on P agar in 24 to 36 h, and these colonies remain very small (1 to 2 mm in diameter).

[f] The slide agglutination test using rabbit or human plasma detects the expression of clumping factor. Use human plasma for *S. lugdunensis* and *S. schleiferi*. Latex agglutination is less reliable for the detection of clumping factor in *S. lugdunensis*.

[g] Hemolysis on bovine blood agar. Symbols and abbreviations: +, wide zone of hemolysis within 24 to 36 h; (+), delayed moderate to wide zone of hemolysis within 48 to 72 h; (*d*), no or delayed hemolysis; −, no or only very narrow (1 mm) zone of hemolysis within 72 h. Some strains designated as negative may produce a slight greening or browning of blood agar.

[h] Catalase and cytochrome synthesis cannot be induced in *S. aureus* subsp. *anaerobius* by the addition of H_2O_2 or hemin to the culture medium. Catalase activity can be induced in *S. saccharolyticus* by hemin supplementation. In this species, cytochromes *a* and *b* are present in small quantities.

[i] Determined by the modified oxidase test to detect the presence of cytochrome *c* (50).

[j] Determined primarily by commercial rapid identification tests (see the text).

[k] Positive (resistant) is defined by an MIC of ≥ 1.6 μg/ml or a growth inhibition zone diameter of ≤ 16 mm with a 5-μg novobiocin disk.

[l] Positive is defined by a growth inhibition zone diameter of < 10 mm with a 300-U polymyxin B disk.

[m] Approximately 6 to 15% of strains of *S. epidermidis* are negative for alkaline phosphatase activity, depending on the population sampled. A low but significant number of clinical isolates are phosphatase negative.

[n] rRNA gene restriction site polymorphism using pBA2 as a probe can distinguish this species from other staphylococcal species, including *S. warneri* (34).

[o] All strains tested are also resistant to penicillin G, methicillin, oxacillin, gentamicin, and streptomycin.

[p] Positive reactions are with the Staph latex agglutination test (Remel) that detects clumping factor and/or protein A.

[q] Positive with the STAPH-ZYM tests but negative with the API STAPH tests (43).

A commercial PYR broth and PYR reagent (Remel) recommended for the identification of streptococci are useful for distinguishing certain staphylococcal species. A loopful of a 24-h agar slant culture or several well-isolated colonies are dispersed in the PYR broth (containing 0.01% PYR) to a turbidity of a 2 McFarland standard. The suspension is incubated at 35°C for 2 h. After incubation, 2 drops of PYR reagent are added to each tube without mixing. The development of red within 2 min is indicative of positive activity. Yellow, orange, or pink is considered a negative result. The basic features of the test have been incorporated into several of the commercial biochemical test panels for the identification of staphylococcal species. *S. haemolyticus, S. lugdunensis, S. schleiferi,* and *S. intermedius* are usually pyrrolidonase positive.

Ornithine Decarboxylase Activity

Positive ornithine decarboxylase activity can identify the species *S. lugdunensis* with considerable accuracy. Ornithine decarboxylase activity can be determined as follows. Decarboxylase basal medium (BD) is prepared according to the instructions of the manufacturer, 1% (wt/vol) L-ornithine dihydrochloride is added, and the final medium is adjusted to pH 6 with 1 N sodium hydroxide before sterilization. The medium is dispensed in 3- to 4-ml amounts into small (13 by 100 mm) screw-cap tubes and autoclaved at 121°C for 10 min. A loopful of an overnight agar slant culture or several well-isolated colonies are dispersed in the test broth, followed by overlaying of each tube with 4 to 5 mm of sterile mineral oil. Inoculated tubes should be incubated at 35 to 37°C for up to 24 h. They can be read initially at as early as 8 h for the positive identification of most strains of *S. lugdunensis;* at this time, *S. epidermidis* will produce negative results. A positive reaction is indicated by alkalinization of the medium, with a change in the initial grayish color or slight yellowing (caused by the initial fermentation of glucose) to violet (caused by decarboxylation of L-ornithine). Yellow at 24 h indicates a negative result.

Urease Activity

Conventional urea broth or agar (BD and Oxoid) can be used to detect urease activity in staphylococcal species. Miniaturization of the broth urease test has been incorporated into several of the commercial biochemical test systems for species-level identification of staphylococci. *S. epidermidis, S. intermedius,* and most strains of *S. saprophyticus* are usually urease positive.

ß-Galactosidase Activity

Detection of high levels of ß-galactosidase activity for the differentiation of certain staphylococcal species can be accomplished by commercial biochemical test systems that use 2-naphthol-ß-D-galactopyranoside as a substrate. Fast blue BB salt in 2-methoxyethanol is added to the test well after an appropriate incubation period to detect free ß-naphthol released by ß-galactosidase. Positive activity is indicated by plum purple. By this assay, *S. intermedius* and most strains of *S. saprophyticus* are ß-galactosidase positive; *S. schleiferi* is delayed or weakly positive.

Acetoin Production

Acetoin production from glucose or pyruvate is a useful alternative characteristic to distinguish *S. aureus* (positive) from another coagulase-positive species, *S. intermedius* (negative), and coagulase-positive strains of *S. hyicus*

TABLE 3 Key tests for identification of the most clinically significant *Staphylococcus* species

Species	Result of test for[a]:								
	Colony pigmentation[b]	Staphylocoagulase	Clumping factor[b]	Heat-stable nuclease	Alkaline phosphatase	Pyrrolidonyl arylamidase[b]	Ornithine decarboxylase	Urease[b]	β-Galactosidase[b]
S. aureus subsp. *aureus*	+	+	+	+	+	−	−	*d*	−
S. epidermidis	−	−	−	−	+	−	(*d*)	+	−
S. haemolyticus	*d*	−	−	−	−	+	−	−	−
S. hyicus (veterinary)	−	*d*	−	+	+	−	−	*d*	−
S. intermedius (veterinary)	−	+	*d*	+	+	+	−	+	+
S. lugdunensis	*d*	−	(+)	−	−	+	+	*d*	−
S. schleiferi subsp. *schleiferi*	−	−	+	+	+	+	−	−	(+)
S. saprophyticus subsp. *saprophyticus*	*d*	−	−	−	−	−	−	+	+

[a] Symbols: +, 90% or more species or strains positive; ±, 90% or more species or strains weakly positive; −, 90% or more species or strains negative; *d*, 11 to 89% of species or strains positive. Parentheses indicate a delayed reaction.
[b] Descriptions are the same as those in Table 2.

(negative). The conventional Voges-Proskauer test tube method with an incubation of 72 h may be used. For speed and convenience, acetoin production can be determined by a miniaturized Voges-Proskauer test incorporated into several of the commercial biochemical test systems for staphylococcal species identification.

Novobiocin Resistance

A disk diffusion test for estimating novobiocin susceptibility and distinguishing *S. saprophyticus* from other clinically important species can be performed using a 5-μg novobiocin disk on Mueller-Hinton agar or tryptic soy sheep blood agar. With an inoculum suspension equivalent in turbidity to a 0.5 McFarland opacity standard and incubation at 35 to 37°C for overnight to 24 h, novobiocin resistance is indicated by an inhibition zone diameter of ≤16 mm with any of these media. Rapid disk elution procedures with either manual or automated instrument interpretation have also been reported to predict reliably novobiocin resistance after only 4 to 5 h of incubation (71). Novobiocin resistance is intrinsic to *S. saprophyticus* and several other species (Table 2) but is uncommon in the other clinically important species.

Polymyxin B Resistance

A disk diffusion test to estimate polymyxin B susceptibility using a 300-U polymyxin B disk can be performed on any of the media mentioned above for estimation of novobiocin resistance, although the largest database has been obtained for tryptic soy sheep blood agar. Test conditions are as described above for novobiocin resistance. The 5-μg novobiocin disk and the 300-U polymyxin B disk can be tested on the same inoculated plate. Polymyxin B resistance is indicated by an inhibition zone diameter of <10 mm. *S. aureus, S. epidermidis, S. hyicus*, and *S. chromogenes* are usually resistant. Some strains of *S. lugdunensis* are also resistant.

Acid Production from Carbohydrates

Acid production from carbohydrates can be detected by the agar plate method of Kloos and Schleifer (92). A wide range of individual diagnostic tests can be performed using Diatabs, which are available from Rosco, Taastrup, Denmark. Carbohydrate reactions are also incorporated into several of the commercial biochemical test systems for staphylococcal species identification. These systems use a more acid-sensitive indicator than the bromcresol purple (pH ≤5.2) in the agar plate method. For this and other reasons, results with conventional carbohydrate tests (Tables 2 and 3) may be slightly different from those obtained with rapid commercial biochemical test systems. The production of acid from maltose and sucrose and the absence of acid production from trehalose and mannitol can distinguish *S. epidermidis* from other novobiocin-susceptible species. Some uncommon strains of this species may produce acid from trehalose. These isolates can be distinguished from other species on the basis of phosphatase activity, anaerobic growth in thioglycolate, polymyxin B resistance, colony morphology, and the absence of ornithine decarboxylase and pyrrolidonase activities. Production of acid from trehalose, mannose, maltose, and sucrose and the absence of acid production from mannitol can identify *S. lugdunensis*. *S. schleiferi* produces acid from mannose and sometimes from trehalose but does not produce acid from mannitol, maltose, or sucrose. The production of acid from sucrose and turanose and the absence of acid production from mannose, xylose, cellobiose, arabinose, and raffinose can distinguish *S. saprophyticus* from other novobiocin-resistant species.

Identification of Species by Commercial Biochemical or Nucleic Acid Test Systems

Commercial kit identification systems and automated instruments (see chapter 15) can identify a number of the *Staphylococcus* species with an accuracy of 70 to >90% with relative speed and simplicity. Since their introduction, systems have been improved and expanded to include more species. *S. aureus, S. epidermidis, S. capitis, S. haemolyticus, S. saprophyticus, S. simulans,* and *S. intermedius* are identified reliably by most of the commercial systems now available. For some systems, reliability depends on additional testing as

Result of test for[a]:										
Acetoin production	Novobiocin resistance[b]	Polymyxin B resistance[b]	Acid production (aerobically) from:							
			D-Trehalose	D-Mannitol	D-Mannose	D-Turanose	D-Xylose	D-Cellobiose	Maltose	Sucrose
+	−	+	+	+	+	+	−	−	+	+
+	−	+	−	−	(+)	(*d*)	−	−	+	+
+	−	−	+	*d*	−	(*d*)	−	−	+	+
−	−	+	+	−	+	−	−	−	−	+
−	−	−	+	(*d*)	+	*d*	−	−	(±)	+
+	−	*d*	+	−	+	(*d*)	−	−	+	+
+	−	−	*d*	−	+	−	−	−	−	−
+	+	−	+	*d*	−	+	−	−	+	+

suggested by the manufacturer. This might include determining coagulase, clumping factor, or ornithine decarboxylase activity, anaerobic growth in thioglycolate, or novobiocin resistance. Identification systems now available include the following: RAPIDEC Staph (identification of *S. aureus, S. epidermidis,* and *S. saprophyticus*), and API STAPH, VITEK, a fully automated microbiology system that uses a gram-positive identification card, and the more recent Vitek 2 (all from bioMérieux); the MicroScan Pos ID panel (read manually or automatically on MicroScan instrumentation) and the MicroScan Rapid Pos ID panel (read by the WalkAway systems; in addition, the ID panels are available with antimicrobial agents for susceptibility testing [Dade MicroScan, Inc., West Sacramento, Calif.]); the Crystal gram-positive identification system, Crystal rapid gram-positive identification system, Pasco MIC/ID gram-positive panel, and BD Phoenix, an automated identification system (BD); the GP MicroPlate test panel (read manually with the Biolog MicroLog system or automatically with the Biolog MicroStation system; Biolog, Hayward, Calif.); the MIDI Sherlock identification system; microbial identification system that automates microbial identification by combining cellular fatty acid analysis with computerized high-resolution gas chromatography (MIDI, Newark, Del.); and the RiboPrinter microbial characterization system (Qualicon, Inc., Wilmington, Del.), based on ribotype pattern analysis. Rapid identification of the species *S. aureus* can be made using the AccuProbe culture identification test for *S. aureus* (Gen-Probe, Inc., San Diego, Calif.). This test is a DNA probe assay directed against rRNA and is reported to be very accurate (≥95% specificity) (2, 172). Coagulase-negative and slide agglutination test-negative strains of *S. aureus* should be identified correctly by the AccuProbe test. Real-time PCR and melt curve analysis based on amplification of a portion of the 16S rRNA gene are reported to give accurate results for nine common staphylococcal species (including *S. aureus* and *S. epidermidis*) (171).

Micrococcus and Related Species, *Alloiococcus,* and *R. mucilaginosa*

Pigment production and colony morphology may be used as simple tests in the presumptive identification of *Micrococcus* species and other related gram-positive cocci. *Micrococcus lylae* can be distinguished from *Micrococcus luteus* by its cream-white or unpigmented colonies, lack of growth on organic nitrogen agar, and lysozyme resistance. However, a small percentage of *Micrococus luteus* strains produce cream-white colonies. *Micrococcus* species can be distinguished from species of the genus *Kocuria* on the basis of their inability to produce acid, aerobically, from D-glucose and β-D-fructose. Furthermore, the species *Kocuria varians* and *Kocuria rosea* can be distinguished from micrococci by the former species' nitrate reduction and negative or only weak oxidase activity, and *Kocuria kristinae* can be distinguished from micrococci by the former's production of acid, aerobically, from glycerol and D-mannose, production of acetoin, and hydrolysis of esculin. The orange-pigmented species *Dermacoccus nishinomiyaensis* can be distinguished from micrococci by the former's small, pale orange colonies, nitrate reduction, and lack of growth on 7.5% NaCl agar. *Kytococcus sedentarius* differs from other micrococci by being resistant to penicillin and methicillin and exhibiting arginine dihydrolase activity. Colonies of this species may produce a brownish water-soluble pigment and grow more slowly than those of micrococci. *Nesterenkonia halobia* can easily be separated from micrococci because it requires at least 5% NaCl for growth.

Alloiococci form small, alpha-hemolytic colonies on blood agar after 48 h of incubation. Isolates can be distinguished from other similar organisms by their inability to utilize carbohydrates, obligate aerobic nature, and negative oxidase activity. On routine blood agar, colonies of *R. mucilaginosa* are mucoid or sticky, transparent to white, and nonhemolytic and often adhere to the agar. This organism is distinguished from other similar organisms by its inability to grow in the presence of 5% NaCl and its ability to hydrolyze gelatin and esculin.

TYPING SYSTEMS

Typing is used in the clinical setting to investigate the relationship between strains of the same bacterial species associated with a cluster of infections (such as during a suspected outbreak of MRSA infections) or to track the spread of strains such as MRSA strains between units or

hospitals. This information is central to the effective functioning of infection control. Typing may also be used for patients with recurrent staphylococcal infections to indicate whether a second episode is due to relapse or recurrence. Identification of the same bacterial species with identical genotype during independent episodes of infection in a given patient has a high probability of representing relapse, although the possibility that a single colonizing strain has caused both infections cannot be excluded.

A wide range of typing techniques have been described, but the most common technique used worldwide is pulsed-field gel electrophoresis (PFGE). This is particularly suitable for the investigation of local outbreaks. It may also be used to compare banding patterns of *S. aureus* between centers, although this has been hampered by a lack of reproducibility both within and between laboratories. Considerable efforts have been made to standardize PFGE methodology for the typing of MRSA (130, 131). Multilocus sequence typing (MLST) is a sequence-based approach that has the advantage of being highly reproducible (48), but it depends on the availability of sequencing facilities, is expensive, and requires highly experienced personnel. Comparison of MLST and PFGE in a microepidemiological setting has demonstrated similar discriminatory abilities (142). Although the use of MLST may become more widespread, it is predominantly a research tool at the present time.

Other typing techniques include ribotyping (186), which can be performed using an automated system such as the RiboPrinter microbial characterization system (Qualicon) (40, 124), and random amplified polymorphic DNA (122, 137, 188). PCR amplification methodology includes analysis of the 16S to 23S rRNA intergenic spacer region (69, 98), restriction digestion or sequencing of *coa* (encoding coagulase) (35, 63, 167), and sequencing of *spa* (encoding protein A) (96, 136). The PCR-based technique termed variable-number tandem repeat analysis has been used to study *S. aureus*. A scheme using five variable-number tandem repeat loci *(sdr, clfA, clfB, ssp,* and *spa)* has been reported to be equivalent to PFGE in terms of discriminatory power and reproducibility (121, 159). GeneChip technology (Affymetrix Inc.) (47) and matrix-assisted laser desorption ionization–time of flight mass spectrometry (10) have been described for the study of *S. aureus* but are currently research tools.

ANTIMICROBIAL SUSCEPTIBILITIES

Staphylococci may be susceptible to a wide range of antimicrobial agents, and testing of a panel of drugs is common. However, the major emphasis in contemporary laboratories is the isolation and identification of MRSA. Methicillin-resistant strains emerged soon after the introduction of methicillin into clinical practice. This was followed from the mid-1970s by outbreaks of MRSA infection in many countries, mostly caused by a single epidemic strain that was transferred between hospitals. The picture of MRSA within the hospital setting is now one of both epidemic and sporadic infection caused by a broader range of strains. In addition to being a nosocomial pathogen, MRSA has become a community pathogen. High-risk groups including intravenous drug users, individuals with a serious underlying disease, those on antimicrobial therapy, and those recently discharged from the hospital accounted for the first reports of MRSA infection in the community (106). Skin and soft tissue infections are the most commonly reported diseases involving community-associated MRSA, particularly in correctional facilities (30), among competitive sports participants (29) and military recruits (207), and in hospital nursery and maternity units (17, 160). Community-associated isolates typically possess the Panton-Valentine leukocidin locus, share a type IV staphylococcal cassette chromosome *mec* (SCC*mec*), and may be categorized into two to three PFGE clonal groups (39, 133, 135, 190). Although the community-associated MRSA isolates typically are susceptible to various antimicrobial classes, sole empiric therapy to treat these infections may prove unsuccessful as resistance to clindamycin and induced resistance to macrolide-lincosamide-streptogramin have been observed (19, 84, 133).

MRSA strains are often heteroresistant to beta-lactam antibiotics in that two subpopulations (one susceptible and the other resistant) coexist within a culture (32). Each cell in the population may carry the genetic information for resistance, but only a small fraction (10^{-8} to 10^{-4}) express the resistant phenotype under in vitro test conditions. The resistant subpopulation usually grows much more slowly than the susceptible subpopulation and may be missed during laboratory testing. The successful detection of heteroresistant strains depends largely on promoting the growth of the resistant subpopulations, which is favored by neutral pH, cooler temperatures (30 to 35°C), the presence of NaCl (2 to 4%), and possibly prolonged incubation (up to 48 h) (18). Cefoxitin disk (30 μg) diffusion is a better indicator of the presence of *mec*A-mediated resistance than the oxacillin disk (149, 192). Detection of oxacillin resistance in staphylococci can be done by the methods recommended by the Clinical and Laboratory Standards Institute (CLSI; formerly the National Committee for Clinical Laboratory Standards [NCCLS]) guidelines (36) and those described in chapters 73 and 74 of this Manual. To increase the accuracy of methicillin susceptibility testing, recent changes in the CLSI guidelines have given *S. lugdunensis* the same MIC breakpoints as *S. aureus* but susceptibility breakpoints separate from those of other CoNS isolates. Difficulties in the differentiation of MRSA isolates from borderline oxacillin-resistant strains of *S. aureus,* which are resistant due to the hyperproduction of beta-lactamase rather than the presence of the *mec*A determinant, may be problematic to many clinical laboratories not routinely utilizing PCR as their standard method of detecting MRSA. However, to date, there have been no reports of treatment failure with penicillinase-resistant penicillins in cases of infection with these organisms.

New chromogenic media, MRSA ID (bioMérieux) and CHROMagar MRSA (BD), have the ability to identify *S. aureus* isolates and differentiate MRSA isolates (44, 72, 145). The application of a cefoxitin disk to CHROMagar Staph aureus and *S. aureus* ID media may be an alternative means to detect MRSA and has the added benefit of preventing growth inhibition of MRSA on the whole agar surface (72).

A signal-amplified, sandwich hybridization kit, EVIGENE MRSA (AdvanDX) (107), is available for the rapid identification of *mec*A from either positive blood cultures or clinical isolates, although it is for research-only use in the United States. Rapid detection of the *mec*A gene product in *S. aureus* is also possible by using a commercial slide latex agglutination test (23, 134, 155). Accurate differentiation of MRSA from borderline oxacillin-resistant *S. aureus* by using a slide latex agglutination test has also been reported (114). Genotypic testing for detecting the presence of *mec*A may be performed using PCR (51, 67, 82, 155, 168, 181). Some

investigators include PCR detection of a second gene such as *femA, femB, orfX,* or *sa442* (80, 155, 168) or the *S. aureus*-specific marker gene *nuc* (thereby providing simultaneous identification) (38), and multiplex PCR may be performed to detect several genes associated with resistance to different antibiotic groups (143). A quadriplex PCR targeting the 16S RNA gene (*Staphylococcus* genus specific), *nuc* (*S. aureus* species specific), *mecA,* and *mupA* (a determinant of mupirocin resistance) (205) demonstrates the potential scope of this rapidly developing technology. The use of real-time PCR is increasing and provides the potential for rapid results. The GenoType MRSA test kit (Hain Lifescience, Nehren, Germany) detects *mecA* plus an *S. aureus*-specific sequence by using PCR and reverse hybridization (138). The probe-based Velogene rapid MRSA identification assay (ID Biomedical Corp., Vancouver, Canada) is based on a chimeric probe to detect the *mecA* gene (115). This test is reported to be accurate and has the advantage that it does not rely on PCR technology.

Antimicrobial susceptibility testing of SCVs of *S. aureus* presents a challenge for the clinical microbiology laboratory. Since SCVs have minimal amounts of ATP available and cannot effectively transport aminoglycosides into the cell, resistance to gentamicin and other aminoglycosides can be expected (152). Resistance to trimethoprim-sulfamethoxazole is observed in thymidine auxotrophs (151). The slow growth of SCVs limits the usefulness of antimicrobials directed against the cell wall. No approved method has been developed to determine the susceptibility profiles of SCVs. Susceptibililty profiles have been determined by using the broth or agar dilution MIC method with low levels of auxotroph supplements, disk diffusion under NCCLS guidelines with MH agar supplemented with blood, and the E test (AB Biodisk, Skolna, Sweden) with Mueller-Hinton (MH) agar supplemented with blood (83, 151).

With the increase in methicillin resistance in *Staphylococcus* species, other antibiotics have been used in the treatment of serious infections caused by this group of bacteria. The glycopeptide vancomycin has been regarded as the drug of choice for the treatment of infections due to methicillin-resistant staphylococci. The appearance of vancomycin-intermediate (MICs, 8 to 16 μg/ml) *S. aureus* (VISA), VRSA (MICs, ⩾32 μg/ml), and resistant CoNS requires the prudent use of vancomycin (12, 28, 31, 184). The Centers for Disease Control and Prevention have developed a recommended algorithm for testing *S. aureus* with vancomycin (see http://www.cdc.gov/ncidod/hip/vanco/vanco.htm). Automated susceptibility systems and disk diffusion are not reliable means to detect VISA or VRSA. A vancomycin-intermediate or -resistant result for a staphylococcal isolate should be verified by repeating a validated MIC method (broth microdilution reference MIC, agar dilution reference MIC, or the E test), and the organism should be identified (36). Detecting vancomycin-heteroresistant populations of staphylococci appears to be a challenge similar to that of detecting methicillin-heteroresistant populations. Heterogeneous VISA poulations include two populations of cells, the majority of which are susceptible to vancomycin (175). The heteroresistant populations are associated with clinical failure of glycopeptide therapy, and the use of other antimicrobials such as linezolid along with surgical intervention may be necessary to effectively treat the infection (78, 109, 156). An evaluation of methods used to detect staphylococcal isolates with reduced susceptibility to glycopeptides suggests that the E test with an inoculum with a 2.0 McFarland standard on brain heart infusion yields the highest sensitivity and specificity, with values of 88 and 88%, respectively (198). Interim guidelines have been established to prevent and control staphylococcal infections associated with reduced susceptibility to vancomycin (27).

Reviews have summarized the antimicrobial susceptibilities of staphylococcal species to various drugs (105, 141). Multidrug resistance is more frequent in *S. haemolyticus, S. epidermidis, S. hominis,* and *S. aureus* than in other staphylococcal species isolated in the clinical laboratory. Increased levels of resistance to antimicrobial agents used for therapy, including aminoglycosides, glycopeptides, quinolones, tetracyclines, macrolides, lincosamides, and trimethoprim-sulfamethoxazole, make the treatment of multidrug-resistant-staphylococcus infections difficult. Recent reviews provide a summary of a variety of new compounds being investigated or utilized for the therapy of MRSA infections, including daptomycin and linezolid (4, 166). Several studies comparing vancomycin and linezolid therapy indicate that linezolid may give a better outcome for patients with complicated skin and soft tissue infections (108, 199), pneumonia (202), and surgical site infections (200) caused by MRSA. It is important to note that clinical failures have occurred with linezolid (148, 157).

Micrococci appear to be susceptible to most antibiotics. Successful treatment has been accomplished using vancomycin, penicillin, gentamicin, clindamycin, or a combination of these antibiotics (120). Strains of alloiococci show resistance to both erythromycin and trimethoprim-sulfamethoxazole and relative resistance to beta-lactams (15). *R. mucilaginosa* appears to be variable in its antimicrobial susceptiblity (194, 195). The observation that *R. mucilaginosa* exhibits poor to no growth on MH agar and MH agar with sheep blood may make susceptibility testing a challenge for clinical microbiology laboratories.

INTERPRETATION AND REPORTING OF RESULTS

The first critical step when interpreting a culture that is positive for staphylococci is to distinguish between *S. aureus* and other species. The laboratory tests required to reach this point are essential in all cases. It is also important to have an appreciation of the quality of the specimen under consideration. A positive culture taken from a sterile site is relatively straightforward to interpret, but one from a contaminated site cannot be interpreted accurately away from the bedside. Clinical features and the results of other investigations should be taken into account during the interpretative process. There is no replacement for good communication between laboratory staff and primary physicians.

Identification of the causative pathogen represents the "gold standard" for the diagnosis of staphylococcal disease; serological testing lacks specificity and predictive accuracy. Isolation of *S. aureus* from a sterile-site culture (such as aspirated pus, blood, or cerebrospinal fluid) should be considered clinically significant unless there is strong evidence to the contrary. Contamination of high-quality samples by *S. aureus* is rare, and further samples should be taken if there is clinical doubt. Interpreting the isolation of *S. aureus* from specimens contaminated with normal flora requires consideration of setting, clinical features, and recent interventions. For example, interpretation of the significance of *S. aureus* in bronchoalveolar lavage fluid from a ventilated patient will require an evaluation of the features of pneumonia such as fever, raised peripheral white blood count, new pulmonary infiltrates upon chest radiography,

increasing oxygen requirements, and increased production of respiratory secretions. Quantitative culture may be helpful, for example, when interpreting a bronchoalveolar lavage or urine sample. Isolation of *S. aureus* from surgical wounds and other sites such as ulcers may represent infection or colonization, and clinical response to the culture should be strongly guided by the presence of features of infection. Colonization alone is an insufficient reason to treat, unless the patient is colonized by MRSA and decolonization is undertaken as part of a specific infection control policy. Susceptibility testing, and in particular the detection of methicillin resistance, should always be carried out following the identification of *S. aureus*.

Interpreting the significance of cultures that are positive for other staphylococcal species is more challenging. It is almost inevitable that samples taken from colonized sites will contain CoNS, and blood samples taken without careful skin cleaning will become contaminated with skin flora. Interpretation of blood cultures positive for CoNS requires knowledge of the presence of prosthetic material in the intravascular compartment, risk factors for true CoNS sepsis such as prematurity or the presence of an immunocompromised immune system, and clinical features of sepsis (the nature of which will vary depending on the clinical scenario). A review of CoNS infections has summarized factors that are helpful in distinguishing between true positive and contaminated cultures taken from a patient with clinical features of infection (89). These include (i) isolation of a strain in pure culture from the infected site or body fluid (most contaminated clinical specimens produce mixed cultures of different strains and/or species; however, some infections may be the consequence of more than one strain or species) and (ii) the repeated isolation of the same strain or combination of strains over the course of the infection. Thus, repeated acquisition of high-quality samples may prove extremely helpful, where clinically possible. If a patient with suspected bacteremia is due to start antibiotics, then independent blood samples may be taken from different sites over a relatively short period of time. The presence of the same CoNS on an intravenous catheter tip and in a blood culture is supportive evidence for intravenous catheter-associated bacteremia. Isolation of CoNS from peritoneal dialysis fluid or cerebrospinal fluid taken from ventricular shunts in a patient suspected of having infection is usually significant. Again, culture of more than one sample may provide helpful confirmation. Cultures of explanted prosthetic material such as pacemakers and joint replacements are often positive for CoNS and may contain mixed flora. When infection is considered likely and treatment is required, each species should be identified and all species should be tested for antimicrobial susceptibilities, after which the choice of therapy may be based on the combined antibiogram. If mixed colony morphology is observed in a potentially significant culture, one or more aged (≥72 h) colonies of a particular morphotype should be isolated from the primary isolation plate for each culture to be identified. The practice of pooling two or more young (24- to 48-h) colonies in the preparation of an inoculum or culture carries the risk of producing a mixed culture, resulting in an erroneous identification and accompanying antibiogram. Selecting only one young colony from a primary isolation plate carries the risk of missing the actual etiologic agent.

Interpretation of other samples will be influenced by sample type. For example, it is recognized that *S. saprophyticus* is a common cause of urinary tract infection, and laboratory tests that distinguish this species are often performed on urine specimens. Colony counts of ≥100,000 CFU/ml in two or more cultures of midstream urine are taken to indicate significant bacteriuria (180), but staphylococci grow relatively slow in urine and lower counts may be significant in the symptomatic patient. Isolation of CoNS from other samples taken from colonized sites rarely represents a clinically significant result. If doubt remains, repeated sampling (if possible, a higher-quality sample) can be performed.

Given the problems of interpreting cultures containing CoNS, it is worthwhile to develop a sampling strategy for complicated patients who probably have a true CoNS infection. This is particularly pertinent when dealing with patients with low-grade infection associated with implanted prosthetic material such as a joint replacement or vascular graft. These patients may require prolonged courses of antibiotics, and optimizing culture techniques may prove crucial to accurate diagnosis, therapy, and cure. As an example, deep-site samples taken by direct visualization from an area of suspected osteomyelitis during an operative procedure, particularly when the lesion communicates with the exterior, should include samples from each anatomical layer or region, and fresh instruments should be used to gather deep-site samples (5).

All CoNS associated with true infection require susceptibility testing. The decision about when to undertake species-level identification of CoNS associated with infection is a matter for debate. It is often helpful to identify to the species level when there are mixed populations (based on colony morphologies) in a clinically significant sample or when samples from different sites or serial samples are positive. The antibiogram is often used as a surrogate marker for the presence of the same or different strains or species, but this is not 100% reliable. It is also prudent to identify organisms from deep-tissue infections and from the bloodstreams of patients with suspected endocarditis, since the identification of *S. lugdunensis* raises the index of suspicion for aggressive disease. However, species-level identification of CoNS rarely leads to an intervention or to changes in therapy, and there is no compelling clinical reason to identify to the species level in many cases. Species-level identification of pathogenic CoNS contributes epidemiological information, but this is probably not sufficient justification to identify all CoNS to the species level in routine diagnostic laboratories. Contaminants do not require susceptibility testing or identification.

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*Streptococcus**

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29

TAXONOMY

The taxonomy of streptococci has experienced a number of changes during the last 20 years mostly due to the application of molecular biology techniques such as DNA-DNA reassociation experiments and 16S rRNA gene sequencing. Among the currently established 17 different genera of catalase-negative gram-positive cocci are several genera that were split off from the genus *Streptococcus* some time ago such as *Enterococcus* and *Lactococcus,* or more recently *Abiotrophia, Granulicatella, Facklamia,* and *Globicatella*. For an excellent review on the topic reflecting many of the recent changes, see reference 34. Molecular 16S rRNA analysis of streptococci has shown that species designation based solely on the hemolysis reaction, colony size, and the presence of Lancefield antigens does not correspond well with the molecular analysis in all cases. Taxonomic changes will certainly continue in the future. However, the traditional streptococcal classification system is well established and still of value to the clinical microbiologist. The phenotypic classification system correlates with clinical syndromes caused by different species and enables a first distinction of broad categories of streptococci that is useful for the choice of further tests and guidance of empirical treatments. The information and the identification schemes presented in this chapter therefore adhere in many aspects to this well-known phenotypic classification system.

The classical phenotypic differentation of streptococci separates the group of beta-hemolytic streptococci, which are discussed first in this chapter, from the group of non-beta-hemolytic streptococcal species. Beta-hemolytic streptococci, also referred to as pyogenic streptococci, include the species *S. pyogenes, S. agalactiae, S. dysgalactiae, S. equi,* and *S. canis*. The designation pyogenic streptococci is more precise, since the group includes species that are non-beta-hemolytic like *S. dysgalactiae* subsp. *dysgalactiae,* which is closely related to several beta-hemolytic species, and the term excludes beta-hemolytic strains of the *S. anginosus* group, which are more appropriately placed into the viridans streptococcal group. The small colony size of streptococci from the anginosus group (<0.5 mm) helps to distinguish them from the large-colony-forming (>0.5 mm) streptococci of the pyogenic group. Species from the pyogenic or beta-hemolytic group are further characterized by the presence of Lancefield antigens, which correlates in the case of group A streptococci fairly well but not precisely with the species designation *S. pyogenes*. While the B antigen appears to be limited to *S. agalactiae,* the Lancefield group A antigen has been detected not only in *S. pyogenes* but also in *S. dysgalactiae* subsp. *equisimilis* isolates (16) and in species from the *S. anginosus* group, (Table 1). Correlation with the other Lancefield antigens is even more complicated, which led to the need for novel species designations, presented below and in Table 1.

The group of nonpyogenic streptococci includes mostly α-hemolytic as well as nonhemolytic and even beta-hemolytic streptococcal species from the large category of viridans streptococci. In a study of the genus *Streptococcus* based on sequence comparisons of small-subunit (16S) rRNA genes, five species groups of viridans streptococci were demonstrated (57) in addition to the pyogenic group (beta-hemolytic, large-colony formers). These nonpyogenic groups were designated the *S. mitis* group, the *S. anginosus* group, the *S. mutans* group, the *S. salivarius* group, and the *S. bovis* group. A few streptococcal species were not unequivocally assigned and remain ungrouped. Descriptions of the species in the nonpyogenic groups follow the discussion on *S. pneumoniae*. Among α-hemolytic streptococci, *S. pneumoniae* can be separated from other streptococci of the viridans group through bile solubility and optochin susceptibility. However, phenotypic characterization and taxonomic considerations place *S. pneumoniae* into the *S. mitis* group (57). The relationship of *S. pneumoniae* to other species of the *S. mitis* group is so close that 16S rRNA gene analysis reveals greater than 99% identity to the nucleotide sequences of *S. mitis* and *S. oralis*. A novel, closely related species, *S. pseudopneumoniae,* has recently been split off from *S. pneumoniae,* following DNA-DNA hybridization studies and phenotypic characterization (1). Strains are nonencapsulated and insoluble in bile, and their clinical significance is currently uncertain.

S. mitis, S. sanguinis, S. parasanguinis, S. gordonii, S. cristatus, S. oralis, S. infantis, S. peroris (58), and *S. pneumoniae* are members of the *S. mitis* group. These species form a group whose classification and nomenclature have been a source of considerable confusion in the past. Among the changes that were made is the reclassification of the original *S. mitis* type strain as an *S. gordonii* strain and the subsequent replacement by a new *S. mitis* type strain (NCTC12261^T) (61). Further

* This chapter contains information presented in chapter 29 by Kathryn L. Ruoff, R. A. Whiley, and D. Beighton in the eighth edition of this Manual.

TABLE 1 Phenotypic characteristics of beta-hemolytic streptococci[a]

Species	Lancefield group	Colony size[e]	Hosts	Bacitracin	PYR[f]	CAMP[g]	VP[i]	Hippurate hydrolysis	Trehalose	Sorbitol
S. pyogenes	A	Large	Humans	+	+	–[h]	–	–	+	–
S. agalactiae	B	Large	Humans, cows	–	–	+	–	+	v	–
S. dysgalactiae subsp. *dysgalactiae*[b]	C	Large	Animals	–	–	–	–	–	+	v
S. dysgalactiae subsp. *equisimilis*	A, C, G, L	Large	Humans	–	–	–	–	–	+	–
S. equi subsp. *equi*	C	Large	Animals	–	–	–	–	–	–	–
S. equi subsp. *zooepidemicus*	C	Large	Animals, humans[c]	–	–	–	–	–	–	+
S. canis	G	Large	Dogs, humans[c]	–	–	+	–	–	v	–
S. anginosus group[d]	A, C, G, F, none	Small	Humans	–	–	–	+	–	+	–
S. porcinus	E, P, U, V, none	Large	Swine, humans[c]	–	+	+	+	v	+	+

[a] Symbols and abbreviations: +, positive; –, negative; v, variable.
[b] *S. dysgalactiae* subsp. *dysgalactiae* is α-hemolytic on sheep blood agar plates.
[c] *S. equi* subsp. *zooepidemicus*, *S. canis,* and *S. porcinus* are primarily animal pathogens that are only rarely isolated from humans.
[d] Species included in the *S. anginosus* group can be beta-hemolytic, alpha-hemolytic, or nonhemolytic on sheep blood agar plates.
[e] Large colony size refers to colonies >0.5 mm after 24 h of incubation, whereas small colony size is <0.5 mm.
[f] Presence of the enzyme pyrrolidonyl aminopeptidase.
[g] CAMP factor reaction (synergistic hemolysis in the presence of *S. aureus* β-hemolysin).
[h] *S. pyogenes* may occasionally yield a false-positive CAMP factor reaction.
[i] Voges-Proskauer test (formation of acetoin from glucose fermentation).

confusion is caused by the use of the term "biotype," especially for the species *S. sanguinis*. While the biotypes differ in phenotypic reactions, biotypes are not included in the approved lists of bacterial names and appear to vary between different studies (7, 61), and in most cases, no type strains are available. Based on phenotypic reactions (especially arginine hydrolysis and esculin tests), the *S. mitis* group can be further subdivided into the *S. sanguinis* and the *S. mitis* groups (34), but based on 16S rRNA analysis, these two groups appear to belong together (57). Since correlation of the renamed streptococcal species with human infections is still difficult, we chose to present these species as part of the *S. mitis* group until further information becomes available.

The small-colony-forming *S. anginosus* group consists of the three distinct species *S. anginosus, S. constellatus,* and *S. intermedius* (118). It includes streptococcal species previously referred to as Lancefield group F streptococci, "*S. milleri*" group or "*S. milleri.*" The species "*S. milleri*" has no standing taxonomically. The *S. mutans* group comprises the species *S. mutans, S. sobrinus, S. criceti,* and *S. ratti* and numerous species that have been identified only from animals thus far *(S. downei, S. ferus, S. macacae,* and *S. hyovaginalis)*.

The human species *S. salivarius, S. vestibularis,* and *S. thermophilus,* which is found in dairy products, belong to the *S. salivarius* group. Whether *S. thermophilus* was a subspecies of *S. salivarius* was controversial, although DNA-DNA reassociation experiments determined that they are two separate species (96). The whole *S. salivarius* group is closely related to the *S. bovis* group. Some streptococcal species that are currently part of the *S. bovis* group *(S. infantarius* and *S. alactolyticus)* (94) were formerly part of the *S. salivarius* group (34).

The *S. bovis* group has experienced extensive taxonomic changes (28, 80, 88, 94, 95). Four DNA clusters are currently recognized. DNA cluster I consists of the species formerly known as animal strains of *S. bovis* and *S. equinus*. Molecular analyses of these strains showed that they belong to a single species (41), and the earlier species name *S. equinus* has been formally adopted. DNA cluster II consists of one species, *S. gallolyticus,* with three subspecies: subspecies *gallolyticus* (formerly *S. bovis* biotype I), subspecies *pasteurianus* (formerly *S. bovis* biotype II.2), and subspecies *macedonicus*. DNA cluster III consists of one species, *S. infantarius* (formerly *S. bovis* biotype II.1), with two subspecies: subspecies *infantarius* and subspecies *coli* (formerly called *S. lutetiensis*). DNA cluster IV consists of one species, *S. alactolyticus*. These changes were made because DNA-DNA reassociation studies revealed considerable heterogeneity among the human isolates described as *S. bovis* biotypes.

DESCRIPTION OF THE GENUS

Bacterial species belonging to the genus *Streptococcus* are catalase-negative, gram-positive cocci of less than 2 μm that tend to grow in chains in liquid media. The cell wall composition is typical for gram-positive bacteria and consists mainly of peptidoglycan with glucosamine and muramic acid as amino sugars and galactosamine as a variable component. A variety of carbohydrates, surface protein antigens, and teichoic acid are attached to the cell wall and are, among other characteristics, responsible for species differences and clonal differences among streptococci. Most species of the genus *Streptococcus* have a low G+C content of DNA that lies in the range of 34 to 46%. Streptococci are facultative anaerobic bacteria. Due to a lack of heme compounds, streptococci are incapable of respiratory metabolism. Some species of the viridans streptococcal group and *S. pneumoniae* require 5% CO_2 levels for adequate growth, and the growth of many streptococcal species is enhanced in the presence of 5% CO_2. The temperature optimum for most streptococci is around 37°C, while some species like *S. uberis* also grow at temperatures as low as 10°C. The complex nutritional

requirements of streptococci are usually provided by the addition of blood or serum to the growth medium. Glucose and other carbohydrates are metabolized fermentatively, with lactic acid as the major metabolic end product. The addition of glucose or other carbohydrates to liquid medium enhances growth but lowers the pH, resulting in growth inhibition unless the medium is highly buffered (e.g., Todd-Hewitt broth [THB]). Leucine aminopeptidase (LAP) is produced by all streptococci and enterococci but can also be found in lactococci, pediococci, and other catalase-negative gram-positive cocci. It helps to distinguish these species from the LAP-negative *Aerococcus* species and *Leuconostoc*. All streptococci are catalase negative upon exposure to 3% hydrogen peroxide. False-positive reactions may occur if bacteria are grown on blood-containing media.

EPIDEMIOLOGY AND TRANSMISSION

Streptococci can cause infections in humans and in many different animal species including mammals and fish. Different species of streptococci are frequently found as commensal bacteria on mucous membranes. Occasionally streptococci are present as transient skin microbiota. Several streptococcal species exhibit high virulence potential, but even the highly virulent streptococcal species are frequently found as colonizing strains without any apparent effect on the host. *S. pneumoniae* was responsible for approximately 40,000 invasive infections in the United States in 2003, leading to an estimated 5,500 deaths (http://www.cdc.gov/abcs), and was found as a colonizing bacterial species in many asymptomatic carriers. The asymptomatic carriage rate for *S. pneumoniae* differs considerably between children and adults. Detection rates of 30 to 70% have been reported for young children, depending on the sampling method, while carriage rates among healthy adults are often reported to be below 5% (47, 51, 90). Evidence for household transmission between parents and their children has been found, demonstrating colonization rates for adults living in a household with young children that are significantly higher than in adults with no contact with preschool children (51).

Due to active bacterial surveillance in the emerging infections program network (99), reliable epidemiologic data on invasive infections due to *S. pneumoniae* (described above), *S. pyogenes* (group A), and *S. agalactiae* (group B) have been obtained for a population of 17 to 30 million people in the United States during 2003. *S. pyogenes* caused an estimated 11,300 cases of invasive disease and 1,800 deaths, with the peak of infections in people older than 65 years. Invasive infections due to *S. agalactiae* were second to those due to *S. pneumoniae,* with an estimated 20,400 cases and 2,200 deaths in 2003. Reflecting the ongoing changes in the epidemiology of group B streptococcal disease, the highest attack rates were observed for patients less than 1 year and adults greater than 65 years of age. Apart from causing invasive infections, both of these streptococcal species are frequently encountered as colonizing strains. While asymptomatic colonization of the nasopharynx with *S. pyogenes* occurs in less than 5% of the adult population in most studies, *S. agalactiae* colonization rates of the urogenital and gastrointestinal tracts can be demonstrated in 10 to 30% of the female as well as the male population. No significant differences are observed in the colonization rates of pregnant and nonpregnant women.

Transmission of streptococcal infections can occur by different routes. Pathogenic species like *S. pyogenes* and *S. pneumoniae* are primarily transmitted through droplets or direct contact. Transmission can first lead to colonization with the potential for the development of a subsequent infection. The tooth decay-causing species *S. mutans* is transmitted from mother to child during early infancy, most probably through oral secretions. Transmission from mother to child is also the typical transmission mode for neonatal invasive *S. agalactiae* infections. Newborns acquire the bacteria usually during delivery, although transmissions, shortly after birth, from the mother or health care personnel to infants have been documented, especially in late-onset neonatal infections. Endogenous infections most often occur by viridans streptococci as part of the oral microbiota.

Streptococcal infections do not represent classical zoonoses, although most species have a preferred host. While occasional animal-to-human transmissions do occur, as in the case of *Streptococcus suis* (67), genotypic and phenotypic analyses of animal and human strains demonstrated that the strains causing human infections were distinct from the strains causing animal infections. For beta-hemolytic group C and G streptococci, such an analysis led to an important change in species designations (111). Human group C and group G streptococcal strains were demonstrated to belong to a separate subspecies, designated *S. dysgalactiae* subsp. *equisimilis,* while animal group C, G, and L streptococci are classified as *S. dysgalactiae* subsp. *dysgalactiae, S. canis, S. equi* subsp. *equi,* and *S. equi* subsp. *zooepidemicus* (27, 40). The reservoir for *S. dysgalactiae* subsp. *equisimilis* strains is the human host, and transmission usually occurs among humans.

CLINICAL SIGNIFICANCE

Streptococcus pyogenes (Group A Streptococci)

S. pyogenes colonizes the human throat and skin and has developed complex virulence mechanisms to avoid host defenses (20, 31). The upper respiratory tract and skin lesions serve as primary focal sites of infections and principal reservoirs of transmission. *S. pyogenes* can cause superficial or deep infections due to toxin- and immunologically mediated mechanisms of disease. *S. pyogenes* is the most common cause of bacterial pharyngitis and impetigo. In the past, *S. pyogenes* was a common cause of childbed fever or puerperal sepsis. *S. pyogenes* is responsible for deep or invasive infections, especially bacteremia; sepsis; deep soft tissue infections, such as erysipelas; cellulitis; and necrotizing fasciitis. Less common presentations include myositis, osteomyelitis, septic arthritis, pneumonia, meningitis, endocarditis, pericarditis, and severe neonatal infections following intrapartum transmission. One or more erythrogenic exotoxins produced by *S. pyogenes* may cause a confluent erythematous sandpaper-like rash characteristic of scarlet fever. While systemic toxic effects occur rarely with scarlet fever, severe clinical manifestations in streptococcal toxic shock syndrome (STSS) may result from massive superantigen-induced cytokine and lymphokine production. Nonsuppurative complications include poststreptococcal glomerulonephritis and acute rheumatic fever. While either of these conditions may follow pharyngitis, only glomerulonephritis is linked with skin infections due to *S. pyogenes*. *S. pyogenes* has also been associated with pediatric autoimmune neuropsychiatric disorders (106).

Causes of the emergence of STSS, frequently accompanied by necrotizing fasciitis, and the resurgence of invasive *S. pyogenes* infections since the mid-1980s are mostly unexplained (105). *S. pyogenes* remains exquisitely sensitive to penicillin. Despite the continuous exposure and subsequent type-specific immunity, the most prevalent

M types associated with STSS continue to be M1 and M3, together accounting for approximately 50% of invasive infections. Since identical strains have accounted for less serious infections (78), host factors and comorbid conditions account for different diseases. The incidence of STSS seems highest among young children, particularly those with varicella, and the elderly. Other persons at risk include those with diabetes mellitus, chronic cardiac or pulmonary diseases, human immunodeficiency virus infection, intravenous drug abuse, and alcohol use. The risk for severe invasive infection in contacts has been estimated to be 200 times greater than for the general population, but most contacts are asymptomatically colonized (23).

Streptococcus agalactiae (Group B Streptococci [GBS])

S. agalactiae was first identified as the cause of bovine mastitis at the end of the 19th century. Since the 1970s it has been increasingly reported as the cause of invasive neonatal infections. Neonatal infections present as two different clinical entities: early-onset neonatal disease, characterized by sepsis and pneumonia within the first 7 days of life; and late-onset disease with meningitis and sepsis between 7 days and 3 months of age. The most important risk factor for the development of invasive neonatal disease is the colonization of the maternal urogenital or gastrointestinal tract by *S. agalactiae,* which is found in 10 to 30% of pregnant women. Prevention of early-onset neonatal infections can be achieved in the majority of cases by administration of intrapartum antibiotic prophylaxis starting at least 4 h before delivery. Official CDC recommendations for the prevention of neonatal *S. agalactiae* infections were first issued in 1996 (100), were revised in 2002 (97), and resulted in a substantial decline of early-onset neonatal GBS disease (98). Invasive *S. agalactiae* infections of adult patients may be observed as postpartum infections or in immunocompromised adult patients with alcoholism, diabetes mellitus, cancer, or human immunodeficiency virus infection (39). The spectrum of infections in adult patients includes pneumonia, bacteremia, endocarditis, urinary tract infections, skin and soft tissue infections, and osteomyelitis.

Streptococcus dysgalactiae subsp. *equisimilis* (Human Group C and G Streptococci)

Human isolates of large-colony-forming beta-hemolytic streptococci harboring the Lancefield group C or group G antigens belong to *Streptococcus dysgalactiae* subsp. *equisimilis,* a novel species described in 1996 (111). While most isolates of this species possess either the Lancefield group C or the G antigen, strains harboring the Lancefield group L as well as the group A antigen (16) have been described. The clinical spectrum of disease caused by *S. dysgalactiae* subsp. *equisimilis* resembles infections caused by *S. pyogenes,* consistent with strains harboring genes similar to virulence factor genes of *S. pyogenes,* such as *emm*-like genes. These organisms are associated with upper respiratory tract infections, skin infections, soft tissue infections, and invasive infections such as necrotizing fasciitis, STSS, bacteremia, and endocarditis. Similar to *S. pyogenes* infections, cases of glomerulonephritis and acute rheumatic fever have been reported (5, 49) following *S. equi* subsp. *zooepidemicus* and *S. dysgalactiae* subsp. *equisimilis* infections.

Streptococcus anginosus Group

Species from the *Streptococcus anginosus* group (*S. anginosus, S. constellatus,* and *S. intermedius)* are regarded generally as harmless commensals of the oropharyngeal, urogenital, and gastrointestinal microbiota. However, these organisms are strongly associated with abscess formation in the brain, the oropharynx, or the peritoneal cavity. A subspecies of *S. constellatus, S. constellatus* subsp. *pharyngis,* has also been described and associated with pharyngitis (119). All species of this group are small-colony-forming bacteria (colony size, <0.5 mm) that can display variable patterns of hemolysis (alpha, beta, or gamma). Since they can also harbor the Lancefield group antigens A, C, F, or G (or none at all), it is especially important to reliably distinguish them from large-colony-forming (>0.5 mm) beta-hemolytic streptococci of the pyogenic group. Association of certain species with specific isolation sites has been reported. Whereas *S. anginosus* is frequently found in specimens from the urogenital or gastrointestinal tracts, *S. constellatus* is commonly isolated from the respiratory tract and *S. intermedius* is most often identified in abscesses of the brain or liver.

Streptococcus pneumoniae

S. pneumoniae is described separately in this section due to its clinical features that distinguish it from other species of the *S. mitis* group. *S. pneumoniae* is the most frequently isolated respiratory pathogen in community-acquired pneumonia. In as many as 30% of community-acquired pneumonia cases, *S. pneumoniae* can be found in peripheral blood cultures of patients. *S. pneumoniae* is also a major cause of meningitis, leading to high morbidity and mortality in pediatric and adult patients. The most frequently observed infection due to *S. pneumoniae* is otitis media, with an estimate of one infection for every child up to the age of 6 in the United States. Other infections due to *S. pneumoniae* include sinusitis, endocarditis, and rare cases of peritonitis. *S. pneumoniae* colonizes the upper respiratory tract commonly in individuals, especially children, without evidence of infection. Prevention of pneumococcal infections can be achieved by immunization with a 23-valent capsular polysaccharide vaccine in adults or the 7-valent conjugate vaccine in children. Widespread use of these vaccines has resulted in a reduction of invasive pneumococcal infections during the past several years.

Streptococcus mitis Group

S. mitis, S. sanguinis, S. parasanguinis, S. gordonii, S. cristatus, S. oralis, S. infantis, S. peroris (58), and *S. pneumoniae* are members of the *Streptococcus mitis* group. The *S. mitis* group members are regular commensals of the oral cavity, the gastrointestinal tract, and the female genital tract. Isolation of these species from blood cultures in asymptomatic patients often does not require antibiotic treatment, if it is due to a transient bacteremia. The *S. mitis* group members can also be found as transient microbiota of the normal skin and often represent contaminants when isolated from blood cultures. At the same time these species are the most frequently isolated bacteria in bacterial endocarditis in native valve and, less frequently, in prosthetic valve infections. Careful evaluation of the clinical situation is therefore crucial to correctly interpret the clinical significance of blood culture isolates from the *S. mitis* group. In neutropenic patients, streptococcal species from the *S. mitis* group are often responsible for life-threatening sepsis and pneumonia cases following immunosuppression by chemotherapy (14).

Streptococcus salivarius Group

Streptococcal species that belong to the *Streptococcus salivarius* group and have been isolated from human sources include *S. salivarius* and *S. vestibularis*. Another species of this

group, *S. thermophilus,* has been identified only from dairy products. *S. salivarius* has been repeatedly reported as a cause of bacteremia, endocarditis, and meningitis (sometimes iatrogenic), whereas *S. vestibularis* has not been clearly associated with human infection. Isolation of *S. salivarius* from blood cultures does correlate to some extent with intestinal neoplasia (93).

Streptococcus mutans Group

S. mutans and *S. sobrinus* belong to the *Streptococcus mutans* group. They are the most commonly isolated species of the group that originate from human clinical specimens, usually obtained from the oral cavity. *S. criceti* and *S. ratti* have occasionally been identified from human sources, while the other streptococcal species of the *S. mutans* group *(S. downei, S. ferus, S. macacae,* and *S. hyovaginalis)* have been identified only in animals. *S. mutans* is the primary etiologic agent of dental caries, and infection is transmissible. By 18 years of age, 85% of the population have at least one caries lesion (103). Permanent colonization with *S. mutans* occurs under normal living conditions in the Western world between the second year and the end of the third year of life (103). Molecular analysis of mother and infant isolates reveals that strains are usually acquired from the mother and that the colonization rate of infants depends on the bacterial load of the mother (17). Analyses of streptococcal blood culture isolates show that *S. mutans* is the most frequently isolated species of this group in cases of bacteremia.

Streptococcus bovis Group

The *Streptococcus bovis* group includes *S. equinus, S. gallolyticus, S. infantarius,* and *S. alactolyticus*. Species from this group are frequently encountered in blood cultures of patients with bacteremia, sepsis, and endocarditis. The clinical significance of blood cultures growing streptococci from the *S. bovis* group lies in the association of *S. gallolyticus* subsp. *gallolyticus* with gastrointestinal cancer and of *S. gallolyticus* subsp. *pasteurianus* with meningitis (4, 43, 63, 82). Extensive taxonomic changes have occurred in this group, and strains formerly known as human *S. bovis* isolates are now designated as different species (see "Taxonomy" above).

Other Streptococci Infrequently Isolated from Human Specimens

Streptococcal species that are primarily animal pathogens are sometimes isolated from human hosts, in most cases from humans that are in close contact with animals. *S. suis, S. porcinus,* and *S. iniae* belong to this category. *S. suis* is a swine pathogen that has occasionally been isolated from cases of human meningitis and bacteremia (67). *S. suis* is encapsulated and appears alpha-hemolytic on sheep blood agar plates, although some strains are beta-hemolytic on horse blood agar. *S. suis* strains are positive for the Lancefield group antigens R, S, or T. These antigens help to distinguish them from the phenotypically similar species *S. gordonii, S. sanguinis,* and *S. parasanguinis*. Similar to *S. suis, S. porcinus* (Lancefield groups E, P, U, and V) is primarily a swine pathogen. Beta-hemolytic *S. porcinus* strains have rarely been isolated from human sources such as peripheral blood, wounds, and the female genital tract (35). *S. porcinus* can be misidentified as *S. agalactiae* due to its isolation from the female genital tract, false-positive reactions with commercially available group B antisera, and a positive CAMP test reaction. *S. porcinus* is PYR (pyrrolidonyl aminopeptidase) positive, in contrast to *S. agalactiae*. *S. iniae* is a fish pathogen that is beta-hemolytic but does not possess any Lancefield group antigens. It has been isolated from soft tissue infections, bacteremia, endocarditis, and meningitis in people handling fish (36, 115). *S. iniae* isolates resemble *S. pyogenes* strains due to the fact that they are PYR positive. Beta-hemolysis of the species can be observed only around agar stabs or under anaerobic culture conditions. Commercial identification systems do not correctly identify the species; the failure to react with Lancefield group antisera is important to notice, since it is rare among beta-hemolytic streptococci.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Specimens suspected of harboring streptococci should be collected by methods outlined elsewhere in this Manual (chapters 5 and 20). Since many streptococcal species lose viability fairly quickly, it is best to place swabs in an appropriate moist transport medium and process specimens rapidly. If transport time is below 1 to 2 h, a special transport system is not absolutely necessary. *S. pyogenes* can safely be transported on dry swabs; desiccation enhances recovery from mixed cultures by suppression of accompanying microbiota (70). Detailed recommendations for collection and storage of swabs from pregnant women to detect *S. agalactiae* colonization have been issued by the U.S. Centers for Disease Control and Prevention. These recommendations are summarized below under "Special Procedures for *S. agalactiae* (GBS)."

DIRECT EXAMINATION

Microscopy

Microscopic examination shows streptococci as gram-positive bacteria growing in chains of varying length. *S. pneumoniae* organisms most often present as gram-positive diplococci with an elongated appearance. In blood culture specimens, *S. pneumoniae* tends to form chains of varying length, similar to other streptococci. Direct identification of streptococci by microscopic methods is most helpful in the case of clinical specimens from sterile body sites, such as cerebrospinal fluid (CSF). Tiny, irregular cocci in clumps of chains seen in abscess- or peritonitis-associated aspirates are suggestive of the *S. anginosus* group. Interpretation of Gram stain results from nonsterile body sites is difficult, due to the residential microbiota of mucous membranes that frequently include streptococci. Thus, for example, throat swabs should not be examined by Gram stain for diagnosis of "strep" throat. Identification of *S. pneumoniae* by direct microscopic evaluation can be aided by the Neufeld Quellung reaction, which can be performed directly on clinical specimens (for instructions, see below). This procedure is not established in most clinical microbiological laboratories today.

Direct Antigen Detection of *S. pyogenes* from Throat Specimens

S. pyogenes is the most common cause of acute pharyngitis and accounts for 15 to 30% of cases of acute pharyngitis in children and 5 to 10% of cases in adults. If diagnosis can be provided rapidly, antibiotic therapy can be initiated promptly to relieve symptoms, to avoid sequelae, and to reduce transmission. Numerous assays for direct detection of the group A-specific carbohydrate antigen in throat swabs by agglutination methods or immunoassays (enzyme, liposome, or optical), also referred to as "rapid antigen assays," have become commercially available during the past 2 decades.

A list of FDA-cleared tests is accessible via the Internet (http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfRL/LSTSimpleSearch.cfdm). Although these tests provide rapid results and allow early treatment decisions, sensitivities and specificities have never equaled those of culture, ranging from 58 to 96% and 63 to 100%, respectively (38, 110). The throat culture remains the "gold standard," and national advisory committees recommend confirmation of negative rapid test results with a conventional throat culture. Currently, the low positive predictive value of rapid group A antigen tests in the adult population frequently results in prescribing unnecessary antimicrobial therapy (83).

Antigen Detection of *S. pneumoniae* in Urine Samples

An immunochromatographic membrane test relying on the detection of the cell wall-associated polysaccharide that is common to all *S. pneumoniae* serotypes (C-polysaccharide antigen) (Binax NOW; Binax Inc., Portland, Maine) has proven helpful for the identification of *S. pneumoniae* infections in adult patients, especially in patients that already received antibiotic treatment. Compared to conventional diagnostic methods, reported sensitivities of antigen detection in urine samples range between 50 and 80% and specificities are approximately 90% (48, 75). Due to the fact that the test is also positive in *S. pneumoniae* carriage without infection, as is often observed among infants (30), it is of limited value for pediatric patients. The test should not be used for children below the age of 6 (30), and comprehensive studies on schoolchildren with lower colonization rates have not been performed. It can currently be recommended only for adults as an addition to conventional diagnostic culture techniques for *S. pneumoniae* (69) and is probably most helpful for patients who received antimicrobial treatment before cultures were obtained.

Antigen Detection of *S. agalactiae* in Urogenital Tract Samples

Several different commercially available antigen detection tests have been developed for the identification of *S. agalactiae* in samples from the urogenital tract. Independent from the technique involved (latex agglutination, enzyme immunoassay, or optical immunoassay), all of the currently available tests lack sufficient sensitivities to detect bacterial colonization with *S. agalactiae* (107). They are not recommended for screening of pregnant women by the CDC. Even though modified protocols with an incubation of the samples in selective broth prior to antigen testing appear to increase assay sensitivities, the current CDC recommendations rely on selective broth culture performed at 35 to 37 weeks of gestation for this purpose (see "Special Procedures for *S. agalactiae* (GBS)."

Streptococcal Antigen Detection in CSF

Commercially available antigen detection tests for the detection of pathogenic microorganisms in CSF samples include reagents for the detection of *S. agalactiae* and *S. pneumoniae*. These tests have also been used on positive blood culture specimens. The tests are not recommended for routine use, as the results should not be used to change decisions about empiric therapy based on clinical and laboratory criteria (108). It has also been shown that the sensitivity of direct antigen detection in CSF is low (<30%) and offers no advantage over a conventional Gram stain (72).

Nucleic Acid Detection Techniques

S. pyogenes

A rapid method for the detection of *S. pyogenes* in pharyngeal specimens is based on a single-stranded chemiluminescent nucleic acid probe assay to identify specific rRNA sequences (Group A Streptococcus Direct Test; Gen-Probe, Inc., San Diego, Calif.). This test has a reported sensitivity and specificity of 89 and 93.5%, respectively, compared with the results of culture (50, 85). Moreover, a real-time PCR assay (LightCycler Strep A assay; Roche Diagnostics, Indianapolis, Ind.) has been recently developed for the detection of *S. pyogenes* in throat swabs (110). Real-time PCR proved to be more sensitive than the standard culture method in one study, and it appears to be unnecessary to perform cultures when results of the real-time PCR are negative. Real-time PCR allows the detection of beta-hemolytic species *S. pyogenes* and *S. dysgalactiae* subsp. *equisimilis*.

S. agalactiae

A rapid method for the detection of *S. agalactiae* colonization in pregnant women at the time of delivery has recently been developed and evaluated (11). The test is based on the detection of the *S. agalactiae cfb* gene (84) by a fluorogenic real-time PCR assay (IDI-Strep B; Becton Dickinson, Sparks, Md.), and results can be obtained in a few hours with a reported sensitivity of 94% and specificity of 95.9% (24). The test has been evaluated and approved by the FDA for combined rectal and vaginal swabs. The IDI-Strep B test is commercially available and performed well in a multicenter evaluation study (24). If real-time PCR is used at the time of delivery, only a vaginal swab (minus the rectal sample) should be tested. A major advantage is that results are available within a short time frame, and the vaginal colonization status can be assessed at the time of delivery. In comparison with the gold standard of selective broth culture, as recommended by the CDC, the test may offer an alternative for the future.

S. pneumoniae

Several different PCR assays have been developed for the identification of *S. pneumoniae* from culture isolates. Tests are based on the detection of the genes for autolysin *lytA*, the pneumococcal surface antigen *psaA*, and the pneumolysin gene *ply*. Comparison of the ability to distinguish difficult-to-identify *S. pneumoniae* strains and closely related atypical streptococci revealed that the *lytA*-based PCR was the most specific method (73). While results based on the detection of *psaA* are also acceptable, the different pneumolysin-targeted methods appear to be relatively nonspecific. Nucleic acid probes for the detection of cultured isolates of *S. pyogenes*, *S. agalactiae*, and *S. pneumoniae* are commercially available (AccuProbe; GenProbe) (21, 25). Detection relies on hybridization of a specific probe to 16S rRNA sequences. These tests are not routinely performed for standard identification procedures but can aid in the identification of atypical streptococcal isolates. Atypical organisms include *S. pneumoniae* isolates with unusual patterns of bile solubility and optochin susceptibility, nonhemolytic *S. agalactiae* strains, or beta-hemolytic streptococci harboring the Lancefield group A antigen with questionable species identification.

ISOLATION PROCEDURES

General Procedures

Streptococci are usually grown on agar media supplemented with blood because the assessment of the hemolytic reaction is important for identification. Growth of streptococci is often enhanced in the presence of an exogenous catalase source. Streptococcal species with low or absent hydrogen peroxide production, such as *S. agalactiae,* can be grown on other commonly used nonselective media without blood.

Agar media selective for gram-positive bacteria (e.g., phenylethyl alcohol-containing agar or Columbia agar with colistin and nalidixic acid) support the growth of streptococci. The optimal incubation temperature range for most streptococcal species lies between 35 and 37°C. Supplemental carbon dioxide (5% CO_2) and anaerobic conditions enhance the growth of many streptococcal species, since streptococci are facultative anaerobes. Although some streptococci grow well in ambient air, incubation in 5% CO_2 is recommended for the culture of *S. pneumoniae* and other streptococcal species of the viridans group.

Special Procedures for *Streptococcus pyogenes* Throat Cultures

A properly performed and interpreted throat culture remains the gold standard for the diagnosis of *S. pyogenes* acute pharyngitis. The recovery of *S. pyogenes* from throat swabs on 5% sheep blood agar with Trypticase soy base and incubated in air is a reliable and well-accepted method (77). Lack of hemolysis, overgrowth and production of toxic bacterial metabolites by normal upper respiratory tract microbiota, or depletion of substrates often leads to false-negative results or delays caused by labor-intensive reisolation steps. More rigorous standards for the isolation of *S. pyogenes* include an additional blood agar plate containing sulfamethoxazole-trimethoprim, which inhibits the normal respiratory microbiota (65). *Streptococcus* selective medium is highly sensitive for isolation of *S. pyogenes,* suppressing the growth of commensal respiratory microbiota including other beta-hemolytic streptococci (116). Incubation in anaerobic or CO_2-enriched atmosphere more frequently leads to the isolation of non-*S. pyogenes* beta-hemolytic streptococci (77). Important details have been summarized (13, 59). The isolation of a few colonies of *S. pyogenes* does not differentiate a carrier from an acutely infected individual and may reflect inadequate specimen collection (12).

After 18 to 24 h of incubation, culture plates should be examined for growth of beta-hemolytic colonies. Negative cultures should be reexamined after an additional 24-h incubation period. Presumptive identification of *S. pyogenes* can be achieved by susceptibility to bacitracin or testing for PYR activity. Other beta-hemolytic streptococci are occasionally positive in one of these tests, but not in both. Definitive diagnosis includes the demonstration of the Lancefield group A antigen by immunoassay. Although other species may rarely possess the group A antigen (Table 1), they lack PYR activity (34).

Special Procedures for *S. agalactiae* (GBS)

Early-onset neonatal group B streptococcal *(S. agalactiae)* (GBS) infections can be prevented by administration of antibiotic prophylaxis during delivery (97). An essential requirement for efficient prophylaxis is the reliable detection of colonization with *S. agalactiae* in pregnant women before delivery. Screening should be performed between weeks 35 and 37 of pregnancy. A lower vaginal swab and a rectal swab (i.e., insert swab through the anal sphincter) should be obtained with either one or two different swabs and placed in an appropriate transport medium (Amies or Stuart's without charcoal; see chapter 20). While culture counts decline to some extent, viability of *S. agalactiae* is preserved in transport medium kept at room temperature or 4°C for up to 4 days. To reduce costs, vaginal and rectal swabs can be placed in a single transport medium tube and cultured together. Swabs should be cultured in selective broth medium for 18 to 24 h at 35 to 37°C in ambient air or 5% CO_2 and subsequently plated on Trypticase soy blood agar plates. Selective broth medium is commercially available (Trans-Vag broth supplemented with 5% sheep blood [Remel Inc., Lenexa, Kans.] or LIM broth [BBL Microbiology Systems, Cockeysville, Md.]). Selective broth can also be prepared by supplementation of Todd-Hewitt broth (THB) with nalidixic acid (15 μg/ml) and colistin (10 μg/ml) or supplementation of THB with nalidixic acid (15 μg/ml) and gentamicin (8 μg/ml). Trypticase soy blood agar plates should be checked for typical colonies (narrow zone of beta-hemolysis) of *S. agalactiae* after 24 and 48 h of incubation at 35 to 37°C. Identification of *S. agalactiae* is then achieved by standard techniques as described below. Selective media relying on the detection of the *S. agalactiae* pigment (StrepB Carrot broth [Hardy Diagnostics, Santa Maria, Calif.] or GBS broth [Northeast Laboratory Services, Waterville, Maine]) are highly specific and sensitive (91, 113). Subculture is needed to detect nonhemolytic strains with pigment-dependent selective media. Methods to detect *S. agalactiae* by nucleic acid probes (AccuProbe; GenProbe) following overnight enrichment have also been published (15, 121). Commercially available antigen detection tests cannot be recommended for the detection of *S. agalactiae* colonization, due to poor sensitivities in comparison to selective broth isolation procedures (107).

IDENTIFICATION

Description of Colonies

Colonies of streptococci usually appear gray or almost white with moist or glistening features. Dry colonies are rarely encountered. Colony size varies between the different beta-hemolytic species and helps to distinguish groups of streptococci. Beta-hemolytic streptococci of the pyogenic group *(S. pyogenes, S. agalactiae,* and *S. dysgalactiae* subsp. *equisimilis)* form colonies of >0.5 mm after 24 h of incubation, in contrast to beta-hemolytic strains of the *S. anginosus* group (formerly called "*S. milleri*" group), which present with pinpoint colonies of <0.5 mm after the same incubation time (Fig. 1). Members of the *S. anginosus* group emit a distinct odor resembling butterscotch or caramel, presumably due to the production of diacetyl (18) by the species belonging to this group. Among the beta-hemolytic species of the pyogenic group, *S. agalactiae* produces the largest colonies with a relatively small zone of hemolysis. Nonhemolytic *S. agalactiae* strains do occur and look like enterococci.

Within the group of alpha-hemolytic streptococci, *S. pneumoniae* has a distinct colony morphology that helps to distinguish pneumococcal isolates from other streptococci of the viridans group. Due to the production of capsular polysaccharide, colonies glisten and appear moist. Colonies may be large and mucoid if large amounts of capsular

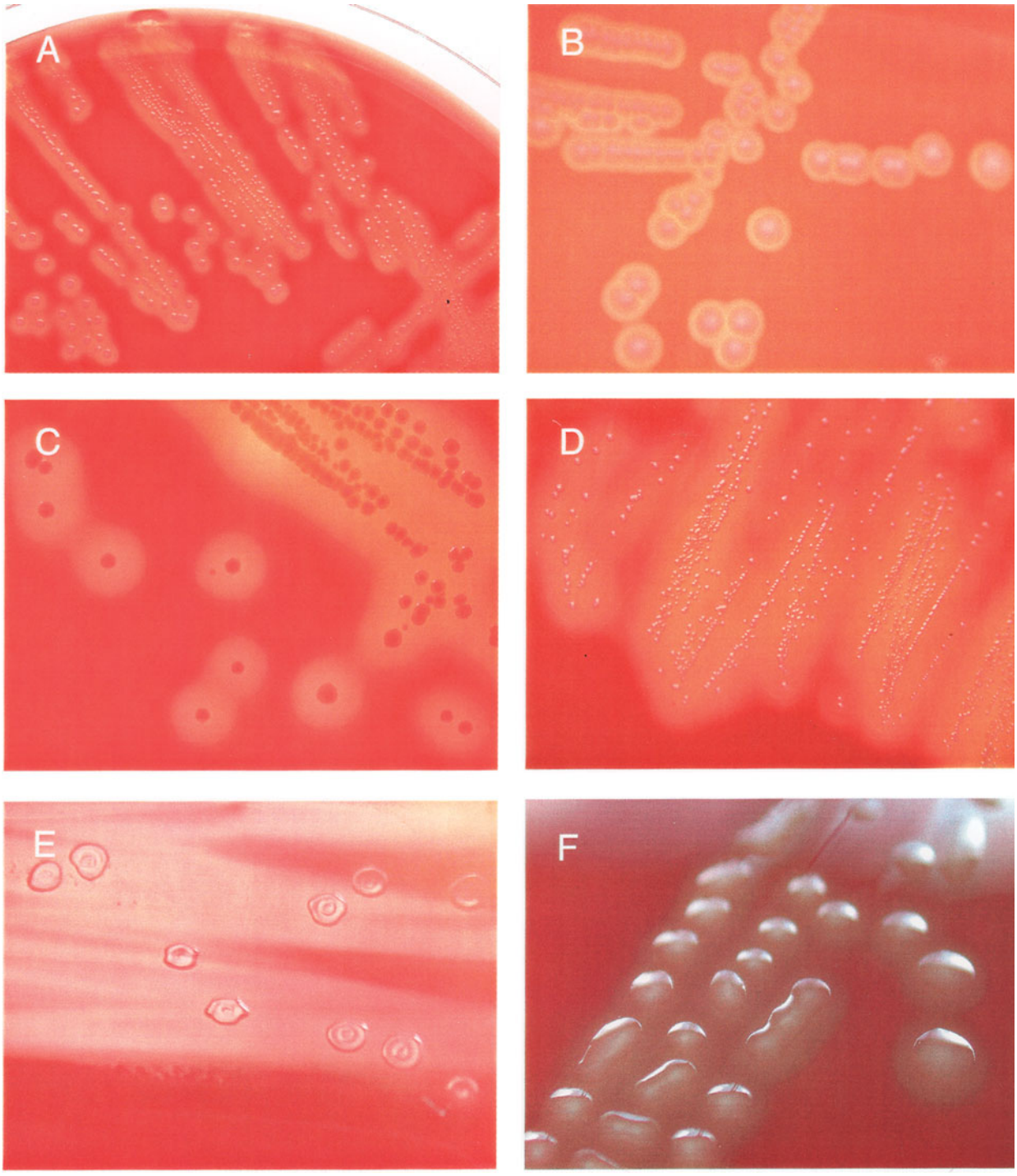


FIGURE 1 Colony morphology of clinical isolates of selected streptococci. (A) *S. pyogenes;* (B) *S. agalactiae;* (C) *S. dysgalactiae* subsp. *equisimilis* (Lancefield group G); (D) *S. anginosus* (note the pinpoint size colonies); (E) *S. pneumoniae* (note the central depression of the colonies); (F) *S. pneumoniae* (note the mucoid appearance of the colonies). (Courtesy of Tim Pietzcker, University of Ulm, Germany.)

polysaccharide are made, a feature often encountered in serotype 3 strains. The mucoid appearance is usually very typical for *S. pneumoniae* but can also occasionally be observed in *S. pyogenes*. Another feature characterizing *S. pneumoniae* isolates is the central navel-like depression of the colonies that is caused by the pneumococcal autolysin. Colonies of other viridans streptococci lack this feature and have a dome-like appearance. Nonhemolytic gray colonies are typical for species of the *S. bovis* and *S. salivarius* groups. Typical streptococcal colony morphologies are presented in Fig. 1.

Identification of Beta-Hemolytic Streptococci by Lancefield Antigen Immunoassays

Commercially available Lancefield antigen grouping sera are primarily used for the differentiation of beta-hemolytic streptococci. Products for rapid antigen extraction and subsequent agglutination can be obtained from many different suppliers. The presence of the Lancefield group B antigen in beta-hemolytic isolates from human clinical specimens correlates with the species *S. agalactiae*. Similarly, the detection of the Lancefield group F antigen in small-colony-forming streptococci from human clinical material allows a fairly reliable identification of a strain as a member of the *S. anginosus* group. The presence of Lancefield group A, C, or G antigens necessitates further testing (Table 1). Beta-hemolytic streptococcal strains not reacting with any of the Lancefield antisera are rare and should be further identified by phenotypic tests or application of nucleic acid probes.

Identification of Beta-Hemolytic Streptococci by Phenotypic Tests

A number of streptococcal identification products incorporating batteries of physiologic tests are commercially available (see chapter 15). In general, these products perform well with commonly isolated streptococci but may lack accuracy for identifying streptococci of the viridans group. For the bulk of pathogenic streptococci isolated in clinical laboratories (e.g., *S. pyogenes, S. agalactiae,* and *S. pneumoniae*), serologic or presumptive physiologic tests (as described below) offer an acceptable alternative to commercially available identification systems.

PYR Test

The presence of the enzyme PYR is often tested to distinguish *S. pyogenes* from other beta-hemolytic streptococci. Hydrolysis of L-pyrrolidonyl-β-naphthylamide by the enzyme to β-naphthylamine produces a red color with the addition of cinnamaldehyde reagent (chapter 21). *S. iniae* and *S. porcinus,* which are also positive with this test, are only rarely identified in human clinical specimens since they are primarily animal-associated species. PYR spot tests are commercially available. It is important to distinguish *Streptococcus* from *Enterococcus* prior to PYR testing, and strains of other related genera may be PYR positive (including the genera *Abiotrophia, Aerococcus, Enterococcus, Gemella,* and *Lactococcus*). However, PYR-positive beta-hemolytic enterococcal isolates typically present with a different colonial morphology, and when combined with other phenotypic characteristics (see chapter 30), may be distinguished from streptococci. To avoid false-positive reactions caused by other PYR-positive bacterial species (for example, staphylococci), the test should be performed only on pure cultures.

Bacitracin Susceptibility

S. pyogenes displays bacitracin susceptibility, in contrast to other beta-hemolytic streptococci found in humans. Together with Lancefield antigen determination, it can be used for the identification of *S. pyogenes* since beta-hemolytic strains of other streptococcal species that may contain the group A antigen are bacitracin-resistant. The test can also be used to distinguish *S. pyogenes* from other PYR-positive beta-hemolytic streptococci *(S. iniae* and *S. porcinus)*. A bacitracin disk (0.04 U) is applied to a sheep blood agar plate that has been heavily inoculated with 3 or 4 colonies of a pure culture of the strain to be tested. After overnight incubation at 35°C in 5% CO_2, any zone of inhibition around the disk is interpreted as indicating susceptibility. Importantly, bacitracin-resistant *S. pyogenes* isolates have been reported and clusters of bacitracin-resistant strains were recently observed in Europe (68, 74).

Voges-Proskauer Test (VP)

The Voges-Proskauer (VP) test detects the formation of acetoin from glucose fermentation. It is performed on streptococci as a modification of the classical VP reaction that is used for the differentiation of enteric bacteria. Small-colony-forming beta-hemolytic streptococci of the *S. anginosus* group that are VP positive may be distinguished from large-colony-forming beta-hemolytic streptococci harboring identical Lancefield antigens (A, C, or G). Streptococci of the *S. mitis* group are VP negative. For the modified VP reaction as described by Facklam in a previous edition of this Manual, the culture growth of an entire agar plate is used to inoculate 2 ml of VP broth and incubated at 35°C for 6 h. Following the addition of 5% α-naphthol and 40% KOH, the tube is shaken vigorously for a few seconds and incubated at room temperature for 30 min. A positive test yields a pink-red color that results from the reaction of diacetyl with guanidine.

β-Glucuronidase Test

Detection of β-glucuronidase (BGUR) activity distinguishes *S. dysgalactiae* subsp. *equisimilis* strains containing Lancefield group antigens C or G from BGUR-negative, small-colony-forming streptococci of the *S. anginosus* group with the same Lancefield group antigens (Table 1). Rapid methods for the BGUR test are commercially available. Alternatively, a rapid fluorogenic assay with methylumbelliferyl-β-D-glucuronide containing MacConkey agar, often used for *Escherichia coli,* has been described (62).

CAMP Test

The CAMP factor reaction was first described in 1944 by Christie, Atkins, and Munch-Petersen and refers to the synergistic lysis of erythrocytes by the beta-hemolysin of *Staphylococcus aureus* and the extracellular CFB protein of *S. agalactiae*. The gene and its expression can be demonstrated in the vast majority (>98%) of *S. agalactiae* isolates, but CAMP-negative mutants do occur. The strain to be tested and an *S. aureus* strain (ATCC 25923) are streaked onto a sheep agar plate at a 90° angle. Plates are incubated in ambient air overnight at 36 ± 1°C. A positive reaction can be detected by the presence of a triangular zone of enhanced beta-hemolysis in the diffusion zone of the beta-hemolysin of *S. aureus* and the CAMP factor (Fig. 2). CAMP factor-positive strains can also be detected by a method using β-lysin-containing disks (Remel Inc.) (120) or by a rapid CAMP factor spot method (89). Despite the fact that close homologs of the CAMP factor gene are present in many *S. pyogenes* strains, beta-hemolytic streptococci other than *S. agalactiae* are usually negative in the above-described CAMP factor test. Several gram-positive rods including corynebacteria and *Listeria monocytogenes* strains may be CAMP factor positive.

Hippurate Hydrolysis Test

The ability to hydrolyze hippurate is an alternative test for the presumptive identification of *S. agalactiae*. A rapid version of the test, as it is used for campylobacters, can be performed by incubating a turbid suspension of bacterial cells in 0.5 ml of 1% aqueous sodium hippurate for 2 h at 35°C. Glycine formed as an end product of hippurate hydrolysis is detected by adding ninhydrin reagent and

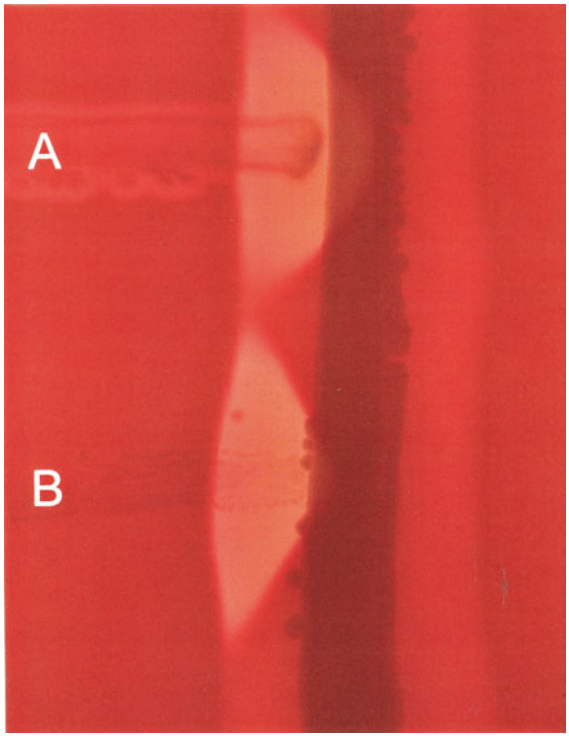


FIGURE 2 CAMP factor test. Arrowhead-shaped zone of hemolysis in the zone of *S. aureus* β-hemolysin. (A) Beta-hemolytic *S. agalactiae* strain O90R; (B) nonhemolytic *S. agalactiae* strain R268.

observing the development of a deep purple color, signifying a positive test (chapter 21) (53). Streptococci other than *S. agalactiae* may also be hippurate hydrolysis positive (114), especially viridans streptococci.

Identification of *S. pneumoniae* and Viridans Group Streptococci

The correct species identification of viridans group streptococci other than *S. pneumoniae* is challenging. Recent taxonomic changes and identification of novel streptococcal species have further complicated matters. The number of recognized species in this group is now greater than 30. The viridans group includes alpha-hemolytic, nonhemolytic (*S. salivarius* group and *S. bovis* group), and beta-hemolytic (*S. anginosus* group) streptococcal strains. All of the viridans streptococci are LAP positive and PYR negative. Conventional microbiologic tests are limited with respect to species identification but are helpful in placing isolates into the correct streptococcal groups (Table 2). Beighton et al. described an identification scheme based on phenotypic tests that allowed the differentiation and correct species identification of the majority of viridans group species (7). The scheme requires the evaluation of enzymatic reactions performed by in-house fluorogenic tests that are not commercially available. Importantly, most clinical laboratories must strive for group, instead of species, classifications with current phenotypic test panels.

The API tests (bioMerieux, Marcy l'Etoile, France) offer species identification of viridans group streptococci. While many species from this group are identified with acceptable accuracy, several species have not been included in the database. Comparisons of molecular species identification by DNA reassociation studies with the results of the API Rapid ID 32 Strep system showed that more than 85% of 156 strains from streptococcal species included in the database were correctly identified (60). However, in the same study, more than 50% of six species not included in the database were incorrectly identified by the test (60). Evaluation studies performed under routine clinical conditions appear to yield less favorable results (46). In conclusion, reliable phenotypic identification of viridans group streptococci can currently be achieved only to the group level.

Molecular methods may offer alternative approaches to conventional phenotypic identification schemes. The most common molecular identification method, 16S rRNA gene sequencing, does not yield reliable species identification for several species including *S. mitis, S. oralis,* and *S. pneumoniae*. The 16S rRNA gene sequences are more than 99% identical (57). Whole-cell protein analyses were unable to yield good correlation with species identification by other methods. Sequence determination of the manganese-dependent superoxide dismutase gene *sodA* appears promising (87, 88). In contrast to 16S rRNA gene sequencing, it allows the differentiation of *S. mitis, S. oralis,* and *S. pneumoniae* and the correct identification of almost 30 different streptococcal species including 16 species from the viridans group.

Descriptions of the species belonging to the nonpyogenic groups are given below, and physiological traits of the groups are shown in Table 2.

TABLE 2 Phenotypic characteristics of viridans streptococcal groups[a]

Streptococcal group	Arginine hydrolysis	Esculin	Mannitol	Sorbitol	Urea hydrolysis	VP
S. mitis	v[b]	v	–	v	–	–
S. anginosus	+	+	–	–	–	+
S. mutans	–	+	+	+	–	+
S. salivarius	–	v	–	–	v[d]	+
S. bovis	–	+	v[c]	–	–	+

[a] Symbols and abbreviations: +, positive; –, negative; v, variable.

[b] The species *S. cristatus, S. gordonii, S. parasanguinis,* and *S. sanguinis* are arginine hydrolysis positive; other species of the *S. mitis* group are arginine hydrolysis negative.

[c] *S. gallolyticus* subsp. *gallolyticus* is positive for the acidification of mannitol, and the other species of the *S. bovis* group are negative for mannitol acidification.

[d] *S. salivarius* is variable for urea hydrolysis, and *S. vestibularis* is positive for urea hydrolysis. All other species of the *S. salivarius* group are negative for urea hydrolysis.

S. mitis Group

Streptococcal species belonging to the *S. mitis* group include *S. mitis, S. sanguinis, S. parasanguinis, S. gordonii, S. cristatus, S. oralis, S. infantis* (58), *S. peroris* (58), and *S. pneumoniae,* which is discussed separately. This group of predominantly alpha-hemolytic streptococci includes several species of known clinical significance together with others for which few or no clinical data have been collected. Among the phenotypic characteristics of the species in this group, extracellular polysaccharide production is negative for *S. mitis* strains but a variable characteristic of *S. oralis* isolates. This feature correlates with the smooth colony surface of many *S. oralis* strains and the rough and dry appearance of *S. mitis* colonies.

S. anginosus Group

The small-colony-forming species *S. anginosus, S. constellatus,* and *S. intermedius* belong to the *S. anginosus* group, which is sometimes referred to as the "*S. milleri*" group. Strains of the *S. anginosus* group may be non-, alpha-, or beta-hemolytic on blood agar plates, with some variations between the species. While *S. constellatus* is frequently beta-hemolytic, most isolates of *S. intermedius* are nonhemolytic. For many strains, growth is enhanced in the presence of CO_2, with some strains requiring anaerobic conditions. *S. anginosus* and *S. constellatus* strains may possess Lancefield group antigens A, C, F, or G. Most *S. constellatus* or *S. intermedius* strains react with antisera against Lancefield group F antigen or are nongroupable.

The species *S. constellatus* has been further subdivided into two subspecies, *S. constellatus* subsp. *constellatus* and *S. constellatus* subsp. *pharyngis* (119). *S. constellatus* subsp. *constellatus* is phenotypically different from *S. constellatus* subsp. *pharyngis,* which usually possesses the Lancefield group antigen C, is beta-hemolytic, and has been associated with pharyngitis. Detailed phenotypic characteristics of the *S. anginosus* group are shown in Table 3.

TABLE 3 Phenotypic characteristics of streptococcal species of the *S. anginosus* group[a]

Test	*S. anginosus*	*S. constellatus*	*S. intermedius*
β-D-Fucosidase	–	–/+[c]	+
β-D-Acetylglucosaminidase	v	–/+[c]	+
β-D-Acetylgalactosaminidase	–	–/+[c]	+
Neuraminidase	–	–	+
α-D-Glucosidase	v	+	+
β-D-Glucosidase	+	–/+[c]	v
α-D-Galactosidase	v	–	–
β-D-Galactosidase	v	v	+
Amygdalin (acidification)	+	v	v
Mannitol (acidification)	v	–	–
Sorbitol (acidification)	–	–	–
Lactose (acidification)	+	v	+
Melibiose (acidification)	v	v	v
Arginine hydrolysis	+	+	+
Esculin hydrolysis	+	v	+
VP[b]	+	+	+
Urease	–	–	–
Hyaluronidase	–	+	+

[a] Symbols and abbreviations: +, positive; –, negative; v, variable.
[b] Voges-Proskauer test (formation of acetoin from glucose fermentation).
[c] The species *S. constellatus* subsp. *pharyngis* is positive for β-D-fucosidase, β-D-acetylglucosaminidase, β-D-acetylgalactosaminidase, and β-D-glucosidase activities. In contrast, *S. constellatus* subsp. *constellatus* is negative for these enzymatic activities.

S. mutans Group

The *S. mutans* group includes *S. mutans, S. sobrinus, S. criceti, S. ratti, S. downei, S. ferus, S. hyovaginalis,* and *S. macacae*. *S. mutans* and *S. sobrinus* are frequently found in human hosts, while the other species are only rarely encountered in humans or represent animal pathogens. The species of the *S. mutans* group are characterized by the production of extracellular polysaccharides from sucrose, which can be tested by culturing the bacteria on sucrose-containing agar and by the ability to produce acid from a relatively wide range of carbohydrates. *S. mutans* strains may present with an atypical morphology for streptococci, forming short rods on solid media or in broth culture under acidic conditions. On blood agar, colonies are often hard, adherent, and usually alpha-hemolytic. On sucrose-containing agar, species from this group form colonies that are rough (frosted glass appearance), heaped, and surrounded by liquid-containing glucan. Under anaerobic growth conditions, some strains are beta-hemolytic. *S. sobrinus* strains are mostly nonhemolytic or occasionally alpha-hemolytic.

S. salivarius Group

Streptococcal species in the *S. salivarius* group are *S. salivarius, S. vestibularis,* and *S. thermophilus*. *S. salivarius* strains are usually non- or alpha-hemolytic on blood agar. On sucrose-containing agar, strains form large mucoid or hard colonies due to the production of extracellular polysaccharides. A high proportion of *S. salivarius* strains react with the Lancefield group K antiserum. Species in this group may also react with the streptococcal group D antiserum. It is unclear if these strains truly possess the group D antigen or yield a nonspecific cross-reaction. *S. vestibularis* is alpha-hemolytic, and the failure of this species to produce extracellular polysaccharides on sucrose-containing agar is helpful in distinguishing *S. vestibularis* from *S. salivarius* strains. *S. thermophilus* is found in dairy products but has not been isolated from clinical specimens.

S. bovis Group

The species belonging to the *S. bovis* group *(S. equinus, S. gallolyticus, S. infantarius,* and *S. alactolyticus)* are non-enterococcal group D streptococci, grow on bile esculin agar, and are unable to grow in 6.5% NaCl. On blood agar, strains are either nonhemolytic or alpha-hemolytic. Strains of the *S. bovis* group share phenotypic characteristics with *S. mutans* strains, such as production of glucan, fermentation of mannitol, and growth on bile esculin agar. However, the *S. bovis* group does not ferment sorbitol, is able to ferment starch or glycogen, and gives a Lancefield group D reaction. Species of the *S. bovis* group may resemble *S. salivarius* strains. Differentiation of these two groups on the basis of the Lancefield group D reaction, ability to grow on bile esculin agar, fermentation of mannitol, and production of inulin, starch, and urease has been suggested (92). Strains from the *S. bovis* group are usually β-galactosidase negative and α-galactosidase positive, in contrast to *S. salivarius*. As described, strains formerly known as *S. bovis* currently belong to several species of the *S. bovis* group.

Physiologic Tests

Optochin Test

Most *S. pneumoniae* isolates are optochin susceptible. Before application of the optochin disk, several colonies of a pure culture are streaked on a sheep blood agar plate. Optochin testing should be performed on plates that are

incubated at 35 to 36°C overnight in 5% CO_2 because up to 8% of strains do not grow under ambient conditions. *S. pneumoniae* isolates show inhibition zones of ≥14 mm with a 6-mm disk (containing 5 μg of optochin) and inhibition zones of ≥16 mm with a 10-mm disk. Incubation in 5% CO_2 yielded increased specificity (1). Optochin-resistant *S. pneumoniae* strains have been reported as well as optochin-susceptible *S. mitis* isolates (especially when tested in ambient conditions). Since optochin testing may miss up to 4% of bile-soluble *S. pneumoniae* isolates (1), strains displaying a smaller zone of inhibition (9 to 13 mm for the 6-mm disk) should be subjected to additional testing for bile solubility to confirm species identification.

Application of an optochin disk onto the primary culture medium may facilitate a rapid presumptive identification but may miss pneumococcal isolates in a mixed culture. The optochin susceptibility test should be repeated with a pure culture in cases of mixed cultures, or additional tests should be performed (e.g., bile solubility).

Bile Solubility Test

Bile solubility can be performed either in a test tube or by direct application of the reagent to an agar plate. For the test tube method, a saline suspension of a pure culture is adjusted to a McFarland standard of 0.5 to 1.0, and 0.5 ml of the suspension is added to a small tube. The bacterial suspension is mixed with 0.5 ml of 10% sodium deoxycholate (bile) and incubated at 35°C. A control containing 0.5 ml of bacterial suspension with 0.5 ml of saline should be prepared for each strain tested. A positive result is characterized by clearing of the bile suspension within 5 to 15 min and allows the identification of a strain as *S. pneumoniae*. For the plate method, one drop of 10% sodium deoxycholate is placed directly on a colony of the strain in question and incubated at 35°C for 15 min in ambient air. It is important to keep the plates in a horizontal position in order to prevent the reagent from washing away the colony. Colonies of *S. pneumoniae* will disappear or demonstrate a flattened colony morphology, while other viridans streptococci will appear unchanged.

Bile Esculin Test

Bile esculin medium (available from commercial sources) in either plates or slants should be inoculated with one to three colonies of the organism to be tested and incubated at 35°C in ambient air for up to 48 h. Optimal results can be achieved by using media supplemented with 4% oxgall (equivalent of 40% bile) (Remel) and a standardized inoculum of 10^6 CFU (19). A definitive blackening of plated media or blackening of at least one-half of an agar slant is considered a positive test, indicative of species belonging to the *S. bovis* group or enterococci. Occasional other viridans streptococci are positive with this test or display weakly positive reactions that are difficult to interpret (92). Isolates from patients with serious infections (e.g., endocarditis) should be more completely characterized.

Arginine Hydrolysis

Arginine hydrolysis is a key reaction for the identification of viridans streptococci. Discrepancies can occur among test methods (117). Two commonly used methods are detailed here. Moeller's decarboxylase broth containing arginine (Becton Dickinson and other sources) should be inoculated with the test organism, overlaid with mineral oil, and incubated at 35 to 37°C for up to 7 days. Degradation of arginine results in elevated pH, indicated by development of a purple color. Negative results are indicated by a yellow color, which is due to acid accumulation from metabolism of glucose only. For the microtiter plate method (8), 3 drops of the arginine-containing reagent are inoculated with 1 drop of an overnight THB culture and incubated for 24 h at 37°C anaerobically. Production of ammonia is detected by the appearance of an orange color following the addition of 1 drop of Nessler's reagent.

Urea Hydrolysis

Christensen urea agar (Becton Dickinson and other sources) is inoculated and incubated aerobically at 35°C for up to 7 days. Development of a pink color indicates a positive reaction. An alternative format is to dispense Christensen's medium without agar into a microtiter tray well and, after inoculation, overlay it with mineral oil prior to incubation.

Voges-Proskauer (VP) Test

The VP test can be performed as described for the identification of beta-hemolytic streptococci. A standard method for performing the VP test, requiring extended incubation, is described in chapter 21.

Esculin Hydrolysis

Esculin agar slants (Becton Dickinson and other sources) are inoculated and incubated for up to 1 week. A positive reaction appears as a blackening of the medium; no change in color indicates a negative esculin hydrolysis test.

Hyaluronidase Production

Hyaluronidase activity can be detected on brain heart infusion agar plates supplemented with 2 mg of sodium hyaluronate per liter (Sigma-Aldrich, St. Louis, Mo.) (104). The strains to be tested are inoculated by stabbing into the agar, and plates are incubated anaerobically at 37°C overnight. After the plate is flooded with 2 M acetic acid, hyaluronidase activity is indicated by the appearance of a clear zone around the stab. A quantitative method for determining hyaluronidase activity can be performed in microtiter trays (52).

Production of Extracellular Polysaccharide

Strains may be isolated as single colonies on sucrose-containing agar. The two most commonly used media are (i) mitis-salivarius agar containing 0.001% (wt/vol) potassium tellurite (Becton Dickinson) and (ii) tryptone-yeast-cystine agar (Lab M, Bury, United Kingdom). Incubation may require up to 5 days at 37°C.

TYPING SYSTEMS

In the majority of cases, typing of streptococci has no immediate clinical or therapeutic consequences. It is most often performed by reference laboratories for the purposes of epidemiologic studies and the evaluation of vaccine efficacy. Although classical antibody-dependent typing systems for capsular serotypes and surface proteins have been used for years, molecular methods have become attractive, since they do not require special techniques or the maintenance of rarely used reagents such as a large antibody panel. Another advantage lies in the fact that the interrogation of DNA sequences is independent of culture conditions and gene expression. For the differentiation of distinct clones, pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) systems have been established for many streptococcal species (32).

S. pneumoniae comprises 90 antigenically distinct capsular serotypes that can be detected by the Neufeld test (Quellung reaction), which is still regarded as the gold standard for epidemiologic studies. Pure cultures of pneumococci are grown on a freshly prepared 5% sheep blood agar plate or a 10% horse blood agar plate at 35 to 37°C and 5% CO_2 for 18 to 24 h. A small amount of bacterial growth (less than a 10-μl loop) is resuspended in a droplet of phosphate-buffered or physiological saline (McFarland standard of approximately 0.5). A few microliters of the saline suspension are mixed with an equal amount of specific pneumococcal rabbit antisera on a glass slide. The specimen is subsequently evaluated for capsular swelling (a clear area surrounding the bacterial cells) by phase-contrast microscopy (×1,000 magnification; oil immersion) (2). The reaction is stable for approximately 30 min. The best results are achieved when 10 to 50 bacterial cells are visible per high-power (×1,000) microscopic field. To increase the visibility of the result, it is possible to add 0.3% aqueous methylene blue in the same amount as the antiserum to the mixture. Following the same principle, commercially available kits (Pneumotest; Statens Serum Institut, Copenhagen, Denmark) allow rapid testing of *S. pneumoniae* serotypes with pooled antisera by a checkerboard method. A rapid antigen detection test using pooled antisera coupled to latex beads (Statens Serum Institut) has been developed (102). Due to strain discrepancies, confirmation by the Neufeld Quellung reaction is recommended. For the distinction of single clones, PFGE (66, 71) or MLST typing schemes (32) have been used in pneumococcal investigations.

Nine different antibody-defined capsular polysaccharides have been described for *S. agalactiae* (Ia, Ib, and II through VIII). The percentage of nontypeable strains can be minimized by optimization of capsular expression (10). In addition to antibody detection of capsular serotypes, recently developed PCR- and DNA sequencing-based techniques allow the detection of capsular serotypes (64). Individual clones of *S. agalactiae* have been detected either by MLST (55) or PFGE (9, 45).

Conventional typing of *S. pyogenes* is based upon the antigenic specificity of the surface-expressed T and M proteins (54). The function of the trypsin-resistant T protein is unknown. The T type can be identified by agglutination with commercially available serologic assays utilizing approximately 20 accepted anti-T sera. M proteins are major antiphagocytic virulence factors of *S. pyogenes* (42). N-terminal sequence variation in genes encoding these highly protective antigens is the basis of the *S. pyogenes* precipitation typing system. At present, 83 M serotypes are unequivocally validated and internationally recognized to be serologically unique and are designated M1 to M93 in the Lancefield classification (37). M serotypes that are not included are from non-*S. pyogenes* organisms or correspond to an existing M serotype.

A molecular serotyping system has been established on the basis of nucleotide sequences encoding the amino termini of M proteins. The *emm* gene sequences encode M proteins and have been correlated with Lancefield M serotypes. This methodology allows assignment to a validated M protein gene sequence *(emm1* through *emm124)* and identification of new *emm*-sequence types and subtypes. Molecular serotyping has evolved into the gold standard molecular methodology of *S. pyogenes* typing (37). A large database of approximately 350 *emm* gene sequences from strains originally used for Lancefield serotyping and including *emm* sequences from beta-hemolytic groups C, G, and L streptococci has been developed by the CDC (http://www.cdc.gov/ncidod/biotech/infotech_hp.html). Recently, MLST has been developed for *S. pyogenes*. Population genetic studies demonstrated stable associations between *emm* type and MLST among isolates obtained decades apart and/or from different continents (33).

In outbreak situations that include *S. pyogenes,* restriction enzyme-mediated digestion of *emm* amplicons is a valuable tool for rapid identification of isolates containing similar *emm* genes (6). For clusters of isolates sharing the same *emm* type, PFGE profiles may be helpful for distinguishing similar strains (79).

SEROLOGIC TESTS

Determination of streptococcal antibodies is indicated for the diagnosis of poststreptococcal disease, such as acute rheumatic fever or glomerulonephritis (101). A fourfold rise in antibody titer is regarded as definitive proof of an antecedent streptococcal infection. Multiple variables, including site of infection, time since the onset of infection, age, the background prevalence of streptococcal infections (3), antimicrobial therapy, and other comorbidities, influence antibody levels. The most widely used antibodies are anti-streptolysin O and anti-DNase B.

Antibodies against streptolysin O (ASO) reach a maximum at 3 to 6 weeks after infection. While ASO responses following streptococcal upper respiratory tract infections are usually elevated, pyoderma caused by *S. pyogenes* does not elicit a strong ASO response. *Streptococcus dysgalactiae* subsp. *equisimilis* can also produce streptolysin O, and thus elevated ASO titers are not specific for *S. pyogenes* infections.

Among the four streptococcal DNases produced, the host response is most consistent against DNase B. Anti-DNase B titers may not reach maximum titers for 6 to 8 weeks, but they remain elevated longer than ASO titers and are more reliable than ASO for the confirmation of a preceding streptococcal skin infection. Moreover, since only 80 to 85% of patients with rheumatic fever have elevated ASO titers, additional anti-DNase B titers may be helpful.

Due to frequent exposure to *S. pyogenes,* ASO and anti-DNase B titers are higher in children in the United States from 2 to 12 years of age. Geometric mean values of 89 Todd U (ASO) and 112 U (anti-DNase B) and 240 Todd U and 640 U, respectively, represent the upper limits of normal values (56). Prompt antibiotic therapy of streptococcal infections can reduce the titer but does not abolish antibody production. Streptococcal carriers do not experience a rise in streptococcal antibody titers.

The hemagglutination-based streptozyme test (Streptozyme; Carter-Wallace, Inc., Cranbury, N.J.) was developed to detect antibodies against multiple extracellular streptococcal products. However, variabilities in test standardization and inconsistent specificities have been reported (44).

ANTIMICROBIAL SUSCEPTIBILITIES

Beta-Hemolytic Streptococci

Penicillin remains the drug of choice for the treatment of streptococcal infections due to beta-hemolytic streptococci, because in contrast to *S. pneumoniae* and other alpha-hemolytic streptococci, beta-hemolytic streptococci remain uniformly susceptible to penicillin. Reports of penicillin-resistant strains of beta-hemolytic streptococci have not been

confirmed by reference laboratories. Due to suspected or confirmed penicillin allergies in more than 10% of patients, macrolides are often given as an alternative treatment. Macrolide resistance rates among isolates of *S. pyogenes* and *S. agalactiae* have been increasing in North America as well as in Europe (26). Resistance rates correlate with the use of macrolides in clinical practice, and geographic differences in resistance rates are often due to differences in macrolide use. In the United States, macrolide resistance among *S. agalactiae* isolates rose from 12 to 20% from 1990 to 2000 (76). Two major mechanisms are responsible for macrolide resistance in streptococci. Resistance encoded by the *mefA* gene results in low-level erythromycin but not clindamycin resistance due to increased drug efflux from the bacterial cell. Methylation of the macrolide binding site within 23S rRNA by methylases encoded by *erm* genes mediates resistance to macrolides, lincosamides, and streptogramin group B (MLS_B) agents. The methylation causes high-level macrolide resistance and is either inducible or constitutively expressed. Strains with inducible MLS_B resistance are resistant to erythromycin and susceptible to clindamycin in vitro. In contrast, strains with constitutive MLS_B resistance demonstrate resistance to both erythromycin and clindamycin. The majority of macrolide-resistant *S. pyogenes* isolates from North America harbor the *mefA* gene, and macrolide resistance due to the presence of *erm* genes is less common in *S. pyogenes*. Among *S. agalactiae* isolates from North America, the predominant macrolide resistance mechanism is methylation of 23S rRNA due to the *erm* methylase genes (*ermTR* and *ermB*), and only a few strains harbor the efflux gene *mefA* (26). Beta-hemolytic streptococcal isolates with a reduced susceptibility to vancomycin have not been reported.

S. pneumoniae and Viridans Streptococci

In view of the development of penicillin resistance in *S. pneumoniae* and other alpha-hemolytic streptococci, penicillin can no longer be recommended as the initial treatment of choice in many countries. Penicillin is considered a preferred antimicrobial agent only for *S. pneumoniae* and other alpha-hemolytic streptococci with demonstrated susceptibilities to penicillin. Penicillin resistance in *S. pneumoniae* is caused by altered penicillin-binding proteins.

Approximately 20% or more of *S. pneumoniae* isolates are not fully susceptible to penicillin in many geographic regions (http://www.cdc.gov/abcs). *S. pneumoniae* infections should be treated according to current guidelines (69). Depending on the clinical situation, treatment options include extended-spectrum cephalosporins, macrolides, fluoroquinolones, and vancomycin. In addition, more than one-third of blood culture isolates of the viridans group collected in the late 1990s in the United States were not susceptible to penicillin (29). Especially *S. mitis* and *S. salivarius* isolates show high percentages of penicillin-resistant strains.

S. pneumoniae was uniformly susceptible to macrolides until the late 1980s in the United States, but macrolide resistance is now evident in 25% of *S. pneumoniae* strains (109). Macrolide resistance in *S. pneumoniae* is caused by ErmB-mediated methylation of 23S rRNA, causing a high-level resistance phenotype, or MefA-mediated efflux of macrolides resulting in a low-level resistance phenotype. In contrast to the United States, where two-thirds of macrolide resistance is mediated by MefA, a higher overall prevalence of macrolide resistance is found in some parts of Europe and most European macrolide-resistant *S. pneumoniae* isolates contain the *ermB* gene (81).

The increased use of fluoroquinolones to treat *S. pneumoniae* infections has been accompanied by a rise in fluoroquinolone-resistant *S. pneumoniae* strains. Resistance occurs in a stepwise fashion and is due to mutations in DNA topoisomerase IV or a subunit of DNA gyrase. While the overall prevalence of fluoroquinolone resistance is below 1% according to ABC surveillance data (http://www.cdc.gov/abcs), the increase in resistant strains during recent years emphasizes the need for close monitoring. Clinical failures of levofloxacin therapy due to resistance have been reported (22). Vancomycin-resistant *S. pneumoniae* isolates have not been described. However, the isolation of a vancomycin-resistant *S. bovis* isolate has been reported (86).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Streptococci from the pyogenic group and *S. pneumoniae* are important human pathogens. Timely identification of these species in clinical specimens is therefore crucial to treat infections adequately and to reduce transmission. Detection of *S. pyogenes* strains in throat specimens allows adequate therapy and reduces nonsuppurative sequelae. Because *S. dysgalactiae* subsp. *equisimilis* (human group C and G streptococci) has been documented as an agent of pharyngitis including cases complicated by nonsuppurative sequelae, the presence of *S. dysgalactiae* subsp. *equisimilis* in throat cultures should be reported. To avoid unnecessary antibiotic treatment, it is important to correctly differentiate these pathogens from the small-colony-forming beta-hemolytic species of the *S. anginosus* group that constitute part of the oropharyngeal microbiota. However, *S. anginosus* group species isolated from wound specimens or abscesses are likely to represent true pathogens. While invasive neonatal *S. agalactiae* infections are declining due to improved prenatal screening and peripartal antibiotic prophylaxis, increased detection of *S. agalactiae* from adult patients has been reported. Thorough identification and reporting of this organism should therefore not be confined to screening swabs during pregnancy or in newborns.

The correct identification of viridans streptococci to the species or group level is often difficult and should be reserved for strains isolated from serious infections, such as endocarditis, abscesses, and infections in neutropenic patients. While in the normal host, transient bacteremia with viridans streptococci is generally cleared without adverse sequelae, prolonged bacteremia, particularly that caused by *S. mitis* in neutropenic patients, has become recognized as a distinct clinical entity that can be complicated by adult respiratory distress syndrome. Severe bacteremic infections with viridans streptococci have also been reported from low-birth-weight term and preterm neonates. Many *S. mitis* isolates are no longer penicillin-susceptible, and special attention should be paid to susceptibility testing. For *S. pneumoniae*, the elevated prevalence of resistance to penicillins, macrolides, ketolides, and fluoroquinolones varies worldwide. Careful identification of, and interpretation of susceptibility testing for, invasive and noninvasive *S. pneumoniae* isolates is required to guide appropriate antimicrobial therapy and to monitor the further spread of resistant pathogens. Due to the association of *S. gallolyticus* subsp. *gallolyticus* with malignancies of the gastrointestinal tract, and in view of the recent taxonomic changes within the *S. bovis* group, reports of novel species designations should include information about former species names (112).

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Enterococcus

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30

TAXONOMY

Microorganisms that are now included in the genus *Enterococcus* were related mainly to the "streptococci of fecal origin" or "enterococci" (26). These microorganisms were considered for a long time to be a major division of the genus *Streptococcus,* differentiated by their higher resistance to chemical and physical agents and accommodating most of the serological group D streptococci. In the last decades, however, the enterococci have undergone considerable changes in taxonomy, which started with the splitting of the genus *Streptococcus,* and the recognition of *Enterococcus* as a separate genus (89). Definite evidence that *Streptococcus faecalis* and *Streptococcus faecium* were sufficiently different from the other members of the genus to merit allocation into a separate genus was provided by studies using molecular approaches (89). The other enterococcal species were then transferred to the new genus, and several new species have been described and proposed for inclusion in the genus *Enterococcus* (25, 26). Some of the proposed new enterococcal species were not validated because they were shown to belong to previously described enterococcal species or did not belong to the genus *Enterococcus* at all (24, 26, 75, 104).

The phylogenetic analysis of genera of catalase-negative gram-positive cocci based on the comparison of the 16S rRNA gene sequences has revealed that *Enterococcus* organisms are more closely related to *Vagococcus, Tetragenococcus*, and *Carnobacterium* than they are to *Streptococcus* and *Lactococcus,* genera to which they have been phenotypically associated (17, 26).

Current criteria for inclusion in the genus *Enterococcus* and for the description of new enterococcal species encompass a polyphasic approach resulting from a combination of different molecular techniques (frequently involving DNA-DNA reassociation experiments, 16S rRNA gene sequencing, and whole-cell protein profiling analysis) and phenotypic tests. Partial or nearly entire sequencing of the 16S rRNA genes is now considered a practical and powerful tool in aiding the identification of enterococccal species, and it has been performed for all recognized species of *Enterococcus*. Figure 1 shows the phylogenetic relationships, based on the analysis of 16S rRNA gene sequences, among the species of *Enterococcus* that have their sequences already available at the GenBank database (as of December 2005). More recently, a variety of other nucleic acid-based methods have been used as additional tools to assess the phylogenetic relationships among enterococcal species and to formulate the descriptions of new species (26, 30, 74, 97, 98), but their use is still limited. The long-chain fatty acid composition of enterococcal cells, as revealed by gas-liquid chromatography analysis, is also of taxonomic value and has been used to discriminate species (30, 42, 89, 109).

DESCRIPTION OF THE GENUS

The members of the genus *Enterococcus* are catalase-negative gram-positive cocci that occur singly or arranged in pairs or as short chains. Cells are sometimes coccobacillary when Gram stains are prepared from growth on solid medium but tend to be ovoid and in chains when grown in thioglycolate broth. After growth on blood agar media for 24 h, colonies are usually between 1 and 2 mm in diameter although some variants may appear smaller. Some (about one-third) cultures of *Enterococcus faecalis* may be β-hemolytic on agar containing rabbit, horse, or human blood but nonhemolytic on agar containing sheep blood. Some cultures of *Enterococcus durans* are β-hemolytic regardless of the type of blood used. All other species are usually α-hemolytic or nonhemolytic. Enterococci are facultative anaerobes with a homofermentative metabolism that follows the Embden-Meyerhof-Parnas pathway resulting in the production of lactic acid as the end product of glucose fermentation. Because of their ability to ferment carbohydrates to lactic acid, the enterococci are referred to as typical lactic acid bacteria. Gas is not produced. These microorganisms are usually able to grow in temperatures ranging from 10 to 45°C, with optimum growth at 35°C. The majority of the species grow in broth containing 6.5% NaCl, and they hydrolyze esculin in the presence of bile salts (bile-esculin [BE] test). They also hydrolyze leucine-β-naphthylamide (LAP) by producing leucine aminopeptidase (LAPase). Most enterococci, apart from *Enterococcus cecorum, Enterococcus columbae* (16), *Enterococcus pallens, Enterococcus saccharolyticus*, and some strains of the recently described species *Enterococcus canintestini*, *Enterococcus devriesei,* and *Enterococcus moraviensis,* hydrolyze L-pyrrolidonyl-β-naphthylamide (PYR) by producing pyrrolidonyl arylamidase (pyrrolidonase [PYRase]). A few species are motile *(Enterococcus casseliflavus* and *Enterococcus gallinarum),* and some are pigmented *(E. casseliflavus, Enterococcus gilvus, Enterococcus mundtii, E. pallens,* and *Enterococcus sulfureus)* (17, 26, 43). Methods used for

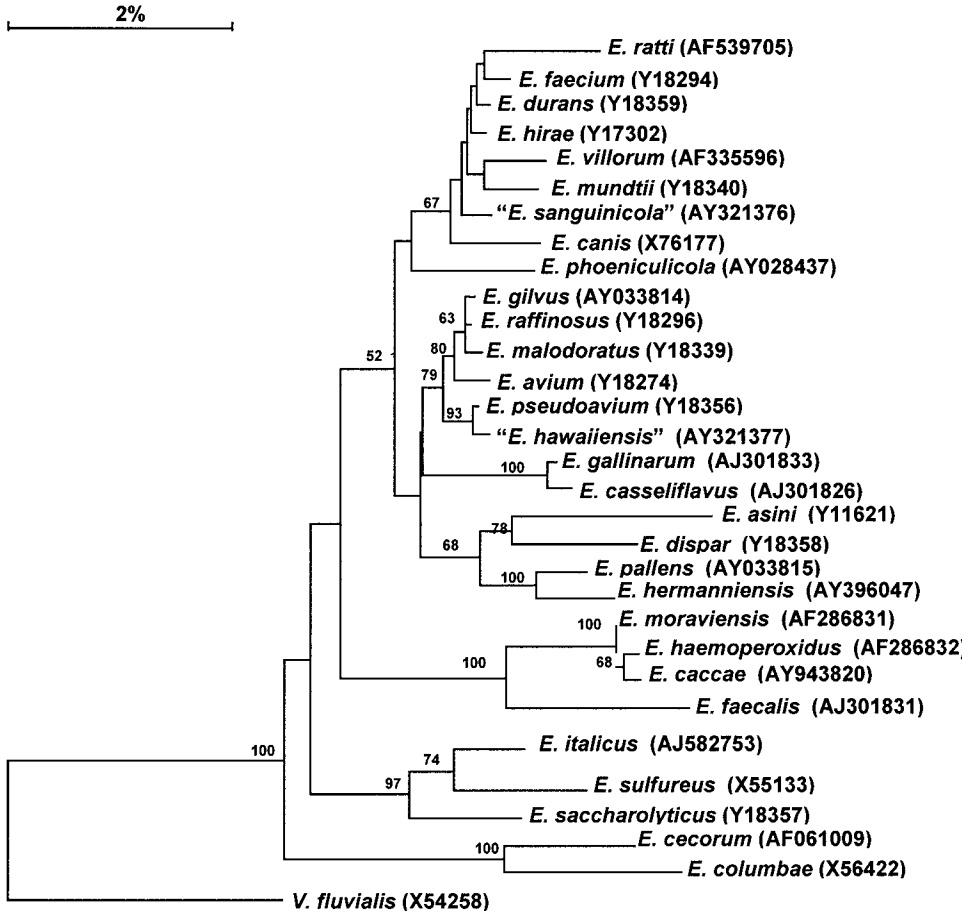


FIGURE 1 Phylogenetic tree based on comparative analysis of 16S rRNA gene sequences, showing the relationships among type strains of species of *Enterococcus*. *Vagococcus fluvialis* was used as an out-group, and bootstrap values at the nodes were displayed as percentages.

detection of enterococcal motility have to be selected carefully, as differences in motility due to the composition of the medium have been demonstrated (111). Enterococci are not able to synthesize porphyrins and, therefore, do not produce cytochrome enzymes (17, 43). However, cytochrome activity is sometimes expressed when strains of *E. faecalis* are grown on blood-containing media, and a weak effervescence is observed in the catalase test. Positive catalase testing has been reported for strains of *Enterococcus haemoperoxidus*, when cultivated on blood-agar media (96). Most enterococcal strains produce a cell wall-associated glycerol teichoic acid that is identified as Lancefield's serological group D antigen. The G+C content of the DNA ranges from 32 to 44 mol%. The genome size is in the range of approximately 2,000 to 3,500 kb (58, 91).

The other genera of catalase-negative gram-positive cocci and the characteristics that distinguish them from the enterococci are discussed in chapters 29 and 31. No phenotypic criteria are available for clearly distinguishing the genus *Enterococcus* unequivocally from other genera, since there are no particular characteristics that are common to all enterococci. However, there are certain characteristics that are usually found in most strains of all the enterococcal species. Accurate presumptive identification of a catalase-negative gram-positive coccus as an *Enterococcus* can be accomplished by demonstrating that the unknown strain is positive for BE, PYR, and LAP tests and grows in the presence of 6.5% NaCl and at 45°C. Because strains of *Lactococcus, Leuconostoc, Pediococcus,* and *Vagococcus* with phenotypic similarities have been isolated from human infections (28, 102), the presumptive identification of enterococci based only on BE reaction and growth in 6.5% NaCl broth can be erroneous. Demonstrating the presence of group D antigen by serological reaction may be helpful in the identification, although antigen is detected in only about 80% of the enterococcal strains. On the other hand, pediococci and leuconostocs (28), as well as some vagococcal strains (102), can also react with anti-group D serum. Reactivity with the AccuProbe *Enterococcus* Culture Identification Test (a genetic probe manufactured by GenProbe, Inc., San Diego, Calif.) can also be used to confirm an unknown strain as an *Enterococcus*. Except for the type strains of *Enterococcus asini*, *Enterococcus canis, E. cecorum, E. columbae, E. haemoperoxidus, E. moraviensis, E. pallens,* and *E. saccharolyticus*, strains of most known species of *Enterococcus* react with this probe. However, *Vagococcus* strains also react with the *Enterococcus* genetic probe (90, 102).

EPIDEMIOLOGY AND TRANSMISSION

Several intrinsic characteristics of the enterococci allow them to grow and survive in harsh environments and to persist almost everywhere. Enterococci are widespread in nature

and can be found in soil, plants, water, food, and in animals, including mammals, birds, insects, and reptiles (1, 16, 17, 100, 101). In humans, as in other animals, they are predominantly inhabitants of the gastrointestinal tract, occurring in high numbers, and are less commonly found in other sites such as the genitourinary tract and the oral cavity (44, 94, 100). The prevalence of the different enterococcal species appears to vary according to the host and is also influenced by age, diet, and other factors that may be related to changes in physiologic conditions, such as underlying diseases and prior antimicrobial therapy. Enterococci are considered to be the most abundant gram-positive coccus colonizing the intestine, with *E. faecalis* being one of the most common bacteria isolated from this site (17, 23, 94, 100). Other species, such as *E. faecium* as well as *E. casseliflavus*, *E. durans,* and *E. gallinarum*, are also found in variable proportions in the gastrointestinal tract of humans. Since the enterococci are opportunistic pathogens, the incidence of each species found in human infections probably reflects the distribution of the different species of *Enterococcus* in the human gastrointestinal tract. This site is believed to represent an important reservoir for strains associated with disease; from this location they may migrate to cause infections and can also disseminate to other hosts and to the environment. Even though the same enterococcal species can be found in several different animal species, the information available on the distribution of distinct enterococcal species in other sources indicates differences from the distribution in humans (1, 17, 100). On the other hand, the occurrence of high numbers of enterococci in the feces, as well as their ability to resist different chemical and physical conditions and to survive in the environment, implies that the enterococci can be used as indicators of fecal contamination and of the hygienic quality of food, milk, and drinking water (31, 33, 93). The occurrence of enterococci in natural nonhuman reservoirs (1), as well as members of the intestinal microbiota of humans (100), and the relationship between the presence of enterococci in foods and human safety (31, 33) have been extensively reviewed recently. Overall, several aspects of the ecology of the enterococci and the real incidence of the different species as members of the microbiota in body sites or in environmental sources merit further evaluation, especially in the light of the changing classification and taxonomic approaches to the genus and of the increasing resistance to antimicrobial agents.

CLINICAL SIGNIFICANCE

The enterococci are commensal microorganisms that act as opportunistic agents of infections, particularly in elderly patients with serious underlying diseases and other immunocompromised patients who have been hospitalized for prolonged periods, have been treated with invasive devices, and/or have received broad-spectrum antimicrobial therapy. From the site of colonization, the microorganism must evade host clearance and produce pathologic changes in the host, either by direct toxic activity or indirectly by inducing an inflammatory response. Several potential virulence factors have been identified in enterococci (13, 22, 39, 44, 47, 66, 84, 94), but none has been established as having a major contribution to virulence in humans. On the other hand, the resistance traits of enterococci may be crucial to allow members of this genus to survive for extended periods of time in the host or environment (44), leading to their persistence and role as prominent nosocomial pathogens. Although the enterococci can cause human infections in the community and in the hospital, these microorganisms began to be recognized with increasing frequency as common causes of hospital-acquired infections in the late 1970s, paralleling the increasing resistance to most currently used antimicrobial agents. As a result, enterococci have emerged as one of the leading therapeutic challenges when associated with serious or life-threatening infections. This trend is likely to continue as the overall population ages and more people become at risk for infection (59). The ubiquitous presence of enterococci, however, requires caution in establishing the clinical significance of a particular isolate. Unnecessary work and potentially misleading laboratory reports should be avoided whenever possible, especially with respect to in vitro susceptibility testing decisions (see "Antimicrobial Susceptibilities" below).

The variety of infections in which enterococci are involved has been thoroughly reviewed and summarized (39, 55, 63, 67, 94). Although the spectrum of infections has remained relatively unchanged since the extensive review by Murray in 1990 (67), trends to increasing prevalence of these organisms as nosocomial pathogens have been frequently observed. Enterococci have become the second or third leading cause of nosocomial urinary tract infections (UTIs), wound infections (mostly surgical, decubitus ulcers, and burn wounds), and bacteremia in the United States (39, 50, 55, 63, 67, 76, 88, 94). UTIs are the most common of the enterococcal infections: enterococci have been implicated in approximately 10% of all UTIs and in up to approximately 16% of nosocomial UTIs (55, 63, 76, 88, 94). Enterococcal bacteruria usually occurs in patients with underlying structural abnormalities and/or in those who have undergone urologic manipulations (63). Intra-abdominal and pelvic infections are the next most commonly encountered infections. However, cultures from patients with peritonitis, intra-abdominal or pelvic abscesses, biliary tract infections, surgical site infections, and endomyometritis are frequently polymicrobial, and the role of enterococci in these settings remains controversial. Enterococci have been considered to be an important cause of endocarditis since early descriptions and are estimated to account for about 20% of cases of native valve bacterial endocarditis and for about 6 to 7% of prosthetic valve endocarditis (59). Whereas endocarditis is a serious enterococcal infection, it is less common than bacteremia. Enterococcal infections of the respiratory tract or the central nervous system, as well as otitis, sinusitis, septic arthritis, and endophthalmitis, may occur but are rare (39, 67, 94). There is evidence for a role of enterococci in dental infections (95). The significance of isolates from some of these sites should be carefully evaluated before any clinical decisions are made.

E. faecalis is the most frequent enterococcal species isolated from human clinical specimens, representing 80 to 90% of the isolates, followed by *E. faecium,* which is found in 5 to 10% of enterococcal infections (6, 27, 35, 64). However, the ratios of isolation of the different enterococcal species can vary according to each setting and can be affected by a number of aspects, including the increasing dissemination of outbreak-related strains such as vancomycin-resistant *E. faecium*. For example, in one recent report, the ratio of *E. faecalis* to *E. faecium* isolates recovered from blood cultures was about 2.5:1.0 (50). Other enterococcal species are identified less frequently, even though clusters of infections associated with *E. casseliflavus* (77) and *E. raffinosus* (113) have been reported. Although less frequently or even rarely, several other enterococcal species, including *E. avium, E. caccae, E. cecorum, E. dispar*, *E. durans, E. gallinarum, E. gilvus,* "*E. hawaiiensis,*" *E. hirae, E. italicus, E. malodoratus, E. mundtii, E. pallens*, *E. pseudoavium*, "*E. sanguinicola*," and *E. faecalis* variant strains, have also been isolated from human sources (12, 27, 35, 42, 62, 64, 109). Isolation of the other species of enterococci has not yet been documented from human sources.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

The standard methods of collecting blood, urine, wound samples, and other secretions or swab specimens are adequate (see chapters 5 and 20). No special methods or procedures are usually necessary for transporting clinical specimens containing enterococci because these microorganisms are easily recovered and are relatively resistant to environmental changes. Transport can be performed on almost any transport media or on swabs that are kept dry. Like most clinical samples, the material should be cultured as soon as possible, preferably within 1 h.

Enterococcal strains can be stored indefinitely when lyophilized. In our experience, cultures frozen at –70°C or less can be stored for several years as heavy cell suspensions made directly in defibrinated sheep or rabbit blood or in a skim milk (10%) solution containing glycerol (10%). Most strains of enterococci survive several months at 4°C on agar slants prepared with ordinary agar bases, such as brain heart infusion and Trypticase soy agar.

DIRECT EXAMINATION

The direct microscopic examination of Gram-stained smears of normally sterile clinical specimens, such as blood, may be useful for the diagnosis of enterococcal infections. Direct examination of certain nonsterile specimens may also be informative, but should not be overemphasized. In any case only a presumptive report of the "presence of gram-positive cocci" should be made, as microscopy by itself cannot differentiate the enterococci from most of the other gram-positive cocci. Culture and appropriate identification techniques should be performed for confirmation.

As the occurrence of vancomycin-resistant enterococci (VRE) represents an important problem worldwide, hospitals should consider the implementation of surveillance programs for VRE detection. In an attempt to overcome the inherent limitations of culture-based methods of detection (discussed in "Isolation Procedures"), conventional PCR and real-time PCR-based methods have been evaluated for direct detection of these microorganisms in clinical and surveillance specimens (80, 86, 92). The LightCycler *vanA/vanB* detection assay (commercially available from Roche Diagnostics, Indianapolis, Ind.) has shown promise for screening of VRE in rectal or perianal swabs and has been evaluated for identification of VRE in blood cultures (92). Relatively few methods for direct detection of enterococci from blood samples or blood cultures have been reported. Results of a recent report indicate the potential usefulness of the commercially available DNA probe (AccuProbe, Gen-Probe, Inc.) for the direct detection of enterococci in blood cultures (54).

ISOLATION PROCEDURES

Trypticase soy–5% sheep blood agar, brain heart infusion–5% sheep blood agar, or any blood agar base containing 5% animal blood supports the growth of enterococci. Enterococci grow well at 35 to 37°C and do not require an atmosphere containing increased levels of CO_2, although some strains grow better in this atmosphere. If the sample to be cultured is likely to contain gram-negative bacteria, commercially prepared media containing bile, esculin, and azide are excellent options for primary isolation. These media have traditionally been used and are supplied under different designations by the various manufacturers. More recently, media containing chromogenic substrates have been proposed for the isolation and presumptive identification of enterococci and their use in clinical laboratories is gaining acceptance (18, 60). The use of selective media for the isolation of enterococci has been previously reviewed (17). An extensive review of the diversity of media used for the isolation of enterococci from fecal and clinical specimens, as well as water, food, feeds, and environmental sources, was recently presented by Domig et al. (18).

The increasing incidence of vancomycin resistance among the enterococci has raised the importance of selective isolation of VRE. Early identification of infection or colonization by VRE is recommended to prevent the spread of these microorganisms (41, 70). Current recommendations for hospital infection control include VRE fecal surveillance cultures, but the optimal methods for these cultures are still unclear. Several different selective agar and/or broth media and procedures have been employed for the isolation of VRE from sources containing commensal microbiota, such as stool samples and rectal or perianal swab specimens (18, 78, 80, 85, 86). Most of them are variations of selective media differing with regard to the antimicrobial agents or the antimicrobial concentrations used. Depending on the specific purposes, direct plating techniques give some indication of the number of VRE present in the sample, but enrichment procedures are mainly applied to detect VRE present in low numbers. Although there is not a single generally accepted screening method for isolation at this point, the use of a selective-enrichment broth to enhance the recovery of VRE seems to be the most effective procedure. Enterococcosel broth (a bile-esculin azide medium supplied by Becton Dickinson Microbiology Systems, Sparks, Md.) has been used in a number of studies as the base medium supplemented with different concentrations of vancomycin, with 6 μg/ml being the most common concentration. In some of the protocols proposed, vancomycin and other antimicrobial agents, such as aztreonam and clindamycin, have been used in the enrichment broth as well as in the subculture medium. It would be prudent, however, to use a nonselective agar as well for subculturing from a selective-enrichment broth, as some strains may be inhibited to the point of very poor or no growth. In some circumstances, it may be necessary to recover VRE from environmental surfaces for epidemiologic studies. Isolation of the organisms from these surfaces can be accomplished by swabbing the surfaces with premoistened swabs and placing them either into a selective-enrichment broth or onto agar plates. Alternatively, the Rodac imprint method may be employed by applying the agar surface directly to the environmental surface to be cultured (37). Overall, culture-based screening methods for VRE may be especially demanding and can take several days to complete, besides having variable degrees of sensitivity, which affects the timely implementation of infection control procedures. Therefore, some microbiology laboratories have recently considered the introduction of molecular techniques to facilitate the rapid and accurate identification of VRE and to improve the ability for detecting this pathogen. However, most of the approaches described in the literature still require bacterial growth in culture prior to detection, requiring 24 h or more to complete. More rapid detection of VRE directly from patient samples is still an area for which there is an important clinical need and relatively little published literature, as already mentioned (see "Direct Examination" above). Because a laboratory report that indicates the presence of VRE may initiate a cascade of infection control events that are time-consuming and costly (10, 41, 70), laboratories should be confident in the epidemiologic importance of any suspected VRE isolate. Transferable and high-level vancomycin resistance, encoded by the *vanA* or the *vanB* genes and most frequently associated with *E. faecalis* and *E. faecium* isolates, is the major focus of infection

control efforts. In contrast, the intrinsically low-level VanC resistance, which is not transferable and is not associated with dissemination of resistant strains, is less likely to be important to infection control surveillance efforts (10, 44, 85, 86). While the isolation of VRE from clinical and surveillance specimens is important, the need for protocols to rapidly determine the likely underlying mechanism of resistance associated with different genetic determinants (see "Antimicrobial Susceptibilities" below; see also chapters 71 and 78) is equally important (15, 85, 86).

IDENTIFICATION

Identification by Conventional Physiologic Testing

Once it is established that an unknown catalase-negative, gram-positive coccus is an *Enterococcus* or closely related genus (see "Description of the Genus"), the conventional tests (see reference 28 and chapters 14, 15, 29, and 31 for methods) listed in Table 1 can be used to identify the species. Most of the information presented here is related to phenotypic characteristics of strains isolated from humans.

TABLE 1 Phenotypic characteristics used for the identification of *Enterococcus* species and some physiologically related species of other gram-positive cocci

Species	Phenotypic characteristic[a]											
	MAN	SOR	ARG	ARA	SBL	RAF	TEL	MOT	PIG	SUC	PYU	MGP
Group I												
E. avium	+	+	−	+	+	−	−	−	−	+	+	V
E. raffinosus	+	+	−	+	+	+	−	−	−	+	+	V
E. gilvus	+	+	−	−	+	+	−	−	+	+	+	−
E. pallens	+	+	−	−	+	+	−	−	+	+	−	+
E. saccharolyticus[b]	+	+	−	−	+	+	−	−	−	+	−	+
E. malodoratus	+	+	−	−	+	+	−	−	−	+	+	V
E. pseudoavium	+	+	−	−	+	−	−	−	−	+	+	+
"*E. hawaiiensis*"	+	+	−	−	+	−	−	−	−	−	+	−
Group II												
E. faecium	+[c]	−	+	+	V	V	−	−	−	+[c]	−	−
E. casseliflavus	+	−	+[c]	+	V	+	−[c]	+[c]	+[c]	+	V	+
E. gallinarum	+	−	+[c]	+	−	+	−	+[c]	−	+	−	+
E. mundtii	+	−	+	+	V	+	−	−	+	+	−	−
E. faecalis	+[c]	−	+[c]	−	+	−	+	−	−	+[c]	+	−
E. haemoperoxidus[b]	+[d]	−	+[d]	−	−	−	−	−	−	+	−	+
"*E. sanguinicola*"	+	−	+	−	−	−	+[e]	−	−	+	−	−
Lactococcus sp.	+	−	+	−	−	−	−	−	−	V	−	−
Group III												
E. dispar	−	−	+	−	−	+	−	−	−	+	+	+
E. hirae	−	−	+	−	−	+	−	−	−	+	−	−
E. durans	−	−	+	−	−	−	−	−	−	−	−	−
E. ratti	−	−	+	−	−	−	−	−	−	−	−	−
E. villorum	−	−	+	−	−	−	−	−	−	−	−	−
Group IV												
E. cecorum[b]	−	−	−	−	−	+	−	−	−	+	+	−
E. phoeniculicola[b]	−	−	−	+	−	+	−	−	−	+	−	+
E. sulfureus	−	−	−	−	−	+	−	−	+	+	−	+
E. asini[b]	−	−	−	−	−	−	−	−	−	+	−	−
E. caccae	−	−	−	−	−	−	−	−	−	+	+	+[d]
Group V												
E. canis[b]	+	−	−	+	−	−	−	−	−	+	+	+
E. columbae[b]	+	−	−	+	+	+	−	−	−	+	+	−
E. moraviensis[b]	+	−	−	+	−	−	−	−	−	+	+	+
E. hermanniensis	+	−	−	−	−	−	−	−	−	−	−	−
E. italicus	V	−	−	−	V	−	−	−	−	+	+	+
Vagococcus fluvialis	+	−	−	−	+	−	−	+	−	+	−	+

[a] Abbreviations and symbols: MAN, mannitol; SOR, sorbose; ARG, arginine; ARA, arabinose; SBL, sorbitol; RAF, raffinose; TEL, 0.04% tellurite; MOT, motility; PIG, pigment; SUC, sucrose; PYU, pyruvate; MGP, methyl-α-D-glucopyranoside; +, 90% or more of the strains are positive; –, 90% or more of the strains are negative; V, variable (11 to 89% of the strains are positive).

[b] Phenotypic characteristics based on data from type strains.

[c] Occasional exceptions occur (<3% of strains show aberrant reactions).

[d] Late positive (3 days of incubation or longer).

[e] Weak reaction.

Enterococcal species can be separated into five physiologic groups of species based on acid formation from mannitol and sorbose, and hydrolysis of arginine (Table 1). Identification of enterococcal species by conventional tests is not rapid and may require up to 10 days of incubation. However, most identifications can be made after 2 days of incubation.

Group I consists of *E. avium, E. gilvus,* "*E. hawaiiensis,*" *E. malodoratus, E. pallens, E. pseudoavium, E. raffinosus,* and *E. saccharolyticus* (Table 1). "*E. hawaiiensis*" is a denomination that has been recently proposed (L. M. Teixeira, M. G. S. Carvalho, A. G. Steigerwalt, R. E. Morey, P. L. Shewmaker, E. Falsen, and R. R Facklam, *Abstr. 2nd Int. ASM-FEMS Conf. Enterococci,* abstr. A15, 2005) for the new species previously designated *Enterococcus* sp. nov. CDC PNS-E3 (7). If the results of conventional tests compare to the results originally reported (98), *E. devriesei,* a recently described species, will be grouped with the enterococcal species in group I.

Group II comprises *E. faecalis, E. faecium, E. casseliflavus, E. gallinarum, E. haemoperoxidus, E. mundtii,* and "*E. sanguinicola.*" These species form acid from mannitol and hydrolyze arginine but fail to form acid from sorbose. The majority of the isolates from human sources belong to species included in this group. Atypical strains that fail to hydrolyze arginine or form acid from mannitol have been documented. *Lactococcus* sp. is also listed in this group because the phenotypic characteristics of some strains can lead to the misidentification as an *Enterococcus*. If nonmotile variants of *E. casseliflavus* and *E. gallinarum* are encountered, production of acid from methyl-α-D-glucopyranoside (MGP) can be used to help in the identification of these species. "*E. sanguinicola*" is the denomination recently proposed (Teixeira et al., *Abstr. 2nd Int. ASM-FEMS Conf. Enterococci*) for the new species previously designated *Enterococcus* sp. nov. CDC PNS-E2 (7).

Group III consists of *E. dispar, E. durans*, *E. hirae*, *E. ratti* (101), and *E. villorum*. Three of these species (*E. durans*, *E. ratti,* and *E. villorum*) have very similar phenotypic profiles in the tests listed in Table 1. They can be differentiated by clotting reactions in litmus milk, hydrolysis of hippurate, and acid formation from trehalose and xylose. *E. durans* forms acid and clot, *E. villorum* forms acid but no clot, and *E. ratti* does not form acid or clot in litmus milk. *E. durans* hydrolyzes hippurate, while *E. villorum* does not. *E. ratti* is variable in the hippurate hydrolysis test. *E. durans* forms acid from trehalose but not from xylose, *E. villorum* forms acid from both trehalose and xylose, and *E. ratti* does not form acid from either trehalose or xylose. The other members of this group are easily identified by the reactions shown in Table 1. Uncommon mannitol-negative variant strains of *E. faecalis* and *E. faecium* resemble species in this group. However, *E. faecalis* strains are positive in the pyruvate test but not for acid formation from arabinose, raffinose, or sucrose, and E. *faecium* variant strains form acid from arabinose. In all likelihood *E. canintestini*, a recently described species (74), will also fall into enterococcal group III.

Group IV is composed by *E. asini, E. caccae, E. cecorum*, *E. phoeniculicola*, and *E. sulfureus* (Table 1). *E. caccae* is a recently proposed new species (8). If the results of conventional tests compare to the results originally reported (97), *E. aquimarinus,* a recently described species, will be grouped with the enterococcal species in group IV. Group V consists of *E. canis*, *E. columbae, E. hermanniensis, E. italicus,* and *E. moraviensis*. Variant strains of *E. casseliflavus*, *E. gallinarum,* and *E. faecalis* that fail to hydrolyze arginine resemble the microorganisms included in this group. However, these variant strains have characteristics similar to those of the strains that hydrolyze arginine and can be differentiated by the same phenotypic tests. *Vagococcus fluvialis* is listed here because the phenotypic characteristics of this species are very similar to those of the genus *Enterococcus* and some strains may be identified as enterococci (102). *E. italicus* corresponds to the new species previously designated *Enterococcus* sp. nov. CDC PNS-E1 (7, 30; Teixeira et al., *Abstr. 2nd Int. ASM-FEMS Conf. Enterococci*).

Identification by Commercial Systems or Molecular Methods

There are several commercially available miniaturized, manual, semiautomated, and automated identification systems for the identification of *Enterococcus* species. Since their introduction, these systems have been updated to improve their performance characteristics and expand their identification capabilities, as investigators have become more aware of inaccuracies (14, 32, 38, 46, 113). In general, these systems are reliable for the identification of *E. faecalis*, and, to a lesser extent, *E. faecium*. Accurate identification of other species, by most systems, depends on additional testing, although improvements have been observed with updated formats and databases. Commercial systems available for the identification of enterococcal species include the API 20S and the API Rapid ID 32 Strep systems (bioMérieux Vitek, Inc., Hazelwood, Mo.), the Crystal Gram-Positive and the Crystal Rapid Gram-Positive identification systems (Becton Dickinson Microbiology Systems), the Gram Positive Identification Card of the Vitek system (bioMérieux), and the Gram-Positive Identification panel of the MicroScan Walkaway system (Dade MicroScan, West Sacramento, Calif.). The accuracy of identification by some of these systems in comparison to identification by molecular techniques has been evaluated (3, 46). In general, a large proportion of enterococcal isolates are accurately identified by any one of these systems; however, the accuracy is dependent on the distribution of species found in each specific setting. Identification of unusual species by a commercial system should be confirmed by a reference method before being reported.

Molecular methods based on the analyses of different target molecules, such as DNA-DNA hybridization and sequencing of the 16S rRNA genes, have been used primarily for taxonomic purposes in special laboratories. In the past 15 years, however, the application of molecular techniques for the identification of *Enterococcus* species has expanded dramatically in an attempt to develop more rapid and accurate identification methods potentially adaptable for use in clinical microbiology laboratories. A variety of molecular procedures have been proposed for the identification of enterococcal species as recently reviewed and summarized (19, 26). These alternate methods include sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of whole-cell protein (WCP) profiles, vibrational spectroscopic analysis, proton magnetic resonance spectroscopic analysis, randomly amplified polymorphic DNA analysis, sequencing analysis of the 16S rRNA gene, restriction fragment-length polymorphism analysis of the PCR-amplified 16S rRNA gene, broad-range amplification of the 16S rRNA gene or of the *groESL* gene, sequencing of the domain V of the 23S rRNA gene, amplification of the tRNA or the rRNA intergenic spacers, amplification of the D-ala:D-ala ligases *(ddl)* and the vancomycin resistance *(van)* genes, sequencing of the *ddl* genes, amplification and probing of the *Enterococcus* protein A *(efaA)* genes or of the *E. faecalis* adhesin for collagen *(ace)* gene, amplification of the elongation factor EF-Tu *(tuf)* or the pEM1225 genes, sequencing of the manganese-dependent superoxide dismutase (*sodA)* gene, sequencing of the chaperonin 60 *(cpn60)* gene, sequencing of the RNA polymerase β subunit *(rpoB)*

gene (21), sequencing of the RNA polymerase α subunit (*rpoA*) and the phenylalanyl-tRNA synthase (*pheS*) genes (72), and sequencing of the α subunit of the ATP synthase (*atpA*) gene (73). Many of these molecular procedures have been performed in only one or a few laboratories and have not been evaluated for the majority of the species of *Enterococcus*. Most of them are potentially applicable to all enterococcal species, and others are species specific.

Among the molecular techniques proposed to identify the different enterococcal species, SDS-PAGE analysis of WCP profiles and sequencing of the 16S rRNA genes have been evaluated in reference laboratories. SDS-PAGE analysis of WCP profiles appears to be a reliable tool for the differentiation and identification of *Enterococcus* strains, since WCP profiles are species specific (7, 26, 61, 101). Apart from minor differences among strains, each of the known enterococcal species corresponds to a unique WCP profile. WCP profiles of related species of *Lactococcus* and *Vagococcus* are also unique and different from those of the enterococci (90, 102-104). Table 1 depicts the phenotypic characteristics of *Enterococcus* species discussed in this chapter, is based on correlations between the WCP profiles and the phenotypic tests, and may include findings of DNA-DNA reassociation experiments and 16S rRNA gene sequencing. Sequencing of the 16S rRNA gene is a frequently used nucleic acid-based method for identification of enterococcal species (81). The 16S rRNA gene sequences are available for comparison purposes via public databanks of nucleotide sequences such as GenBank. Comparisons can be made by using one of the several sequence-comparing software packages, many of which are available for public access. Figure 1 shows a dendrogram generated by comparison of 16S rRNA gene sequences of the type strains of the species included in the genus *Enterococcus*. Accession numbers of the reference sequences used for comparative analyses are indicated in the figure. Clear-cut identification is not obtained for all enterococcal species, since several species differ by only 1 to 3 bases in the entire 16S rRNA gene (approximately 1,500 bp). Together with phenotypic characterization and other alternative molecular methods such as analyses of WCP profiles, DNA sequencing can be an important tool to establish species identity.

TYPING METHODS

The increasing documentation of *Enterococcus* as a leading nosocomial pathogen frequently exhibiting resistance to several antimicrobial agents and the evidence supporting the concept of exogenous acquisition of enterococcal infections have generated demand for strain typing and epidemiologic studies. Besides outbreak analysis, the methods used for epidemiologic investigation of enterococcal isolates must be able to track enterococcal dissemination in different environments and hosts and the evolution of multidrug-resistant strains. Classic phenotypic methods used to investigate the diversity among isolates of a given enterococcal species have frequently failed to adequately discriminate among strains, and they have limited value in epidemiologic studies. However, phenotypic information in association with molecular data can contribute valuable information (19, 26, 115). Discriminatory molecular typing methods demonstrated that strains can be exogenously acquired by direct and indirect contact among patients. Intrahospital transmission and interhospital spread have also been documented for antimicrobial-resistant enterococci (4, 19, 26, 55, 94, 115, 116).

Improved discrimination among enterococcal strains involved analyses of chromosomal DNA restriction endonuclease profiles by pulsed-field gel electrophoresis (PFGE) (4, 19, 26, 34, 36, 69, 83). Multilocus enzyme electrophoresis (9, 107), ribotyping (23, 35, 52, 116), and PCR-based typing methods, such as randomly amplified polymorphic DNA PCR assay and repetitive element sequence-based PCR, have also been used to investigate genetic relationships among enterococcal strains (45, 56). Sequencing of PCR products and restriction fragment length polymorphism analyses of PCR products have been used to trace and determine differences among antibiotic resistance genes in enterococci (20, 51, 82, 114).

Results from a number of investigations indicate that analysis of SmaI restriction digests of genomic DNA by PFGE is widely useful for studying enterococcal species (26), showing definite advantages in strain discrimination. PFGE is considered to be the "gold standard" for the epidemiologic analyses of enterococcal infections. Several protocols for performing PFGE analyses of enterococcal strains have been published. However, the development of standardized protocols as a result of collaborative studies is needed in order to allow for interlaboratory comparisons. Most of the information accumulated during the past several years is related to *E. faecium* and *E. faecalis*. Although PFGE is quite discriminatory, epidemiologic interpretation of PFGE profiles is not always clear-cut. The occurrence of genetic events can be associated with substantial changes in the PFGE profiles, leading to problems in clonality assessment (51, 65, 87). There is not a single definitive typing technique for enterococci. A strong match among the results of several typing techniques, particularly those based on different genomic polymorphisms, can be used to indicate a high degree of relatedness. The use of PFGE in conjunction with at least one additional typing technique or independent PFGE analyses using different restriction enzymes is highly recommended to help in clarifying epidemiologic interpretations. General principles proposed for the interpretation of molecular typing data based on fragment differences are usually applied to interpret PFGE profiles obtained for enterococcal strains (26, 105). Well-characterized control strains should be evaluated along with unknown isolates. For that purpose, two reference strains, *E. faecalis* OG1RF (ATCC 47077) and *E. faecium* GE1 (ATCC 51558), have been proposed (105).

Two additional powerful molecular techniques, multilocus sequence typing (MLST) and multiple-locus variable-number tandem repeat analysis (MLVA), were developed more recently and have already been used to identify clonal complexes within enterococcal populations and for investigating genetic relatedness between strains. MLST typing schemes have been applied to explore the population structure of *E. faecalis* and *E. faecium* (5, 40, 71). Application of MLST typing has revealed the occurrence of host-specific genogroups of *E. faecium* and a specific genetic lineage that seems to have emerged recently worldwide, associated with nosocomial outbreaks. Two simultaneously published studies described the development of MLVA typing schemes for *E. faecalis* (106) and *E. faecium* (108). The data indicate that this technique can achieve a high degree of discrimination and is suitable for easy interlaboratory data exchange. However, neither MLVA nor MLST is currently feasible for most laboratories.

ANTIMICROBIAL SUSCEPTIBILITIES

Resistance to several commonly used antimicrobial agents is a remarkable characteristic of most enterococcal species. Moreover, most of this information is based on studies with *E. faecalis* and *E. faecium*, the two species most frequently associated with human infections. Antimicrobial resistance can be classified as either intrinsic or acquired. Intrinsic

resistance is related to inherent or natural chromosomally encoded characteristics present in all or most of the enterococci. Furthermore, certain specific mechanisms of intrinsic resistance to some antimicrobial agents are typically associated with a particular enterococcal species or groups of species. In contrast, the occurrence of acquired resistance is more variable, resulting from either mutations in existing DNA or acquisition of new genetic determinants found in plasmids or transposons (44, 48, 67, 68, 94). Intrinsic resistance among enterococci involves two major groups of antimicrobial agents, the aminoglycosides and the β-lactams. Because of the poor activity of several antimicrobial agents against enterococci due to intrinsic resistance, the recommended therapy for serious infections (i.e., endocarditis, meningitis, and other systemic infections, especially in immunocompromised patients) includes a combination of a cell wall-active agent, such as a β-lactam (usually penicillin) or vancomycin, combined with an aminoglycoside (usually gentamicin or streptomycin). These combinations overcome the intrinsic resistance exhibited by enterococci, and a synergistic bactericidal effect is generally achieved since the intracellular penetration of the aminoglycoside is facilitated by the cell wall-active agent.

In addition to the intrinsic resistance traits, enterococci have acquired different genetic determinants that confer resistance to several classes of antimicrobial agents, including chloramphenicol, tetracyclines, macrolides, lincosamides and streptogramins, aminoglycosides, β-lactams, glycopeptides, and, more recently, quinolones. During the past several decades, the occurrence of acquired antimicrobial resistance among enterococci, especially high-level resistance (HLR) to aminoglycosides, β-lactams, and resistance to glycopeptides (especially vancomycin), has been increasingly reported. Isolates that are resistant to the cell wall-active agent or have HLR to aminoglycosides are resistant to the synergistic effects of combination therapy and constitute an even more serious problem. Therefore, the detection of resistance to these groups of antimicrobial agents is important in order to predict the likelihood of synergy by using antimicrobial combinations as a therapeutic strategy. Enterococcal isolates exhibiting HLR to one or more aminoglycosides have been described with increasing frequencies (35, 44, 48, 64, 67, 68, 94) and are now present in large proportions in several geographic areas. Strains expressing acquired HLR to aminoglycosides frequently have MICs of >2,000 μg/ml and cannot be detected by diffusion tests with conventional disks. Special tests using high-content gentamicin and streptomycin disks, as well as a single-dilution method, were developed to screen for this type of resistance (see chapters 73 and 74). Strains exhibiting high-level resistance to penicillin and ampicillin due to altered penicillin-binding proteins have also disseminated widely (44, 48, 67, 68, 94), while strains producing β-lactamase have been rarely identified (35, 67).

The emergence of vancomycin resistance as a therapeutic problem in enterococcal strains was first documented in western Europe and in the United States (49, 53, 110). Thereafter, the isolation of VRE has been continuously reported in diverse geographic locations (10, 44, 48, 66, 94). VRE strains have been classified according to phenotypic and genotypic features. Six types of glycopeptide resistance have already been described among enterococci, including three common phenotypes. The VanA phenotype, with inducible high-level resistance to vancomycin as well as to teicoplanin, encoded by the *vanA* gene; the VanB phenotype, with variable (moderate to high) levels of inducible resistance to vancomycin only, encoded by the *vanB (vanB1* and *vanB2)* genes; and the VanC phenotype, encoded by the *vanC* genes, conferring noninducible low-level resistance to vancomycin. VanA and VanB are considered the most clinically relevant phenotypes and are usually associated with *E. faecium* and *E. faecalis* isolates, while VanC resistance is an intrinsic characteristic of *E. gallinarum* (*vanC1* genotype) and *E. casseliflavus* (*vanC2* and *vanC3* genotypes) strains (10, 11, 44, 68, 82). Three additional types of glycopeptide resistance, encoded by the *vanD* (79), *vanE* (29), and *vanG* (57) genes, seem to occur rarely among enterococci. Furthermore, the isolation of vancomycin-dependent (99) and vancomycin-heteroresistant (2) enterococcal strains from clinically significant infections, although still sporadically reported, may represent additional serious threats for the treatment and control of enterococcal infections.

Although β-lactams other than penicillin or ampicillin (e.g., mezlocillin, piperacillin, azlocillin, amoxicillin-clavulanate, ampicillin-sulbactam, and imipenem) do not offer any significant advantages over ampicillin when they are used against enterococci, they may be useful against polymicrobial infections involving enterococci and gram-negative bacilli. Ampicillin testing, along with a test for β-lactamase production, can be used to predict resistance to other β-lactam antibiotics. Susceptibility testing for the other β-lactams is rarely necessary. A positive test for β-lactamase production (see chapter 74) indicates resistance to ampicillin and the acylureidopenicillins (i.e., azlocillin, mezlocillin, or piperacillin). Resistance to ampicillin revealed by disk diffusion or dilution methods indicates the presence of penicillin-binding protein-mediated resistance to these agents, as well as to β-lactam-β-lactamase inhibitor combinations and imipenem (67, 94, 112).

While in vitro methods for detecting vancomycin resistance are discussed in detail in chapters 73 and 74, some aspects regarding VanC-containing species (i.e., *E. gallinarum* and *E. casseliflavus*) need to be emphasized. Resistance usually associated with *vanC* genotypes is not detected by disk diffusion, but VanC strains usually grow on vancomycin agar screen tests. Because the clinical significance of VanC is still uncertain, the implications of susceptibility testing for patient management may be unclear. However, the need to differentiate VanA or VanB strains from VanC strains is quite evident for therapeutic, infection control, and surveillance reasons. Because growth on the vancomycin agar screen fails to help with this important distinction, species identification is necessary. VanC resistance in *E. faecalis* or *E. faecium* is yet to be described, so growth on the vancomycin agar by either of these species is likely due to VanA or VanB resistance. Although rare, the occurrence of the other kinds of vancomycin resistance may also be considered. Additionally, VanA resistance together with VanC resistance has been described for *E. gallinarum* so that identification of a species that usually harbors only VanC resistance does not completely rule out moderate to high levels of vancomycin resistance. In this regard, determining vancomycin MICs is useful as VanC resistance frequently results in MICs of <16 μg/ml, whereas VanA and VanB usually result in MICs of >32 μg/ml. Resistance to other agents such as ampicillin and aminoglycosides also is uncommon among VanC isolates. Because of limited alternatives, VRE isolates may be tested for susceptibilities to chloramphenicol, erythromycin, tetracycline (or doxycycline or minocycline), and rifampin. Testing of quinupristin-dalfopristin, daptomycin, or linezolid is recommended for reporting of vancomycin-resistant *E. faecium*.

Since enterococci are often discussed in terms of their multidrug resistance phenotypes, two of the most problematic resistance profiles, ampicillin and vancomycin resistance, are most commonly associated with *E. faecium* (44, 48). This

is important in terms of resistance surveillance. Because both ampicillin resistance and vancomycin resistance are less commonly seen in *E. faecalis* than in *E. faecium*, widespread emergence and dissemination of these resistance traits in *E. faecalis* would significantly add to the current problem of multiply resistant enterococci. Therefore, enterococcal species identification is important for the purposes of therapy and meaningful surveillance.

Molecular methods (see reference 26 and chapter 78) have been used to detect specific antimicrobial resistance genes and have substantially contributed to the understanding of the spread of acquired resistance among enterococci, especially resistance to vancomycin. However, because of their high specificity, molecular methods do not detect antimicrobial resistance due to mechanisms, including emerging resistance mechanisms, not targeted by testing.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

The diversity and, in some cases, species specificity of emerging antimicrobial resistance traits among enterococcal isolates created an additional need for accurate identification at the species level and for in vitro evaluation of susceptibility to antimicrobial agents. The significance of a particular enterococcal isolate is a major factor in determining when antimicrobial testing should be done. Once the need to test a particular isolate has been established, selection of the appropriate antimicrobial agents for testing must be considered on the basis of the site of infection. Testing of antimicrobial agents to which enterococci are intrinsically resistant is contraindicated. The drugs that should not be tested include aminoglycosides at standard concentrations, aztreonam, cephalosporins, clindamycin, methicillin (or oxacillin), and trimethoprim-sulfamethoxazole. These drugs may appear active for enterococci in vitro but are not effective clinically, and isolates should not be reported as susceptible to these agents. Due to difficulties with some phenotypic methods for detection of HLR to aminoglycosides and resistance to vancomycin, updated guidelines for the selection of antimicrobial agents should be followed for routine testing and reporting. The in vitro methods for detecting antimicrobial resistance in enterococcal isolates were reviewed and summarized by Facklam et al. (26) and are also discussed in detail in chapters 73 and 74.

As already mentioned, synergy testing should be done with any enterococcal isolate implicated in infections for which combination therapy is indicated (e.g., for systemic infections). Enterococci are also frequently encountered in polymicrobial infections associated with the gastrointestinal tract or superficial wounds of hospitalized patients. Their pathogenic significance in such settings is uncertain, but susceptibility testing is warranted when predominant or heavy growth is observed (63). Testing of enterococcal isolates from lower UTIs is optional, as these infections usually respond to therapy with ampicillin. However, many hospital infection control programs require routine testing as a means of surveillance for VRE. For those instances when testing a urinary tract isolate is appropriate, ciprofloxacin, levofloxacin, nitrofurantoin, norfloxacin, or tetracycline could be selected, in addition to ampicillin (44, 55, 94). In cases of treatment failure, testing is always warranted.

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Aerococcus, Abiotrophia, and Other Aerobic Catalase-Negative, Gram-Positive Cocci

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31

TAXONOMY

The bacteria included in this chapter are taxonomically diverse catalase-negative, gram-positive cocci. All, however, share the characteristic of being infrequent clinical isolates found as opportunistic agents of infection in hosts who are usually compromised. Most of these organisms resemble other, more well known clinical isolates (e.g., streptococci and enterococci) and consequently may be mistaken for members of those genera. These bacteria may have been misidentified or overlooked in clinical cultures in the past or may represent emerging pathogens in compromised patient populations. Table 1 lists the organisms included here along with some of their basic characteristics.

Most organisms discussed in this chapter exhibit fairly low G+C contents (30 to 45 mol%) and are not currently affiliated with taxa above the genus level. Two additional infrequently isolated gram-positive cocci (*Rothia mucilaginosa* and *Alloiococcus* sp.) can display positive catalase reactions. The catalase-variable *R. mucilaginosa* (29), formerly called *Stomatococcus mucilaginosus* (G+C content, 50 to 60 mol%), was historically included with staphylococci in the family *Micrococcaceae* (101). The sole species of the catalase-positive genus *Alloiococcus* is *Alloiococcus otitis* (1) (see chapter 28).

The genus *Lactococcus* is composed of organisms formerly classified as Lancefield group N streptococci (102). Motile *Lactococcus*-like organisms with the Lancefield group N antigen (a teichoic acid antigen) have been classified in the genus *Vagococcus* (25, 116). The vagococci also resemble the enterococci, and Facklam and Elliott (51) reported that *Vagococcus* isolates examined at the Centers for Disease Control and Prevention, Atlanta, Ga., gave positive reactions in a commercially available nucleic acid probe test (Gen-Probe, San Diego, Calif.) for enterococci.

The genera *Abiotrophia* and *Granulicatella* have been proposed to accommodate organisms previously known as nutritionally variant or satelliting streptococci (34, 72). These bacteria were initially considered to be nutritional mutants of viridans group streptococcal strains, most notably of the species *Streptococcus mitis*. The work of Bouvet and colleagues (15) suggested that these organisms were really members of two novel streptococcal species given the names *Streptococcus defectivus* and *Streptococcus adjacens*. A comparative analysis of 16S rRNA sequences led Kawamura and coworkers to propose the creation of a new genus, *Abiotrophia*, containing two species, *Abiotrophia defectiva* and *Abiotrophia adiacens,* to accommodate these bacteria (72). A third species from human sources, *Abiotrophia elegans,* was described in 1998 (97). Kanamoto et al. noted the heterogeneity among *Abiotrophia* strains and proposed a fourth species, *Abiotrophia para-adiacens* (71). Most recently, Collins and Lawson proposed a new genus, *Granulicatella*, with *Granulicatella adiacens* and *Granulicatella elegans* encompassing strains formerly called *A. adiacens* and *A. elegans*. *A. defectiva* remains as the sole *Abiotrophia* species (34).

Taxonomic changes among the intrinsically vancomycin-resistant, catalase-negative, gram-positive cocci include the formation of the genus *Weissella* to accommodate the species formerly known as *Leuconostoc paramesenteroides* and related species (38). The vancomycin-susceptible species *Pediococcus halophilus*, formerly included in the otherwise intrinsically vancomycin-resistant *Pediococcus* genus, was reclassified in the genus *Tetragenococcus* (39). The organism formerly called *Enterococcus solitarius* has also been transferred to the *Tetragenococcus* genus as *Tetragenococcus solitarius* (48). Little is known about the role of the tetragenococci in human infection.

The organism we now know as *Gemella morbillorum* was noted in 1917 by Tunicliff (111), who was searching for the etiologic agent of measles. The organism she isolated from blood cultures from numerous measles patients was originally named *Diplococcus rubeolae*. This bacterium was also known as *Diplococcus morbillorum, Peptostreptococcus morbillorum,* and *Streptococcus morbillorum* until a proposal to include it in the genus *Gemella* was made in 1988 (74). *Gemella haemolysans* was originally classified as a *Neisseria* species due to its gram-variable or even gram-negative nature and its cellular morphology (diplococci with flattened adjacent sides). Collins and coworkers described two additional *Gemella* species isolated from human sources, *Gemella bergeri* (originally named *Gemella bergeriae*) (31) and *Gemella sanguinis* (32). The genus *Dolosigranulum* shows phenotypic similarities to *Gemella,* although it is not phylogenetically closely related to *Gemella* strains (3, 79).

Aerococcus urinae, described in 1992, is negative for pyrrolidonyl arylamidase production (PYR) and positive for leucine aminopeptidase production (LAP), showing reactions opposite those of *Aerococcus viridans* in these important identification tests (2). In spite of these phenotypic differences, molecular taxonomic studies suggest that *A. urinae*

TABLE 1 Basic phenotypic characteristics of catalase-negative, gram-positive cocci[a]

Organism (reference[s])	PYR	LAP	NaCl	BE	ESC	MOT	HIP	SAT	ARG	BGUR	MORPH	VAN
Abiotrophia defectiva (21, 34, 72)	+	+	−	−		−	−	+	−	−	Chains	S
Aerococcus christensenii (33, 52)	−	+	+	−	−	−	+	−	−	−	Clusters	S
Aerococcus sanguinicola (52, 82)	+	+	+	+	+	ND	+	−	−	+	Clusters	ND
Aerococcus urinae (22, 23, 52)	−	+	+	−	V	−	+	−	−	+	Clusters	S
Aerococcus urinaehominis (52, 81)	−	−	+	−	+	ND	+	−	−	+	Clusters	ND
Aerococcus viridans[b] (51, 52)	+	−	+	V	+	−	V	−	−	ND	Clusters	S
Dolosicoccus (37)	+	−	−	ND	ND	−	ND	−	ND	ND	Chains	S
Dolosigranulum (3, 52, 79)	+	+	+	−	+[c]	−	−	−	−	−	Clusters	S
Enterococcus[d,e] (51)	+	+	+	+	+	V	ND	−	ND	ND	Chains	S/R
Facklamia languida[f] (79, 80)	+	+	+	ND	−	−	−	−	−	−	Clusters	S
Facklamia spp.[f] (28, 30, 35, 79)	+	+	+	ND	−	−	+	−	V	ND	Chains	S
Gemella haemolysans[g] (51)	+	+	−	−	−	−	ND	−	ND	ND	Clusters	S
Gemella spp.[g] (31, 32, 51)	+	+	−	−	ND	−	ND	−	−	ND	Chains	S
Globicatella (24, 50, 51, 104)	V	−	+	+	+	−	+	−	−	ND	Chains	S
Granulicatella adiacens[h] (21, 34, 72)	+	+	−	−	ND	−	−	+	−	+	Chains	S
Granulicatella elegans[h] (21, 34, 72)	+	+	−	−	ND	−	+	+	+	−	Chains	S
Helcococcus kunzii[b] (26, 51)	+	−	+	−	+	−	−	−	−	−	Clusters	S
Ignavigranum (36, 50, 79)	+	+	+	−	−	−	−	V	−	ND	Chains	S
Lactococcus[e,i] (51)	V	+	V	+	+	−	ND	−	ND	ND	Chains	S
Leuconostoc (51)	−	−	+	V	ND	−	ND	−	ND	ND	Chains	R
Pediococcus (51)	−	+	V	+	ND	−	ND	−	ND	ND	Clusters	R
Streptococcus[i,j] (51)	V	+	V	V	V	−	ND	−	ND	ND	Chains	S
Tetragenococcus (51)	−	+	+	+	ND	−	ND	ND	ND	ND	Clusters	S
Vagococcus[d] (51)	+	+	V	+	+	+	ND	−	ND	ND	Chains	S
Weissella (57, 89)	V	ND	ND	+	+	−	ND	ND	+	ND	Short rods or coccobacilli in pairs and chains	R

[a] Abbreviations and symbols: PYR, production of pyrrolidonyl arylamidase; LAP, production of leucine aminopeptidase; NaCl, growth in 6.5% NaCl; BE, hydrolysis of esculin in the presence of 40% bile; ESC, hydrolysis of esculin; MOT, motility; HIP, hydrolysis of hippurate; SAT, satelliting behavior; ARG, hydrolysis of arginine; BGUR, production of β-glucuronidase; MORPH, cellular arrangement; VAN, susceptibility to vancomycin; +, ≥90% of strains positive; −, ≤10% of strains positive; V, variable (60 to 90% of strains positive); ND, no data; chains, cells arranged primarily in pairs and chains; clusters, cells arranged primarily in clusters, tetrads, or irregular groups; S, susceptible; R, resistant.

[b] Although *H. kunzii* shares some phenotypic traits with *A. viridans*, *H. kunzii* is facultative and usually nonhemolytic, in contrast to *A. viridans*, which favors an aerobic growth atmosphere and is alpha-hemolytic. Two additional species of *Helcococcus* (*H. sueciensis* and "*H. pyogenes*") have been proposed (27, 91), both based on the isolation of single strains. These new species display negative reactions in the PYR test, in contrast to *H. kunzii*.

[c] LaClaire and Facklam (79) note that strains of *Dolosigranulum* are esculin hydrolysis positive when tested on conventional media, but the original description of this organism notes a lack of esculin hydrolysis activity (3).

[d] Most enterococcal strains are capable of growth at 45°C, which differentiates them from vagococci, which may be phenotypically similar. Strains of vagococci have been reported as testing positive with a commercially available nucleic acid probe for members of the genus *Enterococcus*.

[e] Phenotypically similar strains of enterococci and lactococci can be differentiated with a commercially available nucleic acid probe for members of the genus *Enterococcus*.

[f] *F. languida* cells are characteristically arranged in clusters, and cells of other *Facklamia* species are arranged in pairs and chains.

[g] *G. haemolysans* cells are arranged in pairs and clusters, and the cells of other *Gemella* species are arranged in pairs and chains.

[h] Formerly classified as a member of the genus *Abiotrophia*.

[i] Most lactococcal strains are capable of growth at 10°C, which differentiates them from streptococci, which may be phenotypically similar.

[j] *Streptococcus pyogenes* and some strains of *S. pneumoniae* are PYR positive.

should remain in the *Aerococcus* genus. Organisms currently included in the *A. urinae* species are fairly heterogeneous and can probably be subdivided into at least two subspecies (23). *Aerococcus christensenii,* isolated from the human genitourinary tract, was described by Collins and coworkers in 1999 (33) and was joined by the species *Aerococcus sanguinicola* (originally named *Aerococcus sanguicola*) (52, 82) and *Aerococcus urinaehominis* (81) in 2001.

The genera *Facklamia*, *Ignavigranum,* and *Dolosicoccus* are related to, but distinct from, *Globicatella sanguinus*, an organism initially named *Globicatella sanguis*, which was described in 1992 (24). The genus *Facklamia* currently contains four species isolated from human sources: *Facklamia hominis* (28), *Facklamia sourekii* (30), *Facklamia ignava* (35), and *Facklamia languida* (80). The genus *Ignavigranum*, currently consisting of a single species, *Ignavigranum ruoffiae*, was described by Collins and coworkers (36), along with the genus *Dolosicoccus* and its single species, *Dolosicoccus paucivorans* (37).

The genus *Helcococcus* was originally composed of the single species *Helcococcus kunzii* (26), which was joined by a new species isolated from humans, *Helcococcus sueciensis,* in 2004 (27). A third human species, "*Helcococcus pyogenes*," has been proposed but to date has not received official taxonomic standing (91).

The examination of gram-positive cocci with molecular taxonomic methods has encouraged the delineation of new groups of organisms and the refinement of a genetically based taxonomy for the catalase-negative, gram-positive cocci.

DESCRIPTION OF THE GENERA

The organisms included in this chapter form gram-positive coccoid cells, but *G. haemolysans* may appear to be gram variable or gram negative due to the ease with which its cells are decolorized. Cell shape and arrangement can aid in dividing these organisms into two broad groups: those with a streptococcal-like Gram stain morphology (coccobacilli in pairs and chains) and those with a staphylococcal-like Gram stain morphology (more spherical cocci in pairs, tetrads, clusters, or irregular groups). Members of the genera *Abiotrophia* and *Granulicatella* (formerly the nutritionally variant streptococci) form coccobacilli arranged in pairs and chains, but these organisms may also appear pleomorphic, especially when grown under less than optimal nutritional conditions (21). *Weissella* strains form coccobacilli or short rods arranged in pairs and chains (89). Dividing these diverse bacteria into two groups based on cellular shape and arrangement serves only as an aid in identification; no relatedness of organisms is implied by this grouping. With the exception of the infrequently isolated vagococci, these organisms are nonmotile.

Members of most of the genera are catalase-negative facultative anaerobes, but *A. viridans* is classified as a microaerophile that grows poorly if at all under anaerobic conditions. Some strains of *Aerococcus* may exhibit weakly positive catalase reactions due to nonheme catalase activity. None of the members of the genera are beta-hemolytic on routinely employed blood agars, but *G. haemolysans* is described as producing beta-hemolysis on agars supplemented with rabbit or horse blood (95), and some strains of *G. bergeri* and *G. sanguinis* may exhibit hemolytic reactions on horse blood agar (31, 32).

NATURAL HABITATS

Some of the genera discussed here are members of normal flora of the oral cavity or upper respiratory tract (*Gemella*, *Abiotrophia,* and *Granulicatella)* or colonize the skin (*Helcococcus*). Foods and vegetation are normal habitats for lactococci, pediococci, and leuconostocs (59, 60); members of the genera *Lactococcus, Pediococcus,* and *Leuconostoc* may also be found as normal flora of the alimentary tract, but thorough data supporting this contention are lacking. Aerococci are environmental isolates that can also be found on human skin. Although the organisms have been isolated from human sources, the natural habitats of many of the organisms mentioned here are not well characterized.

CLINICAL SIGNIFICANCE

Although the organisms included in this chapter may be present as contaminants in clinical cultures, they are also isolated infrequently as opportunistic pathogens. These bacteria appear to be of low virulence and are usually pathogenic only in compromised hosts. Infection often occurs in previously damaged tissues (e.g., heart valves) or may be nosocomial and associated with prolonged hospitalization, antibiotic treatment, invasive procedures, and the presence of foreign bodies. Specimens likely to yield significant numbers of isolates of these bacteria are blood, cerebrospinal fluid, urine, and wound specimens.

Lactococcus

Difficulties in distinguishing lactococci from either streptococci or enterococci have probably led to the misidentification of clinical *Lactococcus* isolates in the past and may have contributed to the paucity of reports concerning the clinical role of these bacteria. Elliott and coworkers (45) studied the phenotypic characteristics of a number of lactococcal strains isolated from blood, specimens from patients with urinary tract infections, and an eye wound specimen culture. The authors observed that three blood culture isolates were obtained from patients diagnosed with prosthetic valve endocarditis. Other reports have noted cases of lactococcal native valve endocarditis (56, 84, 94), septicemia in an immunosuppressed patient (86), osteomyelitis (69), and liver abscess (4).

Vagococcus

To date, only a handful of *Vagococcus* isolates from human sources have been reported in the literature. Teixeira and coworkers (110) described strains isolated from blood, peritoneal fluid, and a wound specimen. Vagococci are motile organisms that, like lactococci, express the Lancefield group N antigen (51). Difficulties encountered in identifying vagococci may partially account for their infrequent recognition in clinical cultures.

Abiotrophia and *Granulicatella*

Organisms formally known as nutritionally variant streptococci are normal residents of the oral cavity but have been identified as agents of endocarditis involving both native and prosthetic valves (68). These organisms have also been isolated in cases of ophthalmic infections (87, 90), a brain abscess following neurosurgery (14), and iatrogenic meningitis following myelography (100).

Leuconostoc, Pediococcus, and *Weissella*

The vancomycin-resistant genera *Leuconostoc* and *Pediococcus* have been recognized in clinical specimens since the mid-1980s. Handwerger and colleagues (66) noted host defense impairment, invasive procedures breaching the integument, gastrointestinal symptoms, and prior antibiotic treatment as common features among adult patients infected with *Leuconostoc*. They also observed a predisposition to *Leuconostoc* bacteremia among neonates, suggesting that infants may become colonized during delivery by leuconostocs inhabiting the maternal genital tract. In addition to causing bacteremia, leuconostocs have been isolated from cerebrospinal fluid, peritoneal dialysate fluid, and wound specimens. Case reports have implicated leuconostocs as agents of infection in osteomyelitis (119), ventriculitis (43), and postsurgical endophthalmitis (76).

Pediococcus strains have been isolated in cases of bacteremia, sepsis, and hepatic abscess in compromised patients (9, 62, 85, 105). Barros and coworkers (9) noted that *Pediococcus acidilactic*i was isolated from clinical specimens more frequently than *Pediococcus pentosaceus* and was also more commonly isolated in cases of bacteremia. Barton and coworkers noted the role of *Pediococcus* in bacteremia in infants with gastrointestinal malformations requiring surgical correction (10).

Weissella confusa, formerly classified as *Lactobacillus confusus*, has been reported infrequently as an agent of bacteremia and endocarditis (57, 89).

Gemella

G. haemolysans has been isolated as a pathogen in cases of endocarditis (20), meningitis (58), brain abscess (83), and total knee arthroplasty (44). *G. morbillorum* has been isolated (when still classified as a streptococcus; see "Taxonomy") from blood cultures and cultures of respiratory, genitourinary,

wound, and abscess specimens (53). This *Gemella* species has been implicated in cases of empyema and lung abscess (41), septic shock (115), endocarditis (55, 61, 118), brain abscess (108), osteomyelitis (114), peritonitis (6), and infection in an arteriovenous shunt (7). The clinical significance of *G. bergeri* and *G. sanguinis* is not well described, but strains of these species have been isolated from blood cultures, and they may also function as agents of endocarditis (31, 32).

Dolosigranulum

Little is known about the clinical significance of *Dolosigranulum*, a genus that is phenotypically similar, but not closely related to, *Gemella* (3). Strains of *Dolosigranulum pigrum,* the sole species in the genus, have been isolated from blood and eye and respiratory specimens (77), and this organism has been cited as a probable agent in a case of synovitis (65).

Aerococcus

Although aerococci appear as contaminants in clinical cultures, occasional reports have noted a clinically significant role for these organisms in cases of endocarditis and bacteremia (40, 73, 92). Until the early 1990s, *A. viridans* was the only species reported from human specimens, but four additional species isolated from humans have been described to date. *A. urinae* (2, 63) has been implicated as a urinary tract pathogen in patients predisposed to infection (22) and also as an agent of endocarditis (75, 107), lymphadenitis (99), and spondylodiscitis (5). Little is currently known about the clinical significance of *A. christensenii* (isolated from vaginal specimens) (33), *A. urinaehominis* (isolated from urine) (81), and *A. sanguinicola* (isolated from blood and urine) (52, 82).

Globicatella

G. sanguinis, the sole *Globicatella* species isolated from humans, has been implicated in cases of bacteremia, urinary tract infection, and meningitis (24). A second species in the genus, *Globicatella sulfidifaciens*, has been isolated in cases of purulent infections in domestic mammals (113).

Facklamia

Members of the *Facklamia* genus are closely related to, but phenotypically and phylogenetically distinct from, members of the *Globicatella* genus (28). Strains of the four *Facklamia* species isolated from humans have been recovered from blood, wound, and genitourinary sites (28, 30, 35, 80), and a patient with chorioamnionitis (67).

Ignavigranum

Only a few strains of *I. ruoffiae,* the sole species of the genus *Ignavigranum,* have been described to date. Sites of isolation include a wound and an ear abscess (36).

Dolosicoccus

Strains of *D. paucivorans*, the only species currently included in the genus *Dolosicoccus,* have been recovered from blood cultures (37, 50).

Helcococcus

H. kunzii has been recovered from intact skin of the lower extremities (64), as well as from wound specimen cultures (notably those from foot ulcers) containing mixtures of bacteria (26). Consequently, the clinical significance of this organism is difficult to interpret, since it may be present merely as a colonizer of the wound site. The ability of this species to function as an opportunist was, however, suggested by its isolation in pure culture from an infected sebaceous cyst (93) and a breast abscess (19). Classification of two additional species isolated from humans, *H. sueciensis* and "*H. pyogenes,*" is based on single isolates from a wound and a prosthetic joint infection specimen, respectively (27, 91).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

The organisms described in this chapter have all been isolated from routine cultures of clinical specimens, and special requirements for collection and processing of specimens have not been described. Since these bacteria are facultative anaerobes or microaerophiles, aerobic collection, transport, and storage methods as described in chapter 5 of this Manual should allow for their isolation.

ISOLATION PROCEDURES

Generally, there are no special requirements for isolation of the group of bacteria discussed here; general recommendations for the culture of blood, body fluids, and other specimens should be followed (see chapters 5, 6, and 20). These organisms are likely to be isolated on rich, nonselective media (e.g., blood and chocolate agars and thioglycolate broth) since they are nutritionally fastidious. If selective isolation of members of the vancomycin-resistant genera *Leuconostoc* and *Pediococcus* is desired, Thayer-Martin medium may be used to inhibit normal flora or other contaminating microorganisms (98). Members of some of the genera (e.g., *Helcococcus*) grow slowly, forming tiny colonies that may not be visible unless extended incubation (48 to 72 h) is employed. The recovery of members of many of the genera included in this chapter may be enhanced by CO_2 enrichment of the incubation atmosphere.

Members of the genera *Abiotrophia* and *Granulicatella* usually grow on chocolate agar, on brucella agar with 5% horse blood, and in thioglycolate broth but not on Trypticase soy agar with 5% sheep blood. These organisms can be cultured on nonsupportive media that have been appropriately supplemented (see "*Abiotrophia* and *Granulicatella*" under "Additional Procedures for Characterization of Selected Genera with Negative Catalase Reactions" below).

IDENTIFICATION

The phenotypic characteristics detailed here may not always be sufficient for accurate identification of the aerobic catalase-negative, gram-positive cocci encountered infrequently in clinical laboratories. Although Gram stain morphology is prone to subjective interpretation, it has been employed as a major decision point in the identification protocols, with two general categories: Gram stain morphology resembling that of streptococci, meaning cocci or coccobacilli in pairs and chains, and staphylococcal morphology, meaning coccoid cells arranged in pairs, clusters, tetrads, or irregular groups. Broth-grown cells (thioglycolate broth is suitable) should be used for making morphological determinations. The flowcharts in this chapter (Fig. 1 and 2) should not be used for definitive identification. In most cases, additional procedures (Table 1) are recommended before identification to the genus level is made. Identifications of unfamiliar organisms from important specimens should be confirmed by a reference laboratory. Molecular methods such as 16S rRNA

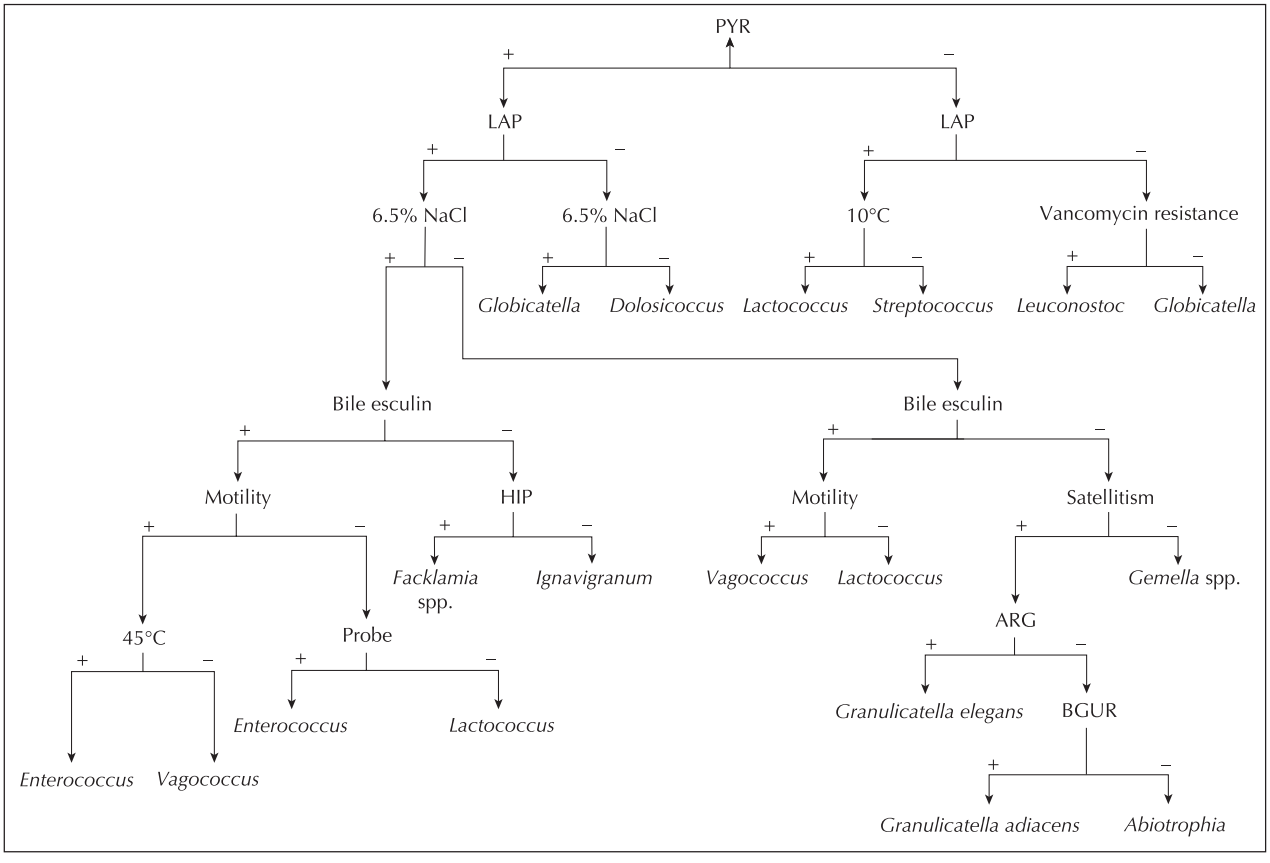


FIGURE 1 Identification of catalase-negative, gram-positive cocci that grow aerobically with cells arranged in pairs or chains. Abbreviations: PYR, pyrrolidonyl arylamidase activity; LAP, leucine aminopeptidase activity; 6.5% NaCl, growth in broth containing 6.5% NaCl; bile esculin, hydrolysis of esculin in the presence of 40% bile; motility, motility in motility test medium; 45°C, growth at 45°C; 10°C, growth at 10°C; probe, reaction with commercially available nucleic acid probe for the genus *Enterococcus;* HIP, hydrolysis of hippurate; satellitism, satelliting growth behavior; ARG, arginine hydrolysis activity; BGUR, β-glucuronidase activity.

gene-based DNA sequencing or the use of alternative genetic targets may be useful for genus- and species-level identification (117).

Procedures for Initial Differentiation of Genera with Negative Catalase Reactions

Initial testing procedures for catalase-negative isolates with streptococcal (Fig. 1) or staphylococcal (Fig. 2) Gram stain morphology are represented in the flowcharts. Note that *Gemella* and *Facklamia* strains may display either type of cellular morphology, depending on the species (see "Additional Procedures for Characterization of Genera with Negative Catalase Reactions" below). Descriptions of tests for these organisms follow.

Catalase Test

If a positive or weakly positive catalase reaction is observed with growth from blood-containing media, growth from a medium devoid of blood (e.g., brain heart infusion agar) should be used to repeat the catalase test. A loopful of growth is transferred onto a microscope slide or empty petri dish and observed for the evolution of bubbles after the addition of a drop of 3% H_2O_2. It may be necessary to use a hand lens to detect weakly positive reactions.

PYR Test

See chapter 21 and reference 51 for a description of the PYR test. Rapid disk tests are commercially available, and an assay for PYR is contained in some commercially available identification kits (e.g., API 20 Strep; bioMerieux, Durham, N.C.).

LAP Test

The LAP test determines the presence of the enzyme leucine aminopeptidase, also called leucine arylamidase. See chapter 21 for a description of this test. An assay for this enzyme is contained in some commercially available identification kits (e.g., API 20 Strep; bioMerieux), and a rapid disk test for LAP is also available commercially (BD Diagnostic Systems, Sparks, Md.; Remel, Lenexa, Kans.). The manufacturer's instructions should be followed when the test is performed (51, 54).

Growth in 6.5% NaCl

Heart infusion broth supplemented with 6.0% NaCl, with or without the acid-base indicator bromcresol purple, may be

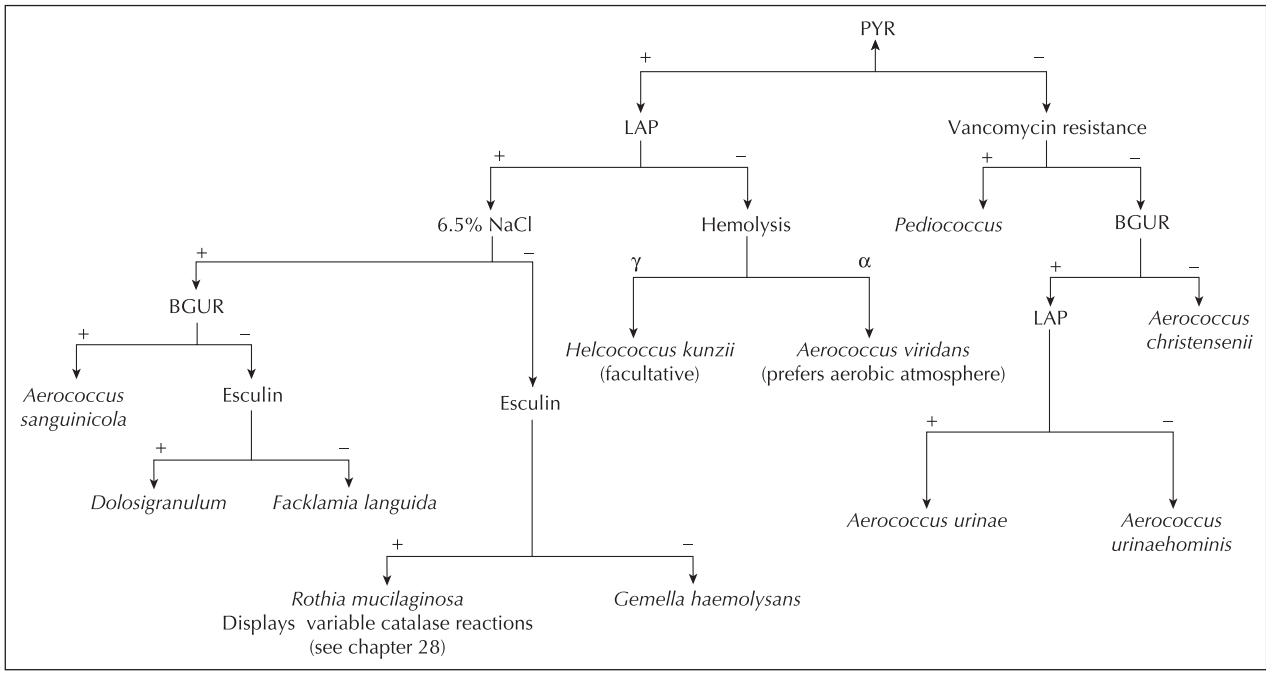


FIGURE 2 Identification of catalase-negative, gram-positive cocci that grow aerobically with cells arranged in pairs, tetrads and clusters, or irregular groups. Abbreviations: PYR, pyrrolidonyl arylamidase activity; LAP, leucine aminopeptidase activity; 6.5% NaCl, growth in broth containing 6.5% NaCl; esculin, hydrolysis of esculin; BGUR, β-glucuronidase activity.

used to test for growth in 6.5% NaCl. The broth is inoculated with two to three colonies of the organism to be tested and incubated at 35°C for up to 72 h. Turbidity with or without a color change from purple to yellow (due to production of acid) indicates growth (51, 54).

Bile Esculin Test

The ability to hydrolyze esculin in the presence of 40% bile is tested by culturing the test organism on bile esculin agar in an ambient-atmosphere incubator at 35°C for up to 48 h. Blackening of the agar indicates a positive reaction (54).

Motility Test

The motility test is performed by stab inoculating modified motility test medium and incubating at 30°C (instead of 35°C) for up to 48 h, according to the method of Facklam and Elliott (51).

Hippurate Hydrolysis Test

Facklam and Elliott recommend a conventional broth medium for determining hippurate hydrolysis (51). This test may also be contained in commercially available identification kits.

Satellitism Test

In a test for satelliting behavior, the strain to be examined is streaked for confluent growth on a medium that fails to support growth or supports only weak growth (e.g., sheep blood agar or brain heart infusion agar). A single cross streak of *Staphylococcus aureus* (ATCC 25923 or another suitable strain) is applied to the inoculated area. After incubation at 35°C in an atmosphere containing an elevated CO_2 level, strains of *Abiotrophia* or *Granulicatella* will grow only in the vicinity of the staphylococcal growth. Alternatively, media can be supplemented with pyridoxal in the form of an aqueous stock solution of filter-sterilized 0.01% pyridoxal hydrochloride. This solution, which can be stored frozen, should be added to media to achieve a final concentration of 0.001%. Disks containing pyridoxal may also be used in the satelliting test and are commercially available (Remel). Some strains of *Ignavigranum* may show satelliting behavior (36).

Arginine Hydrolysis Test

The arginine hydrolysis test can be performed with Moeller's decarboxylase broth containing arginine (51).

β-Glucuronidase Test

A test for β-glucuronidase is included in a number of commercially available identification products or may be performed using commercially prepared disks.

Vancomycin Susceptibility Test

Several colonies are streaked over half of a plate containing Trypticase soy agar with 5% sheep blood. After placing of a 30-μg vancomycin disk in the center of the inoculated area, the plate is incubated overnight in a CO_2-enriched atmosphere at 35°C. Any zone of inhibition indicates susceptibility, and resistant strains exhibit no inhibition zone (51, 54).

Esculin Hydrolysis Test

Esculin agar slants (heart infusion agar containing 0.1% esculin and 0.5% ferric citrate) are employed for the esculin hydrolysis test. After inoculation, slants are incubated at 35°C for up to 7 days. Partial or complete blackening of the agar indicates a positive reaction (51).

Additional Procedures for Characterization of Selected Genera with Negative Catalase Reactions

Lactococcus

Facklam and colleagues (51, 54) recommend growth temperature tests for distinguishing lactococci from streptococci and enterococci. Consult Fig. 1 for growth temperature characteristics of each of the genera. Broths (heart infusion broth containing 1% glucose and bromcresol purple indicator) are inoculated with a single colony or a drop of broth culture of the test strain and incubated at 35°C for up to 7 days. A water bath is recommended for incubation of cultures at 45°C. Turbidity with or without a change in the broth's indicator to yellow indicates a positive test.

If it is important to rule out enterococci, suspicious isolates can be tested with a commercially available nucleic acid probe test for the genus *Enterococcus*. *Lactococcus lactis* and *Lactococcus garvieae* are the *Lactococcus* species most commonly isolated from clinical specimens. Further information on the differentiation of *Lactococcus* isolates to the species level may be found in references 45, 47, and 102.

Abiotrophia and *Granulicatella*

Lists of additional phenotypic traits for *Abiotrophia* and *Granulicatella* species can be found in references 11, 16, 21, and 34. Davis and Peel (42) reported that the API 20 Strep system (bioMerieux) is superior to the Rapid ID 32 Strep system (bioMerieux) for identification of these organisms. They found that accurate results were obtained when a dense inoculum (confluent growth from two blood agar plates) was employed.

Leuconostoc and *Pediococcus*

Members of the genera *Leuconostoc* and *Pediococcus* produce small, alpha- or nonhemolytic colonies on blood agar that can appear similar to those of viridans streptococci. In addition to having differing cellular morphologies (Table 1), members of these vancomycin-resistant genera, along with vancomycin-resistant strains of lactobacilli that form short coccoid cells, may be separated by tests for gas production from glucose and arginine hydrolysis. Leuconostocs produce gas and are always arginine negative. Lactobacilli are variable in both tests, but a positive arginine test for a gas-producing strain would rule out the identification of the organism as a leuconostoc. Pediococci are gas production negative and show variable reactions in the arginine test, although *P. acidilactici* and *P. pentosaceus*, the two species commonly found in clinical material, are arginine positive.

Gas production is measured by inoculating MRS (deMan, Rogosa, Sharpe) broth (BD Diagnostic Systems; Hardy Diagnostics, Santa Maria, Calif.) with the test organism, sealing the culture with melted petrolatum, and incubating for up to 7 days at 35°C. Gas production is evidenced by displacement of the petrolatum plug (51, 54). The arginine hydrolysis test can be performed with Moeller's decarboxylase broth containing arginine (51). The Lancefield group D antigen can be detected in pediococci (54). References 8, 9, 46, 51, 54, and 96 should be consulted for further information on the identification of *Leuconostoc* and *Pediococcus* to the species level.

Tetragenococcus

The organisms formerly known as *P. halophilus* and *E. solitarius* were reclassified in the *Tetragenococcus* genus as *Tetragenococcus halophilus* and *T. solitarius* (39, 48). Although these organisms share some phenotypic characteristics with pediococci, they are vancomycin susceptible. Although little is know about the participation of these organisms in human infection, they should be suspected if vancomycin-susceptible, *Pediococcus*-like strains are encountered.

Weissella

Weissella strains may be misidentified as leuconostocs or lactobacilli. These organisms produce gas from glucose. The few clinical isolates reported in the literature have been described as vancomycin resistant and positive for hydrolysis of arginine. Characteristics useful for the identification of strains of the infrequently isolated *Weissella* genus are found in Table 1 and references 57 and 89.

Gemella

Facklam and Washington (54) state that all *Gemella* species are PYR positive but that *G. morbillorum* displays a weakly positive reaction. Findings of earlier studies (12, 13) generally support these observations, but the previous studies describe some isolates of both *G. haemolysans* and *G. morbillorum* as being negative in the PYR test. Facklam and Washington note that a large inoculum must be used when these bacteria are tested for the pyrrolidonyl arylamidase enzyme. Leucine aminopeptidase is usually absent in isolates of *G. haemolysans* but present in strains of *G. morbillorum* (12, 13). On sheep blood agar media, gemellas form small colonies that are similar in appearance to those of viridans group streptococci. Slow growth of some *Gemella* strains may lead to confusion of these organisms with *Abiotrophia* or *Granulicatella* organisms (formerly called nutritionally variant streptococci). A test for satelliting behavior will separate these two groups of bacteria (54).

Cells of *G. haemolysans* are easily decolorized and resemble those of neisserias, since they occur in pairs with the adjacent sides flattened. *G. haemolysans* favors an aerobic growth atmosphere. *G. morbillorum* cells are gram positive and are arranged in pairs and short chains; individual cells in a given pair may be of unequal sizes. *G. morbillorum* is described as favoring anaerobic growth conditions. Only a small number of strains of *G. bergeri* and *G. sanguinis* have been reported on to date. Information on phenotypic characteristics of these *Gemella* species can be found in references 31 and 32.

Aerococcus

In addition to the traits listed in Table 1, *A. viridans* is characterized by displaying weak or no growth when incubated in an anaerobic atmosphere (49). This trait can be tested by incubating duplicate blood agar plate cultures of the organism in question in an anaerobic and an aerobic atmosphere and comparing growth after 24 to 48 h. When grown aerobically, *A. viridans* forms alpha-hemolytic colonies that may be confused with those of either viridans group streptococci or enterococci. *A. urinae* forms small (0.5 mm in diameter after 24 h of incubation), alpha-hemolytic, convex, shiny, transparent colonies on blood agar media. *A. urinae* is PYR negative and LAP positive, in contrast to *A. viridans* (2). Additional information on the identifying characteristics of *A. urinae* can be found in reference 22, and a second biotype (esculin hydrolysis positive) of this species is described in reference 23. Information on phenotypic traits of the recently described species *A. christensenii*, *A. sanguinicola*, and *A. urinaehominis* can be found in Table 1 and references 33, 52, 81, and 82.

Helcococcus

In addition to the characteristics shown in Table 1, most isolates of *H. kunzii* produce an API 20 Strep (bioMerieux)

profile of 4100413. Colonial morphology (tiny, gray, usually slightly alpha-hemolytic colonies), good growth under anaerobic conditions, and stimulation of growth by the addition of 1% horse serum or 0.1% Tween 80 to the medium differentiate *H. kunzii* from aerococci (26).

ANTIBIOTIC SUSCEPTIBILITIES

The lack of standardized methods and interpretation criteria for antibiotic susceptibility testing results, along with relatively small collections of clinical isolates for some of the genera discussed in this chapter, makes it difficult to accurately assess antimicrobial susceptibility patterns. With the exception of *Leuconostoc*, *Pediococcus,* and *Weissella*, members of all of the genera display susceptibility to vancomycin. Since many of the bacteria presented in this chapter are fairly fastidious, investigators have often employed blood-supplemented Mueller-Hinton medium and, if necessary for good growth, incubation in a CO_2-enriched atmosphere. Pyridoxal hydrochloride (0.001%) should also be added to blood-supplemented media for testing strains of *Abiotrophia* and *Granulicatella*. Most studies have employed streptococcal (other than *Streptococcus pneumoniae*) interpretative criteria for determining susceptibility. Details of susceptibility testing methodology and interpretive criteria can be found in reference 88.

Limited information on the in vitro antimicrobial susceptibilities of *L. lactis* and *L. garvieae* strains isolated from humans suggests that *L. garvieae* isolates are less susceptible to penicillin and cephalothin than are strains of *L. lactis*. The uniform resistance of *L. garvieae* to clindamycin contrasts with the uniform susceptibility of the *L. lactis* strains examined by Elliott and Facklam (47) and led them to propose a test for clindamycin susceptibility as an aid in differentiation of these two species. In clinical practice, patients with lactococcal endocarditis have been successfully treated either with penicillin alone or with penicillin and gentamicin (84, 94).

Teixeira and colleagues examined a small collection of *Vagococcus* isolates and observed that all strains tested were susceptible to ampicillin, cefotaxime, and trimethoprim-sulfamethoxazole. All strains were resistant to clindamycin, lomefloxacin, and ofloxacin. Variable results were observed with other antimicrobial agents (110).

Members of the vancomycin-resistant genera *Leuconostoc* and *Pediococcus* are also resistant to teicoplanin. Although they are usually susceptible to imipenem, minocycline, chloramphenicol, and gentamicin, MICs of penicillin for these strains correspond to the moderately susceptible category (109).

There is a range of MICs of penicillin for *Abiotrophia* and *Granulicatella* isolates, with the majority of strains classified as either susceptible or relatively resistant. There is also variability in susceptibilities to aminoglycosides, but no cases of high-level resistance have been reported. A synergistic effect between beta-lactam agents and aminoglycosides has been demonstrated for isolates of *Abiotrophia* (70). Tuohy and colleagues examined a collection of 27 *G. adiacens* and 12 *A. defectiva* strains, noting susceptibilities of all isolates to clindamycin, rifampin, levofloxacin, ofloxacin, and quinupristin-dalfopristin. These authors noted that the percentage of isolates of *G. adiacens* and *A. defectiva* examined that were susceptible to other agents tested was as follows: penicillin, 55 and 8%; amoxicillin, 81 and 92%; ceftriaxone, 63 and 83%; and meropenem, 96 and 100%, respectively (112). Zheng and coworkers reported high rates of beta-lactam and macrolide resistance in a collection of pediatric *Abiotrophia* and *Granulicatella* isolates (120).

A. viridans and *G. haemolysans* appear to be susceptible to penicillin and display a low level of resistance to aminoglycosides (17, 18). Buu-Hoi and colleagues (17) noted that although *A. viridans* seems to be naturally susceptible to macrolides, tetracyclines, and chloramphenicol, resistance to these agents has been observed. *A. urinae* has been described as susceptible to penicillin, amoxicillin, piperacillin, cefipime, rifampin, and nitrofurantoin but resistant to sulfonamides and netilmicin. Isolates display variable susceptibilities to trimethoprim and cotrimoxazole (22, 103, 106). *A. sanguinicola* isolates display susceptibilities to penicillin, amoxicillin, cefotaxime, cefuroxime, erythromycin, chloramphenicol, quinupristin-dalfopristin, rifampin, linezolid, and tetracycline (52). Buu-Hoi and coworkers (18) demonstrated a synergistic effect of penicillin and gentamicin against *G. haemolysans*.

A collection of 27 clinical isolates of *D. pigrum* studied by LaClaire and Facklam (77) all exhibited susceptibilities to penicillin, amoxicillin, cefotaxime, cefuroxime, clindamycin, levofloxacin, meropenem, quinupristin-dalfopristin, rifampin, and tetracycline. Various susceptibilities to erythromycin were noted, and one of the 27 strains was resistant to trimethoprim-sulfamethoxazole. A small number of *Helcococcus* isolates displayed susceptibilities to penicillin and clindamycin, and most strains were resistant to erythromycin (19, 93). MICs of a variety of antibiotics for strains of *Facklamia* varied (78). A study of 27 strains of *G. sanguinis* reported susceptibilities of all isolates to amoxicillin but various levels of resistance to other antimicrobials tested (104).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Since the gram-positive cocci included in this chapter may appear in clinical cultures as contaminants or part of normal microbiota, efforts to identify them should be made only when isolates are considered to be clinically significant (e.g., when isolated repeatedly, when grown in pure culture, or when recovered from normally sterile sites). It should be remembered that these bacteria are opportunists: isolation from an immunocompetent patient may not have the same significance as isolation from a compromised host. Communication with clinicians should guide the microbiology laboratory in evaluating the significance of these infrequently isolated organisms.

Vancomycin susceptibility testing should be performed routinely on significant isolates. Documenting resistance to this antibiotic will not only guide therapy but also aid in identification of the isolates. The method mentioned in this chapter, using a nonstandardized inoculum, seems to be fairly reliable for determining susceptibility to this drug for identification purposes. Since standardized susceptibility testing methods do not exist for these infrequently isolated gram-positive cocci, caution should be observed in interpretation of in vitro susceptibility test results. A reference laboratory should be consulted for identification or confirmation of the identity of unfamiliar organisms.

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Bacillus and Other Aerobic Endospore-Forming Bacteria*

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32

TAXONOMY

Molecular taxonomic methods have had a huge impact on the classification of aerobic endospore-forming bacteria. The 1986 edition of *Bergey's Manual of Systematic Bacteriology* listed 40 valid *Bacillus* species, and since then 218 further species have been newly described or revived among *Bacillus* and the genera derived from it: *Alicyclobacillus* (112), *Paenibacillus* (5), *Brevibacillus* (93), *Aneurinibacillus* (93), *Virgibacillus* (50), *Gracilibacillus* (107), *Salibacillus* (107; subsequently merged with *Virgibacillus* [51]), *Geobacillus* (75), *Marinibacillus* (114), and *Ureibacillus* (37). Three species formerly classified within *Bacillus, B. pasteurii, B. globisporus*, and *B. psychrophilus*, have been transferred (113) to the long-established genus of aerobic endospore-forming cocci, *Sporosarcina,* which now contains six species. Furthermore, 15 new genera containing 33 new species have been proposed to accommodate novel aerobic endospore formers not previously assigned to *Bacillus* (over one-half of these genera have only one species, and over one-half of the species were proposed on the basis of single isolates); they are, in order of validation, *Sulfobacillus*, *Amphibacillus*, *Halobacillus*, *Ammoniphilus*, *Anoxybacillus*, *Thermobacillus*, *Filobacillus*, *Jeotgalibacillus*, *Lentibacillus*, *Oceanobacillus*, *Paraliobacillus*, *Cerasibacillus*, *Pontibacillus*, *Tenuibacillus,* and *Salinibacillus*. Overall, therefore, there have been proposals for 25 new genera containing 234 new or revived species or new combinations, and yet only six proposals for merging species were made in that time. Unfortunately, taxonomic progress has not always revealed readily determinable features characteristic of each genus. They show wide ranges of sporangial morphologies and phenotypic test patterns. Many novel species represent genomic groups disclosed by DNA-DNA pairing experiments or single isolates whose distinction from existing species rests chiefly upon 16S rRNA gene sequence analysis, and routine phenotypic characters for distinguishing some of them are very few and of unproven value.

Bacillus continues to accommodate the best-known species such as *B. subtilis* (the type species), *B. anthracis, B. cereus, B. licheniformis, B. megaterium, B. pumilus, B. sphaericus,* and *B. thuringiensis.* It still remains a large genus, with over 100 species, as losses to other genera have been balanced by new species proposals. Members of the *B. cereus* group, *B. anthracis, B. cereus,* and *B. thuringiensis,* are considered to be pathovars of a single species (104).

DESCRIPTIONS OF THE GENERA

Although the production of resistant endospores in the presence of oxygen remains the defining feature for *Bacillus* and the new genera derived from it, the definition was undermined by the discovery of *Bacillus infernus* and *B. arseniciselenatis,* which are strictly anaerobic, and spores have not been detected in *B. infernus* and *B. subterraneus*.

However, those members likely to be isolated in a clinical laboratory are gram-positive (in young cultures) but sometimes gram-variable or clearly gram-negative, rod-shaped, endospore-forming organisms which may be aerobic or facultatively anaerobic. They are mostly catalase positive and may be motile by means of peritrichous flagella. Most species are mesophilic, but *Bacillus* contains some thermophiles and psychrophiles, and *Paenibacillus* contains one psychrophilic species. *Alicyclobacillus, Gracilibacillus, Marinibacillus,* and *Ureibacillus* strains and members of the 15 new genera not derived from *Bacillus* are unlikely to be encountered in a clinical laboratory, and clinical isolates of *Geobacillus* and *Sporosarcina* have not been reported, so these genera will not be considered further.

NATURAL HABITATS

Most aerobic endospore formers are saprophytes widely distributed in the natural environment, but some species are opportunistic or obligate pathogens of animals, including humans, other mammals, and insects. The main habitats are soils of all kinds, ranging from acid to alkaline, hot to cold, and fertile to desert, and the water columns and bottom deposits of fresh and marine waters. Their spores readily survive distribution in soils, dusts, and aerosols from these natural environments to a wide variety of other habitats; for example, strains of *B. fumarioli* showing similar phenotypic behavior and substantial genotypic similarity have been isolated from volcanic soils in continental Antarctica and from Candlemas Island, which is some 5,600 km distant in the South Sandwich archipelago, and from gelatin production plants in Belgium, France, and the United States (28). Dried foods such as spices, milk powders, and farinaceous products

*This chapter contains information presented in chapter 32 by Niall A. Logan and Peter C. B. Turnbull in the eighth edition of this Manual.

are often quite heavily contaminated with spores. *B. anthracis* is, to all intents and purposes, an obligate pathogen of animals and humans. Its close relative, *B. cereus*, is now well established as an opportunistic pathogen, and other aerobic endospore formers can also be opportunistic pathogens occasionally. Six organisms are important as insect pathogens: *B. thuringiensis* (another close relative of *B. anthracis*), *B. popilliae, B. lentimorbus*, *B. sphaericus*, *P. larvae* subsp. *larvae,* and *P. larvae* subsp. *pulvifaciens*.

CLINICAL SIGNIFICANCE AND INTERPRETATION OF RESULTS

The majority of aerobic endospore-forming species apparently have little or no pathogenic potential and are rarely associated with disease. The principal exceptions to this are *B. anthracis*, the agent of anthrax, and *B. cereus*, but a number of other species, particularly *B. licheniformis*, have been implicated in food poisoning and other human and animal infections. The resistance of the spores to heat, radiation, disinfectants, and desiccation also results in aerobic endospore formers being troublesome contaminants in the operating room, on surgical dressings, in pharmaceutical products, and in foods.

Apart from *B. anthracis*, the majority of species are common environmental contaminants, and isolation from a single clinical specimen is generally not a sufficient basis for incriminating one of these organisms as the etiological agent. Moderate or heavy growth of aerobic endospore formers from wounds is usually significant, however, and *B. cereus* infections of the eye are emergencies which should always be taken seriously and reported to the physician immediately.

In the clinical laboratory, the most important questions to ask about an aerobic spore-forming isolate are as follows. Was it isolated in pure culture or at least apparently dominating the flora? Was it isolated in large numbers? Was it isolated more than once? Low-level contamination of foodstuffs by aerobic endospore formers is common, as is asymptomatic transient fecal carriage. Therefore, in foodborne-illness investigations, qualitative isolation tests are insufficient. The ideal criteria for establishing that an aerobic endospore former is the etiological agent are (i) isolation of significant numbers ($>10^5$ CFU/g) of the organism from the epidemiologically incriminated food (and, in the case of suspected *B. cereus* food poisoning, detection of emetic toxin and/or enterotoxin) and (ii) recovery of the same strain (biovar, serovar, phage type, plasmid type, etc.) in significant numbers from acute phase specimens (feces or vomitus) from the patients, but not from healthy controls.

B. anthracis continues to be generally regarded as an obligate pathogen; its continued existence in the ecosystem appears to depend on a periodic multiplication phase within an animal host, and its environmental presence reflects contamination from an animal source at some time rather than self-maintenance within the environment. In human and animal specimens, it is usually sought only when the case history suggests that it is reasonable to suspect anthrax. Demonstration of capsulating *B. anthracis*, even in low numbers, confirms the clinical suspicion of anthrax, because the bacterium is rapidly destroyed by putrefactive processes after the host's death.

Bacillus anthracis

Anthrax remains the most widely recognized clinical condition caused by a *Bacillus* species. It is primarily a disease of domestic or wild animals, and prior to the availability of an effective veterinary vaccine in the late 1930s, anthrax was one of the foremost causes worldwide of mortality in cattle, sheep, goats, and horses. Humans almost invariably contract anthrax directly or indirectly from animals. The use of veterinary and human vaccines together with improvements in factory hygiene and sterilization procedures for imported animal products and the increased use of man-made alternatives to animal hides or hair have resulted over the past half-century in a marked decline in the incidence of anthrax in both animals and humans. Nevertheless, the disease continues to be endemic in many countries, particularly those that lack efficient vaccination policies (77). Because anthrax spores remain viable in soil for many years and their persistence does not depend on animal reservoirs, *B. anthracis* is exceedingly difficult to eradicate from an area where it is endemic; regions of nonendemicity must be constantly on the alert for the arrival of *B. anthracis* in imported animal products. Anthrax is not contagious, and transmission to humans is usually restricted to direct contact with infected animals or contaminated fomites, including such oddities as communal loofahs and contaminated syringes (84). Direct animal-to-animal transmission within a species (i.e., excluding scavengers feeding on anthrax carcasses) is also very rare.

Circumstantial evidence shows that humans are moderately resistant to anthrax compared with obligate herbivores. Human anthrax has traditionally been classified as either (i) nonindustrial, resulting from close contact with infected animals or their carcasses after death from the disease, or (ii) industrial, i.e., acquired by those employed in processing wool, hair, hides, bones, or other animal products. Dependent on the route of infection, there are three major clinical forms of anthrax: cutaneous, inhalation, and gastrointestinal. Anthrax meningitis can develop as a complication of any of these forms (92). Only a few reports of laboratory-acquired infections exist (18). A major outbreak of anthrax occurred in April 1979 in the city of Sverdlovsk, former USSR (now Yekaterinburg, Russia), in the Urals as a result of the accidental release of spores from a military production facility.

B. anthracis has been subjected to military research, development, and occasional deployment in several countries over many years, following attacks on livestock during the First World War, and it has remained high on the list of agents that could be used in biological warfare or bioterrorism. The natural disease is readily controllable, but the 2001 bioterrorism-related anthrax outbreak in the United States increased public concern about this disease (see chapter 9).

Cutaneous anthrax accounts for about 99% of naturally acquired human anthrax worldwide—an estimated 2,000 cases are reported annually. Infection occurs through a break in the skin. Following the incubation period of usually 2 to 3 days, a small papule appears, progressing over the next 24 h to a ring of vesicles, with subsequent ulceration and formation of a characteristic blackened eschar. Subsequent eschar formation may become thick and surrounded by extensive edema. Fever and pus and pain at the site are normally absent; their presence probably indicates a secondary bacterial infection. Before the availability of antimicrobial agents and vaccines, 10 to 20% of untreated cases of cutaneous anthrax were fatal. Less than 1% of cases are fatal today, and they are due mainly to obstruction of the airways by the edema that accompanies lesions on the face or neck or to the progression of the cutaneous disease into systemic infection.

Gastrointestinal anthrax is not uncommon in regions of endemicity worldwide, where socioeconomic conditions are

poor and people eat raw or undercooked meat of animals that have died suddenly (77, 96); asymptomatic infections and symptomatic infections with recovery may not be uncommon. The symptoms of gastrointestinal anthrax are the result of ulcerations developing primarily in the cecal and terminal ileal mucosae: vomiting, nausea, and abdominal pain, accompanied by fever. This can rapidly progress to bloody diarrhea and systemic infection. Mortality ranges from 4 to 50% (96), greatly due to the nonspecific nature of the early symptoms and late initiation of antimicrobial therapy.

Prior to the bioterrorist attack of 2001, 18 cases of naturally acquired inhalation anthrax had been recorded in the United States since 1900, with 16 (88.9%) of them being fatal (12); figures in the United Kingdom show a similar picture. Among the 22 cases of the bioterrorism-related outbreak in the United States in late 2001, in which spores were delivered in mailed letters and packages, early recognition and treatment of the 11 patients with confirmed inhalation anthrax resulted in 60% survival (9, 58). In inhalation anthrax the inhaled spores are ingested by macrophages and carried from the lungs to the lymphatic system, where the infection progresses. During transit to lymph nodes, spores germinate into vegetative cells, begin to replicate, and produce the capsule and toxins that lead to bacteremia and associated hemorrhage and necrosis. The replacement of the older name for this form of the disease, "pulmonary anthrax," with the newer name, "inhalation anthrax," is a reflection of the fact that active infection occurs in the lymph nodes rather than the lung itself. Analysis of 10 of the cases associated with the bioterrorist events of 2001 (58) revealed a median incubation period of 4 days (range, 4 to 6 days). All 11 patients with inhalation anthrax had severe illness and were hospitalized (6). Their clinical presentation included fever or chills ($n = 11$), fatigue or malaise ($n = 11$), minimal or nonproductive cough ($n = 10$), dyspnea ($n = 9$), nausea or vomiting ($n = 9$), chest pain ($n = 7$), and sweats ($n = 7$). All patients had abnormal chest X-ray images with pleural effusion ($n = 8$), infiltrates ($n = 7$), and mediastinal widening ($n = 7$).

Regardless of the form of the diseases, the generalized symptoms that are usually mild (fatigue, malaise, fever, and/or gastrointestinal symptoms) can rapidly develop into the fulminant state, characterized by dyspnea, cyanosis, severe pyrexia, and disorientation followed by circulatory failure, shock, coma, and death (57, 58). Depending on the host, there is a rapid buildup of the bacteria in the blood over the last few hours to terminal levels of 10^7 to 10^9 organisms/ml in the most susceptible species. Enhancing clinical and laboratory expertise and conducting prospective surveillance are critical components of rapid anthrax diagnosis and of preparedness for bioterrorism (40).

Opportunistic Pathogens

Opportunistic infections with *Bacillus* species other than *B. anthracis* have been reported since the late 19th century. It is important to assess isolates of *Bacillus* in the light of any other species cultured and the clinical context and to be wary of dismissing them as mere contaminants.

Bacillus cereus Group

Bacillus cereus is next in importance to *B. anthracis* as a pathogen of humans (and other animals), causing foodborne illness and opportunistic infections, and its ubiquity ensures that cases are not uncommon.

In relation to foodborne illness, *B. cereus* is the etiological agent of two distinct food poisoning syndromes (31, 62): (i) the diarrheal type, characterized by abdominal pain with diarrhea 8 to 16 h after ingestion of the contaminated food and associated with a diversity of foods from meats and vegetable dishes to pastas, desserts, cakes, sauces, and milk; and (ii) the emetic type, characterized by nausea and vomiting 1 to 5 h after eating the offending food, predominantly oriental rice dishes, although occasionally other foods such as pasteurized cream, milk pudding, pastas, and reconstituted formulas have been implicated. One outbreak followed the mere handling of contaminated rice in a children's craft activity, and fulminant liver failure associated with the emetic toxin has been reported. Both syndromes arise as a direct result of the fact that *B. cereus* spores can survive normal cooking procedures; under conditions of improper storage after cooking, the spores germinate and the vegetative cells multiply. Strains of *B. thuringiensis*, which are close relatives of *B. cereus,* may also produce the diarrheal toxin (24).

The toxigenic basis of *B. cereus* food poisoning and other *B. cereus* infections has begun to be elucidated, and a complex picture is emerging (14). A toxin that is possibly associated with *B. licheniformis* food poisoning has been identified (70), but in general, for species outside the *B. cereus* group, toxins or virulence factors associated with foodborne illness have not been identified.

B. cereus is also a destructive ocular pathogen. Endophthalmitis may follow penetrating trauma of the eye or hematogenous spread and evolve very rapidly. Loss of both vision and the eye is likely if appropriate treatment is instituted too late (26). *B. cereus* keratitis associated with contact lens wear has also been reported (80). Other *B. cereus* infections occur mainly, though not exclusively, in persons predisposed by neoplastic disease, immunosuppression, alcoholism and other drug abuse (including a case associated with contaminated heroin), the presence of catheters (49) or implants such as fluid shunts, or some other underlying condition, and fatalities occasionally result. Reported conditions include bacteremia, septicemia, fulminant sepsis with hemolysis, meningitis, brain hemorrhage, ventricular shunt infections, endocarditis (23), pneumonia, exacerbation of bronchiectasis, empyema, pleurisy, lung abscess, brain abscess (23), liver abscess, osteomyelitis, salpingitis, urinary tract infection, and primary cutaneous infections. Wound infections, mostly in otherwise healthy persons, have been reported following surgery (associated, in one report, with contaminated incontinence pads), road traffic and other accidents, scalds, burns, plaster fixation, drug injection, and close-range gunshot and nail bomb injuries; some became necrotic and gangrenous (25, 66). A fatal inflammation was caused by a blank firearm injury; blank cartridge propellants are commonly contaminated with the organism. Neonates also appear to be particularly susceptible to *B. cereus* (100), especially with umbilical stump infections; respiratory tract infections associated with contaminated ventilation systems have also occurred (105). Recently, a near-fatal *B. cereus* pneumonia in an otherwise normal patient was reported (54). Such severe infection in healthy individuals is more typical of *B. anthracis*. Interestingly, the isolate from this case was found to harbor a plasmid (pBCXO1) that was 99.6% similar to the pXO1 virulence plasmid of *B. anthracis* (54); its role, if any, in the virulence of this isolate is not known. A hospital pseudo-outbreak of *B. cereus* infection was associated with contaminated ethanol used as a skin disinfectant.

There have been reports of wound, burn, and ocular infections with *B. thuringiensis* (24), but there is as yet no evidence of infections associated with the use of this organism as an insecticide. The safety of using *B. thuringiensis* as a biopesticide on crop plants has been reviewed (11). Strains

of this species commonly carry genes for *B. cereus* enterotoxins, but it was found that the main pesticide strains assayed produced low titers of enterotoxin. Occupational exposure to the organism has been connected with the presence of the organism in feces but without gastrointestinal symptoms. Cases of illness caused by *B. thuringiensis* may have been diagnosed as caused by *B. cereus*, as the former may not produce its characteristic insecticidal toxin crystals when incubated at 37°C, owing to the loss of the plasmids carrying the toxin genes (44). Strains of *B. cereus* and *B. thuringiensis* have been isolated in association with periodontitis (47). *B. cereus* also causes infections in domestic animals. It is a well-recognized agent of mastitis and abortion in cattle and can cause these conditions in other livestock.

Other Species

Reports of infections with non-*B. cereus* group species are comparatively rare but very diverse (see reference 66 for the earlier literature), and there have been several hospital pseudoepidemics associated with contaminated blood culture systems. *B. licheniformis* has been reported from ventriculitis following the removal of a meningioma, cerebral abscess after penetrating orbital injury, septicemia following arteriography, bacteremia associated with indwelling central venous catheters, bacteremia during pregnancy with eclampsia and acute fibrinolysis, peritonitis in a chronic ambulatory peritoneal dialysis patient and in a patient with volvulus and small-bowel perforation, ophthalmitis, and corneal ulcer after trauma. Most bizarre, perhaps, are two reports of bacteremia in cases of Munchausen's syndrome: one case followed self-inoculation with organic drain cleaner (46), and in another *B. pumilus* and *Paenibacillus polymyxa* were also isolated (39). There have also been reports of L-form organisms, phenotypically similar to *B. licheniformis,* occurring in human blood and other body fluids (66). *B. licheniformis* can cause foodborne diarrheal illness and has been associated with an infant fatality (70). This organism is frequently associated with bovine abortion and has been shown to have a tropism for the bovine placenta; it has also been associated with abortion in water buffalo and occasionally with bovine mastitis. These types of *B. licheniformis* and *B. cereus* infections are associated with wet and dirty conditions during winter housing, particularly when the animals lie in spilled silage, and in one outbreak a water tank contaminated with *B. licheniformis* was implicated.

The name *B. subtilis* was often used in the past to mean any aerobic, endospore-forming organism, but since 1970 there have been reports of infection in which the identification of this species appears to have been made accurately. They include cases of pneumonia, bacteremia, and septicemia associated with neoplastic disease; breast prosthesis and ventriculoatrial shunt infections; isolations from surgical wound drainage sites; endocarditis in a drug abuser; meningitis following a head injury; bacteremia associated with trauma; cholangitis associated with kidney and liver disease; and isolation from dermatolymphangioadenitis associated with filarial lymphedema. The administration of a probiotic preparation marketed for the treatment or prevention of intestinal disorders and allegedly containing *B. subtilis* led to a fatal septicemia in an immunocompromised patient (76). Subsequently, the organism concerned was identified as *B. clausii* (97). The authors of the latter report reported another *B. clausii* infection, cholangitis in polycystic kidney disease in a 15-year-old French boy who had undergone renal transplant, but the source of the organism was unclear. *B. subtilis* has also been associated with cases of bovine mastitis and ovine abortion (66). *B. subtilis* has been implicated in foodborne illness: vomiting has been the commonest symptom, but with accompanying diarrhea frequently reported; the onset periods have been short (ranging from 10 min to 14 h; median; 2.5 h), the bacterial loads of the organism were high (10^5 to 10^9 CFU/g), and the implicated foods were often prepared dishes in which meat or fish was served with cereal-based components such as bread, pastry, rice, or stuffing (63). *B. amyloliquefaciens*, a close relative of *B. subtilis*, is widely used industrially for enzyme and amino acid production, but human consumption of L-tryptophan manufactured in an organism genetically engineered from a strain of this species was associated with a large epidemic of eosinophilia-myalgia syndrome with 37 deaths (71); the causative agent has not been identified with certainty (71). Environmental strains of this species producing a heat-stable, nonprotein toxin have been isolated in association with building-related health problems (71).

Organisms identified as *B. circulans* have been isolated from cases of meningitis, a cerebrospinal fluid shunt infection, endocarditis, a wound infection in a cancer patient, a bite wound, endophthalmitis, and epidemic endophthalmitis associated with a contaminated product used during cataract surgery (89). It must be noted, however, that many isolates previously identified as *B. circulans* might have been misallocated (see comments on *B. circulans* below). *B. coagulans* has been isolated from corneal infection, bacteremia, and bovine abortion. *B. pumilus* has been found in cases of pustule and rectal fistula infection, bacteremia in an immunosuppressed patient, bacteremia in a patient with Munchausen's syndrome (39) and in association with bovine mastitis. Toxigenic strains of *B. pumilus* have been isolated in association with foodborne illness and from clinical and environmental specimens (98). *B. sphaericus* has been implicated in a fatal lung pseudotumor and in meningitis (66). Among 18 cancer patients with 24 bacteremic episodes, Banerjee et al. (7) isolated *B. cereus* (eight cases), *B. circulans* (three cases), *B. subtilis* (two cases), *B. coagulans* (one case), *B. licheniformis* (one case), *B. pumilus* (one case), *B. sphaericus* (one case), and six unidentified aerobic endospore formers. *B. megaterium* (eight isolates), *B. pumilus* (six isolates), *Brevibacillus brevis* (five isolates), *B. licheniformis* (two isolates), and *B. subtilis* (one isolate), all from chewing tobacco, were found to produce potent exogenous virulence factors that caused plasma exudation and tissue dysfunction in an animal model (90).

Bacillus brevis has been isolated from corneal infection and implicated in several incidents of food poisoning; since the time of these reports, the species has been split (see "Taxonomy" above) and transferred to the new genus *Brevibacillus*. Strains of the new species, *Brevibacillus agri,* have been isolated in association with an outbreak of waterborne illness in Sweden; *Brevibacillus centrosporus* was isolated from bronchoalveolar lavage fluid, *Brevibacillus parabrevis* was found in a breast abscess, and both species have been isolated from human blood (68). *Brevibacillus laterosporus* has been reported in association with a severe case of endophthalmitis (66).

Paenibacillus alvei has been isolated from cases of meningitis and endophthalmitis, from a prosthetic hip infection in a patient with sickle cell anemia, from a wound infection, and, in association with *Clostridium perfringens,* from a case of gas gangrene. *P. macerans* has been isolated from a wound infection following removal of a malignant melanoma, from a brain abscess following penetrating periorbital injury, from a catheter-associated infection in a leukemic patient, and from bovine abortion, and *P. polymyxa* has been isolated from ovine abortion (66) and a bacteremic case of Munchausen's

syndrome (39). *P. popilliae* has been reported from endocarditis, and a new *Paenibacillus* species, *P. hongkongensis*, was discovered in a child with neutropenic fever and pseudobacteremia (99), a case in which the organism was found in only one of four blood cultures and was considered to be an environmental contaminant.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Clinical specimens for isolation of *Bacillus* species other than *B. anthracis* can be handled without special precautions. *Bacillus* species will normally survive transport in freshly collected specimens or in a standard transport medium. Local transport of specimens (over a few hours) can be done at room temperature or at 2 to 8°C for most specimens, including serum. Generally, if specimens such as swabs, stool, sputum, pleural fluid, and blood are to be shipped overnight or longer, they should be sent at 2 to 8°C. Fresh tissue and serum samples should be shipped frozen, whereas formalin-fixed tissues can be sent at room temperature primarily for detection using immunohistochemistry and (much less suitable) for PCR. For blood specimens in which PCR will be used to detect *B. anthracis* DNA, collection tubes containing EDTA or citrate as anticoagulants are preferable to those containing heparin.

Safety Aspects in Relation to Anthrax

The infectious doses in human anthrax are considered to be high, with the 50% lethal dose as high as 2,500 to 55,000 (34, 57) but as low as 1 to 3 spores (79) or 2 to 9 spores (34). In general, precautions need to be sensible, not extreme. When collecting specimens for suspected anthrax, personnel should wear disposable gloves, disposable apron or overalls, and boots which can be disinfected after use; for dusty samples that might contain many spores, the use of personal protective equipment such as a face shield and/or a respirator should be considered. It should be noted that although hand washing with soap and water or with chlorhexidine gluconate and the use of hypochlorite-releasing towels may all reduce endospore contamination of the skin, waterless rubs containing ethanol are ineffective at removing spores; the model organism in this study was *B. atrophaeus*, a close relative of *B. subtilis* (110). Disposable items should be discarded into suitable containers for autoclaving. Nonautoclavable items should be immersed overnight in 10% formalin (5% formaldehyde solution), glutaraldehyde (5%), a pH 7-adjusted 1:10 dilution of household bleach, or an aqueous solution of chlorine dioxide (500 mg/liter). Items that cannot be immersed should be bagged and sent for formaldehyde fumigation. Ethylene oxide and hydrogen peroxide vapor are also effective fumigants, but the latter is inappropriate if organic matter is being treated. The best disinfectant for specimen spills is formalin. In cases where this is considered impractical, a 1:10 dilution of household bleach (6,000-mg/liter hypochlorite solution) (http://www.epa.gov/pesticides/factsheets/chemicals/bleachfactsheet.htm) can be used, although its limitations should be appreciated; it is rapidly neutralized by organic matter, and it corrodes metals. Other strong oxidizing agents, such as hydrogen peroxide (5%) and peracetic acid (1%), are also effective but likewise inactivated by organic matter.

When working with pure cultures of *B. anthracis*, direct and indirect contact of broken skin with cultures and contaminated laboratory surfaces, accidental parenteral inoculation, and rarely, exposure to infectious aerosols are the primary hazards to laboratory personnel. Isolation and presumptive identification of *B. anthracis* can be performed safely in the routine clinical microbiology laboratory, provided that the usual good laboratory practice is observed; vaccination is not required for minimal handling of the organism (20).

All of the other species of aerobic endospore-forming bacteria that may be isolated from clinical specimens can be handled safely on the open bench. Efforts should be made to avoid methods that produce aerosols. Any procedures that have the potential to generate aerosols should be done in a biological safety cabinet. In addition, all centrifuging should be done using an aerosol-tight rotor and rotors should be opened within the biological safety cabinet. Centrifuges are the most frequently contaminated pieces of laboratory equipment. Laboratories that frequently centrifuge *B. anthracis* suspensions should use an aerosol-tight rotor that can be repeatedly autoclaved; the rotor and rotor lid should be swabbed regularly to monitor for contamination, and contaminated rotors can be autoclaved before reuse. Biosafety level 2 practices, containment equipment, and facilities are recommended for activities using clinical materials and diagnostic quantities of infectious cultures. Biosafety level 3 practices, containment equipment, and facilities are recommended for work involving production quantities or concentrations of cultures and for activities likely to produce aerosols (20).

Specimens from Suspected Anthrax Patients

In all cases, specimens from possible sources of infection (carcass, hides, hair, bones, etc.) should be sought in addition to patient specimens.

Cutaneous Anthrax

For cutaneous anthrax, collect sufficient vesicular fluid with swabs to allow both culture and a smear for visualizing the capsule. For immunohistochemical analysis of cutaneous lesions, a full-thickness punch biopsy fixed in 10% buffered formalin from a papule or vesicle lesion and including adjacent skin should be taken. Biopsy specimens should also be taken from both vesicle and eschar if present (94).

Intestinal Anthrax

Intestinal anthrax will be suspected only if an adequate history of the patient is known. If the patient is not severely ill, a fecal specimen may be collected, but isolation may not be successful. If the patient is severely ill, blood should also be cultured, although isolation may not be possible after antimicrobial treatment; treatment should not await laboratory results. A blood smear may reveal the capsulated rods or, if treatment has started, capsule "ghosts."

Postmortem blood collected by venipuncture (a characteristic of anthrax is nonclotting blood at death [103]) should be examined by smear (for capsule) and culture. Any hemorrhagic fluid from the nose, mouth, or anus should be cultured. If these are positive, no further specimens are needed. If they are negative, specimens of peritoneal fluid, spleen, and/or mesenteric lymph nodes, aspirated by techniques avoiding spillage of fluids, may be collected for smear and culture.

Inhalation (Pulmonary) Anthrax

As with the intestinal form, inhalational anthrax will be suspected only if the patient's history suggests it. If the patient is severely ill, blood smear and culture should be done. Following the bioterrorist attacks of 2001 in the United States, PCR on pleural fluid specimens was very useful, even when the specimens were collected after antimicrobial therapy had begun;

specimens from three patients were negative by culture but still had positive PCR results even when taken ≥24 h after treatment had begun (53), but results depend somewhat on previous treatment. Serology is also useful for the diagnosis of cases when culture fails due to previous treatment. Sera should be taken <7 days after symptoms appear (or after exposure, if known) and again at >14 days. Postmortem, the approach given above for intestinal anthrax should be followed. If the patient is not severely ill, immediate specimen collection is likely to be unfruitful and the person should be treated and simply observed; paired sera (when first seen and >10 days later) may be useful for confirmation of diagnosis.

Specimens from Animals with Suspected Anthrax

Anthrax should be considered as the possible cause of death in herbivorous animals which have died suddenly and unexpectedly, particularly if hemorrhage from the nose, mouth, or anus has occurred and if death has taken place at a site with a history of anthrax (even several decades previously).

Carcasses 1 to 2 Days Old

Due to the nonclotting nature of blood in anthrax victims, in the case of 1- or 2-day-old carcasses it is usually possible to aspirate a few drops of blood from a vein for (i) M'Fadyean-stained smear and (ii) direct plate culture on blood agar.

Pigs frequently do not develop the enormous terminal bacteremia seen in herbivores, and the capsulated rods may not be visible in blood smears. When cervical edema is present, smears and cultures should be made of fluid aspirated from the enlarged mandibular and suprapharyngeal lymph nodes. In porcine intestinal anthrax, possibly obvious only at necropsy, rods are usually visible in stained smears made from mesenteric lymph nodes.

Older Putrefying Carcasses

B. anthracis competes poorly with putrefactive organisms and may not be seen in smears after 2 to 3 days, so culture is necessary for diagnostic confirmation for older putrefying carcasses. Sections of tissue or any blood-stained material should be collected. If the animal has been opened, spleen or lymph node specimens should be taken. With putrefied and very old carcasses, swabs of the nostrils and eye sockets are likely to yield *B. anthracis*, but the best specimens may be samples of contaminated soil beneath the nose and anus.

Other Specimens

Tests for the presence of *B. anthracis* may be requested on a variety of other specimens, such as animal products (e.g., wool, hides, hair, or bonemeal) from regions of endemicity, soil or other materials from old burial sites or tannery or laboratory sites due for redevelopment, or other environmental materials associated with outbreaks (e.g., sewage sludge). At present, culture by the selective agar techniques described below is the only approach. Suitably equipped laboratories are beginning to use PCR techniques for rapid detection of *B. anthracis* in such samples, but at present it is advisable to confirm positives by conventional methods.

Potential Bioterrorism-Related Specimens

In 1999, a Laboratory Response Network (LRN) was established in the United States by the Centers for Disease Control and Prevention (CDC) in partnership with the Association of Public Health Laboratories, the Federal Bureau of Investigation, and the United States Army Medical Research Institute of Infectious Diseases (USAMRIID) to provide the public health laboratory response to acts of bioterrorism (74). This network links local laboratories (sentinel) to laboratories with more specialized testing and increased biosafety level at the state (reference) and federal (national) level. There are reference level laboratories in all 50 states able to detect agents, including *B. anthracis*, rapidly. State public health laboratories are part of the LRN and will be able to provide guidance, or the LRN can be accessed using the Internet (http://www.bt.cdc.gov/lrn/). State and territorial public health laboratory contact information and sentinel laboratory guidelines are also available on the American Society for Microbiology website at http://www.asm.org. Emergency response guidelines for state, local, and tribal public health personnel can be found at http://www.bt.cdc.gov/ under "Additional Topics and Resources" and a 24-h hotline number for urgent advice is 770-488-7100. Although initially limited to the United States, there are now 152 national and international laboratories within the LRN, including Canada, Great Britain, Australia, Germany (U.S. military base), and South Korea (U.S. military base), which are capable of providing a rapid response to acts of biological terrorism, emerging infectious diseases, and other public health threats and emergencies.

ISOLATION PROCEDURES

Specimens with Mixed Microbiota

In specimens submitted for food-poisoning investigations or for isolation of *B. anthracis* from old carcasses, animal products, or environmental specimens, the organisms will be present mostly as spores. Heating (62.5 to 80°C for 10 to 30 min) will both heat shock the spores and effectively destroy nonspore-forming contaminants. A variety of approaches are used to process solid samples prior to heat treatment. Direct plate cultures are made on blood, nutrient, or, selective agars, as appropriate, by spreading up to 100-μl volumes from undiluted and 10- and 100-fold dilutions of the treated sample.

Enrichment procedures are generally inappropriate for isolations from clinical specimens, but when searching for *B. cereus* in stools ≥3 days after a food-poisoning episode, nutrient or tryptic soy broth with polymyxin (100,000 U/liter) may be added to the heat-treated specimen. There is no effective enrichment method for *B. anthracis* in old animal specimens or environmental samples; isolation from these is best done with polymyxin-lysozyme EDTA-thallous acetate (PLET) agar (61; see also chapter 21). A differential/selective chromogenic medium has recently been introduced by R&F Laboratories, Downers Grove, Il. (R&F Anthracis Chromogenic Medium), but it has yet to be thoroughly evaluated. Aliquots (100 μl) of the undiluted and 1:10 and 1:100 dilutions of heat-treated suspension of the specimen are spread across PLET plates, which are read after incubation for 36 to 40 h at 37°C. Roughly circular, creamy white colonies, 1 to 3 mm in diameter, with a ground-glass texture are subcultured on (i) blood agar plates to test for gamma phage and for hemolysis and (ii) directly or subsequently in blood to look for capsule production by using M'Fadyean's stain or, less reliably, India ink negative stain (the ink coagulates the blood and makes interpretation difficult); 2.5 ml of blood (defibrinated horse blood seems best; horse or fetal calf serum is quite good) is inoculated with a pinhead quantity of growth from the suspect colony, incubated statically for 6 to 18 h at 37°C, and then stained. PCR-based methods are being used increasingly for the direct detection of *B. anthracis* in clinical specimens and environmental samples (53).

Several media have been designed for isolation, identification, and enumeration of *B. cereus*. They exploit the organism's egg yolk reaction positivity and acid-from-mannitol negativity;

pyruvate and polymyxin may be included for selectivity. Three satisfactory formulations are MEYP or MYP (mannitol, egg yolk, polymyxin B agar [MEYP]; Oxoid, Basingstoke, United Kingdom; MYP; Difco, BD, Franklin Lakes, N.J.), PEMBA (polymyxin B, egg yolk, mannitol, bromthymol blue agar; Oxoid), and BCM (*Bacillus cereus* Medium; LabM, Bury, United Kingdom) (106) (see chapter 21). There are no selective media for other *Bacillus* species, but spores can be selected for by heat treating part of the specimen, as described above; the vegetative cells of both spore formers and non-spore-formers will be killed, but the heat-resistant spores not only will survive but also may be heat shocked into subsequent germination. The other part of the specimen is cultivated without heat treatment in case spores are very heat sensitive or absent. Heat treatment is not appropriate for fresh clinical specimens, in which spores are usually sparse or absent.

IDENTIFICATION OF *B. ANTHRACIS*

Gram Stain

Inevitably the first examination of smears and cultures will be with the Gram stain. In the past, it has been regarded as being of limited value in anthrax diagnosis because it does not reveal the capsule. In recent bioterrorism cases such preparations were clearly considered to be of great value (Fig. 1a), but caution is still necessary. In a well-developed country, it is unlikely that large numbers of gram-positive bacteria in the blood at death are going to be anything but *B. anthracis*, particularly when supported by the recent events. In other circumstances, and in animals in particular, the blood or other specimen may not be collected soon after death and before putrefactive organisms appear; *B. anthracis* may then be indistinguishable without the use of the proper capsule stain.

The M'Fadyean polychrome methylene blue staining test dates from 1903 and has proved to be a remarkably successful rapid diagnostic test over the decades. However, reliable stain and adequate quality control of its performance are becoming hard to guarantee. A rapid immunochromatographic on-site test has been developed (13) but is not commercially available.

It is generally easy to distinguish virulent *B. anthracis* from other members of the *B. cereus* group. *B. anthracis* isolates are characterized by a typical microscopic appearance (Fig. 1c and 2a) and colonial morphology (Fig. 3a): colonies are white or gray, nonhemolytic or only weakly hemolytic, susceptible to the diagnostic "gamma phage" (inquiries

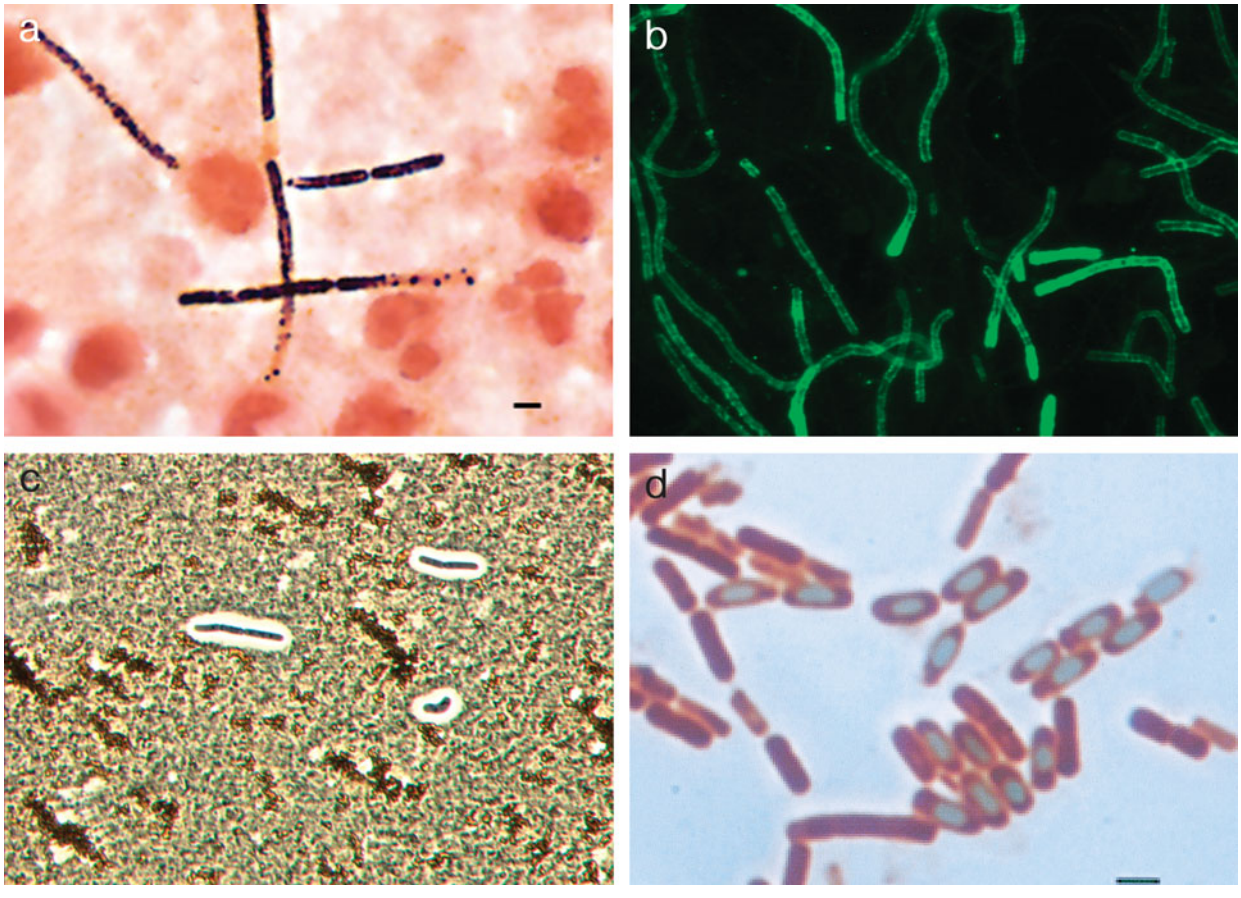


FIGURE 1 (a) Gram stain of *B. anthracis*, associated with a bioterrorism attack, showing gram-positive rods in peripheral blood buffy coat following admission of patient; bar, 3 μm. Courtesy of H. Masur. (b) DFA-stained preparation of *B. anthracis* using an antibody specific for the poly-γ-D-glutamic acid capsule. (c) India ink stain of *B. anthracis* incubated in horse blood. Clear zones surrounding the rods are due to the exclusion of the India ink by the capsule. (d) Spore-stained preparation of *B. cereus* sporangia, viewed by bright-field microscopy. Spores are stained green and vegetative cells are counterstained red. Bar, 2 μm. (Photograph kindly provided by M. Rodriguez-Diaz.)

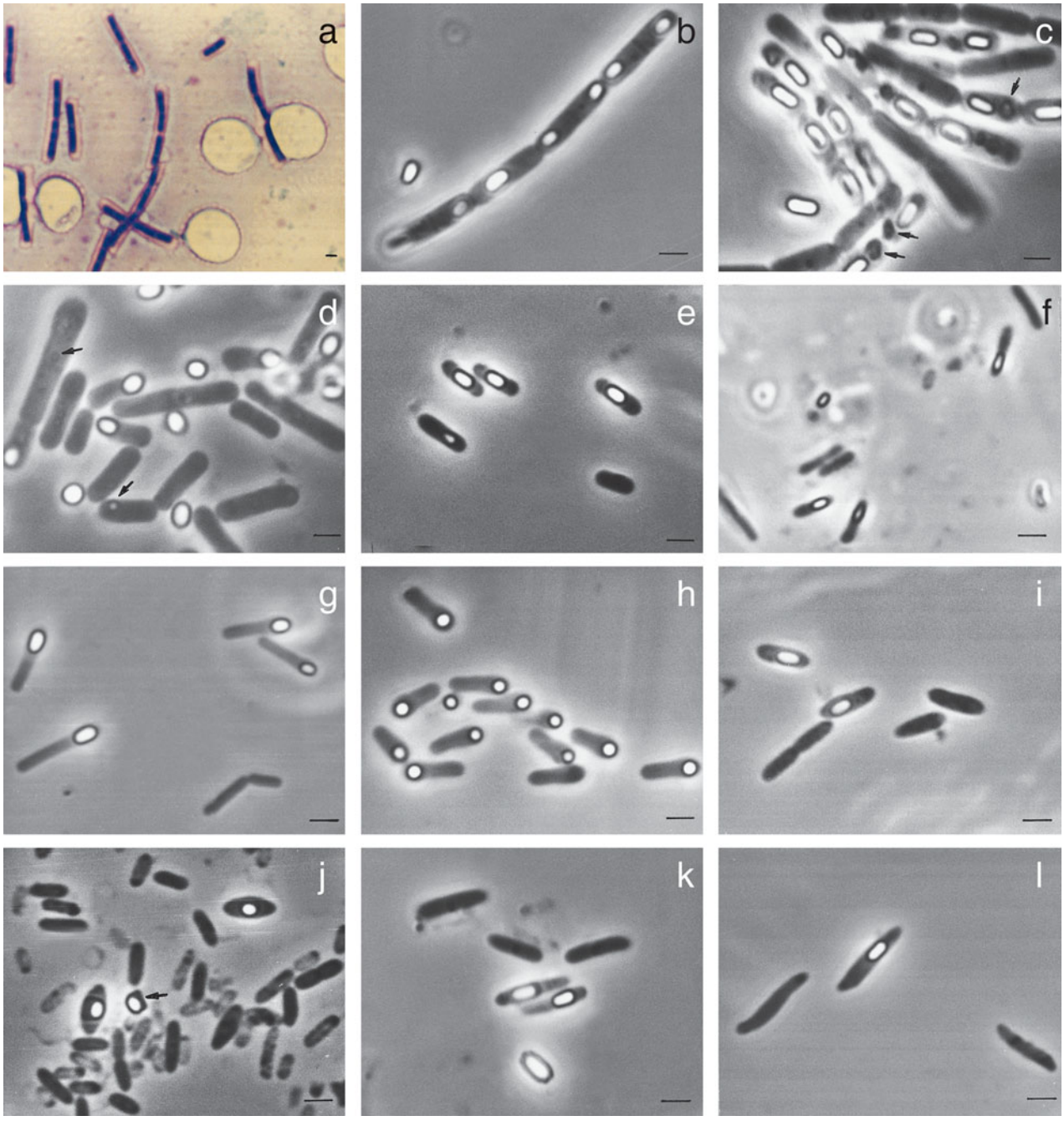


FIGURE 2 Photomicrographs of endospore-forming bacteria viewed by bright-field (a) and phase-contrast (b to l) microscopy. Bars, 2 μm. (a) *B. anthracis,* M'Fadyean stain showing capsulate rods in guinea pig blood smear; (b) *B. cereus,* broad cells with ellipsoidal, subterminal spores, not swelling the sporangia; (c) *B. thuringiensis,* broad cells with ellipsoidal, subterminal spores, not swelling the sporangia, and showing parasporal crystals of insecticidal toxin (arrowed); (d) *B. megaterium,* broad cells with ellipsoidal and spherical, subterminal and terminal spores, not swelling the sporangia, and showing poly-β-hydroxybutyrate inclusions (arrowed); (e) *B. subtilis,* ellipsoidal, central and subterminal spores, not swelling the sporangia; (f) *B. pumilus,* slender cells with cylindrical, subterminal spores, not swelling the sporangia; (g) *B. circulans,* ellipsoidal, subterminal spores, swelling the sporangia; (h) *B. sphaericus,* spherical, terminal spores, swelling the sporangia; (i) *Brevibacillus brevis,* ellipsoidal, subterminal spores, one swelling its sporangium slightly; (j) *Brevibacillus laterosporus,* ellipsoidal, central spores with thickened rims on one side (arrowed), swelling the sporangia; (k) *Paenibacillus polymyxa,* ellipsoidal, paracentral to subterminal spores, swelling the sporangia slightly; (l) *Paenibacillus alvei,* cells with tapered ends, ellipsoidal, paracentral to subterminal spores, not swelling the sporangium.

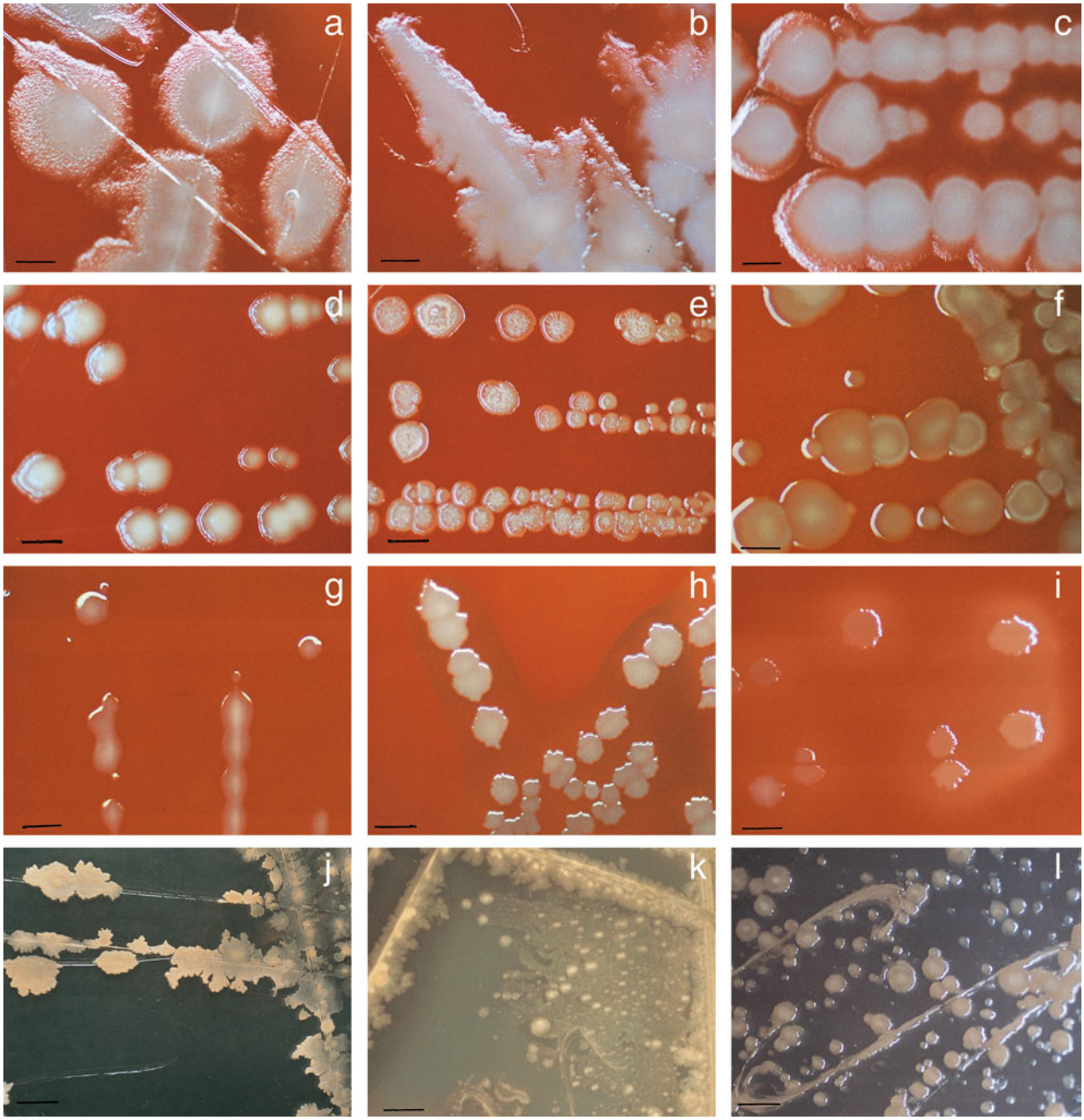


FIGURE 3 Colonies of endospore-forming bacteria on blood agar (a to i) and nutrient agar (j to l) after 24 to 36 h at 37°C. Bars, 2 mm. (a) *B. anthracis;* (b) *B. cereus;* (c) *B. thuringiensis;* (d) *B. megaterium;* (e) *B. pumilus;* (f) *B. sphaericus;* (g) *Brevibacillus brevis;* (h) *Brevibacillus laterosporus;* (i) *Paenibacillus polymyxa;* (j) *B. subtilis;* (k) *Paenibacillus* strain formerly assigned to *B. circulans;* (l) *Paenibacillus alvei.*

about gamma phage should be addressed to the Diagnostics Systems Division, USAMRIID, Fort Detrick, Frederick, MD 21702-5011), generally susceptible to penicillin, nonmotile, and able to produce the characteristic capsule, as shown by M'Fadyean staining (Fig. 2a) or India ink staining (103) (Fig. 1c). As an alternative to culture in blood, the capsule of virulent *B. anthracis* can be demonstrated on nutrient agar containing 0.7% sodium bicarbonate, incubated overnight under 5 to 7% CO_2 (candle jars perform well). Colonies of the capsulated organism appear mucoid, and the capsule can be visualized by M'Fadyean or India ink staining of smears or by direct fluorescent antibody (DFA) staining (Fig. 1b; see below) or indirect antibody staining (inquiries about indirect fluorescent antibody capsule staining should be addressed as above to the Diagnostics Systems Division, USAMRIID).

In addition to phenotypic analysis, molecular and antigenic detection assays are available for the rapid identification of *B. anthracis*. The LRN PCR (restricted to LRN laboratories; see "Potential Bioterrorism-Related Specimens" above) targets three distinct loci on the *B. anthracis* chromosome, pXO1

virulence plasmid, and pXO2 virulence plasmid (54). Using several loci increases specificity and allows for the detection of avirulent strains (lacking pXO1 or pXO2). The anthrax toxin genes (*pagA*, *lef*, and *cya*) are located on pXO1, whereas the genes required for capsule biosynthesis (*capBCA*) are located on pXO2. Isolates lacking pXO2 or both plasmids are found mostly in the environment and are frequently mistaken for *B. cereus,* due to the lack of a capsule, and discarded (102). A commercial kit is also available for the detection of the *B. anthracis* toxin gene, *pagA*, and the capsule gene, *capB* (Roche, Mannheim, Germany) (8). These genes have been widely used as *B. anthracis* specific gene targets; however, there have been recent reports of these genes being found in species other than *B. anthracis* (41, 54). Recently, several laboratories have developed specific PCR assays for *B. anthracis* that target chromosomal genes such as *rpoB*, *gyrA*, and *plcR* (30, 56, 82).

A two-component DFA assay has been used to identify encapsulated vegetative cells of *B. anthracis* (27, 33). This assay uses two different monoclonal antibodies specific for a *B. anthracis* cell wall antigen and the *B. anthracis* capsule (Fig. 1b). Neither antigen is 100% specific for *B. anthracis;* however, only *B. anthracis* has been found to be positive for both antigens, and thus the assay is 100% specific when both cell wall and capsule components are used together; it was heavily used at the CDC during the 2001 bioterrorism-associated outbreak for the rapid (<4-h) identification of isolates (27).

Tetracore, Inc. (Gaithersburg, Md.) has produced a rapid (within 15 min) immunochromatographic test (RedLine Alert) utilizing an antibody specific for one of the *B. anthracis* S-layer proteins. This assay has been approved by the Food and Drug Administration (FDA) for use on nonhemolytic *Bacillus* species colonies cultured on sheep blood agar plates. Manufacturer's data suggest that the test was 98.6% sensitive when tested on 145 *B. anthracis* isolates and 45 nonhemolytic, non-*B. anthracis* isolates; however, such identification of *B. anthracis* is considered presumptive and it should not be used as a stand-alone test.

Molecular Characterization of *B. anthracis*

Although *B. anthracis* is a genetically monomorphic species, the development of a multiple-locus variable-number tandem repeat analysis (MLVA) has allowed for effective strain differentiation (59). This method was relied on during the 2001 bioterrorism-associated outbreak in the United States, and MLVA of the attack strain implicated the Ames laboratory strain (52). Recently, Keim et al. proposed adding canonical single nucleotide polymorphisms, expanding MLVA from 8 to 15 loci, and analyzing four single nucleotide repeats to increase genotyping accuracy and resolution (60). In addition, although not in widespread use, the availability of genome data has led to the use of microarray technology to detect differences between strains of *B. anthracis* and other *Bacillus* species (86, 116) (see also "Molecular Typing" below).

Direct Detection of *B. anthracis* in Clinical Specimens

In addition to culturing *B. anthracis,* there are molecular and antigen-based detection methods available for direct detection in clinical and environmental samples. These are essential when cultures fail, as they particularly do after the initiation of antimicrobial therapy. Several methods, including a *B. anthracis*-specific LRN PCR assay, immunohistochemical (IHC) assays, and serology, were useful for confirmation of cases during the 2001 bioterrorism-associated outbreak, particularly when culture failed (45, 53, 83, 85, 94). At least two such tests need to be positive for a case to be considered laboratory confirmed.

The most widely used and available detection method in the U.S. public health system is the LRN PCR as previously described (53). Following the 2001 anthrax attacks in the United States, this assay was negative on all specimens (n = 142) from patients in whom anthrax was excluded (100% specific). In addition, testing of specimens from inhalation anthrax patients produced a positive result in 33% (29/87) of the specimens which were culture negative, with pleural fluid appearing to be the best specimen even after the initiation of antimicrobial therapy. The IHC assay, as performed at the CDC, uses the same antibodies as the DFA assay (specific to cell wall antigen or the capsule) to detect *B. anthracis* in formalin-fixed, paraffin-embedded tissues. This method was particularly useful in the diagnosis of cutaneous cases during the 2001 bioterrorism-associated outbreak. Skin biopsy specimens from cutaneous lesions from 8 of 10 patients were positive for both the capsule and cell wall antigens (94).

The many reports on the use of different PCR assays to detect or identify *B. anthracis* cannot be summarized here. Although PCR is currently the most widely used detection technology (29), there are increasing reports on novel technologies or improvements to existing ones such as mass spectrometry, flow cytometry, time-resolved fluorescent assays, high-performance liquid chromatography, and even the use of engineered B-cells to detect *B. anthracis* (2, 55, 87, 111, 115). With further improvements and validations, these assays may increasingly be used to detect *B. anthracis* in the future.

Serological Detection of Anthrax

Serological assays for the detection of antibody response against the anthrax toxin protein, protective antigen (PA), were used in combination with PCR or IHC assay results to confirm anthrax cases when culture failed. A quantitative human anti-PA immunoglobulin G (IgG) enzyme-linked immunosorbent assay was performed at the CDC during the 2001 outbreak and was positive only on sera from individuals with anthrax or vaccinated with Anthrax Vaccine Adsorbed (BioThrax; BioPort, Lansing, Mich.) (85). More recently, a commercially available, FDA-approved, qualitative kit (QuickELISA Anthrax-PA Kit) from Immunetics (Boston, Mass.) has become available for the detection of anti-PA IgG and IgM antibodies in human serum. Serological assays aided in the effort to confirm cases in the 2001 attack, particularly cutaneous ones; however, the time to seroconversion after infection limits its usefulness in terms of the rapid diagnosis necessary for treatment and a public health response.

IDENTIFICATION OF SPECIES OTHER THAN *B. ANTHRACIS*

Remember that these organisms do not always stain gram positive. Before attempting to identify to the species level, it is important to establish that the isolate really is an aerobic endospore former and that other inclusions are not being mistaken for spores. A Gram-stained smear showing cells with unstained areas suggestive of spores can be stripped of oil with acetone or alcohol, washed, and then stained for spores. Spores are stained in heat-fixed smears by flooding with 10% aqueous malachite green for up to 45 min (without heating), followed by washing and counterstaining with 0.5% aqueous safranin for 30 s; spores are green within pink/red cells at a magnification of ×1,000 (Fig. 1d). Phase-contrast microscopy (at a magnification of ×1,000) should be used if

available, as it is superior to spore staining and more convenient. Spores are larger, more phase bright, and more regular in shape, size, and position than other kinds of inclusion such as polyhydroxybutyrate (PHB) granules (Fig. 2d), and sporangial appearance is valuable in identification (Fig. 2).

Members of the *B. cereus* group and *B. megaterium* produce large amounts of storage material when grown on carbohydrate media, but on routine media this vacuolate or foamy appearance is rarely sufficiently pronounced to cause confusion. Isolates of other organisms have often been submitted to reference laboratories as *Bacillus* species because they were large, aerobic gram-positive rods, even though sporulation had not been observed, or because PHB granules or other storage inclusions had been mistaken for spores.

Bacillus contains facultative anaerobes as well as strict aerobes, which can be a valuable characteristic in identification. For example, *B. licheniformis* and *B. subtilis,* which have very similar colonial (Fig. 3j) and microscopic morphologies (Fig. 2e), are facultatively anaerobic and strictly aerobic, respectively; likewise, the two large-celled species *B. cereus* and *B. megaterium* (Fig. 2b and d) are facultatively anaerobic and strictly aerobic, respectively.

The most widely used diagnostic schemes use traditional phenotypic tests (42) or miniaturized tests of the API 20E and 50CHB kits used together (67) (bioMérieux, Marcy l'Etoile, France). The API 20E and 50CHB kits can be used for the presumptive distinction of *B. anthracis* from other members of the *B. cereus* group within 48 h. bioMérieux also offers identification cards for *Bacillus* and related genera for the Vitek and Vitek Compact automated identification systems. As many new species have been proposed since these schemes were established, updated API and Vitek databases have been prepared. Biolog Inc. (Hayward, Calif.) also offers a *Bacillus* database. The effectiveness of such kits can vary with the genera and species of aerobic endospore formers concerned, but they are improving with continuing development and enlarged databases (67). The many proposals for new species, often on the basis of single isolates, make the satisfactory expansion of such databases problematic; for a database to be effective in identifying a particular species, its entry for that species needs to reflect the characterization of at least 10 authentic strains from a range of sources, but these requirements can be very difficult or impossible to fulfill. It is stressed that the use of these kits should always be preceded by the basic characterization tests described below.

Other approaches include chemotaxonomic fingerprinting by fatty acid methyl ester profiling, polyacrylamide gel electrophoresis analysis, pyrolysis mass spectrometry, and Fourier-transform infrared spectroscopy. All these approaches have been successfully applied either across the genera or to small groups. As with genotypic profiling methods, large databases of authentic strains are necessary; some of these are commercially available, such as the Microbial Identification System software (Microbial ID Inc., Newark, Del.) database for fatty acid methyl ester analysis.

For diagnostic purposes, the aerobic endospore formers comprise two groups: the reactive ones, which give positive results in various routine biochemical tests and which are therefore easier to identify, and the nonreactive ones, which give few if any positive results in such tests. Nonreactive isolates tend to dominate the identification requests sent to reference laboratories. Table 1 shows reactions for some species belonging to the former group, and the phenotypic test scheme outlined above may be used in conjunction with it. Guidance on identifying members of the nonreactive group is found in reference 68.

Identification of *B. cereus* and *B. thuringiensis*

Colonies of *B. cereus* and relatives are very variable but readily recognized (Fig. 3a, b, and c): they are characteristically large (2 to 7 mm in diameter) and vary in shape from circular to irregular, with entire to undulate, crenate, or fimbriate edges; they have matte or granular textures. Smooth and moist colonies are not uncommon, however. The optimum growth temperature is about 37°C with minima and maxima of 15 to 20°C and 40 to 45°C, respectively. Although colonies of *B. anthracis* and *B. cereus* can be similar in appearance, those of the former are generally smaller and nonhemolytic, may show more spiking or tailing along the lines of inoculation streaks, and are very tenacious compared with the usually more butyrous consistency of *B. cereus* and *B. thuringiensis* colonies, so that they may be pulled into standing peaks with a loop. *Bacillus mycoides* produces characteristic rhizoid or hairy-looking, adherent colonies which readily cover the whole agar surface.

There are key characteristics for recognizing and distinguishing the *B. cereus* group organisms. They display a typical colonial morphology (Fig. 3a, b, and c); large cells are observed, often in chains, producing ellipsoidal spores not swelling the sporangia (Fig. 2b and c), usually within 48 h and often apparent after 24 h; they are facultative anaerobes and positive for the egg yolk reaction (i.e., lecithinase positive). Negative or very weak hemolysis and lack of motility distinguish *B. anthracis* and *B. mycoides* from *B. cereus* and *B. thuringiensis*. *B. cereus, B. mycoides, B. thuringiensis,* and, to a lesser extent, *B. anthracis* synthesize lecithinases, forming opaque zones of precipitation around colonies on egg yolk agar as the colonies grow (i.e., usually after overnight or perhaps 24 h of incubation). Recognition of *B. thuringiensis* is largely dependent on observation of its cuboid or diamond-shaped parasporal crystals in sporulated cultures (after 2 to 5 days) by phase-contrast microscopy (Fig. 2c) or by staining with malachite green counterstained with carbol fuchsin or safranin.

Other Species

Other species show a very wide range of colonial morphologies, both within and between species after 24 to 48 h (Fig. 3). They vary from moist and glossy (Fig. 3f to i) through granular to wrinkled (Fig. 3e); shapes vary from round to irregular (Fig. 3d to i), sometimes spreading (Fig. 3k and l), with entire through undulate or crenate to fimbriate edges (Fig. 3d to j); sizes range from 1 to 5 mm; color commonly ranges from buff or creamy gray to off-white, but some strains may produce an orange pigment; hemolysis may be absent, slight or marked, partial or complete (Fig. 3h); elevations range from effuse through raised to convex; the consistency is usually butyrous, but mucoid and dry, adherent colonies are not uncommon. Despite this diversity, *Bacillus* colonies are not generally difficult to recognize, and some species have characteristic yet seemingly infinitely variable colonial morphologies, as does the *B. cereus* group (Fig. 3a to c).

B. subtilis and *B. licheniformis* produce similar colonies, which are exceptionally variable in appearance and often appear to be mixed cultures (Fig. 3j); colonies are irregular in shape and of moderate diameter (2 to 4 mm), and they range from moist and butyrous or mucoid, with margins varying from undulate to fimbriate through membranous with an underlying mucoid matrix, with or without mucoid beading at the surface, to rough and crusty as they dry. The "licheniform" colonies of *B. licheniformis* tend to be quite adherent.

Rotating and migrating microcolonies, which may show spreading growth (Fig. 3k), have been observed macroscopically

TABLE 1 Characters for differentiating some species of *Bacillus, Geobacillus, Paenibacillus*, and *Virgibacillus*[a]

Character[b]	*Bacillus*								
	B. subtilis group				*B. cereus* group				*B. megaterium*
	B. subtilis	*B. amyloliq-uefaciens*	*B. licheniformis*	*B. pumilus*	*B. cereus*[c]	*B. anthracis*	*B. thuringiensis*	*B. mycoides*	
Rod mean diameter (μm)	0.8	0.8	0.8	0.7	1.4	1.3	1.4	1.3	1.5
Chains of cells	(−)	(+)	(+)	−	+	+	+	+	+
Motility	+	+	+	+	+	−	+	−	+
Sporangia[d]									
Spore shape	E	E	E (C)	C, E	E (C) [E]	E	E (C)	E	E, S
Spore position	S, C	S, T	S, C	S, C	S, C	S	S	S (C)	S, C
Sporangium swollen	−	−	−	−	−	−	−	−	−
Parasporal crystals	−	−	−	−	−	−	+	−	−
Parasporal bodies	−	−	−	−	−	−	−	−	−
Anaerobic growth	−	−	+	−	+	+	+	+	−
Growth at:									
50°C	v	v	+	v	−	−	−	−	−
65°C	−	−	−	−	−	−	−	−	−
Egg yolk reaction	−	−	−	−	+	+	+	+	−
Casein hydrolysis	+	+	+	+	+	+	+	+	+
Starch hydrolysis	+	+	+	−	+	+	+	+	+
Arginine dihydrolase	−	−	+	−	v[(−)]	−	+	v	−
Indole production	−	−	−	−	−	−	−	−	−
Gelatin hydrolysis	+	+	+	+	+	(+)	+	+	+
Nitrate reduction	+	+	+	−	(+)[+]	+	+	(+)	−
Gas from carbohydrates	−	−	−	−	−	−	−	−	−
Acid from:									
D-Arabinose	−	−	−	−	−	−	−	−	−
Glycerol	+	+	+	+	+[v]	−	+	+	+
Glycogen	+	+	+	−	+[−]	+	+	+	+
Inulin	(+)	−	v	−	−	−	−	−	+
Mannitol	+	+	+	+	−	−	−	−	+
Salicin	+	+	+	+	+[−]	−	(+)	(+)	+
D-Trehalose	+	+	+	+	+	+	+	+	+

[a] Symbols and abbreviations: +, ≥85% positive; (+), 75 to 84% positive; v, variable (26 to 74% positive); (−), 16 to 25% positive; −, 0 to 15% positive.
[b] Arginine dihydrolase, indole production, gelatin hydrolysis, and nitrate reduction reactions were determined using tests in the API 20E strip (bioMérieux). Acid from carbohydrate reactions was determined using the API 50CHB System (bioMérieux).

in about 13% of strains received as *B. circulans*, but this very heterogeneous species continues to undergo radical taxonomic revision, and such spreading strains are now assigned to *Paenibacillus* species (Fig. 3l).

Other species that have been encountered in the clinical laboratory include *B. coagulans, B. megaterium, B. pumilus,* and *B. sphaericus; B. brevis* and *B. laterosporus* (now both in *Brevibacillus*); and *B. macerans* and *B. polymyxa* (now both in *Paenibacillus*), and they do not produce a particularly distinctive growth (Fig. 3d to i).

Microscopic morphologies, particularly of sporangia (Fig. 2), are much more helpful for distinguishing between species. Vegetative cells are usually round-ended, but those of *P. alvei* may be tapered (Fig. 2l). The large cells of *B. megaterium* may accumulate PHB (Fig. 2d) and appear vacuolate or foamy when grown on glucose nutrient agar. Overall, cell widths vary from about 0.5 to 1.5 μm, and cell lengths vary from 1.5 to 8 μm. Most strains of these species are motile. Spore shapes vary from cylindrical (Fig. 2f) through ellipsoidal (Fig. 2b, c to e, g, and i to l) to spherical (Fig. 2d and h); bean- or kidney-shaped, curved-cylindrical, and pear-shaped spores are also seen occasionally. Spores may be terminally (Fig. 2h), subterminally (Fig. 2b, c, f, g, and i), or centrally (Fig. 2e and j) positioned within sporangia and may distend them (Fig. 2g to k). Despite within-species and within-strain variation, sporangial morphologies tend to be characteristic of species and may allow tentative identification by the experienced worker. One species, *Brevibacillus laterosporus,* produces very distinctive ellipsoidal spores that have thickened rims on one side, so that they appear to be laterally displaced in the sporangia (Fig. 2j).

All these species are mesophilic and grow well between 30 and 37°C. Minimum growth temperatures lie mostly between 5 and 20°C, and maxima are mostly between 35 and 50°C. Strains of *B. coagulans* may show slight thermophily and grow up to 55 to 60°C.

GENE SEQUENCING AND SPECIES IDENTIFICATION

Although the sequencing of several genes, such as *rpoB* and *gyrA,* has been used to aid in the identification of bacterial species, the16S rRNA gene remains the one most commonly used, and it provides an abundance of data for comparison

Bacillus				*Geobacillus*		*Paenibacillus*				*Virgibacillus pantothenticus*
B. circulans group										
B. circulans	*B. firmus*	*B. lentus*	*B. coagulans*	*G. stearothermophilus*	*G. thermodenitrificans*	*P. polymyxa*	*P. alvei*	*P. macerans*	*P. validus*	
0.8	0.8	0.8	0.8	0.9	0.8	0.9	0.8	0.7	0.8	0.6
−	−	(+)	v	−	v	−	(−)	−	−	+
+	+	+	+	+	−	+	+	+	+	+
E	E	E	E	E	E	E	E (C)	E	E	E, S
S, T	S (C)	S, C	S, T	S, T	S	S, C	S, C	S, T	S, T	S, T
+	v	v	+	+	−	+	+	+	+	+
−	−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−	−
+	−	−	+	−	−	+	+	+	−	+
−	−	−	+	+	+	−	−	v	v	v
−	−	−	−	+	+	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−	−
−	+	v	v	(+)	(−)	+	+	−	−	+
+	+	+	+	+	+	+	+	+	+	+
(−)	−	−	v	−	−	−	−	−	−	(−)
−	−	−	−	−	−	−	+	−	−	−
−	v	v	−	+	+	+	+	v	−	+
v	(+)	(+)	(−)	v	(+)	v	−	−	v	v
−	−	−	−	−	−	+	−	+	−	−
−	−	−	−	−	−	−	−	+	−	+
v	−	v	+	(+)	v	+	+	+	+	+
+	−	v	−	+	−	+	v	+	v	−
(+)	−	(−)	−	−	−	+	−	+	v	−
+	v	(+)	v	−	v	+	−	+	+	−
+	−	+	+	(−)	v	+	v	+	−	+
+	v	(+)	+	+	+	+	v	+	+	+

[c] Reactions shown in brackets are for the biotype isolated particularly in connection with outbreaks of emetic-type food poisoning and for strains of serovars 1, 3, 5, and 8, which are commonly associated with such outbreaks.

[d] Spore shape: C, cylindrical; E, ellipsoidal; S, spherical. Spore position: C, central or paracentral; S, subterminal; T, terminal. The commonest shapes and positions are listed first, and those shown in parentheses are infrequently observed.

(4, 73, 108). Many species, however, including species within the *B. cereus* group, can be extremely difficult to differentiate on the basis of a single gene sequence. Sacchi et al. reported that while *B. anthracis* and *B. cereus* could be differentiated by 16S rRNA gene sequencing, some strains of *B. cereus* and *B. anthracis* differed at only a single nucleotide position; furthermore, a more recent report identified a *B. anthracis* strain with a sequence identical to that of some *B. cereus* strains (3, 91). With differences so small, the quality of the sequence and its analysis are becoming more critically important. Nonetheless, even if sequencing of a gene, such as the 16S rRNA gene, does not always provide identification to the species level with 100% confidence, it is a very helpful component of the identification process and of phylogenetic analyses.

TYPING AND STRAIN DIFFERENTIATION OF SPECIES OTHER THAN *B. ANTHRACIS*

A strain differentiation system for *B. cereus* based on flagellar (H) antigens is available at the Food Safety Microbiology Laboratory, Health Protection Agency Centre for Infections, Colindale, London, United Kingdom, for investigations of food-poisoning outbreaks or other *B. cereus*-associated clinical problems. *B. thuringiensis* strains are classified on the basis of their flagellar antigens; 82 serovars have been recognized. This classification is done at the Institut Pasteur, Paris, France.

Toxin and Antitoxin Detection

The three protein components of anthrax toxin (protective antigen [PA], lethal factor, and edema factor) and antibodies to them can be used in enzyme immunoassay systems. For routine confirmation of anthrax infection, or for monitoring response to anthrax vaccines, antibodies against PA alone appear to be satisfactory; they have proved useful for epidemiological investigations in humans and animals. In human anthrax, however, early treatment sometimes prevents antibody development. PA is available commercially from List Biological Laboratories, Inc., Campbell, Calif. (http://www.listlabs.com). The current human vaccine in the United States is an aluminum hydroxide-adsorbed vaccine strain culture filtrate containing a relatively high proportion of PA and relatively low amounts of lethal factor and edema factor (101).

In countries of the former USSR, a skin test utilizing Anthraxin (trade mark), a heat-stable extract from a noncapsulate strain of *B. anthracis,* which has been licensed for human and animal use since 1962, is widely acclaimed for the retrospective diagnosis of anthrax (95). The delayed-type hypersensitivity is interpreted as indicating cell-mediated immunity to anthrax and can be used to diagnose anthrax retrospectively or to evaluate the vaccine-induced immune status after periods of several years. Anthraxin does not contain highly specific anthrax antigens and depends on the nature of anthrax rather than the specificity of the antigens involved. This is also true of the Ascoli test, which, dating from 1911, must be one of the oldest antigen detection tests in microbiology. It is a precipitin test using hyperimmune serum raised to *B. anthracis* whole-cell antigen to provide rapid retrospective evidence of anthrax infection in an animal from which the material being tested was derived. The test is still in use in Eastern Europe and Central Asia.

The enterotoxin complex responsible for the diarrheal type of *B. cereus* food poisoning has been increasingly well characterized. Two commercial kits are available for its detection in foods and feces, the Oxoid BCET-RPLA (Oxoid Ltd.) and the TECRA VIA (TECRA Diagnostics, Roseville, NSW, Australia). However, these kits detect different antigens, and there is some controversy about their reliabilities. Other assays, based on tissue culture, have also been developed (36). The emetic toxin of *B. cereus* has been identified as a dodecadepsipeptide, and it may be assayed in food extracts or culture filtrates using HEp-2 cells (35) or boar semen (1).

Molecular Typing

Pulsed-field gel electrophoresis has been applied to differentiate between strains of *Bacillus sphaericus* and between very closely related species such as *Bacillus anthracis*, *Bacillus cereus, Bacillus mycoides,* and *Bacillus thuringiensis*, and two studies dealt with infrequently reported clinical infections by *B. cereus* (47, 65).

Amplified fragment length polymorphism is based upon a specific combination of PCR and restriction methodologies, and although it is much more complex than random amplified polymorphic DNA and repetitive extragenic palindromic-PCR methods, it is also much more reproducible. It has been used in epidemiological studies of *B. cereus* (88) and for the genetic comparison of *B. anthracis* and its closest relatives (104). For other members of the *B. cereus* group, which are not as monomorphic as *B. anthracis*, several multilocus sequence typing (MLST) schemes have recently been reported (48, 81), and a *B. cereus* MLST website that allows for viewing of data and submission of new data is available at http://pubmlst.org/bcereus/. These methods include the partial sequencing and comparison of seven housekeeping genes to differentiate strains.

MAINTENANCE OF STRAINS

All the clinically significant isolates reported to date are of species that grow, and often sporulate, on routine laboratory media at 37°C. It seems unlikely that many clinically important, but more fastidious, strains are being missed for the want of special media or growth conditions. Maintenance is simple if spores can be obtained, but it is a mistake to assume that a primary culture or subculture on blood agar will automatically yield spores if stored on the bench or in the incubator. It is best to grow the organism on nutrient agar containing 5 mg of manganese sulfate per liter for a few days and refrigerate the culture when microscopy shows that most cells have sporulated. For most species, sporulated cultures on slants of this medium, sealed after incubation, can survive in a refrigerator for years. Alternatively, cultures (preferably sporulated) can be frozen or lyophilized.

B. anthracis is defined as a select agent and an "overlap" agent on the HHS/CDC and USDA/APHIS select agent lists. Thus, possession of the agent in the United States requires registration of the laboratory with either CDC or APHIS. When *B. anthracis* is identified in an unregistered clinical or diagnostic laboratory, the identification of this agent must be reported to CDC or APHIS within 7 days and to other authorities as required by federal, state, or local laws. When *B. anthracis* is isolated in an unregistered laboratory, the organism must either be destroyed on-site by a recognized sterilization or inactivation process or be transferred to a registered laboratory within 7 days. Shipping of this agent requires completion of the APHIS/CDC Form 2 and prior approval from either CDC or APHIS.

ANTIMICROBIAL SUSCEPTIBILITIES

B. anthracis

Most strains of *B. anthracis* remain susceptible to penicillin (22, 72). Of 25 genetically diverse isolates from around the world, 3 strains were resistant to penicillin but were negative for β-lactamase production. Most strains give variable susceptibility results for cephalosporins; in vitro results, even if susceptible, may not predict clinical efficacy, particularly for expanded- and broad-spectrum cephalosporins (22). In a study of 50 historical isolates from humans and animals and 15 clinical isolates from the 2001 bioterrorist attack in the United States, the majority of strains could be regarded as not susceptible to the broad-spectrum cephalosporin ceftriaxone, and three strains were resistant to penicillin (72). Tetracyclines, fluoroquinolones, and chloramphenicol are suitable for the treatment of patients allergic to penicillin (but these are not good choices clinically, regardless of in vitro results); most strains in the previously mentioned study showed only intermediate susceptibility to erythromycin (72). Ciprofloxacin and the newer quinolone gatifloxacin had good in vitro activities against 40 Turkish isolates, but for another new quinolone, levofloxacin, it was observed that MICs were high for 10 strains (32). Other in vitro studies have shown novel fluoroquinolones and a ketolide to be of potential therapeutic value (38). Standards for antimicrobial susceptibility testing of *B. anthracis* have been recently adopted (21).

Postexposure prophylaxis is needed for the prevention of inhalation anthrax following a bioterrorist attack; the recommended regimen is 60 days of antibiotic therapy and three doses of anthrax vaccine (19), and recommended antimicrobial agents include ciprofloxacin, doxycycline, and levofloxacin. Amoxicillin is recommended as an option in cases where the *B. anthracis* strain has been demonstrated to be susceptible to penicillins and when other antimicrobial agents are not considered safe, as in the treatment of children and pregnant or lactating women (16, 17). The use of penicillins for postexposure prophylaxis or for treatment of inhalation anthrax following the use of *B. anthracis* as a bioweapon gives cause for concern, owing to the presence of β-lactamases in *B. anthracis* isolates, and the poor penetration of β-lactams into macrophages, the site of spore germination (9). Combination intravenous antibiotic therapy with two or more antibiotics, begun early, such as with ciprofloxacin and one or more other antibiotics to which the organism is sensitive, appeared to improve survival during

the treatment of cases during the 2001 outbreak in the United States (58). Following that outbreak, the recommendation for initial treatment of inhalation anthrax is ciprofloxacin or doxycycline along with one or more agents to which the organism is normally susceptible (15).

B. cereus

There have been rather few studies of the antibiotic sensitivity of *B. cereus*, and most information has to be gleaned from the reports of individual cases or outbreaks. *B. cereus* and *B. thuringiensis* produce a broad-spectrum β-lactamase and are thus resistant to penicillin, ampicillin, and cephalosporins; they are also resistant to trimethoprim. An in vitro study of 54 isolates from blood cultures by disk diffusion assay found that all strains were susceptible to imipenem and vancomycin and that most were sensitive to chloramphenicol, ciprofloxacin, erythromycin, and gentamicin (but a small number of strains showed moderate or intermediate sensitivity), while 22 and 37% of strains showed only moderate or intermediate susceptibilities to clindamycin and tetracycline, respectively (109). Although strains are almost always susceptible to clindamycin, erythromycin, chloramphenicol, vancomycin, and the aminoglycosides and are usually sensitive to tetracycline and sulfonamides, there have been several reports of treatment failures with some of these drugs: a fulminant meningitis which did not respond to chloramphenicol (69); a fulminant infection in a neonate which was refractory to treatment that included vancomycin, gentamicin, imipenem, clindamycin, and ciprofloxacin (100); failure of vancomycin to eliminate the organism from cerebrospinal fluid in association with a fluid shunt infection (10); and persistent bacteremias with strains showing resistance to vancomycin in two hemodialysis patients (A. von Gottberg and W. van Nierop, personal communication). Oral ciprofloxacin has been used successfully in the treatment of *B. cereus* wound infections, bacteremia, and pulmonary infection. Clindamycin with gentamicin, given early, appears to be the best treatment for ophthalmic infections caused by *B. cereus,* and experiments with rabbits suggest that intravitreal corticosteroids and antibiotics may be effective in such cases.

Other Species

Information on treatment of infections with other species is sparse. Gentamicin was effective in treating a case of *B. licheniformis* ophthalmitis, and cephalosporin was effective against *B. licheniformis* bacteremia/septicemia. Resistance to macrolides appears to occur naturally in *B. licheniformis*. *B. subtilis* endocarditis in a drug abuser was successfully treated with cephalosporin, and gentamicin was successful against a *B. subtilis* septicemia. Penicillin, or its derivatives, or cephalosporins probably form the best first choices for treatment of infections attributed to other *Bacillus* species. In the study by Weber et al. (109), over 95% of isolates of *B. megaterium*, *B. pumilus*, *B. subtilis*, *B. circulans*, *B. amyloliquefaciens,* and *B. licheniformis*, along with strains of *B.* (now *Paenibacillus*) *polymyxa* and three unidentified strains from blood cultures, were susceptible to imipenem, ciprofloxacin, and vancomycin; between 75 and 90% were susceptible to penicillins, cephalosporins, and chloramphenicol. Isolates of "*B. polymyxa*" and *B. circulans* were more likely to be resistant to the penicillins and cephalosporins than strains of the other species—it is probable that some or all of the strains identified as *B. circulans* might now be accommodated in *Paenibacillus*, along with "*B. polymyxa.*" An infection of a human bite wound with an organism identified as *B. circulans* did not respond to treatment with amoxicillin and flucloxacillin but was resolved with clindamycin (43).

A strain of *B. circulans* showing vancomycin resistance has been isolated from an Italian clinical specimen (64). Vancomycin resistance has been reported for *Paenibacillus popilliae*, a biopesticide, and isolates of this species have been shown to carry genes resembling those responsible for high-level vancomycin resistance in enterococci (78). Of two South African vancomycin-resistant clinical isolates, one was identified as *P. thiaminolyticus* and the other was unidentified but considered to be related to *B. lentus;* the latter was isolated from a case of neonatal sepsis and has been shown to have inducible resistance to vancomycin and teicoplanin; this is in contrast to the *B. circulans* and *P. thiaminolyticus* isolates mentioned above, in which expression of resistance was found to be constitutive (von Gottberg and van Nierop, personal communication).

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Listeria and *Erysipelothrix**

JACQUES BILLE

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LISTERIA

Taxonomy

Listeria and *Brochothrix* form one of several sublines within the *Clostridium* subdivision. The *Listeria-Brochothrix* subline is approximately equidistant from the *Bacillus* and *Enterococcus-Carnobacterium* sublines. On the basis of 23S rRNA gene sequences, *Listeria* is most similar to *Bacillus* and *Staphylococcus*. Phylogenetically, *Listeria* is sufficiently remote from *Lactobacillus* to justify its exclusion from the family *Lactobacillaceae* and formation of a separate *Listeria-Brochothrix* family (20). The phylogenetic position of *Listeria* is consistent with the low G+C content of its DNA (36 to 42 mol%) (20, 49, 82).

Listeria monocytogenes is the type species and one of six species in the genus *Listeria*. The other species are *L. ivanovii*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, and *L. grayi* (73). Two subspecies of *L. ivanovii* have been described: *L. ivanovii* subsp. *ivanovii* and *L. ivanovii* subsp. *londoniensis*. Based on the results of DNA-DNA hybridization, multilocus enzyme analysis, and 16S rRNA gene sequencing, the six species in the genus *Listeria* are divided into two lines of descent: (i) *L. monocytogenes* and its closely related species, namely, *L. innocua*, *L. ivanovii* (subspecies *ivanovii* and *londoniensis*), *L. welshimeri*, and *L. seeligeri*, and (ii) *L. grayi* (12, 13, 20, 71, 73, 74). Within the genus *Listeria*, only *L. monocytogenes* and *L. ivanovii* are considered to be pathogenic, as evidenced by their 50% lethal doses in mice and their ability to grow in mouse spleen and liver. *L. monocytogenes* is a human pathogen of high public health concern; *L. ivanovii* is primarily an animal pathogen.

Description of the Genus

Members of the genus *Listeria* are asporogenous, nonbranching, regular, short (0.5 to 2 by 0.4 to 0.5 μm), gram-positive rods that occur singly or in short chains. Filaments of 6 to 20 μm long may occur in older or rough cultures. The organisms are motile at 28°C by means of one to five peritrichous flagella but are much less motile at 37°C. Colonies are small (1 to 2 mm in diameter after 1 or 2 days of incubation at 37°C), smooth, and blue-gray on nutrient agar when examined with obliquely transmitted light. The optimum growth temperature is between 30 and 37°C, but growth occurs at 4°C within a few days. *Listeria* spp. are facultatively anaerobic. Catalase is produced except in a few strains (24), and the oxidase test is negative. Acid is produced from D-glucose and other sugars. The Voges-Proskauer and methyl red tests are positive. Esculin is hydrolyzed in a few hours. Urea and gelatin are not hydrolyzed. Neither indole nor H_2S is produced. The cell wall contains a directly cross-linked peptidoglycan based on *meso*-diaminopimelic acid, as well as lipoteichoic acid, but no mycolic acid; the major menaquinone contains seven isoprene units (MK-7). The two predominant cellular fatty acids are $C_{ai15:0}$ and $C_{ai17:0}$ (branched-chain type) (5).

Natural Habitats

Listeria species are widely distributed in the environment. They have been isolated from soil, decaying vegetable matter, silage, sewage, water, animal feed, fresh and frozen poultry, fresh and processed meats, raw milk, cheese, slaughterhouse waste, and asymptomatic human and animal carriers (26). *L. monocytogenes* has been isolated from numerous species of mammals, birds, fish, crustaceans, and insects (25). Nevertheless, the primary habitats of *L. monocytogenes* are considered to be the soil and decaying vegetable matter, in which it survives and grows saprophytically. Because of its widespread occurrence, *L. monocytogenes* has many opportunities to enter food production and processing environments and, because of its ability to grow at 4°C, to cause human disease in persons ingesting colonized food (25, 74).

Clinical Significance

In nonpregnant human adults, *L. monocytogenes* causes primarily meningitis, encephalitis, and/or septicemia (55, 80). Elderly patients or persons with predisposing conditions that lower cell-mediated immunity, such as transplants, lymphomas, and human immunodeficiency virus infection, are especially susceptible. On rare occasions, patients have no known predisposing conditions. The tropism of *L. monocytogenes* for the central nervous system leads to severe disease, often with high mortality (20 to 50%) or with neurologic sequelae among survivors (17). In pregnant women, *L. monocytogenes* often causes an influenza-like bacteremic illness that, if untreated, may lead to placentitis and/or amnionitis and infection of the fetus, resulting in abortion, stillbirth, or premature birth, because the pathogen is able to cross the placenta (46) Early diagnosis can be made in some

* This chapter contains information presented in chapter 33 by Jacques Bille, Jocelyne Rocourt, and Bala Swaminathan in the eighth edition of this Manual.

cases by detecting *L. monocytogenes* in maternal blood cultures; at birth, the diagnosis is made by detecting the organism in cerebrospinal fluid (CSF), blood, amniotic fluid, respiratory secretions, placental or cutaneous swabs, gastric aspirate, or meconium of the neonate. Direct microscopic visualization of gram-positive rods in these specimens may be invaluable in early diagnosis of the disease.

Focal infections rarely occur after an episode of bacteremia. However, primary cutaneous listeriosis with or without bacteremia has been reported among veterinarians and abattoir workers, who acquire the illness through contact with infected animal tissues (53). Endocarditis, arthritis, osteomyelitis, intraabdominal abscesses, endophthalmitis, and pleuropulmonary infections have been described infrequently (48).

The incubation period and infective dose have not been firmly established. Reported incubation times vary from a few days to 2 to 3 months. Gastrointestinal symptoms such as diarrhea have been observed in some individuals with systemic listeriosis. A transient healthy carrier state exists in 2 to 20% of animals and humans (25, 35). In the past decade, several outbreaks of febrile gastroenteritis caused by *L. monocytogenes* have been documented (38). Implicated foods were chocolate milk, rice salad, corn-and-tuna salad, cold smoked trout, corned beef and ham, delicatessen meat, and cheese. These gastroenteritis outbreaks differ from the invasive outbreaks in several respects. They affect persons with no known predisposing risk factors for listeriosis. Illness typically occurs 24 h after ingestion of a large inoculum of bacteria (1.9×10^5 to 1×10^9 CFU/g or ml) and usually lasts 2 days. Common symptoms include fever, watery diarrhea, nausea, headache, and pain in joints and muscles (63). Therefore, the possibility of infection with *L. monocytogenes* should be considered in investigations of gastroenteritis in which routine enteric pathogens have been ruled out. Cervicovaginal carriage in women (including pregnant ones) seems to be nonexistent.

Listeriosis is observed mainly in industrialized countries. It can occur sporadically or epidemically; in both cases, contaminated foods are the primary mode of transmission. A few limited, non-food-related nosocomial outbreaks, mainly in nurseries, have been described (51) due to cross-infection and on one occasion caused by contaminated mineral oil for bathing (79). The number of sporadic cases of listeriosis in countries that report the illness is typically in the range of 0.5 to 0.8 cases per 100,000 persons (75); during foodborne-disease outbreaks, the incidence may rise to 5 cases per 100,000 persons (8). Foods implicated as vehicles of infection are ready-to-eat food stored at refrigeration temperatures and able to sustain *Listeria* growth, including coleslaw (cabbage), soft cheeses, paté, poultry, turkey frankfurters, mushrooms, milk, pork tongue in jelly, and smoked fish. Large numbers of organisms ($>10^3$ CFU/g) were detected in foods quantitatively assayed for the organism (25).

Major progress has been made recently in the understanding of *Listeria* pathogenesis. After contaminated food has been ingested, the development of an invasive infection in some individuals depends on several factors: host susceptibility, gastric acidity, inoculum size, virulence factors of the organism, and the type of food. After penetrating the epithelial barrier of the intestinal tract, *L. monocytogenes* can grow within hepatic and splenic macrophages, due to a number of virulence factors (21), and then spread to the central nervous system or pregnant uterus, due to the interaction between internalin, a major *Listeria* virulence factor, and E-cadherin, a placental receptor (46). Immunity to listeriosis relies mainly on T-cell-mediated activation of macrophages by lymphokines; the role of humoral defenses is not fully understood (74).

Determination of Pathogenicity

Methods using laboratory animals for evaluation of the virulence potential of *Listeria* isolates are available but are not used routinely. Such tests include intraperitoneal inoculation of mice, inoculation of the chorioallantoic membranes of embryonated eggs, and inoculation of the conjunctivas of rabbits (Anton test). Also, cell culture cytotoxicity assays using the human intestinal epithelial cell line Caco-2 have been developed to determine the virulence potential of *Listeria* isolates in vitro (74). Although the results generally agree with those of animal tests, cytotoxicity assays do not provide as quantitative a measure of virulence (50% lethal dose) as the animal tests do. Also, some outbreak-associated *L. monocytogenes* isolates show very little cytotoxicity in the Caco-2 cell assays (66, 83).

Collection, Transportation, and Storage of Specimens

Laboratory Safety

The infectious dose for listeriosis has not been determined, and it may depend, in part, on the susceptibility of the host. Therefore, laboratorians working with *L. monocytogenes* should be made aware of this potential risk and advised to be particularly cautious when working with this organism. Because *L. monocytogenes* takes advantage of the localized immunosuppression at the maternal-fetal interface and attacks the fetus with devastating consequences (stillbirths and abortions) while causing only mild, flu-like symptoms in the mother, pregnant women should be particularly careful working in a laboratory where *L. monocytogenes* is propagated or handled (18).

Specimens

Clinical

L. monocytogenes is readily isolated from clinical specimens obtained from normally sterile sites (blood, CSF, amniotic fluid, placenta, or fetal tissue). These specimens should be immediately cultured at 35°C or stored at 4°C for up to 48 h. Stool specimens are more productive than rectal swabs when epidemiologic studies of carriage rates are undertaken. One gram of stool can be inoculated into 100 ml of a selective University of Vermont (UVM) enrichment broth or polymyxin-acriflavin-lithium chloride-ceftazidime esculin-mannitol (PALCAM) (87) (see chapter 21) and then shipped at room temperature by overnight mail. If this is not possible, stools should be shipped frozen on dry ice by overnight mail. Other nonsterile-site specimens may be stored at 4°C for 24 to 48 h. To avoid overgrowth of *L. monocytogenes* by contaminating microflora during longer periods of storage, freezing of specimens at −20°C is recommended. Routine stool cultures performed in clinical laboratories should not include *Listeria* detection.

Foods

Food samples must be collected aseptically in sterile containers. Whenever possible, foods packaged in original containers must be collected. Attempts should be made to collect at least 100 g of a sample. Samples may be placed in sterile bags and shipped on ice by overnight mail. Ice cream and other frozen products are best transported in the frozen state in the original container and must be thawed immediately before analysis. Although *L. monocytogenes* is relatively resistant to freezing, repeated freezing and thawing may adversely affect the viability of the bacteria.

Isolates

Cultures of *Listeria* spp. may be shipped to a distant laboratory on a non-glucose-containing agar slant (such as heart infusion agar or tryptic soy agar) packaged to conform with the requirements for interstate shipment of etiologic agents (e.g., U.S. Code of Federal Regulations, 42CFR, part 72).

Isolation Procedures

Culture

Clinical specimens from normally sterile sites can be plated directly onto tryptic soy agar containing 5% sheep, horse, or rabbit blood. Samples for blood culture can be inoculated into conventional blood culture broth. Clinical specimens obtained from nonsterile sites and foods and environmental specimens should be selectively enriched for *Listeria* spp. before being plated.

The U.S. Department of Agriculture method and The Netherlands Government Food Inspection Service method are used together at the Centers for Disease Control and Prevention to isolate *L. monocytogenes* from nonsterile-site clinical specimens and foods (37). Individually, the two methods are approximately 75% sensitive; in conjunction with each other, they are 90% sensitive. The U.S. Department of Agriculture method involves enrichment of the specimen in UVM primary selective enrichment broth (1 part sample plus 9 parts of broth) at 30°C. After 24 h, 0.1 ml of the enrichment culture is plated onto lithium chloride-phenylethanol-moxalactam (LPM) (47) agar and Oxford or modified Oxford agar (see chapter 21). Another 0.1 ml of the enrichment culture is added to 10 ml of UVM secondary selective enrichment broth, which is then incubated for an additional 24 h. The secondary enrichment culture is plated as described above. The plates are incubated at 35°C and examined after 24 and 48 h. All of the media named above are described in the compendium by Atlas and Parks (2) and in chapter 21 of this Manual.

The Netherlands Government Food Inspection Service method involves enrichment of the specimen in liquid PALCAM-egg yolk broth (see chapter 21) (87) at 30°C and plating of the enrichment culture onto PALCAM agar at 24 and 48 h. PALCAM agar is incubated at 30°C for 48 h under microaerobic conditions (5% oxygen, 7.5% carbon dioxide, 7.5% hydrogen, and 80% nitrogen).

LPM agar (47) was developed as a highly selective but nondifferential medium for the isolation of *Listeria* species. Colonies on LPM agar are examined under a stereozoom microscope (magnification, ×15 to ×25), with oblique lighting directed to the microscope stage by a concave mirror positioned at a 45° angle to the incident light (Henry illumination). *Listeria* colonies appear blue, and colonies of other bacteria appear yellowish or orange.

Oxford and PALCAM agars contain selective differential chemicals that eliminate the need for examination under oblique lighting (87). On Oxford and modified Oxford agars, *Listeria* colonies appear black, are 1 to 3 mm in diameter, and are surrounded by a black halo after 24 to 48 h of incubation at 37°C. Color formation is due to the hydrolysis of esculin by *Listeria* spp. and the formation of black iron-phenol compounds in the medium. On PALCAM agar, *Listeria* colonies appear gray-green, are approximately 2 mm in diameter, and have black sunken centers; esculin, ferric iron, D-mannitol, and phenol red contribute to this color formation. New chromogenic media improve the isolation of *L. monocytogenes* (6, 68). They rely on the production by *L. monocytogenes* —but not by other species of *Listeria*—of a phosphatidylinositol-specific phospholipase C which produces an opaque halo around the colonies. Several formulations are available: ALOAgar (Biolife, Milano, Italy) (89), BCM *L. monocytogenes* (Biosynth, Staad, Switzerland) (69), and CHROMagar (BD, Sparks, Md.) (28). To rule out *Listeria* gastroenteritis in an outbreak situation, at least one selective agar plate medium specific for *Listeria* spp. (Oxford or PALCAM) and/or for *L. monocytogenes* (ALOAgar or CHROMagar) should be added to conventional media used for bacterial stool culture.

Rapid Detection

Many rapid and/or automated methods have been developed to speed up the recovery and identification of *L. monocytogenes* from food. They rely on genus- or species-specific immunoassays, as well as on DNA or RNA (indicating cell viability) amplification methods (6). These kits are for use with food products only; they are not designed for the analysis of clinical specimens or for diagnosis and/or treatment.

L. monocytogenes DNA in CSF and tissue (fresh or in paraffin blocks) can be specifically detected by PCR-based tests (42, 79). The PCR assay is highly sensitive and specific and may be particularly useful when prior administration of antimicrobial agents compromises culture.

Identification

Genus Identification

A simplified identification is based on the following tests: Gram staining, observation of tumbling motility in a wet mount, and tests for a positive catalase reaction and esculin hydrolysis. Acid production from D-glucose and positive Voges-Proskauer and methyl red reactions are confirmatory test results.

Differentiation of the Genus *Listeria* from Other Genera

Because they share some characteristics, *Listeria* spp. and some other gram-positive bacteria may be confused. *Streptococcus* and *Enterococcus* spp. may be differentiated from *Listeria* spp. on the basis of Gram stain morphology, motility, and catalase activity. *Erysipelothrix* spp. differ from *Listeria* spp. in motility, catalase reaction, and ability to grow at 4°C (*Erysipelothrix* spp. do not grow at that temperature). Among background microflora of foods, *Lactobacillus* spp. are usually nonmotile and catalase negative, *Brochothrix* spp. are unable to grow at 37°C, and *Kurthia* spp. are strictly aerobic and esculin negative.

Species Identification

The scheme for identification of *Listeria* species is shown in Table 1. Identification of *Listeria* isolates to the species level is crucial, because all species can contaminate foods but only *L. monocytogenes* is of public health concern. Identification is based on a limited number of biochemical markers, among which hemolysis is essential to differentiating between *L. monocytogenes* and the most frequently isolated nonpathogenic *Listeria* species, *L. innocua*.

Hemolysis

Only three species, *L. monocytogenes*, *L. seeligeri,* and *L. ivanovii*, are hemolytic on sheep blood agar plates. Recent studies indicated hemolysin to be the major virulence factor of *L. monocytogenes;* however, hemolysis alone cannot be used as an indicator of the presence of a virulent species because *L. seeligeri* is hemolytic but nonpathogenic. *L. monocytogenes*

TABLE 1 Biochemical differentiation of species in the genus *Listeria*[a]

Characteristic	*L. grayi*	*L. innocua*	*L. ivanovii* subsp. *ivanovii*	*L. ivanovii* subsp. *londoniensis*	*L. monocytogenes*	*L. seeligeri*	*L. welshimeri*
Beta-hemolysis	−	−	++[b]	++	+	+	−
CAMP[c] test reaction							
S. aureus	−	−	−	−	+	+	−
R. equi	−	−	+	+	V	−	−
Acid production from:							
Mannitol	+	−	−	−	−	−	−
α-Methyl-D-mannoside	+	+	−	−	+	−	+
L-Rhamnose	V	V	−	−	+	−	V
Soluble starch	+	−	−	−	−	ND	ND
D-Xylose	−	−	+	+	−	+	+
Ribose	V	−	+	−	−	−	−
N-Acetyl-β-D-mannosamine	ND	ND	V	+	ND	ND	ND
Hippurate hydrolysis	−	+	+	+	+	ND	ND
Reduction of nitrate	V	−	−	−	−	ND	ND
Associated serovar(s)	S	4ab, US, 6a, 6b	5	5	1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4ab, 4b, 4c, 4d, 4e, 7	1/2a, 1/2b, 1/2c, 4a, US, 4b, 4d, 6b	1/2b, 4c, 6a, 6b, US

[a] See references 13 and 82. Symbols and abbreviations: +, ≥90% of strains are positive; −, ≥90% of strains are negative; ND, not determined; V, variable; US, undesignated serotype; S, specific.
[b] ++, usually a wide zone or multiple zones.
[c] See text and Fig. 2.

and *L. seeligeri* produce narrow zones of hemolysis that frequently do not extend much beyond the edges of the colonies, whereas *L. ivanovii* exhibits a wide zone of hemolysis (Fig. 1).

The CAMP (Christie, Atkins, Munch-Peterson) test uses a β-lysin-producing *Staphylococcus aureus* or *Rhodococcus equi* strain streaked in one direction on a sheep blood agar plate and test cultures of *Listeria* spp. streaked at right angles to (but not touching) the *S. aureus* and *R. equi* lines. According to *Bergey's Manual of Systematic Bacteriology* (82), hemolysis of *L. monocytogenes* and *L. seeligeri* is enhanced in the vicinity of the *S. aureus* streak, and *L. ivanovii* hemolysis is enhanced in the vicinity of *R. equi* (resulting in a shovel shape) (Fig. 2). However, because many investigators have reported observing a synergistic hemolysis reaction between *L. monocytogenes* and *R. equi,* this CAMP reaction must be interpreted with caution. A β-lysin disk (Remel, Lenexa,

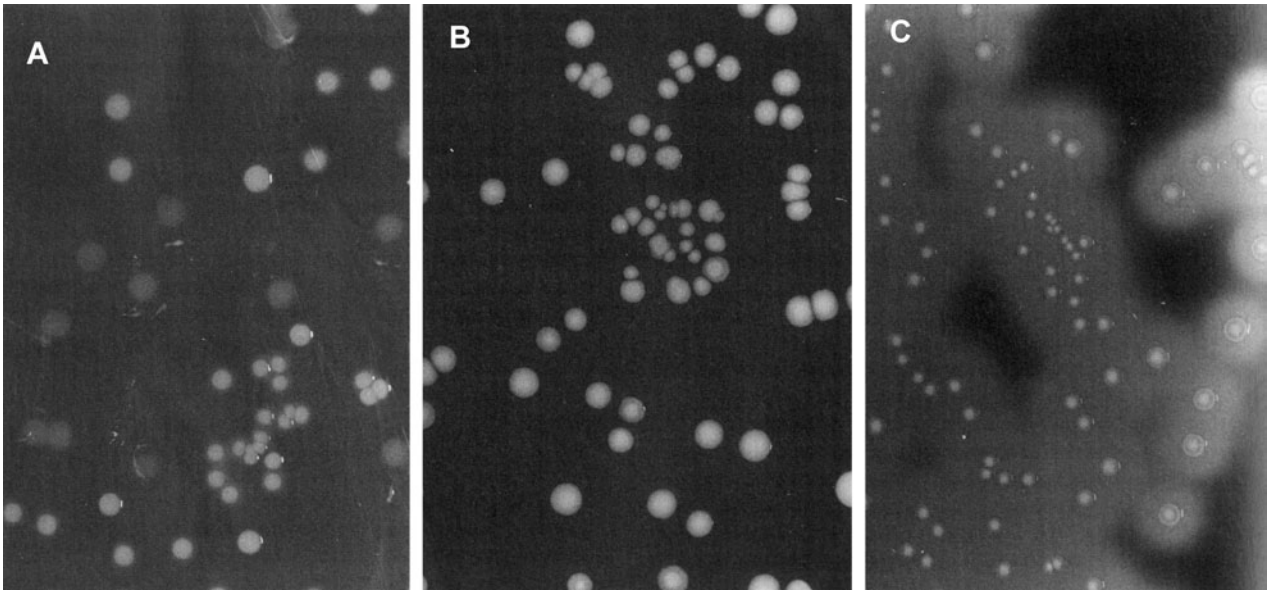


FIGURE 1 Macroscopic view of colonies on 5% human blood agar plates after 24 h of incubation. (A) *L. monocytogenes:* discrete zone of beta-hemolysis under the removed colonies. (B) *L. innocua*: no hemolysis. (C) *L. ivanovii:* wide zone of beta-hemolysis around the colonies.

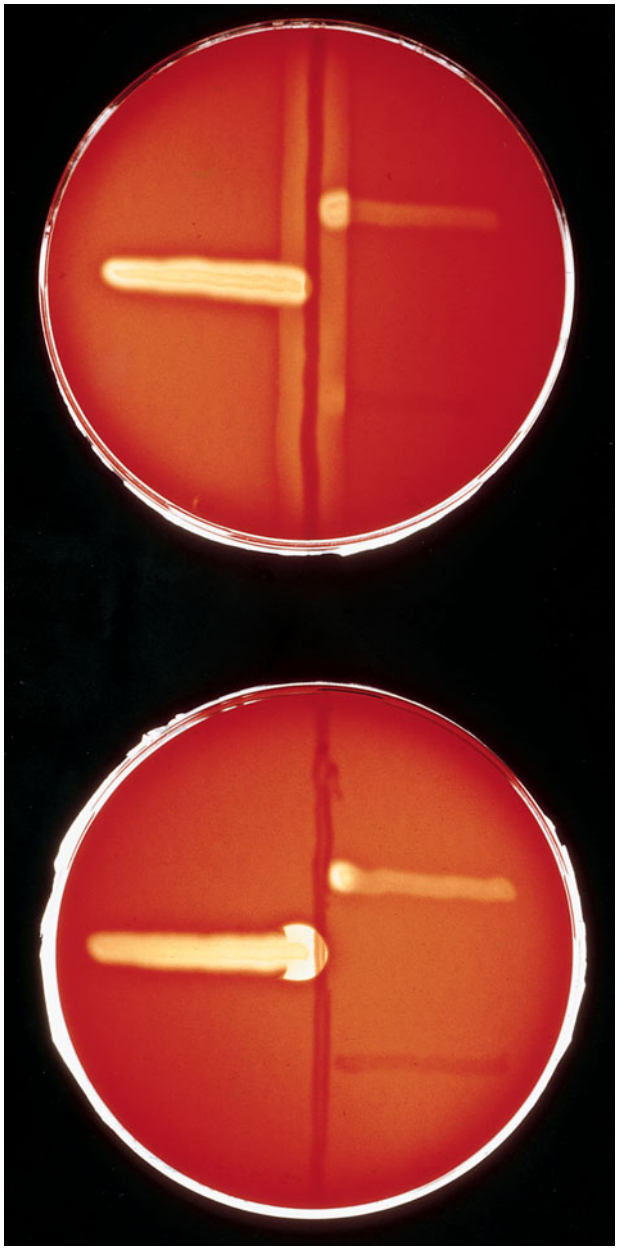


FIGURE 2 CAMP test done with *S. aureus* CIP 5710 (top plate) and *R. equi* CIP 5869 (bottom plate) after 24 h of incubation. Upper right, *L. monocytogenes*; lower right, *L. innocua;* middle left, *L. ivanovii*.

Kans.) may be used to observe enhancement of *L. monocytogenes* with β-lysin from *S. aureus*.

Acid Production from Carbohydrates

L. monocytogenes is always D-xylose negative and α-methyl-D-mannoside positive. Rare atypical strains may be L-rhamnose negative. Test tubes are incubated for 7 days at 37°C in an aerobic incubator.

Miniaturized Biochemical Tests

The API-Listeria test (bioMérieux Vitek, Inc., Hazelwood, Mo.) was specifically designed for *Listeria* and includes 10 biochemical differentiation tests in a microtube format. It includes a patented DIM test, based on the absence or presence of arylamidase, which distinguishes between *L. monocytogenes* (negative) and *L. innocua* (positive) without further tests for hemolytic activity (9). The API Coryne System (bioMérieux) correctly identifies *Listeria* isolates to the genus level (29).

Automated Identification Systems

Both Vitek 2 (bioMérieux) and Phoenix (BD) automated systems have *Listeria* spp. on the list of organisms for their gram-positive identification cards; Vitek 2 (with the new colorimetric gram-positive card) but not Phoenix is able to differentiate *Listeria* colonies to the species level. No independent evaluation is currently available on their performance in identifying *Listeria* spp. at the species or genus level.

DNA Probe Assay for Colony Confirmation

A 30-min chemiluminescence DNA probe assay is available (Gen-Probe, San Diego, Calif.) for the rapid confirmation of *L. monocytogenes* from colonies on primary isolation plates. This assay was highly specific for *L. monocytogenes* in two independent evaluations (56, 62).

Typing Systems

Serotyping

Strains of *Listeria* species are divided into serotypes on the basis of somatic ("O") and flagellar (H) antigens (81). Thirteen serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7) of *L. monocytogenes* are known; 4bX, a variant of serotype 4b, was implicated in a listeriosis outbreak traced to contaminated paté in England (52). Serotyping antigens are shared among *L. monocytogenes*, *L. innocua*, *L. seeligeri*, and *L. welshimeri*. Most human disease is caused by serotypes 1/2a, 1/2b, and 4b; therefore, serotyping alone is not sufficiently discriminating for subtyping purposes. Nevertheless, serotyping is useful as a first-level discriminator. Also, unlike those of other subtyping methods, serotype designations are universal. Determination of the serotype also facilitates selection of appropriate controls for molecular subtyping methods. A commercial kit (Denka Seiken, Tokyo, Japan) which relies on the slide agglutination method (78) has been adapted to an enzyme-linked immunosorbent assay format (64). Recently, a multiplex PCR assay has been designed to separate the four major *L. monocytogenes* serovars (1/2a, 1/2b, 1/2c, and 4b) into distinct groups (23).

Phage Typing

Because of the poor discriminating ability of serotyping, phage typing was the only means of distinguishing strains of the same serotype before the introduction of molecular methods (72) which have largely replaced it today. In addition, phage typing is hampered by the nontypeability of some strains.

MLEE

Multilocus enzyme electrophoresis (MLEE) has been extremely useful for taxonomic studies and for characterization of the evolution of strains within the species (7, 11, 12, 65, 79); however, it does not have adequate discriminatory power for use as the sole subtyping method for epidemiologic investigations.

DNA Microrestriction Pattern Analysis

Characterization of chromosomal DNA by restriction endonuclease analysis or ribosomal DNA gene restriction

patterns (ribotyping) has been used to differentiate *L. monocytogenes* strains of different serotypes and those within a given serotype, in particular serotype 4b, which is most frequently involved in outbreaks. Microrestriction patterns generated by high-frequency-cutting restriction enzymes (e.g., EcoR1) have proved useful in epidemiologic investigations (3, 57), although the complexity of patterns makes it difficult to compare the patterns of several strains. Ribotyping simplifies the microrestriction patterns by rendering visible only the DNA fragments containing part or all of the ribosomal genes, but its discriminating ability, particularly for serotype 4b, may not be adequate (3, 33, 40, 41, 58). A completely automated RiboPrinter system (Qualicon, Wilmington, Del.) facilitates rapid and easy subtyping of *L. monocytogenes* within 8 h. Although ribotyping alone does not have adequate discriminating power for epidemiologic investigations of outbreaks, the automated RiboPrinter is invaluable for preliminary recognition of disease clusters and for identification of transient and long-term-resident strains of *L. monocytogenes* in food processing plants and their environments (22, 59).

DNA Macrorestriction PFGE Pattern Analysis

Brosch et al. (16) evaluated the pulsed-field gel eletrophoresis (PFGE) method for the World Health Organization multicenter international typing study of *L. monocytogenes*. Four laboratories participated in using PFGE to analyze 80 coded strains. Two restriction endonucleases (ApaI and SmaI) were used by all laboratories; one laboratory used an additional restriction endonuclease (AscI). Agreement of typing data among the four laboratories ranged from 79 to 90%. Sixty-nine percent of the strains were placed in exactly the same genomic groups by all four laboratories; most of the epidemiologically related strains were correctly identified by all four laboratories. This study validated the previous claims that PFGE is a highly discriminating and reproducible method for subtyping *L. monocytogenes* and is particularly useful for subtyping serotype 4b isolates, which are not typed satisfactorily by most other typing methods.

In the United States, the Centers for Disease Control and Prevention has established a network (PulseNet) of public health and food regulatory laboratories that routinely subtype foodborne pathogenic bacteria to rapidly detect foodborne disease clusters that may have a common source. PulseNet laboratories use highly standardized protocols for subtyping of bacteria by PFGE and are able to quickly compare PFGE patterns of foodborne pathogens from different locations within the country via the Internet (84) (PFGE@cdc.gov). A 1-day standardized protocol for PFGE subtyping of *L. monocytogenes* was added to PulseNet in 1998 (34). Routine and timely subtyping of *L. monocytogenes* by participating PulseNet laboratories has significantly enhanced the ability to recognize and investigate outbreaks. For several years now, PFGE has been the standard subtyping method to detect listeriosis outbreaks.

RAPD

Random amplification of polymorphic DNA (RAPD) analysis is a rapid and relatively simple technique for epidemiological typing of *L. monocytogenes* isolates by using several short (10-mer) primers (14, 91). Despite its simplicity and high discriminating ability, RAPD suffers from inconsistent reproducibility of patterns.

MLST

Multilocus sequence typing (MLST) is currently being established for *L. monocytogenes* typing. It is based on the comparative sequencing of housekeeping genes or genes coding for virulence factors. The discriminatory power of MLST is very high, and sequence data comparisons are easily done (70, 76, 93).

Present Status of Subtyping

The vast majority of *L. monocytogenes* strains causing sporadic infections or outbreaks belong to serotypes 1/2a, 1/2b, and 4b. Strains of serotype 1/2a are highly heterogeneous and thus are easily differentiated by any of the molecular methods. In contrast, strains of serotype 4b are more closely related and probably necessitate the combined use of several methods to be optimally differentiated (10). PFGE is considered to be the most effective typing method; it is time and cost efficient and highly discriminative (30).

Serodiagnosis

Serologic responses to whole-cell antigens cannot be used for diagnosis because of antigenic cross-reactivity between *L. monocytogenes* and other gram-positive bacteria such as staphylococci, enterococci, and *Bacillus* species. Furthermore, patients with culture-confirmed listeriosis have had undetectable antibody levels (79). Determination of levels of antibody to listeriolysin O may be of value both for invasive listeriosis and for febrile gastroenteritis (4). Although a serologic method based on the detection of antibodies against recombinant truncated forms of listeriolysin O may be more specific (31), serological tests are not recommended at the present time.

Antimicrobial Susceptibilities

The pattern of antimicrobial susceptibility and resistance of *L. monocytogenes* has been relatively stable for many years (86). In vitro, the organism is susceptible to penicillin, ampicillin, gentamicin, erythromycin, tetracycline, rifampin, and chloramphenicol (77, 92) but only moderately susceptible to quinolones (39). However, many of these antimicrobial agents are only bacteriostatic. Penicillin or ampicillin with or without an aminoglycoside is usually recommended for the treatment of listeriosis. Studies in vitro and in animal models have shown that an aminoglycoside enhances the activity of penicillin against *L. monocytogenes* (54). Trimethoprim-sulfamethoxazole and aminoglycosides are among the few anti-infective agents that are bactericidal to *L. monocytogenes;* only trimethoprim-sulfamethoxazole has been used occasionally with success. Resistance plasmids conferring resistance to chloramphenicol, macrolides, and tetracyclines have been found in several clinical isolates of *L. monocytogenes* and have raised concern for the future (36). *L. monocytogenes* appears to be susceptible in vitro to newly released antibiotics such as linezolid, daptomycin, and tigecyclin, but few strains have been tested. Cephalosporins are ineffective in vivo, although in vitro tests may indicate susceptibility, and should never be administered when listeriosis is suspected.

Evalutation, Interpretation, and Reporting of Results

Colonies from blood, CSF, or other normally sterile-site specimens that show subdued beta-hemolysis on blood agar should be subjected to motility tests, Gram staining, catalase testing, and esculin hydrolysis to confirm identification. They may resemble group B streptococcal colonies on blood agar plates. The CAMP test is not necessary on a routine basis, and the β-lysin test may be substituted for it. Use of the API-Listeria test may eliminate the need for enhanced hemolysis testing altogether. If *L. monocytogenes* is present in low numbers in

CSF, the direct examination of Gram-stained clinical specimens may be of little or no value. Also, Gram-stained *Listeria* cells closely resemble other gram-positive bacteria, such as streptococci, enterococci, and corynebacteria. If antimicrobial therapy was initiated before a CSF specimen was obtained, culture results may be negative. In these instances, Gram staining may be useful, as well as molecular detection of DNA by PCR. Although numerous PCR-based detection methods have been developed and tested with food products, a very limited number of applications to clinical samples have been described, using either a single PCR or multiplex PCR (30).

ERYSIPELOTHRIX

Taxonomy

Included at one time in the coryneform group, *Erysipelothrix* is now classified with the regular non-spore-forming gram-positive rods, a group that comprises the genera *Listeria* and *Lactobacillus*. The genus *Erysipelothrix* has two validated species, *E. rhusiopathiae* and *E. tonsillarum* (85). A third species, *E. inopinata*, has been recently proposed (88). *E. rhusiopathiae*, which is widely distributed in nature and can be carried by a variety of animals, has been recognized for more than 100 years as the agent of swine erysipelas and occasionally causes erysipeloid, a human cutaneous infection usually localized to the hands and fingers (67).

Description of the Genus

Erysipelothrix organisms are mesophilic, facultatively anaerobic, non-spore-forming, non-acid-fast, gram-positive bacteria that appear microscopically as short rods (0.2 to 0.5 μm by 0.8 to 2.5 μm) with rounded ends and occur singly, in short chains, or in long, nonbranching filaments (60 μm or more in length). They are nonmotile and grow in complex media at a wide range of temperatures (5 to 42°C; optimum, 30 to 37°C) and at alkaline pHs (pH 6.7 to 9.2; optimum, pH 7.2 to 7.6). Like *Listeria* organisms, they can grow in the presence of high concentrations of sodium chloride (up to 8.5%). Metabolically, *Erysipelothrix* organisms are catalase negative and oxidase negative, do not hydrolyze esculin, and weakly ferment glucose without the production of gas. They are methyl red and Voges-Proskauer negative and do not produce indole or hydrolyze urea but distinctively produce H_2S in triple sugar iron agar. Key fatty acids are $C_{16:0}$ and $C_{18:cis9}$ (5).

Natural Habitats

E. rhusiopathiae is widespread in nature and is remarkably persistent under environmental conditions such as low temperature and alkaline pH and within organic matter favoring survival. The organism is parasitic on mammals, birds, and fish but is most frequently associated with pigs. Contamination of water and soil from the feces and urine of sick and asymptomatic animals often occurs.

E. tonsillarum has been recovered from water and from the tonsils of healthy swine.

Clinical Significance

Infection with *E. rhusiopathiae* is a zoonosis. Many animal species, especially turkeys and swine, carry the organism in their digestive tracts or tonsils. *E. rhusiopathiae* causes chronic or acute swine erysipelas. Erysipelas can present in several clinical manifestations: skin infection, arthritis, septicemia, and endocarditis. Other domestic and wild animals and birds also can be affected, in particular sheep, rabbits, cattle, and turkeys. Infection is most likely acquired by ingestion of contaminated matter (67).

In humans, *E. rhusiopathiae* causes mostly erysipeloid, a localized cellulitis developing within 2 to 7 days around the inoculation site. The disease is contracted through skin abrasion, injury, or a bite on the hands or arms of individuals handling animals or animal products. Erysipeloid is an occupational disease, occurring most frequently among veterinarians, butchers, and particularly fish handlers. The lesion usually is violaceous and painful, indurated with edema and inflammation but without suppuration, and clearly delineated at the border. Regional lymphangitis may be present, as well as an adjacent arthritis. Dissemination and endocarditis can occur, especially in immunocompromised patients; their prognosis is generally poor (32). Healing of erysipeloids usually takes 2 to 4 weeks, sometimes months, and relapses are common. No apparent immunity develops after an episode of erysipeloid.

E. tonsillarum has not yet been recovered from humans.

Collection, Transport, and Storage of Specimens

Biopsy specimens from erysipeloid lesions are the best source of *E. rhusiopathiae*. Care should be taken to cleanse and disinfect the skin before sampling. The organisms typically are located deep in the subcutaneous layer of the leading edge of the lesion; hence, a biopsy of the entire thickness of the dermis at the periphery of the lesion should be taken for Gram staining and culture. Swabs from the surface of the skin are not useful. In disseminated disease, the organism can be cultured from blood without special procedures.

Isolation Procedures

Microscopic Examination

Generally, direct examination of Gram-stained biopsy specimens is of little value. However, the presence of long, slender, gram-positive rods in tissue from an individual with a consonant history is suggestive of erysipeloid.

Culture

Biopsy specimens should be plated onto blood agar or chocolate blood agar, placed in tryptic soy or Schaedler broth, and incubated at 35°C aerobically or in 5% CO_2 for 7 days. Blood from patients with septicemia or endocarditis can be plated directly onto blood agar plates for primary isolation or inoculated into commercial blood culture systems. *E. rhusiopathiae* colonies generally develop in 1 to 3 days, appearing as pinpoints (<0.1 to 0.5 mm in diameter) on blood agar plates after 24 h of incubation; at 48 h, two distinct colony types can be observed. The smaller, smooth colonies are 0.3 to 1.5 mm in diameter, transparent, convex, and circular with entire edges. Larger, rough colonies are flatter and more opaque and have a matte surface and an irregular, fimbriated edge. A zone of greenish discoloration frequently develops underneath the colonies on blood agar plates after 2 days of incubation (43).

Rapid Detection

PCR initially developed for rapid diagnosis in swine (50) has been used with human samples (15). The target is the 16S rRNA or the 23S rRNA gene.

Identification

Gram Staining of Colonies from a 24-h Blood Agar Plate

Cells stain gram positive but can decolorize and appear gram negative, with gram-positive granules giving a beaded effect. Cells from smooth colonies appear as rods or coccobacilli,

sometimes in short chains. Cells from rough colonies appear as long filaments, often more than 60 μm.

Biochemical Identification of *E. rhusiopathiae*

E. rhusiopathiae is catalase negative; glucose, lactose, and H_2S positive; and nitrate, urease, esculin, gelatin, xylose, mannose, maltose, and sucrose negative. *E. tonsillarum* differs biochemically from *E. rhusiopathiae* by being sucrose positive. Vitek automated systems, as well as API Coryne, usually identify *E. rhusiopathiae* correctly.

Differentiation of *Erysipelothix* from Related Genera

Genera that have morphological and physiological characteristics in common with *Erysipelothrix* include *Lactobacillus*, *Listeria*, *Brochothrix*, and *Kurthia*. All are regular nonpigmented, non-spore-forming, gram-positive rods (44). A major discriminatory characteristic is that *E. rhusiopathiae* produces H_2S in triple sugar iron whereas species of the other genera do not. Furthermore, *Listeria*, *Brochothrix*, and *Kurthia* species are catalase positive. In addition, *Listeria* isolates are motile, are esculin positive, and are not alpha-hemolytic. *Brochothrix* isolates strongly ferment carbohydrates, are Voges-Proskauer positive, and do not grow at above 30°C. *Kurthia* species are strict aerobes and are motile and nonhemolytic (45). Corynebacteria and streptococci also can be confused with *E. rhusiopathiae*, but careful examination of cell morphology should facilitate the distinction. The production of H_2S in triple sugar iron by a gram-positive bacterium is usually indicative of *E. rhusiopathiae* because very few gram-positive bacteria of clinical origin produce H_2S. Exceptions include some *Bacillus* strains, but they are easily differentiated from *E. rhusiopathiae* by cellular morphology, spore formation, and catalase reaction. An additional trait highly characteristic of *E. rhusiopathiae* is its "pipe cleaner" pattern of growth in gelatin stab cultures incubated at 22°C (43, 90).

Typing Systems

Twenty-two serovars of *E. rhusiopathiae* have been identified on the basis of heat-stable somatic antigens. Although most isolates are serovar 1 or 2, no serotyping schemes are available for routine use in clinical laboratories. Both MLEE and ribotyping methods have been applied to *Erysipelothrix* strains and have shown an enormous genetic diversity (1, 19, 61). PFGE with SmaI seems to be the best method for epidemiological studies of *E. rhusiopathiae* isolates (60). In these studies, serotyping was unreliable for use as an epidemiologic tool.

Pathogenicity Testing

Most strains of *E. rhusiopathiae* are virulent for mice in a mouse protection test (43).

Serological Tests

Since humans apparently do not develop immunity after an episode of erysipeloid, there are no serological tests for routine use to demonstrate antibodies to *E. rhusiopathiae*. Active immunization of animals with a live attenuated vaccine protects against erysipelas (67); however, a natural erysipeloid infection in humans does not prevent relapses or reinfection from occurring.

Antibiotic Susceptibility

E. rhusiopathiae isolates are generally susceptible to penicillin, cephalosporins, clindamycin, imipenem, tetracycline, chloramphenicol, erythromycin, and the fluoroquinolones; they are usually resistant to aminoglycosides, sulfonamides, and vancomycin (27). Penicillin is the treatment of choice for both localized and systemic infections (32).

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Coryneform Gram-Positive Rods

GUIDO FUNKE AND KATHRYN A. BERNARD

34

This chapter deals with aerobically growing, asporogenous, irregularly shaped, non-partially acid-fast, gram-positive rods generally called "coryneforms." The term "coryneform" is actually somewhat misleading, since only true *Corynebacterium* spp. exhibit a typical club-shaped ("*coryne,*" meaning "club" in ancient Greek) morphology, whereas all the other bacteria discussed in this chapter show an irregular morphology. However, in our experience, the term "coryneforms" is a common and convenient expression used by many clinical microbiologists, and therefore, the term will be used in this chapter.

The coryneform bacteria which were, for didactical reasons, not included in this chapter comprise *Actinomyces* spp. (in particular, the most frequently encountered species on aerobic plates, *A. europaeus, A. neuii, A. radingae,* and *A. turicensis*), *Actinobaculum* spp., *Propionibacterium* spp., and *Propioniferax innocua* (see chapter 56), whereas *Arcanobacterium* spp. are included. *Gardnerella vaginalis* is included in this chapter but is discussed separately. Regularly shaped aerobically growing gram-positive rods (*Bacillus, Listeria* and *Erysipelothrix, Lactobacillus,* and *Clostridium tertium)* are covered in chapters 32, 33, 56, and 57, respectively. Taxa which might be initially misidentified as coryneform bacteria also include partially acid-fast bacteria and other actinomycetes (see chapter 35) as well as rapidly growing mycobacteria (see chapter 38).

GENERAL TAXONOMY

The bacteria discussed in this chapter all belong to the class *Actinobacteria*, the genera of which are characterized by specific 16S rRNA gene signature nucleotides (134). All the genera described in this chapter except *Exiguobacterium* and *Gardnerella* belong to the lineage of the gram-positive bacteria with high guanine-plus-cytosine (G+C) content. The coryneform bacteria are most diverse and are differentiated by chemotaxonomic features (Table 1). Phylogenetic investigations, in particular 16S rRNA gene sequencing, have, in general, confirmed the framework set by chemotaxonomic investigations. The 16S rRNA gene sequencing data demonstrate that the genera *Corynebacterium* and *Turicella* are more closely related to the partially acid-fast bacteria and to the genus *Mycobacterium* than to the other coryneform organisms covered in this chapter (62, 104, 123). The genus *Arthrobacter*, which contains rods, is phylogenetically intermixed with the genus *Micrococcus* (and genera formerly called *Micrococcus*), which contains cocci (44, 79). The genus *Rothia* contains both rod-forming organisms, represented by *Rothia dentocariosa*, and a coccus-forming species, *Rothia mucilaginosa,* the former *Stomatococcus mucilaginosus* (19). Other genera which are phylogenetically closely related include *Oerskovia, Cellulosimicrobium,* and *Cellulomonas* (8, 55, 125, 133), as well as *Arcanobacterium* and *Actinomyces* (105).

DESCRIPTIONS OF THE GENERA

Genus *Corynebacterium*

The genus *Corynebacterium* is presently composed of 67 species (and two taxon groups), 40 (and the two taxon groups) of which are medically relevant. Five of the *Corynebacterium* species have been defined since the last edition of this Manual (Table 2).

The cell wall of corynebacteria contains *meso*-diaminopimelic acid (*m*-DAP) as the diamino acid as well as short-chain mycolic acids with 22 to 36 carbon atoms (17). *C. amycolatum, C. atypicum,* and *C. kroppenstedtii* are the only genuine *Corynebacterium* species which do not possess mycolic acids (16, 18, 69). The corynebacterial cell wall also contains arabinose and galactose (17). Palmitic ($C_{16:0}$), oleic ($C_{18:1\omega9c}$), and stearic ($C_{18:0}$) acids are the main cellular fatty acids (CFAs) in all corynebacteria, and tuberculostearic acid (TBSA) can also be found in some species like *C. urealyticum, C. confusum,* and *C. appendicis* (3, 52, 151). The G+C content of *Corynebacterium* spp. varies from 46 to 74 mol%, indicating the enormous diversity within this genus. The phylogenetic relationships within the genus *Corynebacterium* have been outlined (104, 123), creating an extensive and reliable database for future comparative 16S rRNA gene studies, e.g., for the delineation of new species.

Gram staining of corynebacteria shows slightly curved, gram-positive rods with sides not parallel and sometimes slightly wider ends, giving some of the bacteria a typical club shape (Fig. 1a). Corynebacteria whose morphologies differ from this morphology include *C. durum, C. matruchotii,* and *C. sundsvallense* (see below under each species). Cells infrequently stain unevenly. If *Corynebacterium* cells are taken from fluid media, they are arranged as single cells, in pairs, in V forms, in palisades, or in clusters with a so-called Chinese letter appearance. It is again emphasized that the club-shaped form of the rods is observed only for true

TABLE 1 Some chemotaxonomic features of the bacteria covered in this chapter

Genus	Major CFAs	Mycolic acids	Peptidoglycan diamino acid[a]	Acyl type
Corynebacterium	18:1ω9*c*, 16:0, 18:0	+[b]	*m*-DAP	Acetyl
Turicella	18:1ω9*c*, 16:0, 18:0	−	*m*-DAP	Glycolyl
Arthrobacter	15:0*ai*, 17:0*ai*, 15:0*i*	−	LYS	Acetyl
Brevibacterium	15:0*ai*, 17:0*ai*, 15:0*i*	−	*m*-DAP	Acetyl
Dermabacter	17:0*ai*, 15:0*ai*, 16:0*i*	−	*m*-DAP	ND[c]
Rothia	15:0*ai*, 17:0*ai*, 16:0*i*	−	LYS	ND
Exiguobacterium	17:0*ai*, 15:0*ai*, 16:0, 13:0*i*	−	LYS	ND
Oerskovia	15:0*ai*, 15:0*i*, 17:0*ai*	−	LYS	Acetyl
Cellulomonas	15:0*ai*, 16:0, 17:0*ai*	−	ORN	Acetyl
Cellulosimicrobium	15:0*ai*, 15:0*i*, 17:0*ai*	−	LYS	Acetyl
Microbacterium	15:0*ai*, 17:0*ai*, 16:0*i*	−	LYS, ORN	Glycolyl
Curtobacterium	15:0*ai*, 17:0*ai*, 16:0*i*	−	ORN	Acetyl
Leifsonia	17:0*ai*, 15:0*ai*, 16:0*i*	−	DAB	ND
Janibacter	17:1, 16:0*i*, 17:0	−	*m*-DAP	Acetyl
Pseudoclavibacter	15:0*ai*, 17:0*ai*, 16:0*i*, 16:0	−	DAB	Acetyl
Brachybacterium	15:0*ai*, 16:0*i*, 17:0*ai*	−	*m*-DAP	Acetyl
Knoellia	17:1*i*, 15:0*i*, 16:0*i*, 17:0*i*	−	*m*-DAP	Acetyl
Arcanobacterium	18:1ω9*c*, 16:0, 18:0	−	LYS	ND
Gardnerella	16:0, 18:1ω9*c*, 14:0	−	LYS	ND

[a] *m*-DAP, *meso*-diaminopimelic acid; LYS, lysine; ORN, ornithine; DAB, diaminobutyric acid.
[b] Exceptions: *C. amycolatum*, *C. atypicum*, and *C. kroppenstedtii*.
[c] ND, no data.

Corynebacterium spp. Corynebacteria are always catalase positive, and the medically relevant species are all nonmotile. The genus *Corynebacterium* includes both fermenting and nonfermenting species.

Genus *Turicella*

The genus *Turicella* is phylogenetically closely related to *Corynebacterium* but contains *T. otitidis* as the only species. The cell wall contains *m*-DAP, but mycolic acids are not present (62). The main CFAs for *T. otitidis* are the same as those for *Corynebacterium* spp., but all *T. otitidis* strains also contain significant amounts of TBSA (2 to 10% of all CFAs) (62). *T. otitidis* is the only coryneform bacterium that has a polar lipid profile without glycolipids. The G+C content varies between 65 and 72 mol% (62).

Gram staining shows relatively long gram-positive rods (Fig. 1b). *T. otitidis* is catalase positive, nonmotile, and an oxidizer.

Genus *Arthrobacter*

The genera *Arthrobacter* and *Micrococcus* are so closely related phylogenetically that it has been stated that micrococci are, in fact, arthrobacters which are unable to express rod forms (79). Presently, the genus *Arthrobacter* contains over 50 species, of which only a few have been recovered from human clinical specimens. Lysine is the diamino acid of the cell wall, and $C_{15:0ai}$ is the overall dominating CFA, which represents more than 50% of all CFAs in most *Arthrobacter* species. The G+C content varies between 59 and 70 mol%, indicating the diversity within this genus.

Gram staining may demonstrate a rod-coccus cycle (i.e., rod forms in younger cultures and cocci in older colonies) when cells are grown on rich media (e.g., Columbia base agar). Jointed rods (i.e., rods in a rectangular form, which contributed to the designation of this genus, as "*arthros*" means "joint" in ancient Greek) may also be observed in younger cultures (i.e., after 24 h) but may not be demonstrable for every species. Arthrobacters are catalase positive, motility is variable, and they are always oxidizers.

Genus *Brevibacterium*

The genus *Brevibacterium* presently comprises 17 species, of which 7 species are medically relevant. *m*-DAP is the

TABLE 2 Chronology of recently proposed or reclassified medically relevant coryneform bacteria since publication of the previous edition of this Manual

Yr of definition	Taxon (reference[s])
2002	*Corynebacterium appendicis* (151)
2003	*Brevibacterium luteolum*[a] (146), *Corynebacterium atypicum* (69), *Microbacterium paraoxydans* (82)
2004	*Pseudoclavibacter/Zimmermannella* (87, 90), black-pigmented CDC fermentative group 4 bacteria as *Corynebacterium aurimucosum* and *Rothia dentocariosa* (21), *Corynebacterium tuberculostearicum* (35), *Brevibacterium sanguinis* (147)
2005	*Curtobacterium* spp. (38), *Arthrobacter scleromae* (75), *Janibacter* spp. (31, 88), *Brachybacterium* spp. (K. A. Bernard, unpublished observation), *Knoellia* sp. (K. A. Bernard, unpublished observation), *Corynebacterium resistens* (103), *Cellulomonas denverensis* (7)
2006	*Corynebacterium tuscaniae* (114), *Cellulosimicrobium funkei* (8)

[a] This species had originally been designated *Brevibacterium lutescens*.

diamino acid of the cell wall. $C_{15:0ai}$ and $C_{17:0ai}$ usually represent more than 75% of all CFAs (40). The G+C content varies between 60 and 70 mol%.

Gram staining demonstrates relatively short rods which may develop into cocci when cultures are getting older (after 3 days). Brevibacteria are catalase positive, nonmotile, and oxidizers.

Genus *Dermabacter*

The genus *Dermabacter* presently comprises only one species, *D. hominis*. *m*-DAP is the diamino acid of the cell wall, and $C_{15:0ai}$ and $C_{17:0ai}$ usually account for 40 to 60% of all CFAs. The G+C content range is between 60 and 62 mol% (76).

Gram staining shows very short rods (Fig. 1c), which are often initially misinterpreted as cocci. *D. hominis* strains are catalase positive, nonmotile, and glucose fermenters.

Genus *Rothia*

For didactical reasons, the genus *Rothia* is also included in this chapter because some species are rod-like. The genus *Rothia* belongs to the family *Micrococcaceae*. Collins and colleagues reclassified *Stomatococcus mucilaginosus* as *Rothia mucilaginosa* (19). Since *Rothia mucilaginosa* exhibits coccoid forms in the Gram stain, the genus *Rothia* is also covered in chapter 31 (on the catalase-positive, gram-positive cocci). However, the species *R. dentocariosa* clearly exhibits mainly rod forms and is therefore covered in this chapter on coryneform bacteria.

Lysine is the diamino acid of the cell wall, and $C_{15:0ai}$ and $C_{17:0ai}$ usually represent 40 to 60% of all CFAs. The G+C content ranges between 47 and 56 mol%. *Rothia* strains can be quite pleomorphic by Gram staining, but filamentous forms are normally not observed. They have a variable catalase reaction, are nonmotile, and exhibit a fermentative metabolism.

Genus *Exiguobacterium*

The genus *Exiguobacterium* is phylogenetically related to the so-called group 2 bacilli. Seven species are included in this genus, of which only *E. acetylicum* has been mentioned in any publication as being isolated from human clinical material. Lysine is the diamino acid of the cell wall, and $C_{15:0ai}$ and $C_{17:0ai}$ represent only about 30 to 40% of the total CFAs. *E. acetylicum* contains significant amounts of $C_{13:0}$ and $C_{13:0ai}$, which are not found in any other coryneform taxon (3). The G+C content is about 47 mol%.

Exiguobacteria present as relatively short rods in young cultures. Strains are catalase positive and motile and have a fermentative metabolism.

Genus *Oerskovia*

In older textbooks, oerskoviae were assigned to the nocardioform group of organisms due to their morphological features. This includes branching vegetative substrate hyphae and penetration into agar, but they have no aerial hyphae. However, there is now phylogenetic evidence that *Oerskovia*, including the reclassified *Promicromonospora* (133), is more closely related to genera like *Cellulomonas* than to the mycolic acid-containing genera like *Nocardia*. Representatives of the type species, *O. turbata*, were recovered from soil, but human pathogens originally identified as *O. turbata* have now been placed in *Cellulosimicrobium funkei* (8). Lysine is the diamino acid of the cell wall, and $C_{15:0ai}$ is the main CFA in oerskoviae. The G+C content is 70 to 75 mol%.

Gram staining shows coccoid to rod-shaped bacteria which originate from the breaking up of mycelia. Oerskoviae strains are catalase positive, motility is variable, and they are fermentative.

Genus *Cellulomonas*

The genus *Cellulomonas* presently comprises 13 species, of which only *C. hominis* and *C. denverensis* have been described as being isolated from humans (7, 55). Ornithine is the diamino acid of the cell wall, and $C_{15:0ai}$ and $C_{16:0}$ are the main CFAs. The G+C content is 71 to 76 mol%.

Gram staining shows small, thin rods. All *Cellulomonas* spp., except *C. fermentans* and *C. humilata,* are catalase positive, their motility is variable, the environmental *Cellulomonas* strains are cellulolytic (whereas *C. hominis* did not hydrolyze cellulose in the test system used [55]), and they have a fermentative metabolism.

Genus *Cellulosimicrobium*

The genus *Cellulosimicrobium* presently comprises three species. The medically relevant species are *C. funkei* and *C. cellulans*, which had been designated *Cellulomonas cellulans* or *Oerskovia xanthineolytica* in the past (125). The reason for removing *C. cellulans* from the genus *Cellulomonas* was that the topology of the 16S rRNA gene dendrogram indicated that the branching point of this taxon was outside *Cellulomonas* proper. In addition, the chemotaxonomic characteristic of lysine as diamino acid supported the reclassification. Predominant CFAs include $C_{15:0ai}$, $C_{15:0i}$, $C_{16:0i}$, and $C_{16:0}$. The major menaquinone (MK) is MK-9(H_4), and the G+C content is 74 mol%. It should be noted that the genus *Cellulosimicrobium* is related to the genus *Oerskovia* but nevertheless distinct.

In young cultures, a mycelium is produced that fragments later into irregular, curved, and club-shaped rods. Catalase activity is detected, and strains are nonmotile. All strains have a fermentative metabolism.

Genus *Microbacterium*

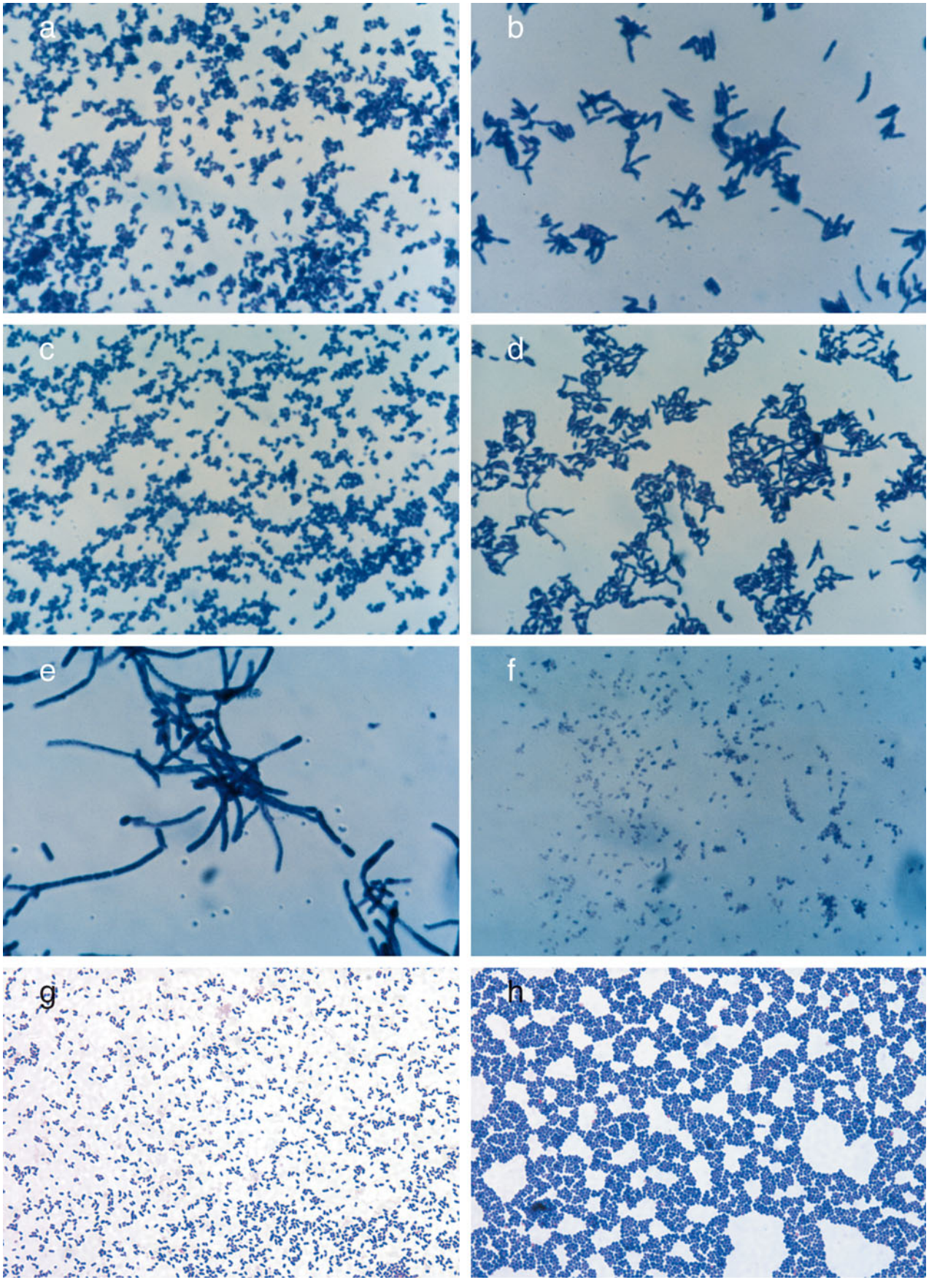
It had been known since the mid-1990s that the genera *Microbacterium* and *Aureobacterium* are phylogenetically intermixed (109). The diamino acid in the third position of the tetrapeptide of the peptidoglycan was considered one of the most important chemotaxonomical markers. L-Lysine is present in microbacteria, and D-ornithine is present in the former aureobacteria. Because in some genera (e.g., *Propionibacterium* and *Bifidobacterium*) there is not a good correlation between the type of the diamino acid in their peptidoglycan and their phylogenetic trees and because a set of signature nucleotides within the 16S rRNA gene of both microbacteria and aureobacteria was demonstrated, it has been proposed to unify the two genera in a redefined genus, *Microbacterium* (135).

To date, over 35 *Microbacterium* species have been validated, but only a minority of them have been demonstrated to be of clinical importance. Microbacteria are frequently encountered in environmental specimens (e.g., soil). $C_{15:0ai}$ and $C_{17:0ai}$ are the two main CFAs, often representing up to 75% of the total CFAs (42, 65, 135). The G+C content of *Microbacterium* spp. is 65 to 76 mol%, indicating the diversity within the genus.

Gram staining often shows thin or short rods with no branching. Catalase activity and motility are variable. Microbacteria can be either fermenters or oxidizers.

Genus *Curtobacterium*

Curtobacterium spp., like microbacteria, belong to the peptidoglycan type B actinomycetes (i.e., cross-linkage between positions 2 and 4 of the two peptide subunits). Ornithine is the diamino acid and the only amino acid composing the interpeptide bridge. Curtobacteria have an acetyl peptidoglycan acyl-type and menaquinone MK-9 as major MK, whereas


a
b
c
d
e
f
g
h

microbacteria possess a glycolyl type and MK-11,12 (Table 1). For most curtobacteria, $C_{15:0ai}$ and $C_{17:0ai}$ represent more than 75% of all CFAs (38). The G+C contents range from 68 to 75 mol%. Six *Curtobacterium* species are validly described.

Gram staining shows small and short rods with no branching. Catalase activity is positive, motility is observed in most strains, and all strains show a respiratory metabolism which proceeds slowly in oxidizing carbohydrates.

Genus *Leifsonia*

The former "*Corynebacterium aquaticum*" has been transferred into the new genus *Leifsonia* as *L. aquatica* (34). This species is the only medically relevant species in this genus. *L. aquatica* strains belong to the peptidoglycan B type actinomycetes and therefore cannot be true corynebacteria, which actually possess an A type of peptidoglycan (i.e., cross-linkage between positions 3 and 4 of the two peptide subunits). Diaminobutyric acid is the diamino acid of the cell wall peptidoglycan, and $C_{15:0ai}$ and $C_{17:0ai}$, as in microbacteria, are the main CFAs but represent <75% of all CFAs (65). The G+C content is about 70 mol%.

Gram staining shows thin rods. The strains are catalase and oxidase positive (the latter is an untypical feature for coryneform bacteria), always motile, and oxidizers.

Genus *Janibacter* and Other Unusual Coryneforms

Recent examinations of coryneform bacteria by using primarily sequence-based identification approaches have shown that additional genera could be recovered from human clinical materials.

Genus *Janibacter*

Strains of the genus *Janibacter* (91) were found to be associated with bacteremia and recovered from blood cultures (31, 88; Bernard, unpublished) and are the first medically relevant coryneforms reported from the family *Intrasporangiaceae*. *Janibacter* strains can have gram-variable or gram-positive coccoidal to rod forms in singles, pairs, or irregular clumps. The DNA base composition is 69 to 73 mol% G+C, with an unusual CFA profile consisting of significant volumes of CFAs $C_{16:0i}$, $C_{17:1}$, and $C_{17:0}$. These bacteria are described as oxidizers, with white, creamy, or yellowish pigments. They are nonmotile, and optimal growth may occur at 25 to 30°C.

Genus *Pseudoclavibacter*

The genus *Pseudoclavibacter* was described in 2004 for a previous "*Brevibacterium helvolum*" strain (90). The novel genus *Zimmermannella,* described shortly afterwards, is a later synonym of *Pseudoclavibacter* (87). *Z. alba* strains were recovered from urine, and *Z. bifida* strains were recovered from blood cultures and wounds. By Gram staining, these species were found to be short or medium gram-positive rods, with *Z. bifida* demonstrating some rudimentary branching. The DNA base composition is 62 to 68 mol% G+C. Major CFAs are $C_{15:0ai}$, $C_{16:0}$, $C_{16:0i}$, and $C_{17:0ai}$. These strains are oxidizers, with white or yellowish colonies. Optimal growth occurs at 30°C.

Genera *Brachybacterium* and *Knoellia*

Two isolates from the genus *Brachybacterium* and one strain from the genus *Knoellia*, all recovered from blood cultures, have been characterized (K. A. Bernard, unpublished observations). *Brachybacterium* spp. (15) are members of the familiy *Dermabacteraceae* and so are most closely related to the genus *Dermabacter*. Members of this genus grow at 37°C, exhibit gram-positive coccoidal and rodlike forms, and have a G+C content of 68 to 73 mol%. The *Brachybacterium* blood culture isolates were metabolically fermentative and had branched-chained-type CFAs. The genus *Knoellia* (68), like the genus *Janibacter,* is a member of the family *Intrasporangiaceae*. Cells are irregular gram-positive rods or cocci, with major CFAs of the branched-chain type, and the G+C content is 68 to 69 mol%. The single *Knoellia* blood culture isolate was capnophilic, growing best in 5% CO_2 at 37°C.

Genus *Arcanobacterium*

The genus *Arcanobacterium* presently contains six species, of which *A. haemolyticum, A. bernardiae,* and *A. pyogenes* have been recovered from human clinical specimens. Lysine is the diamino acid of the cell wall, whereas in the phylogenetically closely related *Actinomyces* spp. lysine or ornithine is found. Arcanobacteria contain menaquinones of the MK-9(H_4) type, whereas the *Actinomyces* spp. examined so far have MK-10(H_4). The main CFAs of arcanobacteria are $C_{16:0}$, $C_{18:1\omega9c}$, and $C_{18:0}$ (as in *Corynebacterium* spp. and *T. otitidis*), but in contrast to corynebacteria significant amounts of $C_{10:0}$, $C_{12:0}$, and $C_{14:0}$ may also be detected (3). The G+C content is 48 to 52 mol%.

Gram staining of arcanobacteria shows irregular gram-positive rods. All clinically relevant arcanobacteria are catalase negative, nonmotile, and fermenters.

NATURAL HABITAT

Many species of the corynebacteria are part of the normal flora of the skin and mucous membranes in humans and mammals. The habitat for some medically irrelevant corynebacteria (e.g., *C. terpenotabidum* and *C. halotolerans*) is the environment. It is noteworthy that not all corynebacteria are equally distributed over skin and mucous membranes but many of them occupy a specific niche. *C. diphtheriae* can be isolated from the nasopharynx as well as from skin lesions, which actually represent a reservoir for the spread of diphtheria. Important opportunistic pathogens like *C. amycolatum, C. striatum,* and *D. hominis* are part of the normal human skin flora but have thus far not been recovered from throat swabs from healthy individuals (144). Coryneform bacteria prominent in the oropharynx include *C. durum* and *R. dentocariosa* (144). *C. auris* and *T. otitidis* seem to have an almost exclusive preference for the external auditory canal (73). In nearly every instance that *C. macginleyi* has been isolated, it has been recovered from eye specimens (53). Another *Corynebacterium* species with a distinctive niche is *C. glucuronolyticum,* which is almost exclusively isolated from genitourinary specimens from humans (39) and also from animals (24). *C. urealyticum,* another genitourinary pathogen, has, like *C. jeikeium,* also been cultured from the inanimate hospital environment.

The natural habitat of arcanobacteria is not fully understood, but *A. haemolyticum* is recovered from throat as well as from wound swabs, whereas *A. bernardiae* has been found mainly in abscesses adjacent to skin (G. Funke, unpublished

FIGURE 1 Gram stain morphologies of *Corynebacterium diphtheriae* ATCC 14779 after 48 h of incubation (a), *Turicella otitidis* DSM 8821 (48 h) (b), *Dermabacter hominis* ATCC 51325 (48 h) (c), *Corynebacterium durum* DMMZ 2544 (72 h) (d), *Corynebacterium matruchotii* ATCC 14266 (24 h) (e), *Gardnerella vaginalis* ATCC 14018 (48 h) (f), *Corynebacterium aurimucosum* HC-NML 91-0032 (24 h) (g), and a black-pigmented *Rothia dentocariosa* HC-NML 77-0298 (24 h) (h).

observation). It is unclear whether the two species are part of the normal skin and/or the gastrointestinal flora. *A. pyogenes* is found on mucous membranes of cattle, sheep, and swine. Brevibacteria can be found on dairy products (e.g., cheese) but are also inhabitants of the human skin (40). Arthrobacters are some of the most frequently isolated bacteria when soil samples are cultured, but at least *Arthrobacter cumminsii* also seems to be present on human skin (54). Members of the genera *Exiguobacterium, Oerskovia, Cellulomonas, Cellulosimicrobium,* and *Microbacterium* have their habitats in the inanimate environment (e.g., soil and activated sludge). *Microbacterium* spp. have also been recovered from hospital environments (42). Curtobacteria are primarily plant pathogens (38).

CLINICAL SIGNIFICANCE

Estimating the clinical significance of coryneform bacteria isolated from clinical specimens is often confusing for clinical microbiologists. This is in part due to the natural habitat of coryneform bacteria, which may lead to their recovery if specimens were not taken correctly. The reader is referred to the guidelines on minimal microbiological requirements in publications on disease associations of coryneform bacteria (64).

Coryneform bacteria should be identified to the species level if they are isolated (i) from normally sterile body sites, e.g., blood (except if only one of multiple specimens became positive); (ii) from adequately collected clinical material if they are the predominant organisms; and (iii) from urine specimens if they are the only bacteria encountered and the bacterial count is $>10^4$/ml or if they are the predominant organisms and the total bacterial count is $>10^5$/ml.

The clinical significance of coryneform bacteria is strengthened by the following findings: (i) multiple specimens are positive for the same coryneform bacteria; (ii) coryneform bacteria are seen in the direct Gram stain, and a strong leukocyte reaction is also observed; and (iii) other organisms recovered from the same material are of low pathogenicity.

For a comprehensive summary of case reports on individual coryneform bacteria, the reader is referred to review articles (2, 64). The most frequently reported coryneforms, as well as their established disease associations, are listed in Table 3.

Historically, diphtheria caused by *C. diphtheriae* (or *C. ulcerans*) is the most prominent infectious disease for which coryneform bacteria are responsible. Therefore, special attention is given to that disease in this chapter. Due to immunization programs, the disease has nearly disappeared in countries with high socioeconomic standards. However, the disease is still endemic in some subtropical and tropical countries as well as among individuals of certain ethnic groups (e.g., indigenous peoples in the Americas and Australia). In the 1990s, diphtheria reemerged in the states of the former Soviet Union. However, despite increased global travel activities, only a few imported cases have been reported by countries with well-developed health care systems.

TABLE 3 Most frequently reported disease associations of coryneform bacteria in humans

Taxon	Disease or disease association	Reference(s)[a]
C. amycolatum	Wound infections, foreign body infections, bacteremia, sepsis, urinary tract infections, respiratory tract infections	46, 83
C. aurimucosum	Genitourinary tract infections (mainly females)	126, 127
CDC group F-1	Urinary tract infections	
CDC group G/*C. tuberculostearicum*	Catheter infections, bacteremia, endocarditis, wound infections, eye infections	
C. diphtheriae (toxigenic)	Throat diphtheria, cutaneous diphtheria	30
C. diphtheriae (nontoxigenic)	Endocarditis, foreign body infections, pharyngitis	25, 37, 111
C. glucuronolyticum	Genitourinary tract infections (mainly males)	39
C. jeikeium	Endocarditis, bacteremia, foreign body infections, wound infections	115
C. kroppenstedtii	Granulomatous lobular mastitis	106
C. macginleyi	Eye infections	53
C. minutissimum	Wound infections, urinary tract infections, respiratory tract infections	154
C. pseudodiphtheriticum	Respiratory tract infections, endocarditis	
C. pseudotuberculosis	Lymphadenitis (occupational)	
C. resistens	Bacteremia	103
C. riegelii	Urinary tract infections (females)	49
C. striatum	Wound infections, respiratory tract infections, foreign body infections	
C. ulcerans (toxigenic)	Respiratory diphtheria	
C. urealyticum	Urinary tract infections, bacteremia, wound infections	130
Arthrobacter spp.	Bacteremia, foreign body infections, urinary tract infections	44, 54
Brevibacterium spp.	Bacteremia, foreign body infections, malodorous feet	40
D. hominis	Wound infections, bacteremia	61
Rothia spp.	Endocarditis, bacteremia, respiratory tract infections	
Cellulomonas spp.	Bacteremia, wound infections	7, 55
Cellulosimicrobium sp.	Foreign body infections, bacteremia	8, 93
Microbacterium spp.	Bacteremia, foreign body infections, wound infections	42, 82, 85
A. bernardiae	Abscess formation (together with mixed anaerobic flora)	57
A. haemolyticum	Pharyngitis in older children, wound and tissue infections	89
A. pyogenes	Abscess formation, wound and soft tissue infections	
G. vaginalis	Bacterial vaginosis, endometritis, postpartum sepsis	10

[a] References for taxa without references are our observations. For further information, see references 2 and 64.

The main manifestation of diphtheria is an upper respiratory tract illness with a sore throat, dysphagia, lymphadenitis, low-grade fever, malaise, and headache. A nasopharyngeal adherent membrane which may occasionally lead to obstruction is characteristic. The severe systemic effects of diphtheria include myocarditis, neuritis, and kidney damage caused by the *C. diphtheriae* exotoxin, which is encoded by a bacteriophage carrying the *tox* gene. *C. diphtheriae* may also cause cutaneous diphtheria or endocarditis (with either toxin-positive or toxin-negative strains). Some people with poor hygienic standards (e.g., drug and alcohol abusers) are prone to colonization (on the skin more often than in the pharynx) by *C. diphtheriae* strains, which are often nontoxigenic.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

In general, coryneform bacteria do not need special handling when samples are collected.

C. diphtheriae

The diagnosis of diphtheria is primarily a clinical one. The physician should notify the receiving laboratory immediately of suspected diphtheria. In case of respiratory diphtheria, material for culture should be obtained on a swab (either a cotton- or a polyester-tipped swab) from the inflamed areas in the nasopharynx. Multisite sampling (nasopharynx) is thought to increase sensitivity. If membranes are present and can be removed (swabs from beneath the membrane are most valuable), they should also be sent to the microbiology laboratory (although *C. diphtheriae* might not be culturable from those in every instance). Nasopharyngeal swabs should be obtained from suspected carriers. It is preferable that the swabs be immediately transferred to the microbiology laboratory for culturing. If the swabs must be sent to the laboratory, semisolid transport media (e.g., Amies) ensure the maintenance of the bacteria. All coryneform bacteria are relatively resistant to drying and moderate temperature changes. Material from patients with suspected cases of wound diphtheria can be obtained by swab or aspiration.

After the appropriate isolation media have been inoculated (see "Isolation Procedures and Incubation" below), the swabs taken from diphtheritic membranes may be subjected to Neisser or Loeffler methylene blue staining (positive if metachromatic granules [polar bodies] are seen). However, it is noteworthy that the sensitivity of the microscopic examination is limited.

A PCR-based direct detection system for diphtheria toxin has been described by the Centers for Disease Control and Prevention (CDC) (98). Their system had the highest sensitivity when Dacron polyester-tipped swabs were used and when silica gel packages were stored at 4°C rather than at room temperature. The approach of a PCR-based direct detection system was successfully used in a retrospective study for which only formalin-fixed clinical specimens were available (80). However, direct detection for diphtheria toxin as the sole, primary test for clinical specimens has not been recommended, and microbiological culture is essential for confirming diphtheria (29).

Long-term preservation in skim milk at −70°C is applicable to all coryneform bacteria. The same skim milk tube except for those containing lipophilic corynebacteria can be thawed and put into the freezer again, and this can be done several times (Funke, unpublished). For nonlipophilic coryneforms, good results were also observed with Microbank tubes (Pro Lab Diagnostics, Austin, Tex.) (Funke, unpublished). The advantage of using these tubes is that individual beads can be taken out of the tube. Coryneform bacteria can also be stored for decades when they are kept lyophilized in an appropriate medium (e.g., 0.9% NaCl containing 2% bovine serum albumin).

ISOLATION PROCEDURES AND INCUBATION

Coryneform bacteria including *C. diphtheriae* can be readily isolated from a 5% sheep blood agar (SBA)-based selective medium containing 100 μg of fosfomycin per ml (plus 12.5 μg of glucose-6-phosphate per ml), since nearly all coryneforms (except *Actinomyces* spp. and *D. hominis*) are highly resistant to this compound (139, 144). It is also possible to put disks containing 50 μg of fosfomycin (plus 50 μg of glucose-6-phosphate [already incorporated in the disk]) (BD Diagnostics, Sparks, Md.) on a SBA plate and then examine the colonies which grow around the disk. Selective media for coryneform bacteria containing 50 to 100 μg of furazolidone/ml (Sigma, St. Louis, Mo.) have also been described. If lipophilic corynebacteria like *C. jeikeium* or *C. urealyticum* are sought, then 0.1 to 1.0% Tween 80 (Merck, Darmstadt, Germany) should be added to a SBA plate (add Tween 80 before pouring the medium). It is also possible to streak sterile filtered Tween 80 with a cotton swab onto SBA plates. Coryneform bacteria do not grow on MacConkey agar. However, if "coryneform" bacteria are recovered from this medium, they should be examined carefully to rule out rapidly growing mycobacteria.

With very few exceptions (some arthrobacters, microbacteria, and curtobacteria, which have optimal growth temperatures of between 30 and 35°C), the medically relevant coryneform bacteria all grow at 37°C. It is desirable to culture specimens for coryneform bacteria in a CO_2-enriched atmosphere, since some taxa, e.g., *Rothia* and *Arcanobacterium* spp., grow much better under those conditions. Nearly all medically relevant coryneform bacteria grow within 48 h, so that primary culture plates should not be incubated longer than that. However, if liquid media are used (e.g., for specimens from normally sterile body sites), these should be checked after 5 days by Gram staining for the presence of coryneform bacteria (only if growth is observed with the naked eye) before they are discarded.

It is recommended that urine specimens be incubated for longer than 24 h to check for the presence of *C. urealyticum* but only when patients are symptomatic or have alkaline urine or struvite crystals in their urine sediment.

C. diphtheriae

The primary plating media for the cultivation of *C. diphtheriae* should be SBA plus one selective medium (e.g., Cystine-Tellurite blood agar [CTBA] or freshly prepared Tinsdale medium) (29, 30). If silica gel is used as a transport medium, the desiccated swabs need to be additionally incubated overnight in broth (supplemented with either plasma or blood), which should then be streaked onto the primary plating medium. The plates are read after 18 to 24 h of incubation at 37°C, preferably in a 5% CO_2-enriched atmosphere. Tellurite inhibits the growth of many noncoryneform bacteria, but even a few *C. diphtheriae* strains are sensitive to potassium tellurite and will therefore not grow on CTBA but may grow on SBA. It is noteworthy that growth on CTBA and tellurite reduction are not specific for *C. diphtheriae,* since many other coryneforms may also produce black (albeit smaller) colonies. The best medium for direct culturing of *C. diphtheriae* is probably Tinsdale medium (30).

However, the limitations of Tinsdale medium are its relatively short shelf life (<4 weeks) and the necessity to add horse serum to it. On Tinsdale plates, both tellurite reductase activity (as shown by black colonies) and cystinase activity (as shown by a brown halo around the colonies) can be observed (see Fig. 3i). If neither CTBA nor Tinsdale medium is available, colistin-nalidixic acid blood agar plates are recommended for the isolation of C. *diphtheriae* or any other coryneform bacterium. It is necessary to pick multiple colonies from CNA plates to rule out C. *diphtheriae* (first Gram staining, then subculturing, and then subsequent biochemical testing). Nonselective Loeffler serum slants are no longer recommended for the primary isolation of C. *diphtheriae* because of overgrowth by other bacteria (but C. *diphtheriae* cells with polar bodies are produced on Loeffler or Pai slants only).

IDENTIFICATION AND TYPING SYSTEMS

Basic tests available in every microbiology laboratory are of great value for the identification of coryneform bacteria. The Gram staining morphology of the cells can exclude the assignment to many genera and may even lead to the assignment to the correct genus (e.g., to the genus *Corynebacterium, Turicella*, or *Dermabacter*) (Fig. 1). Morphology, size, pigment, odor, and hemolysis of colonies are also valuable criteria in the differential diagnosis of coryneform bacteria.

von Graevenitz and Funke (143) had outlined a biochemical identification system for coryneform bacteria which was based on previous results from the CDC Special Bacteriology Reference Laboratory (71). This system includes the following reactions: catalase, test for fermentation or oxidation (in our experience, this is best observed in semisolid cystine Trypticase agar medium [rather than on triple sugar iron or oxidation-fermentation media], with fermentation indicated by acid or alkali production in the entire tube and oxidation found at the surface of the tube); motility; nitrate reduction (24-h incubation); urea hydrolysis (24-h incubation); esculin hydrolysis (up to 48 h of incubation); acid production from glucose, maltose, sucrose, mannitol, and xylose (48-h incubation); CAMP reaction (24-h incubation) with a β-hemolysin-producing strain of *Staphylococcus aureus* (e.g., strain ATCC 25923), i.e., positive reaction indicated by an augmentation of the effect of *S. aureus* β-hemolysin on erythrocytes, resulting in a complete hemolysis in an arrowhead configuration (Fig. 2); and a test for lipophilia (24-h incubation), which is performed only for catalase-positive colonies <0.5 mm in diameter. For the test for lipophilia, colonies are subcultured onto ordinary SBA and onto a 0.1 to 1% Tween 80-containing SBA plate. Lipophilic corynebacteria develop colonies up to 2 mm in diameter after 24 h on Tween-supplemented agar. It has also been suggested that growth in brain heart infusion broth with and without supplementation of 1% Tween 80 be compared and strains which grow only in the supplemented broth can be called lipophilic. The identification protocols given in this chapter are, in principle, based on the identification system of von Graevenitz and Funke (143) mentioned above (Tables 4 and 5).

The currently recommended identification systems include the API (RAPID) Coryne system (bioMérieux, Marcy l'Etoile, France) and the RapID CB Plus system (Remel, Lenexa, Kans.). The API Coryne system contains 49 taxa in its present database (version 2.0). In a comprehensive multicenter study, it was found that 90.5% of the strains belonging to the taxa included were correctly identified, with additional tests needed for correct identification for 55.1% of all strains tested (60). The results were highly reproducible if the manufacturer's recommendations for use were rigorously followed. It was concluded that the system is a useful tool for the identification of the diverse group of coryneform bacteria encountered in the routine clinical laboratory. The RapID CB Plus system correctly identified 80.9% of the strains to the genus and the species levels, and an additional 12.2% strains were correctly identified to the genus level but with less accurate species designations; it was also concluded that this system may perform well under the conditions of a routine clinical laboratory (58). However, it is always important to question critically the identifications provided by any commercial identification system and to correlate the results with simple basic characteristics such as macroscopic morphology and Gram staining results. Furthermore, it is important to note that for both commercially available identification systems the databases have not been updated since 1997 and therefore the recently described taxa are not covered.

For some identifications, the commercial API 50CH system (bioMérieux) has been found to be useful. For example, when applying the AUX medium (usually attached to the kit

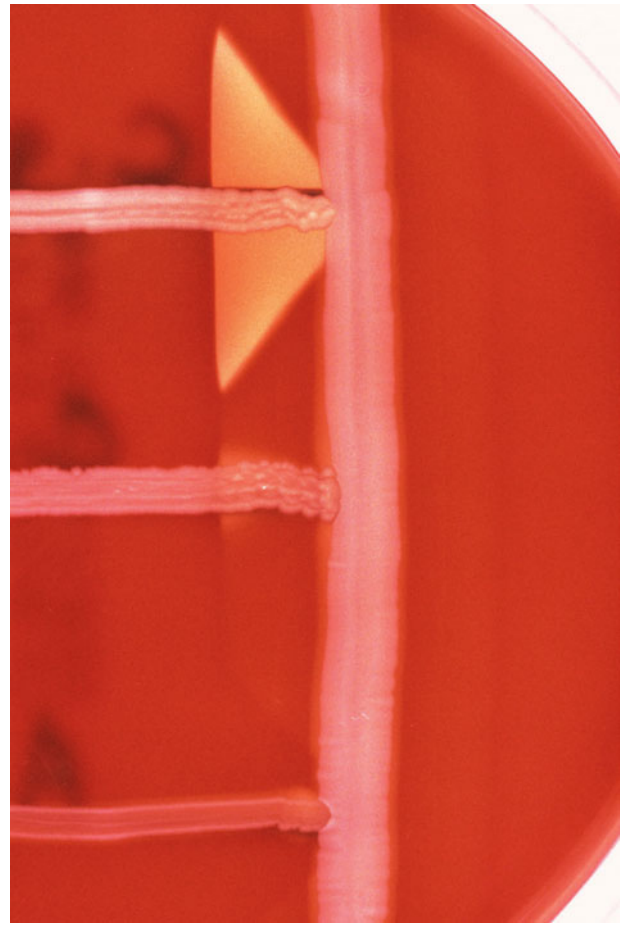


FIGURE 2 CAMP reactions of different coryneform bacteria after 24 h. (Top) *C. glucuronolyticum* DMMZ 891 (positive reaction). (Middle) *C. diphtheriae* ATCC 14779 (negative reaction). (Bottom) *A. haemolyticum* ATCC 9345 (CAMP inhibition reaction). The vertical streak is *S. aureus* ATCC 25923.

TABLE 4 Identification of medically relevant *Corynebacterium* spp.[a]

Species	Fermentation/ oxidation	Lipophilism	Nitrate reduction	Urease	Esculin hydrolysis	Pyrazin-amidase	Alkaline phos-phatase	Acid production from:					CAMP reaction	Other traits
								Glucose	Maltose	Sucrose	Mannitol	Xylose		
C. *accolens*	F	+	+	−	−	V	−	+	−	V	V	−	−	
C. *afermentans* subsp. *afermentans*	O	−	−	−	−	+	+	−	−	−	−	−	V	
C. *afermentans* subsp. *lipophilum*	O	+	−	−	−	+	+	−	−	−	−	−	V	
C. *amycolatum*	F	−	V	V	−	+	+	+	V	V	−	−	−	Most O/129 resistant, propionic detected
C. *appendicis*	F	+	−	+	−	+	+	+	+	−	−	−	ND	Huge amounts of TBSA present
C. *argentoratense*	F	−	−	−	−	+	V	+	−	−	−	−	−	Chymotrypsin may be positive; propionic detected[b]
C. *atypicum*	F	−	−	−	−	−	−	+	+	+	−	−	ND	Pinpoint colonies, β-glucuronidase positive
C. *aurimucosum*	F	−	−	−	V	+	+	+	+	+	−	−	ND	Most strains exhibit grayish black pigment, some pitting agar
C. *auris*	O	−	−	−	−	+	+	−	−	−	−	−	+	Slight adherence to agar, cleaved mycolics
C. *bovis*[c]	F	+	−	−	−	−	+	+	−	−	−	−	−	TBSA positive; fructose positive
C. *confusum*	F	−	+	−	−	+	+	(+)	−	−	−	−	−	Tyrosine negative, propionic detected
C. *coyleae*	F	−	−	−	−	+	+	(+)	−	−	−	−	+	
CDC group F-1	F	+	V	+	−	+	−	+	+	+	−	−	−	
CDC group G	F	+	V	−	−	+	+	+	V	V	−	−	−	Fructose positive, anaerobic growth positive
C. *diphtheriae* biotype *gravis*	F	−	+	−	−	−	−	+	+	−	−	−	−	Glycogen positive, propionic detected
C. *diphtheriae* biotype *intermedius*	F	+	+	−	−	−	−	+	+	−	−	−	−	Propionic detected

(Continued on next page)

TABLE 4 Identification of medically relevant *Corynebacterium* spp.[a] *(Continued)*

Species	Fermentation/ oxidation	Lipophilism	Nitrate reduction	Urease	Esculin hydrolysis	Pyrazin-amidase	Alkaline phos-phatase	Acid production from:					CAMP reaction	Other traits
								Glucose	Maltose	Sucrose	Mannitol	Xylose		
C. *diphtheriae* biotype *mitis* and *belfanti*	F	−	+/−[d]	−	−	−	−	+	+	−	−	−	−	Glycogen negative, propionic detected
C. *durum*	F	−	+	(V)	(V)	+	−	+	+	+	V	−	−	Adherent to agar, propionic detected
C. *falsenii*	F	−	−	(+)	−	(+)	+	(+)	V	−	−	−	−	Yellowish
C. *freneyi*	F	−	V	−	−	+	+	+	+	+	−	−	ND	α-Glucosidase positive, grows at 20 and 42°C
C. *glucuronolyticum*	F	−	V	V	V	+	V	+	V	+	−	V	+	β-Glucuronidase positive, propionic detected
C. *imitans*	F	−	−	−	−	(+)	+	+	+	(+)	−	−	+	Tyrosine negative, O/129 resistant
C. *jeikeium*	O	+	−	−	−	+	+	+	V	−	−	−	−	Fructose negative, anaerobic growth negative
C. *kroppenstedtii*	F	+	−	−	+	+	−	+	V	+	−	−	−	Lacking mycolic acids, propionic detected
C. *lipophiloflavum*	O	+	−	−	−	+	+	−	−	−	−	−	−	Yellow
C. *macginleyi*	F	+	+	−	−	−	+	+	−	+	V	−	−	
C. *matruchotii*	F	−	+	−	V	+	−	+	+	+	−	−	−	"Whip handle" (upon Gram staining); propionic detected
C. *minutissimum*	F	−	−	−	−	+	+	+	+	V	V	−	−	Tyrosine positive
C. *mucifaciens*	O	−	−	−	−	+	+	+	−	V	−	−	−	Very mucoid yellowish colonies
C. *propinquum*	O	−	+	−	−	V	V	−	−	−	−	−	−	Tyrosine positive
C. *pseudodiphtheriticum*	O	−	+	+	−	+	V	−	−	−	−	−	−	
C. *pseudotuberculosis*	F	−	V	+	−	−	V	+	+	V	−	−	REV	Propionic detected
C. *resistens*	F	+	−	−	−	−	+	+	−	−	−	−	−	Slow growth in anaerobic atmosphere
C. *riegelii*	F	−	−	+	−	V	V	−	(+)	−	−	−	−	
C. *singulare*	F	−	−	+	−	+	+	+	+	+	−	−	−	Tyrosine positive
C. *simulans*[e]	F	−	+	−	−	V	+	+	−	+	−	−	−	Reduces nitrite
C. *striatum*	F	−	+	−	−	+	+	+	−	V	−	−	V	Tyrosine positive
C. *sundsvallense*	F	−	−	+	−	V	V	+	+	+	−	−	−	Sticky colonies
C. *thomssenii*	F	−	−	+	−	+	+	+	+	+	−	−	−	*N*-Acetyl-β-glucosaminidase positive, sticky colonies

C. tuberculostearicum	F	+	V	–	–	+	V	+	V	V	–	–	ND	
C. tuscaniae	O	–	–	–	–	+	+	+	+	–	–	–	–	Hippurate positive, tyrosine negative
C. ulcerans	F	–	–	+	–	–	+	+	+	–	–	–	REV	Glycogen positive, propionic detected
C. urealyticum	O	+	–	+	–	+	V	–	–	–	–	–	–	
C. xerosis	F	–	V	–	–	+	+	+	+	+	–	–	–	O/129 susceptible, propionic not detected

[a] Abbreviations and symbols: F, fermentation; O, oxidation; +, positive; –, negative; V, variable; ( ), delayed or weak reaction; ND, no data; REV, CAMP inhibition reaction.
[b] Propionic acid as a glucose fermentation product.
[c] Blood culture isolate (4) was also ONPG positive, oxidase positive, weakly maltose positive but negative by API Coryne, propionic acid was not detected; β-galactosidase was not observed using two methods (API Coryne, API Zym); API Coryne code obtained, 0101104.
[d] *C. diphtheriae* biotype *mitis* is nitrate reductase positive, and *C. diphtheriae* biotype *belfanti* is nitrate reductase negative.
[e] *C. simulans* (145) is a strong nitrite reducer at low and high concentrations; nitrate reduction may appear to be negative unless further tested using zinc dust; one strain was catalase negative (4).

for gram-negative nonfermenters [bioMérieux]) to the API 50CH system, utilization reactions which allow the differentiation of *Brevibacterium* spp. or some *Arthrobacter* spp. can be observed (40, 44).

A reference laboratory would also use chromatographic techniques for further characterization of coryneform bacteria. The presence of mycolic acids and their chain lengths can be detected by thin-layer chromatography (TLC), gas chromatography and mass spectrometry, or high-performance liquid chromatography (22). These methods can be useful for the differentiation of *Corynebacterium* spp. (mycolic acids of 22 to 36 carbon atoms) from the partially acid-fast bacteria (mycolic acids of 30 to 78 carbon atoms) but may also provide evidence that a coryneform bacterium is not a *Corynebacterium* (exceptions are *C. amycolatum*, *C. atypicum*, and *C. kroppenstedtii*) if mycolic acids are not detected. The detection of the diamino acid of the peptidoglycan by one-dimensional TLC is of certain value for determining the genus to which a particular strain belongs (Table 1). In some cases, partial hydrolysates of the peptidoglycan are separated by two-dimensional TLC to reveal the interpeptide bridge of the peptidoglycan in order to distinguish between genera having the same diamino acid in the peptide moiety. For example, some of the yellow-pigmented microbacteria and all curtobacteria have ornithine as their diamino acids, but microbacteria have (glycine)$_{1-3}$-ornithine as the interpeptide bridge, whereas curtobacteria possess ornithine only.

The analysis of CFAs by means of gas-liquid chromatography with the Sherlock system (MIDI Inc., Newark, Del.) is an extremely useful method for the identification of coryneform bacteria. This system is, in general, able to identify coryneform bacteria to the genus level, but identification to the species level is in most cases impossible, although the commercial database suggests that it is possible. This is because of the very closely related CFA profiles of coryneform bacteria belonging to the same genus (3) and because the quantitative profiles observed depend strongly on the incubation conditions. When a laboratory creates its individual database based on its own entries, species identification becomes possible in some cases (Bernard, unpublished). The mycolic acids of some corynebacteria (e.g., *C. auris*) are cleaved at the temperature (300°C) produced in the injection port of the system, resulting in fatty acids which were identified as, e.g., $C_{17:1\omega6c}$ to $C_{\omega9c}$, by the Sherlock system (47).

Molecular genetic-based identification systems for coryneform bacteria have been outlined in recent years. Restriction fragment length polymorphism analysis of the partly amplified and digested 16S rRNA gene has been demonstrated to be of use for the identification of species within the genera *Corynebacterium* and *Brevibacterium* (9, 140). Some corynebacteria may also be identified to the species level by examination of the length of the 16S-23S rRNA intergenic spacer region (1). rRNA gene restriction fragment polymorphism analysis (ribotyping) has been demonstrated to allow the identification of corynebacteria if three different restriction enzymes (BstEII, SmaI, and SphII) are used (5). Another interesting approach for the identification of true corynebacteria is the sequencing of a 434- to 452-bp fragment of the *rpoB* gene (using primers designated C2700F and C3130R), since this particular region of the gene displays a high degree of polymorphism within the genus *Corynebacterium* (77, 78). This approach for molecular identification needs to be evaluated with other genera. For pure taxononomic investigations of coryneform bacteria and in cases of growth of coryneform bacteria from difficult to obtain clinical material (122), full-length 16S rRNA gene

TABLE 5 Identification of medically relevant coryneform bacteria other than *Corynebacterium* spp.[a]

Taxon	Catalase	Fermentation/ oxidation	Motility	Nitrate reduction	Urease	Esculin hydrolysis	Acid production from:					Other traits
							Glucose	Maltose	Sucrose	Mannitol	Xylose	
Turicella otitidis	+	O	−	−	−	−	−	−	−	−	−	CAMP reaction positive, long rods
Arthrobacter spp.	+	O	V	V	V	V	V	V	V	−	−	
Brevibacterium spp.	+	O	−	V	−	−	V	V	V	−	−	Odor cheese-like
Dermabacter hominis	+	F	−	−	−	+	+	+	+	−	V	Small rods
Rothia dentocariosa	V	F	−	+	−	+	+	+	+	−	−	Some strains adherent, grayish-black-pigmented strains exist
Exiguobacterium acetylicum	+	F	+	V	−	+	+	+	+	+	−	Golden yellow pigment
Oerskovia turbata	+	F	V	+	−	+	+	+	+	−	+	Xanthine not hydrolyzed
Cellulomonas spp.	+	F	V	+	−	+	+	+	+	V	+	
Cellulosimicrobium spp.	+	F	V	V	V	+	+	+	+	−	+	Hydrolysis of xanthine
Microbacterium spp.	V	F/O	V	V	V	V	+	+	V	V	V	
Curtobacterium spp.	+	O	V	−	−	+	+	V	V	V	+	
Leifsonia aquatica	+	O	+	V	−	V	+	V	V	+	+	
Arcanobacterium haemolyticum	−	F	−	−	−	−	+	+	V	−	−	CAMP inhibition reaction
Arcanobacterium pyogenes	−	F	−	−	−	V	+	V	V	V	+	
Arcanobacterium bernardiae	−	F	−	−	−	−	+	+	−	−	−	Glycogen positive
Gardnerella vaginalis	−	F	−	−	−	−	+	+	V	−	−	Decolorized cells in Gram stain

[a] Abbreviations and symbols: +, positive reaction; −, negative reaction; V, variable reaction; O, oxidation; F, fermentation.

sequencing might be indicated. Determination of the complete 16S rRNA gene sequence is a rational approach for identifying corynebacteria, since nearly all established species exhibit 3% or greater divergence except for *C. afermentans, C. coyleae,* and *C. mucifaciens* (<2%), *C. aurimucosum, C. minutissimum,* and *C. singulare* (<2%), *C. sundsvallense* and *C. thomssenii* (<1.5%), and *C. ulcerans* and *C. pseudotuberculosis* (<2%) (2). In very few selected cases, quantitative DNA-DNA hybridizations might be necessary but will be clearly restricted to the reference laboratory. Because of the ever-growing number of coryneform taxa encountered in clinical specimens, it becomes more difficult to readily differentiate all taxa biochemically, so it appears that sequencing studies are most likely to replace some of the biochemical testing in the near future.

The commercial MicroSeq 500 16S bacterial sequencing kit (Perkin-Elmer, Foster City, Calif.) has been applied to the identification of coryneform bacteria, but discordant results (with extensive phenotyping as the "gold standard") were observed for >30% of the *Corynebacterium* isolates, mainly because of the present database of the commercial system (136). However, due to the short running time of approximately 15 to 19 h, this and other similar systems will further spread to the routine clinical laboratory, in which case direct costs should drop and databases will be improved.

It is emphasized that unidentifiable, clinically significant coryneform bacteria should be sent to an established reference laboratory experienced in corynebacterial identification.

DESCRIPTIONS OF GENERA AND SPECIES

Genus *Corynebacterium*

C. accolens

C. accolens (101) is found in specimens from eyes, ears, the nose, and the oropharynx. Endocarditis of native aortic and mitral valves due to this agent has been described. Colonies are, as for all other lipophilic corynebacteria, convex, smooth, and <0.5 mm in diameter on SBA. *C. accolens* strains had initially been described to exhibit satellitism in the vicinity of *S. aureus* strains, attributable to its lipophilism (for the recommended method to demonstrate lipophilism, see "Identification and Typing Systems" above). *C. accolens* has a variable pyrazinamidase reaction but is negative for alkaline phosphatase, thus differentiating it from the morphologically and biochemically closely related CDC group G bacteria (Table 4). The API Coryne and RapID CB Plus systems correctly identify *C. accolens* (58, 60). *C. accolens* strains are susceptible to a broad spectrum of antibiotics.

C. afermentans subsp. *afermentans*

C. afermentans subsp. *afermentans* (116) is part of the normal human skin flora and has so far been isolated mainly from blood cultures. Colonies are whitish, convex with regular edges, creamy, and about 1 to 1.5 mm in diameter after 24 h of incubation. *C. afermentans* subsp. *afermentans* has an oxidative metabolism. The API Coryne system provides the numerical code of 2100004 for this species. About 60% of all strains of this taxon are CAMP reaction positive. *C. afermentans* subsp. *afermentans* can be differentiated from *C. auris* and *T. otitidis* (both of which give the same API numerical code) by the consistency of its colonies (*C. auris* is slightly adherent to agar) and morphology on Gram staining (*T. otitidis* has longer cells). Further differential reactions include the carbohydrate utilization reactions tested with

either the Biolog GP plate (Biolog, Hayward, Calif.) or the bioMérieux biotype 100 gallery (47). By chemotaxonomic means, both *C. afermentans* subspecies and *C. auris* contain mycolates, whereas *T. otitidis* lacks them, but these techniques are not applicable in a routine clinical laboratory. *C. afermentans* subsp. *afermentans* is generally susceptible to β-lactam antibiotics.

C. afermentans subsp. *lipophilum*

Strains belonging to the species *C. afermentans* subsp. *lipophilum* (116) have been isolated mainly from blood cultures as well as from superficial wounds. Colonies are, typically for lipophilic corynebacteria, convex, smooth, and <0.5 mm in diameter after 24 h. *C. afermentans* subsp. *lipophilum* has an oxidative metabolism and does not produce acid from any of the carbohydrates usually tested (Table 4). It is the only species of lipophilic corynebacteria which may exhibit a positive CAMP reaction. *C. afermentans* subsp. *lipophilum* is not included in the API Coryne database. The numerical profile observed for the species is 2100004, and so by that method it cannot be discerned from more robustly growing *C. afermentans* subsp. *afermentans, C. auris,* or *T. otitidis*. Strains are usually susceptible to β-lactam antibiotics.

C. amycolatum

C. amycolatum is part of the normal human skin flora but was not recovered from throat swabs from healthy persons (144). *C. amycolatum* is the most frequently encountered *Corynebacterium* species in human clinical material (64). It is also the most frequently isolated nonlipophilic *Corynebacterium* in dairy cows with mastitis (74). *C. amycolatum* strains are often multidrug resistant (59). Colonies are very typically dry, waxy, and grayish white with irregular edges and are 1 to 2 mm in diameter after 24 h of incubation. *C. amycolatum* actually has a fermentative metabolism, but when CTA media are used for the observation of acid production from carbohydrates, *C. amycolatum* appears to resemble an oxidizer (i.e., main acid production at the surface of the medium). Strains of *C. amycolatum* are remarkable for their variability in basic biochemical reactions (Table 4) and had been often misidentified in the past as the biochemically similar species *C. xerosis, C. striatum,* or *C. minutissimum* (46, 148, 154). These four species can be differentiated by the following reactions: *C. amycolatum* and *C. minutissimum* do not grow at 20°C, but *C. xerosis* and *C. striatum* do; in addition, *C. xerosis* does not ferment glucose at 42°C whereas the other three species do, and *C. minutissimum* and *C. striatum* produce alkali from formate but *C. amycolatum* and *C. xerosis* do not (148). When tested on Mueller-Hinton agar supplemented with 5% sheep blood, nearly all *C. amycolatum* strains were resistant to the vibriocidal compound O/129 (150-μg disks) (Oxoid, Basingstoke, United Kingdom), as indicated by no zone of inhibition around the disk (46). In contrast, only 4% of all *C. amycolatum* strains were resistant to O/129 when tested on Mueller-Hinton agar with 5% horse blood (83). The API Coryne system identifies this species very well, but in every case additional reactions must be carried out in order to confirm the identification of *C. amycolatum* (60). All *C. amycolatum* strains produce propionic acid as the major end product of glucose metabolism. In contrast to many other corynebacteria, *C. amycolatum* exhibits only weak or no leucine arylamidase activity. The identification may also be suggested by the absence of mycolic acids. In addition, it may be shown that acyl phosphatidylglycerol is a major phospholipid in *C. amycolatum,* in contrast to other *Corynebacterium* spp., in which other phospholipids are predominant.

C. appendicis

The one strain of *C. appendicis* described in the literature was isolated from a patient with appendicitis accompanied with abscess formation (151). This lipophilic species contains huge amounts of TBSA (up to 50% of all CFAs) not seen in any other *Corynebacterium* species. It is differentiated from CDC coryneform group F-1 bacteria by a positive alkaline phosphatase reaction but negative reactions for nitrate reduction and sucrose fermentation.

C. argentoratense

C. argentoratense (118) has been isolated from the human throat as well as, in one instance, from a blood culture (4). Colonies are cream colored, nonhemolytic, slightly rough, and 2 mm in diameter after 48 h of incubation. Phenotypically, *C. argentoratense* may appear to be very similar to (rare) ribose negative strains of *C. coyleae*. However, glucose fermentation by *C. argentoratense* is quite rapid compared to the slowly fermenting species *C. coyleae.* As well, CAMP-negative *C. argentoratense* produces propionic acid as a fermentation product, but CAMP-positive *C. coyleae* does not (4, 56). *C. argentoratense* is the only medically relevant *Corynebacterium* species expressing α-chymotrypsin activity, which can be observed in the API ZYM (bioMérieux) system; however, the blood culture isolate was not observed to produce that enzyme (4). Although *C. argentoratense* is phylogenetically closely related to *C. diphtheriae,* it does not harbor the *tox* gene coding for the diphtheria toxin.

C. atypicum

Although this species clearly belongs to the genus *Corynebacterium,* corynomycolic acids, like in *C. amycolatum* and *C. kroppenstedtii,* are not detected (69). *C. atypicum* is not lipophilic but shows only pinpoint colonies after 48 h of incubation. It is the only medically relevant *Corynebacterium* not expressing pyrazinamidase but β-glucuronidase activity.

C. aurimucosum

The initial description of *C. aurimucosum* was based on a single strain which exhibited slightly yellow and sticky colonies on 5% SBA plates but on Trypticase soy agar without blood had colorless and slimy colonies (150). The basic biochemical profile of this particular *C. aurimucosum* strain was similar to that of *C. minutissimum*. The number of *C. aurimucosum* strains was significantly enhanced when it was demonstrated that some former CDC coryneform group 4 bacteria actually belong to *C. aurimucosum* (21). It is important to note that many strains of *C. aurimucosum* exhibit a grayish black pigment that is not seen in any other true *Corynebacterium*. Strains that were originally designated "*C. nigricans*" (126) were shown to be a later synonym of *C. aurimucosum* (21). Some strains of *R. dentocariosa* can also exhibit a charcoal black pigment (21); these strains are differentiated from *C. aurimucosum* by being constantly nitrate reductase positive, a possible negative catalase reaction, and having branched-chain CFAs as opposed to straight-chain-type CFAs for *C. aurimucosum*. API Coryne codes for pigmented *C. aurimucosum* strains include 0000125, 2000125, and 2100327 (126).

C. auris

C. auris (47) has been isolated almost exclusively from the ear region. Colonies are dryish, are slightly adherent to but do not penetrate agar, become slightly yellowish with time, and have diameters ranging from 1 to 2 mm after 48 h of incubation. *C. auris* does not produce acid from any carbohydrates usually tested. However, utilization reactions applying either

the Biolog GP plate or the bioMérieux biotype 100 system may help in distinguishing *C. auris* from *C. afermentans* subsp. *afermentans* and *T. otitidis,* but in the clinical routine laboratory this can also be achieved by morphologic differentiation (see "*C. afermentans* subsp. *afermentans*"). All *C. auris* strains are strongly CAMP test positive. The API Coryne system provides the numerical code 2100004 for this species. Abundant degradation products of mycolic acids are indirectly observed when CFA patterns are determined with the Sherlock system (47). It is noteworthy that the MICs of β-lactam antibiotics for *C. auris* strains are elevated, but the molecular mechanism for this is not known at present (59).

C. bovis

Occasionally, but not in the recent era, human infections had been attributed to the lipophilic bovine species, *C. bovis*. Characterization of lipophilic-like corynebacteria based solely on the use of phenotypic tests was probably incorrect in the absence of modern polyphasic methods or identification schemes such as those found in Table 4. This species had not been definitively recovered for many years from human clinical material, as previously reviewed (64). Recently, however, a human blood culture isolate of *C. bovis* was identified based on a polyphasic approach, including phenotypic, chemotaxonomic, and genotypic characteristics, with an API Coryne code of 0101104 (4).

C. confusum

C. confusum has been isolated from patients with foot infections, a blood culture (52), and a breast abscess (4). Colonies are whitish, glistening, convex, creamy, and up to 1.5 mm in diameter after 48 h. Acid from glucose is produced only very weakly, becoming visible in the API Coryne or the API 50CH gallery only after 48 to 72 h. Weak growth under anaerobic conditions corresponds to slow fermentative acid production. It is advisable to incubate the API Coryne system after 24 h for another day in those cases in which the results for acid production are ambiguous (i.e., only a slight change in the color of the indicator). After 48 h of incubation, the APl Coryne system provides the numerical code 3100304 for this species; the breast abscess strain had a code of 3100104. Interestingly, the breast abscess strain was also CAMP positive, making it potentially more difficult to discern from *C. coyleae* isolates (4). *C. confusum* is correctly identified by the RapID CB Plus system (58). If glucose fermentation is judged to be negative, *C. confusum* strains can be misidentified as *C. propinquum.* However, in contrast to that species, *C. confusum* does not hydrolyze tyrosine and contains small amounts of TBSA (1 to 3%), whereas *C. propinquum* hydrolyzes tyrosine but does not contain TBSA. *C. confusum* is differentiated from *C. coyleae* and *C. argentoratense* by its ability to reduce nitrate.

C. coyleae

C. coyleae (56) has been isolated mainly from cultures of blood and other normally sterile body fluids, but it may also be recovered from genitourinary specimens (4; Funke, unpublished). Colonies are whitish and slightly glistening with entire edges and are about 1 mm in diameter after 24 h. The consistency of the colonies is either creamy or sticky. A slow fermentative acid production from glucose and a strongly positive CAMP reaction are the most significant phenotypic characteristics. *C. coyleae* is positive for cystine arylamidase, which is not observed for many other corynebacteria. Various API Coryne numerical codes have been observed, especially 2100304 and 6100304. *C. coyleae* is always positive for ribose fermentation, whereas the biochemically similar species *C. argentoratense* is variable for this reaction. The API Coryne database lists only 6% glucose-fermenting *C. coyleae* strains, and therefore, when applying this commercial identification system, the clinical microbiologist may not receive a correct identification (60). However, the two numerical profiles given above combined with a positive CAMP reaction are highly indicative of *C. coyleae.* This species is correctly identified by the RapID CB Plus system (58).

CDC Group F-1 Bacteria

CDC group F-1 bacteria (119) have not been given a species name. Although genetically distinct, no distinguishing phenotypic markers that clearly allow their separation from other defined *Corynebacterium* spp. have been found. The characteristics of the CDC group F-1 bacteria are consistent with the definition of the genus *Corynebacterium* in all respects. The strains are lipophilic and are the only lipophilic fermentative *Corynebacterium* species able to hydrolyze urea. Of note is the negative alkaline phosphatase reaction (Table 4). CDC group F-1 strains are usually susceptible to penicillin but are often resistant to macrolides.

CDC Group G Bacteria

CDC group G bacteria possess all chemotaxonomic features of true corynebacteria but cannot be given a species name since it has so far been impossible to find phenotypic traits allowing for a unanimous definition (119). At least some strains of CDC group G have been found to be consistent with *C. tuberculostearicum* (Bernard, unpublished). These lipophilic strains can be separated from *C. jeikeium* by anaerobic growth and fermentative acid production from fructose (115). Further biochemical features of CDC group G bacteria are given in Table 4. The API Coryne system correctly identifies CDC group G bacteria. They might be multidrug resistant, but the most frequently observed resistance is to macrolides and lincosamides.

C. diphtheriae

In 2003, the complete genome sequence of a *Corynebacterium diphtheriae* strain representative for the diphtheria outbreak in the former Soviet Union states in the 1990s was determined (11). The genome consists of a single circular chromosome of 2,488,635 bp with no plasmids. A complete set of enzymes for the glycolysis, gluconeogenesis, and pentose-phosphate pathways are present, as well as all the de novo amino acid biosynthesis pathways. Fimbrial and fimbria-related genes and sialidase (neuraminidae) genes, as well as iron uptake systems, have been detected as pathogenicity factors.

C. diphtheriae is commonly divided into four biotypes, *gravis, mitis, belfanti*, and *intermedius*; biotype differentiation is recommended by WHO (29, 30), although biotypes cannot be assigned separate subspecies status (120), nor is biotyping satisfactory for epidemiologic tracking. Initially, these biotypes were defined by differences in colony morphology and biochemical reactions (Table 4). However, only *C. diphtheriae* biotype *intermedius* can be identified on the basis of colonial morphology (small, gray, or translucent lipophilic colonies) (20) as well as positive dextrin fermentation. Other *C. diphtheriae* biotypes produce larger (up to 2 mm after 24 h) white or opaque colonies (Fig. 3b), which are indistinguishable from one another. The lipophilic *C. diphtheriae* biotype *intermedius* occurs only rarely in clinical infections, and *C. diphtheriae* biotype *belfanti* strains almost never harbor the diphtheria toxin gene.

Presumptive identification of *C. diphtheriae* (as well as of *C. pseudotuberculosis* and *C. ulcerans*) may be made by testing suspicious gram-positive rods for the presence of cystinase (as detected by using Tinsdale medium or diagnostic tablets [Rosco, Taastrup, Denmark]) and the absence of pyrazinamidase (diagnostic tablets are available from Key Scientific Products, Round Rock, Tex.). The API Coryne system identifies *C. diphtheriae* strains, with additional tests needed for the differentiation of *C. diphtheriae* biotype *mitis,* *C. diphtheriae* biotype *belfanti,* and *C. diphtheriae* biotype *intermedius* (60). Usually, *C. diphtheriae* strains do not ferment sucrose, but in Brazil sucrose-positive strains have been described (23). Large amounts of propionic acid are produced as the end product of glucose metabolism (36). *C. diphtheriae* strains are distinct from all other coryneform bacteria (except *C. pseudotuberculosis* and *C. ulcerans*) in their CFA patterns by the presence of a large volume of $C_{16:1\omega7c}$ (3).

Diphtheria Toxin Testing

It is recommended that at least 10 colonies of *C. diphtheriae* and related species be tested for diphtheria toxin by the Elek method, modified as described by Engler (32), in a laboratory with personnel skilled in performing the test and in interpreting the test results. The modified Elek method described by the WHO Diphtheria Reference Unit was initially used to characterize strains from the 1990s epidemic in Russia and Ukraine and was found to be faster and less technically problematic than the original version. Antitoxins from various suppliers (e.g., Swiss Serum and Vaccine Institute, Bern, Switzerland; Pasteur Mérieux, Lyon, France; CDC Biological Products Division, Atlanta, Ga.), applied to blank filter disks at 10 IU/disk, have been successfully used with the modified Elek test (32), and precipitin lines can be read as early as 24 h (Fig. 4). A similar modification of the Elek test, which can test up to 24 isolates on the same plate, has been described (112). A 3-h enzyme-linked immunosorbent assay for the detection of diphtheria toxin from clinical isolates of *Corynebacterium* spp. has been developed by the WHO Diphtheria Reference Unit (33).

PCR-based methods for the detection of the diphtheria toxin gene *(tox)* in isolated bacteria have been developed and validated (29, 70). Recently, a real-time fluorescence PCR assay for detecting the A and B subunits of the *tox* gene has been described and evaluated directly with clinical specimens (96). *tox* PCR assays applied directly to clinical specimens are acceptable, particularly because isolation is not always possible for patients already receiving antibiotics. However, a PCR-positive patient from whom bacteria are not isolated or without a histopathologic diagnosis and without an epidemiologic linkage to a patient with a laboratory-confirmed case of diphtheria should be classified as having a "probable case" of diphtheria, since to date there are insufficient data to conclude that a PCR-positive result always implies diphtheria. Also, detection of the toxin gene in samples by PCR cannot automatically be attributed to one species, because *C. diphtheriae* as well as *C. ulcerans* and *C. pseudotuberculosis* may harbor the bacteriophage which carries the diphtheria toxin gene. Furthermore, *tox*-containing, nontoxigenic isolates have been described and characterized further (12). A comprehensive review on the biology and molecular epidemiology of the diphtheria toxin has been given by Holmes (72). Nontoxigenic strains of *C. diphtheriae,* i.e., those which do not express toxin in the Elek test or those which lack a detectable diphtheria toxin gene by PCR, have caused serious disease such as cases or outbreaks of skin disease and endocarditis and occasional mortality among homeless people, alcoholics, and intravenous drug abusers (37, 64, 111). For nontoxigenic *C. diphtheriae* strains circulating in the United Kingdom, it has been shown that the diphtheria toxin repressor *(dtxR)* genes are functional, so that if these strains are lysogenized by a bacteriophage, they could represent a reservoir for toxigenic *C. diphtheriae* (28).

Typing Methods

Outbreaks of *C. diphtheriae* in the states of the former Soviet Union and other locations have been studied by whole-cell peptide analysis, whole-genome restriction fragment length polymorphism analysis, ribotyping, pulsed-field gel electrophoresis, PCR-single-strand conformation polymorphism analysis of *tox* and *dtxR* (i.e., the regulatory element of the diphtheria toxin) as well as of the 16S-23S rRNA spacer region, amplified fragment length polymorphisms, random amplification of polymorphic DNA (RAPD), and multilocus enzyme electrophoresis (26, 27, 99, 108). An international database for *C. diphtheriae* ribotypes using endonuclease BstEII has been established (67). Recently, a spoligotyping system (similar to the spacer oligonucleotide typing for *Mycobacterium tuberculosis*) checking for the presence or absence of 21 different spacers has been described for *C. diphtheriae* (95). Sequencing studies with *C. diphtheriae* strains from the epidemic in the former Soviet Union have shown that point mutations within the *tox* gene were silent mutations, whereas multiple point mutations (which even led to amino acid substitutions) were observed for the *dtxR* gene, corresponding to the heterogeneity of outbreak strains as revealed by PCR-single-strand conformation polymorphism analysis (97). Molecular epidemiologic studies using RAPD have been used to rapidly screen a large number of *C. diphtheriae* strains to identify the epidemic clonal group associated with the outbreak in the former Soviet Union. Isolates derived from specific populations in the United States and Canada and characterized using multilocus enzyme electrophoresis, ribotyping, and RAPD were found to be members of persistent endemic strains, rather than being imported from other countries where diphtheria is endemic.

Antibiotic treatment is required to eliminate *C. diphtheriae* and prevent its spread; however, it is not a substitute for antitoxin prevention, with antibiotics of choice being penicillins or macrolides. Sporadic isolates of *C. diphtheriae* resistant to erythromycin or rifampin have been reported. Penicillin and some of the newer ketolides were tested against a large collection of geographically diverse strains and were found to generally demonstrate significant efficacy against *C. diphtheriae* but reduced ketolide activity against some Southeast Asian strains. It is believed that between 20 and 60% of adults in the United States lack protective antibodies to diphtheria toxin because of declining antibody titers in immunized persons and because many individuals were never immunized, which could pose a potentially significant public health risk and could result in the reemergence of this disease.

C. durum

C. durum (117) was originally described as being exclusively isolated from respiratory tract specimens. Well-characterized isolates have now been recovered from additional sites, including the gingiva, blood cultures, or abscesses (110). *C. durum* strains were originally isolated after 2 or 3 days from nonselective charcoal-buffered yeast extract plates inoculated with sputa or bronchial washings. *C. durum* is the

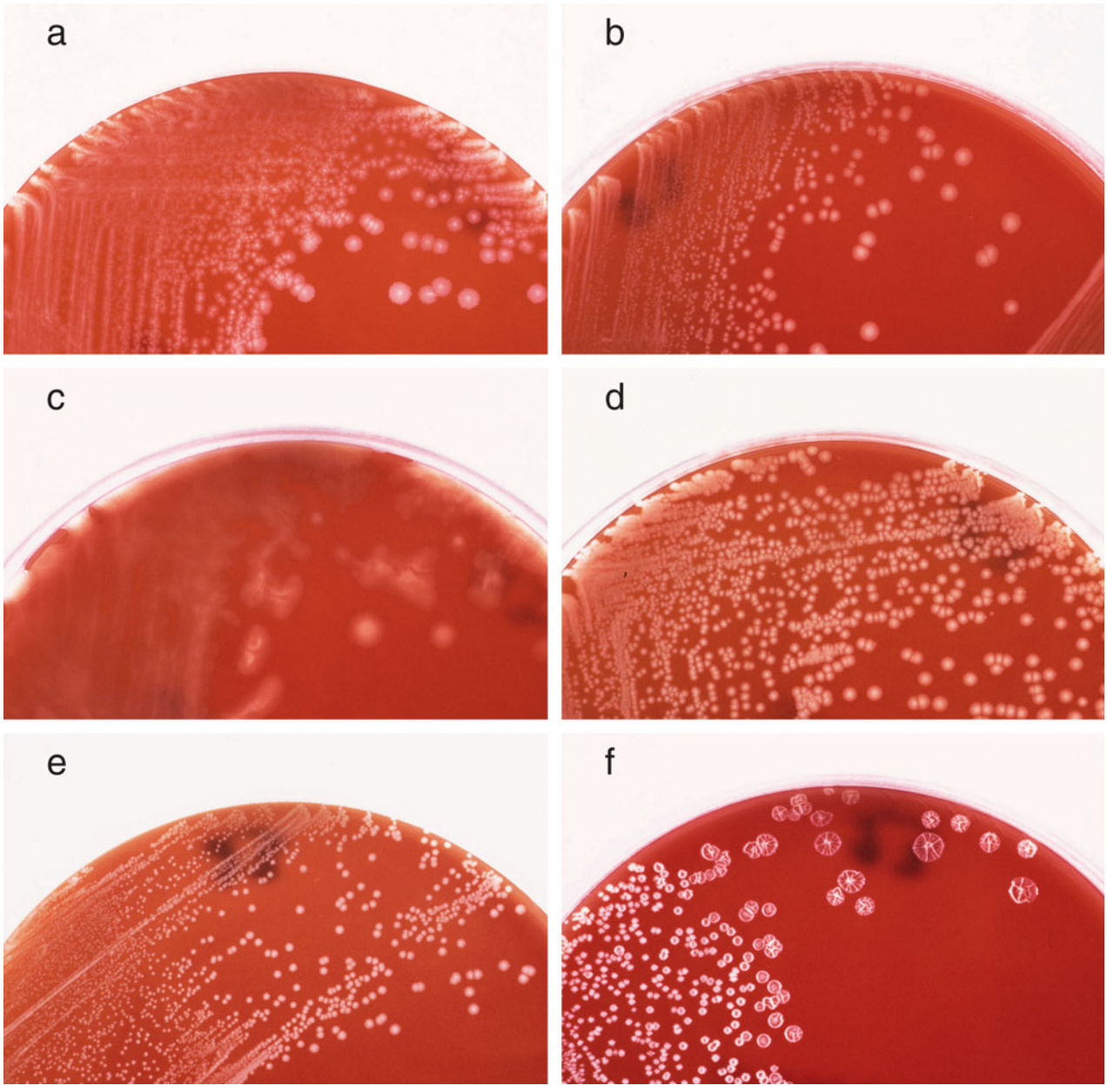


FIGURE 3 Colony morphologies of different coryneform bacteria after 48 h of incubation on SBA. (a) *C. amycolatum* LCDC 91-0077; (b) *C. diphtheriae* ATCC 14779; (c) *C. mucifaciens* LCDC 97-0202; (d) *C. striatum* ATCC 6940; (e) *D. hominis* ATCC 51325; (f) *R. dentocariosa* LCDC 95-0154; (g) *C. aurimucosum* HC-NML 91-0032 (after 96 h); (h) black-pigmented *Rothia dentocariosa* HC-NML 77-0298 (after 96 h); (i) *C. diphtheriae* biotype *gravis* colonies on a Tinsdale agar plate. Panel i was kindly provided by C. Hinnebusch and M. Cohen, UCLA School of Medicine, Los Angeles, Calif.

most frequent *Corynebacterium* isolated from throat swabs from healthy persons (144). Its pathogenic potential is unclear at present. *C. durum* is a peculiar nonlipophilic organism that forms colonies of only 0.5 to 1 mm in diameter after aerobic incubation for 72 h. The original description of this bacterium cited beige and rough colonies with convolutions, with an irregular margin, and strongly adhering to agar if grown under aerobic conditions (117). However, strains described in a later publication were found to be sometimes smoother and not necessarily adherent to agar (110). Gram staining of aerobic cultures shows long and filamentous rods, with occasional "bulges," but true *C. durum* isolates do not have *C. matruchotii*-like "whip handles" (Fig. 1d and e). Long forms are not otherwise found among other *Corynebacterium* species, nor are they observed for *C. durum* when cells are grown in a 10% CO_2-enriched

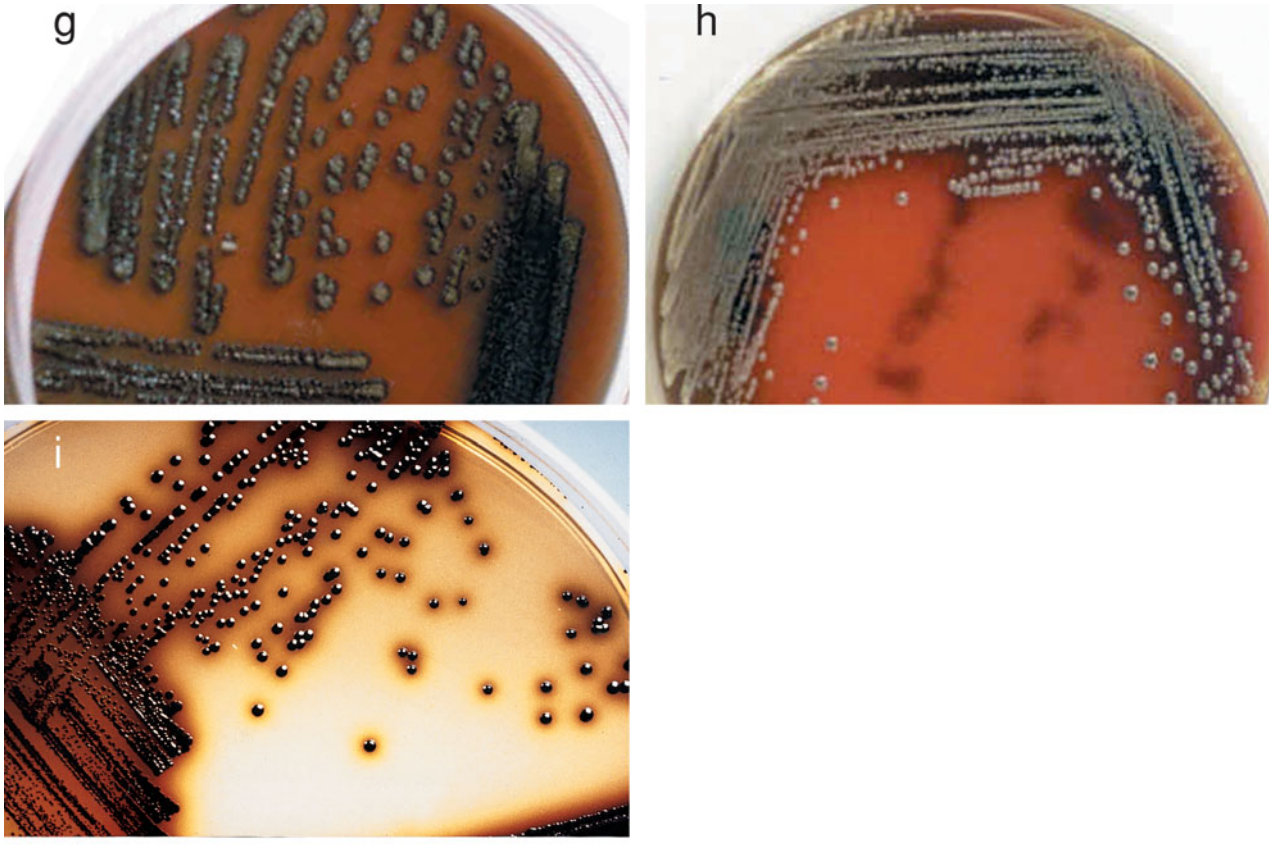


FIGURE 3 *(Continued)*

atmosphere (117). Strains grow only weakly under anaerobic conditions. They always reduce nitrate, and some may exhibit weak and delayed urease and esculinase activities. The majority (but not all [144]) of *C. durum* strains ferment mannitol, which is another very unusual feature for true corynebacteria (Table 4). API Coryne codes observed for *C. durum* include 3000135, 3001135, 3040135, 3400115, 3400135, 3400305, 3400325, and 3400335 (117), as well as 3040325, 3040335, 3440335, and 3441335 (110). This suggests that most strains are negative for alkaline phosphatase and all appear to be negative for pyrrolidonyl arylamidase. Only a small number of *C. durum* strains have been tested with the RapID system, and all were correctly identified (58). It is most likely that some strains identified as *C. matruchotii* in the past may actually have been *C. durum* strains and that differentiation can be difficult if phenotypic methods alone are used. Both species produce propionic acid as a fermentation product (4). *C. durum* usually ferments galactose and very often mannitol, whereas *C. matruchotii* is usually negative for those sugars. The *C. matruchotii* type strain exhibits α-glucosidase activity, which is not observed in *C. durum* (117). It has been shown that some *C. durum* strains also express β-galactosidase activity and ferment ribose (144).

C. falsenii

C. falsenii strains (129) have so far been isolated only from sterile body fluids. Colonies are whitish, glistening, and smooth with entire edges and are 1 to 2 mm in diameter after 24 h. After 72 h, most strains described to date

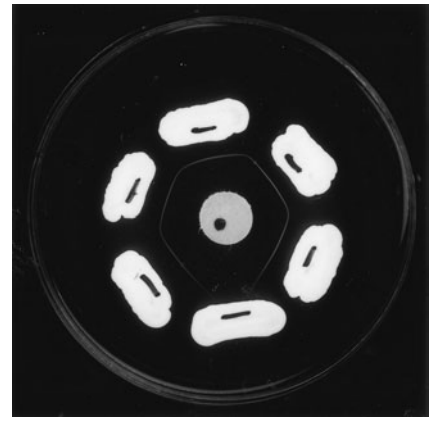


FIGURE 4 Modified Elek test (see the text) with antitoxin disk in the center. Strains are (clockwise starting at noon) NCTC 3984 (weakly toxin-positive *C. diphtheriae* biotype *gravis*), NCTC 10648 (strongly toxin-positive *C. diphtheriae* biotype *gravis*), a test strain (which was found to be a toxin producer), NCTC 10356 (nontoxigenic *C. diphtheriae* biotype *belfanti*), another test strain (also a toxin producer), and (again) NCTC 10648. The photo was kindly supplied by K.-H. Engler (WHO Diphtheria Reference Unit, Central Public Health Laboratory, London, United Kingdom).

exhibit a yellowish pigment that becomes even more intense after 120 h. This pigment is not observed in any other nonlipophilic *Corynebacterium* encountered in clinical specimens, except in the rarely found species *C. xerosis* (the colonies of the latter are dry, in contrast to *C. falsenii* colonies). The most characteristic biochemical features of *C. falsenii* are a slow but fermentative acid production from glucose, a weak pyrazinamidase reaction, and a weak urease activity which becomes visible in either Christensen's urea broth or the API Coryne system after overnight incubation only. API Coryne codes observed for *C. falsenii* are 2101104 and 2101304 (4, 129).

C. freneyi

This species has been outlined based on the study of three strains (113). All these strains had been isolated from skin-related material. There is now evidence that *C. freneyi* is also isolated from genitourinary specimens (43). *C. freneyi* is phylogenetically closely related to *C. xerosis*. Colonies of *C. freneyi* are whitish, dry, and rough, have irregular edges, and are 0.5 to 1 mm in diameter after 48 h of incubation, but *C. freneyi* strains are nonlipophilic. The basic biochemical profile (Table 4) is also similar to that of *C. xerosis*. All *C. freneyi* strains studied so far exhibit α-glucosidase activity, which is not frequently observed in other *Corynebacterium* species (very few *C. amycolatum* and all *C. xerosis* strains express this enzyme). *C. freneyi* can be differentiated from *C. xerosis* by glucose fermentation at 42°C and growth at 20°C, whereas *C. xerosis* is negative for these two reactions.

C. glucuronolyticum

C. seminale is a junior synonym of *C. glucuronolyticum* (24, 39). This species is probably part of the normal genitourinary flora of males, while its presence in females is uncertain. Colonies are whitish-yellowish, convex, and creamy, and they measure 1 to 1.5 mm in diameter after 24 h. The fermentative species *C. glucuronolyticum* is remarkable for its variability in basic biochemical reactions (Table 4). It is the only medically relevant, large-colony *Corynebacterium* species exhibiting β-glucuronidase activity. When urease activity is present, it is abundant in Christensen's urea broth, becoming positive after only 5 min of incubation at room temperature (39). *C. glucuronolyticum* is also one of the very few corynebacteria which are able to hydrolyze esculin. All *C. glucuronolyticum* strains are CAMP reaction positive (Fig. 2). With the exception of strains which are alkaline phosphatase positive, the API Coryne strip identifies *C. glucuronolyticum* well (60), although the profiles obtained from human strains may differ from those of animal isolates (24). Propionic acid is one of the major end products of glucose metabolism. *C. glucuronolyticurn* strains are often tetracycline resistant and may also exhibit resistance to macrolides and lincosamides (59). 16S rRNA gene sequences derived from fluids of patients with prostatitis have been found to be homologous with sequences derived for this species, indicating that *C. glucuronolyticum* might be involved in selected cases of prostatitis (137).

C. imitans

C. imitans was originally isolated from a nasopharyngeal specimen of a child suspected of having throat diphtheria, as well as from three adult contacts (41). This was the first well-documented case of person-to-person transmission of a *Corynebacterium* other than *C. diphtheriae* in a nonhospital setting. Additional strains of *C. imitans* have been recovered from blood cultures (4). Colonies are whitish-grayish and glistening, with entire edges; are creamy; and measure 1 to 2 mm in diameter. The strain did not produce a brown halo on Tinsdale medium but was tellurite reductase positive. Interestingly, Neisser staining was positive for polar bodies. Pyrazinamidase activity was weak only, as was fermentation of sucrose, which may lead to the initial misidentification as an atypical *C. diphtheriae* strain. It is not unlikely that *C. imitans* may also have been misidentified as *C. minutissimum* in the past, since the basic biochemical reactions of the two taxa are quite similar (Table 4). However, *C. imitans* is CAMP reaction positive and does not hydrolyze tyrosine, whereas the opposite reactions are observed for *C. minutissimum.* The API Coryne system provided the numerical codes 1100325, 2100324, and 3100325 for *C. imitans,* indicating a negative α-glucosidase reaction, whereas all *C. diphtheriae* strains express this enzyme. *C. imitans* strains do not produce propionic acid as a fermentation product, unlike *C. diphtheriae* (4), and the CFA composition profiles for each species qualitatively differ, as *C. diphtheriae* and closely related species have a unique pattern among the *Corynebacterium* spp. Diphtheria toxin assays using the Elek test or a PCR for the *tox* gene were all negative for *C. imitans* strains (4, 41). *C. imitans* is resistant to O/129, while *C. diphtheriae* is not.

C. jeikeium

C. jeikeium is one of the most frequently encountered corynebacteria in clinical specimens (64). Nosocomial transmission has been described. Recently, the complete genome sequence of a *C. jeikeium* strain has been determined (138), indicating that the lipophilic phenotype of *C. jeikeium* originates from the absence of fatty acid synthase. *C. jeikeium* is often resistant to multiple antibiotics (including penicillin and gentamicin), but this cannot be used as a taxonomic characteristic because the phenotypically closely related CDC group G bacteria may also demonstrate multidrug resistance. Quantitative DNA-DNA hydridization experiments had shown that *C. jeikeium* includes two genomospecies for which penicillin and gentamicin MICs are low, but as they could otherwise not be differentiated phenotypically from the resistant *C. jeikeium* strains, they were not proposed as independent species (115). Colonies of *C. jeikeium* are tiny, low, entire, and grayish white. *C. jeikeium* is a strict aerobe which may oxidatively produce acid from glucose and sometimes from maltose but not from fructose (CDC group G bacteria are positive for acid production from fructose). The RapID CB Plus system correctly identifies *C. jeikeium,* as does the API Coryne system if ancillary tests are used (58, 60). As for all other lipophilic corynebacteria, imperfectly cleaved mycolic acids coeluting with CFAs at or near equivalent chain lengths of 14.966 to 15.000 or of 16.7 to 16.8 have never been observed among *C. jeikeium* strains (3; Bernard, unpublished).

C. kroppenstedtii

C. kroppenstedtii (18) is a rarely recovered species originally isolated from the sputum of a patient with pulmonary disease. Additional strains have been isolated from a lung biopsy specimen, sputum, a breast abscess, and patients with granulomatous lobular mastitis (4, 106). Apart from *C. amycolatum* and *C. atypicum,* it is the only *Corynebacterium* species lacking mycolic acids. Colonies are grayish, translucent, slightly dry, and less than 0.5 mm in diameter after 24 h of incubation at 37°C. *C. kroppenstedtii* is lipophilic and is one of the few medically relevant *Corynebacterium* species exhibiting esculinase activity. Other biochemical characteristics are given in Table 4. API Codes of *C. kroppenstedtii* are 0101104,

2040104, and 2040105 (4). It can be differentiated from *C. durum*, *C. matruchotii*, and *C. glucuronolyticum* by its colony morphology and from *C. glucuronolyticum* also by its negative CAMP reaction.

C. lipophiloflavum

C. lipophiloflavum (45) is represented by only a single strain, which has been isolated from vaginal discharge from a patient with bacterial vaginosis. It has the same biochemical screening pattern as *C. urealyticum*, except that it exhibits a strong yellow pigment and weaker urease activity (Table 4). In contrast to most *C. urealyticum* strains, the *C. lipophiloflavum* strain observed was not multidrug resistant.

C. macginleyi

C. macginleyi (119) has been isolated almost exclusively from eye specimens, whether from diseased (53) or healthy conjunctiva. Colonies are typical for lipophilic corynebacteria (see above). When grown on Tween 80-SBA plates (better growth is usually found on plates supplemented with 0.1% Tween 80 than on those supplemented with 1.0% Tween 80), some *C. macginleyi* strains exhibit a rose pigment which is not seen for any other lipophilic *Corynebacterium* species. *C. macginleyi* is one of the very few *Corynebacterium* species not expressing pyrazinamidase activity (Table 4). Most strains ferment mannitol, while the majority of other corynebacteria are unable to do so. The API Coryne system correctly identifies *C. macginleyi* (60). Strains belonging to this species are susceptible to a broad spectrum of antibiotics (53).

C. matruchotii

C. matruchotii is thought to be a natural inhabitant of the oral cavity, particularly on calculus and plaque deposits, and so has been much studied by oral microbiologists (110). Otherwise, it is a very rare human pathogen. Microcolonies appear flat, filamentous, and spider-like, but macrocolonies have a variable appearance. *C. matruchotii* demonstrates a very unusual appearance by Gram staining in that so-called whip handles (i.e., filamentous bacteria with a single short bacillus adjacent to the end of the filament, creating the illusion of a whip) are observed (Fig.1e). This microscopic presentation is consistent even when isolates that had been preserved for many years in a culture collection are stained. It has recently been demonstrated that heterogeneity existed among *C. matruchotii* strains obtained from international culture collections and that some strains represented were misidentified *C. durum* isolates (110). *C. matruchotii* strains are consistently negative for galactose, whereas *C. durum* strains can be positive. The API Coryne system database does not contain *C. matruchotii;* the numerical codes observed for *C. matruchotii* include 7000325, 7010325, and 7050325.

C. matruchotii-Like Strain

This species is represented by a single strain, ATCC 43833 (110). It had been deposited in ATCC as *C. matruchotii,* but it is a distinct species as revealed by dot-blot hybridization and 16S rRNA gene sequencing data. Colonies are pinpoint to 0.1 mm in diameter, grayish white, with a smooth, nonadherent texture. Biochemical screening reactions are similar to those of *C. minutissimum,* except that strain ATCC 43833 exhibits esculinase activity in the API Coryne system. The numerical API Coryne profile for this unvalidated taxon is 2140325.

C. minutissimum

C. minutissimum is part of the normal human skin flora. Its association with erythrasma is highly questionable (154). Colonies of *C. minutissimum* are whitish-grayish, shiny, moist, convex, and circular; have entire edges; and are about 1 to 1.5 mm in diameter after 24 h. Most of the colonies are creamy, but some may also have a sticky consistency. *C. minutissimum* strains have a fermentative metabolism and produce acid from sucrose variably. Very few *C. minutissimum* strains are also able to produce acid from mannitol (154). The API Coryne system identifies *C. minutissimum,* with additional tests being necessary for most of the strains (60). Many *C. minutissimum* strains are pyrrolidonyl arylamidase positive. *C. minutissimum* strains exhibit DNase activity (154), and nearly all strains hydrolyze tyrosine, whereas only a very few strains exhibit a positive CAMP reaction. Lactic and succinic acids are the major end products of glucose metabolism (36, 154). Some isolates possess TBSA in their cell membranes. Nearly all *C. minutissimum* strains are susceptible to O/129 (150-μg disk); i.e., they exhibit an inhibition zone around the disk (usually between 20 and 35 mm in diameter).

C. mucifaciens

C. mucifaciens (48) has been isolated mainly from blood cultures and other sterile body fluids, but it has also been recovered from abscesses, soft tissue, and dialysate (4). Colonies are very distinct because they are slightly to overtly yellow and very mucoid (Fig. 3c) (with very few strains not being mucoid [Funke, unpublished]). *C. mucifaciens* is the only presently known *Corynebacterium* species exhibiting such mucoid colonies; this characteristic strongly reminds the bacteriologist of *Rhodococcus equi* colonies. An extracellular substance (probably polysaccharides) causing connective filaments between the cells has been demonstrated as the ultrastructural correlate of the mucoid colonies. Colonies are about 1 to 1.5 mm after 24 h of incubation and have entire edges. They appear less mucoid after extended incubation for 96 h. *C. mucifaciens* has an oxidative metabolism. It consistently produces acid from glucose, but acid production from sucrose is variable. The API Coryne numerical codes 2000004, 2000104, 2000105, 2100104, 2100105, 6000004, 6100104, and 6100105 have been observed for *C. mucifaciens,* suggesting that occasionally glucose oxidation may be too slow to be observed by that method. *C. mucifaciens* is enzymatically less active than *R. equi,* which exhibits α- and β-glucosidase activities not observed for *C. mucifaciens.* In addition, *C. mucifaciens* produces acid from fructose and may produce acid from glycerol and mannose, but acid production from these sugars is not seen in *R. equi* strains. Tuberculostearic acid can be detected in amounts of 1 to 2% of the total CFAs. β-Lactam antibiotics and aminoglycosides show very good activities against *C. mucifaciens.*

C. propinquum

C. propinquum is the closest phylogenetic relative of *C. pseudodiphtheriticum* (104, 123) and has the same niche (i.e., the oropharynx) as *C. pseudodiphtheriticum.* Colonies are whitish and somewhat dryish, have entire edges, and measure 1 to 2 mm in diameter after 24 h of incubation. This species reduces nitrate and hydrolyzes tyrosine but does not hydrolyze urea (Table 4). The API Coryne system and the RapID CB Plus system correctly identify *C. propinquum* strains (58, 60).

C. pseudodiphtheriticum

C. pseudodiphtheriticum is part of the normal oropharyngeal flora. As described in Table 3, this species has been well

documented to cause pneumonia in various patient populations. Colonies are whitish and slightly dry, have entire edges, and measure 1 to 2 mm in diameter after 48 h of incubation. This nonfermenting species reduces nitrate and hydrolyzes urea but does not produce acid from any of the commonly tested carbohydrates (Table 4). Some strains hydrolyze tyrosine. The API Coryne system and the RapID CB Plus system correctly identify *C. pseudodiphtheriticum* strains (58, 60). For this species, imperfectly cleaved mycolic acids coeluting with CFAs have been demonstrated (3). *C. pseudodiphtheriticum* strains are susceptible to β-lactam antibiotics, but resistance to macrolides and lincosamides has been observed.

C. pseudotuberculosis

C. pseudotuberculosis is phylogenetically closely related to *C. diphtheriae* (104, 123), may harbor the diphtheria toxin gene, produces propionic acid as a fermentation product, and contains large amounts of the CFA $C_{16:1\omega7c}$ (3). Colonies are yellowish white, opaque, convex, and about 1 mm in diameter after 24 h. Like *C. ulcerans,* *C. pseudotuberculosis* is positive for urease and the CAMP inhibition test (complete inhibition of the effect of *S. aureus* β-hemolysin on sheep erythrocytes is achieved by streaking the presumed *C. pseudotuberculosis* strain in a right angle toward *S. aureus* and overnight incubation; a β-hemolysin inhibition zone in the form of a triangle is observed, as is the case for *A. haemolyticum* [Fig. 2]). *C. pseudotuberculosis* is not susceptible to O/129, whereas *C. ulcerans* strains are (74). *C. pseudotuberculosis* is variable for both nitrate reduction and sucrose fermentation. The API Coryne system and the RapID CB Plus panel correctly identify this species (58, 60).

C. resistens

This recently defined species (103) has entire, grayish-whitish, and glistening colonies and is lipophilic. It is unusual in having a negative pyrazinamidase reaction, which differentiates it from the phenotypically related *C. jeikeium* or CDC coryneform group G strains. In addition, *C. resistens* grows slowly under anaerobic conditions, whereas *C. jeikeium* is unable to do so. The *C. resistens* strains reported in the literature were resistant to penicillin, cephalosporins, aminoglycosides, clindamycin, and ciprofloxacin but remained susceptible to glycopeptides. It is presently unknown whether true *C. resistens* strains are often misidentified as *C. jeikeium* in the routine laboratory.

C. riegelii

C. riegelii strains were originally described as being isolated from females with urinary tract infections (49), but additional strains have been recovered from blood cultures, including cord blood (4). Colonies are whitish, glistening, and convex; have entire margins; and measure up to 1.5 mm in diameter after 48 h of incubation. Some colonies are of a creamy consistency, whereas others are sticky. *C. riegelii* strains exhibit a very strong urease activity with Christensen's urea broth, becoming positive within 5 min after inoculation at room temperature. A very peculiar characteristic of *C. riegelii* is the slow fermentation of maltose but not glucose. No other defined *Corynebacterium* exhibits this feature (Table 4). The weak anaerobic growth of *C. riegelii* corresponds to the weak fermentative metabolism. The API Coryne system codes observed for *C. riegelii* include 0101224, 2001224, and 2101224.

C. simulans

This species has been delineated from some *C. striatum*-like strains (145). The three strains described in the original publication came from skin-related specimens (foot abscess, lymph node biopsy specimen, and boil). Two additional strains have been characterized, one from bile and one from a blood culture (4). Colonies of *C. simulans* (grayish white, glistening, creamy, and 1 to 2 mm in diameter) are very similar to those of *C. minutissimum, C. singulare,* and *C. striatum,* all of which are the closest phylogenetic neighbors. *C. simulans* is the only valid *Corynebacterium* species described to date that reduces nitrite. Further characteristics which differentiate *C. simulans* from the closely related nonlipophilic, fermentative corynebacteria are the inability to acidify ethylene glycol and the inability to grow at 20°C (in contrast to *C. striatum*). API Coryne profiles include 0100305, 2100105, 2100301, 2100305, and 3000125 (including the falsely negative nitrate reduction reaction because of the strong nitrite reduction).

C. singulare

C. singulare colonies are circular and slightly convex with entire margins and are of a creamy consistency, as observed for *C. minutissimum* and *C. striatum* (121). Key biochemical reactions are like those for *C. minutissimum,* except that urease activity is observed (Table 4). The numerical API Coryne system profile is 6101125, indicative that pyrrolidonyl arylamidase activity is observed. Like *C. minutissimum* and *C. striatum*, *C. singulare* also hydrolyzes tyrosine. *C. singulare* can be differentiated from the much more frequently isolated *C. minutissimum* with use of the bioMérieux biotype 100 gallery, but this is not a usual clinical microbiology laboratory test. *C. singulare* does not produce propionic acid as a fermentation product, differentiating it from *C. amycolatum*.

C. striatum

C. striatum is part of the normal human skin flora. Nosocomial transmission of *C. striatum* has been documented (86). Colonies are convex, circular, shiny, moist, and creamy with entire edges and are about 1 to 1.5 mm in diameter after 24 h of incubation. Some investigators have described *C. striatum* colonies as somewhat like those of small coagulase-negative staphylococci. *C. striatum* has a fermentative metabolism, and acid production from sucrose is variable. The API Coryne system identifies *C. striatum,* but additional tests are needed in most of the cases (60). All *C. striatum* strains hydrolyze tyrosine, and some strains are CAMP reaction positive; however, the CAMP reaction of *C. striatum* strains is usually not as strong as that of other CAMP test-positive species (e.g., *C. auris* or *C. glucuronolyticum*). Lactic and succinic acids are the major end products of glucose metabolism (36). All *C. striatum* strains are susceptible to O/129. Resistance to macrolides and lincosamides due to the presence of an rRNA methylase has been described. *C. striatum* may also be resistant to quinolones and tetracyclines.

C. sundsvallense

C. sundsvallense (4, 14) is a newly described species that has been isolated from blood cultures, a vaginal swab, and a sinus drainage from an infected groin. Colonies of this nonlipophilic species are buff or slightly yellowish and adherent to agar and have a sticky consistency. Gram staining shows bulges or knobs at the ends of some rods, and these are not seen in any other corynebacteria. Fermentation of glucose, lactose, and sucrose is slow (Table 4). *C. sundsvallense* can be

differentiated from *C. durum* by its positive α-glucosidase reaction and its inability to ferment galactose. It is further differentiated from *C. matruchotii* by expressing urease but not nitrate reductase activity and by not producing propionic acid as an end product of glucose metabolism (4, 14).

C. thomssenii

C. thomssenii (153) is a rarely found species, originally repeatedly isolated from a patient with pleural effusion; a second strain was recovered from the environment in Canada (4). This species is fastidious and slowly growing, resulting in colonies <0.5 mm after 48 h, but it is not lipophilic. After 96 h, colonies are molar tooth-like, very sticky, and slightly adherent to agar. The clinical strain of *C. thomssenii* is the only *Corynebacterium* species expressing *N*-acetyl-β-glucosaminidase activity, which can be observed in either the API Coryne or the API ZYM system. Acid is slowly and fermentatively produced from glucose, maltose, and sucrose, and the resulting API Coryne code for *C. thomssenii* is 2121125.

C. tuberculostearicum

C. tuberculostearicum was recently revived for a never validly published taxon and also included a strain of the unvalidated species "*C. pseudogenitalium*" (35). This lipophilic species has some variable biochemical key reactions (Table 4) and can be differentiated from CDC group G and *C. accolens* only through the presence of TBSA. However, as CFA analysis cannot be routinely performed in every clinical laboratory, it is unknown how many tentatively identified CDC group G strains are in fact *C. tuberculostearicum* strains. Unfortunately, the published report on *C. tuberculostearicum* did not provide any further features to differentiate this species from the phenotypically closely related CDC group G bacteria.

C. tuscaniae

C. tuscaniae, a recently described species, has been isolated from blood cultures of a patient suffering from endocarditis (114). *C. tuscaniae* does not grow under anaerobic conditions, which distinguishes this species from the phenotypically closely related *C. minutissimum*. In addition, *C. tuscaniae* hydrolyzes hippurate but not tyrosine, whereas *C. minutissimum* has opposite reactions (Table 4). *C. tuscaniae* colonies are rounded and regular, in contrast to the biochemically closely related *C. amycolatum* colonies, which exhibit irregular edges.

C. ulcerans

Phylogenetically, *C. ulcerans* (120) is, together *with C. pseudotuberculosis*, the closest relative of *C. diphtheriae* (104, 123) and contains significant amounts of $C_{16:1\omega7c}$. *C. ulcerans* can harbor the diphtheria toxin gene, but differences in the receptor-binding and translocation domains have been described, and a *C. ulcerans* diphtheria toxin-specific PCR has been developed (128). Disease associated with this bacterium is rare, but if the organism is recovered from pseudomembranous material, the disease must be treated like a case of diphtheria (29, 30). Recent reports have described the transmission of *C. ulcerans* from domestic dogs and cats to humans (84). Colonies are somewhat dry, waxy, and gray-white with light hemolysis, being 1 to 2 mm in diameter after 24 h. *C. ulcerans* may be differentiated from *C. diphtheriae* by urease activity and a CAMP inhibition reaction (Table 4). Strains of *C. ulcerans* are positive for glycogen, starch, and trehalose fermentation. The API Coryne system and the RapID CB Plus identification strip correctly identify *C. ulcerans* (58, 60).

C. urealyticum

C. urealyticum is one of the more frequently isolated, clinically significant corynebacteria in clinical specimens (64). *C. urealyticum* is strongly associated with urinary tract infections. Recovery of this bacterium is often associated with urine with an alkaline pH, resulting in struvite crystals. As for all other lipophilic corynebacteria, colonies are pinpoint, convex, smooth, and whitish-grayish on regular SBA. *C. urealyticum* is a strict aerobe and has very strong urease activity (Table 4). Commercial identification systems correctly identify *C. urealyticum. C. urealyticum* is almost always multidrug resistant (64, 130), but rare penicillin-susceptible strains have also been described.

C. xerosis

Colonies are dry, granular, and yellowish, have irregular edges, and are 1 to 1.5 mm in diameter after 24 h. It must be emphasized that nearly all "*C. xerosis*" strains that appeared in the literature before 1996 may have been misidentified *C. amycolatum* strains (46). *C. striatum* strains were also misidentified as *C. xerosis* in the past. *C. xerosis* has a fermentative metabolism, is variable for the presence of nitrate reductase, but always expresses α-glucosidase as well as leucine arylamidase activities. Because *C. xerosis* was thought to be rarely encountered in clinical specimens, it was not included in the API Coryne system version 2.0 database. The numerical profiles observed for *C. xerosis* strains include 2110325 and 3110325. The RapID CB Plus system correctly identifies *C. xerosis* (58). Lactic acid is the major end product of glucose metabolism, and strains are susceptible to O/129.

Biochemical Reactions for Other Genera

The key biochemical reactions for the genera other than *Corynebacterium* are given in Table 5.

Genus *Turicella*

T. otitidis is almost exclusively isolated from clinical specimens from the ear region, but it does not cause otitis media with effusion in children (73). Colonies are whitish, convex, and creamy, have entire edges, and measure 1 to 1.5 mm in diameter after 48 h of incubation. Some young colonies show a greenish appearance when taken away from the plates with a swab. The distinctive Gram stain morphology of *T. otitidis* is shown in Fig. 1b. Differentiation from *C. auris* and *C. afermentans* subsp. *afermentans* is readily achieved by morphologic features, but utilization reactions may also assist in the differentiation of these taxa (47). All *T. otitidis* strains are strongly CAMP reaction positive and give the numerical code 2100004 in the API Coryne system. The MICs of β-lactam antibiotics for many strains are very low; some strains might be resistant to macrolides and clindamycin (59).

Genus *Arthrobacter*

Arthrobacter spp. might be part of the indigenous normal human flora, but their main habitat is soil. *A. cumminsii* seems to be a normal commensal in humans and appears to be the most frequently isolated *Arthrobacter* species in human clinical specimens (54). *Arthrobacter* colonies are usually whitish-grayish, slightly glistening, and creamy and are 2 mm or greater in diameter after 24 h. *A. cumminsii* is slightly smaller than the other arthrobacters and may also exhibit a sticky consistency (54). *Arthrobacter* spp. usually do not oxidize any of the carbohydrates routinely tested and do not express a cheese-like smell, as is often found for the phenotypically closely related brevibacteria. Some arthrobacters are motile,

whereas brevibacteria are always nonmotile. Like brevibacteria, *Arthrobacter* spp. express DNase and have gelatinase activity (44). The identification of arthrobacters to the species level might be achieved by carbohydrate utilization tests, but this is recommended for the reference laboratory only. The recently described *A. albus* is phylogenetically closely related to *A. cumminsii* but might be differentiated phenotypically by being resistant to desferrioxamine, whereas *A. cumminsii* is susceptible. *A. cumminsii* has a distinctive CFA pattern, with $C_{14:0i}$ and $C_{14:0}$ each representing 2 to 4% of all CFAs (54). The penicillin MICs for most *Arthrobacter* strains are low (44). Aminoglycosides and quinolones show only very weak activities against *A. cumminsii* strains (54).

Genus *Brevibacterium*

Some *Brevibacterium* spp. are part of the normal human skin flora. Colonies are whitish-grayish (or yellowish like *B. luteolum* [146]), convex, and mostly creamy, and are 2 mm or greater in diameter after 24 h. *B. mcbrellneri* colonies have a more granular appearance and are dryer than those of other brevibacteria. Some brevibacteria may develop a yellowish or greenish pigment after prolonged incubation. Many *Brevibacterium* strains isolated from human clinical material give off a distinctive cheese-like odor. Brevibacteria are nonmotile, are halotolerant (6.5% NaCl), and form methanethiol from methionine, but this test is specific for brevibacteria only when it is read within 2 h (40). Brevibacteria can be identified to the species level by carbohydrate utilization tests. More than 90% of all clinical *Brevibacterium* isolates are *B. casei* (40). *B. sanguinis* is very similar to *B. casei* and can be differentiated from this species by susceptibility to thallium acetate (147). The MICs of β-lactam antibiotics for brevibacteria are often elevated (59, 139).

Genus *Dermabacter*

D. hominis strains are part of the normal skin flora. Colonies are whitish, convex, of a creamy or sticky consistency, and 1 to 1.5 mm in diameter after 48 h (Fig. 3e). *D. hominis* strains are sometimes mistaken for small-colony coagulase-negative staphylococci. The Gram stain result is distinctive, with coccobacillary or coccoidal forms (Fig. 1c). The key biochemical reactions are given in Table 5. *D. hominis* is one of the few coryneform bacteria with a variable reaction for xylose fermentation. It is the only catalase-positive coryneform bacterium (except *Actinomyces neuii* [36]) that is able to decarboxylate lysine and ornithine (61). The API Coryne system and the RapID CB Plus panel correctly identify this species (58, 60). *D. hominis* strains may be resistant to aminoglycosides (61, 139).

Genus *Rothia*

This genus presently comprises five validated species, of which only two are clinically relevant: *R. mucilaginosa* (formerly designated *Stomatococcus mucilaginosus*), *R. dentocariosa* (the type species), as well as *R. dentocariosa* genomovar II (19, 81). The last organism has not been formally named because it has not been possible to biochemically distinguish it from authentic *R. dentocariosa* sensu stricto (genomovar I) (81). Some of the former CDC coryneform group 4 bacteria (Fig. 3h) were also shown to be representatives of *R. dentocariosa* (21). These grayish strains were isolated primarily from respiratory materials, pus, or blood cultures, in contrast to *C. aurimucosum* strains, which were derived from the female urogenital tract.

Colonies of *R. dentocariosa* are whitish (or more rarely grayish black), raised, and smooth or rough or have a "spoke wheel" form (Fig. 3f), and they are up to 2 mm in diameter after 48 h. *Rothia* strains usually grow slightly better in a CO_2-enriched atmosphere. The biochemical features of *R. dentocariosa* are given in Table 5. The API Coryne system correctly identifies *R. dentocariosa* (60). Its CFA composition is of the branched-chain type (3), which allows differentiation from the biochemically similar species *C. durum, C. matruchotii,* and *Actinomyces viscosus* (see chapter 56), all of which also occupy the oropharynx. *R. dentocariosa* may also be confused with *D. hominis* and *Propionibacterium avidum* (see chapter 55) (142), both of which, in contrast, always exhibit smooth colonies. As shown in a study on the pharyngeal bacterial flora of healthy adults, one-third of all *R. dentocariosa* strains isolated were negative for the key biochemical reaction catalase (144). The MICs of aminoglycosides for some *R. dentocariosa* strains are elevated, whereas penicillins usually show good in vitro activities against *Rothia* strains.

Genus *Exiguobacterium*

It is not known whether exiguobacteria are part of the indigenous bacterial flora of humans. Colonies of *E. acetylicum* are smooth, golden-yellow to orange, and up to 2 mm in diameter after 24 h of incubation. Acid from carbohydrates is rapidly produced by fermentative metabolism. Exiguobacteria are motile and often oxidase positive. They might be confused with microbacteria, but CFA analysis provides a clear-cut distinction between the two genera (Table 1). The pathogenic potential of *E. acetylicum* seems to be rather low, since it has been isolated from different sources (e.g., skin, wounds, and cerebrospinal fluid [71]), but case reports on infectious diseases due to *E. acetylicum* are not extant. Cases of pseudobacteremia due to *E. acetylicum* have been observed.

Genus *Oerskovia*

O. turbata is usually acquired from the environment (e.g., soil). Colonies are pale yellow to phosphorous yellow, convex, and creamy; they penetrate agar; and they are approximately 1 to 2 mm in diameter after 24 h. *O. turbata* rapidly produces acid from sugars by fermentation; it also exhibits a very strong esculin reaction. The genus is well identified by the API Coryne system (60). *O. turbata* does not hydrolyze xanthine or hypoxanthine, whereas the related *C. cellulans* is able to do so.

Genus *Cellulomonas*

Cellulomonas strains are usually acquired from the environment. Colonies are first whitish or pale or bright yellow, but after 7 days nearly all *Cellulomonas* strains are somewhat yellow. Colonies vary between 0.5 and 1.5 mm in diameter after 24 h, are convex and creamy, and have entire edges. *Cellulomonas* spp. are variable for the fermentation of mannitol. Other key biochemical reactions of *Cellulomonas* spp. are given in Table 5. The majority of *Cellulomonas* strains express cellulase activity, demonstrated by incubating a heavy bacterial suspension (McFarland no. 6 standard) with a piece of sterile copy paper in a 0.9% NaCl solution for 10 days, resulting in dissolution of the paper (55). The recently described *C. denverensis* can be differentiated from *C. hominis* by its positive reaction for D-sorbitol fermentation (7).

Genus *Cellulosimicrobium*

Colonies of *C. cellulans* are similar to those of *O. turbata* (see above) and also pit the agar. In addition, *C. cellulans* exhibits

a biochemical screening profile which is very similar to that of *O. turbata* (Table 5). However, *C. cellulans* hydrolyzes either xanthine or hypoxanthine whereas *O. turbata* does not, and *O. turbata* strains might be motile whereas *C. cellulans* strains are not (71, 125). The recently described *C. funkei* strains can be differentiated from *C. cellulans* (former *O. xanthinolytica*) by their negative inulin and raffinose fermentation reactions (8); in addition, *C. funkei* strains are motile but *C. cellulans* strains are not.

Genus *Microbacterium*

Microbacteria account for the majority of yellow-pigmented coryneform bacteria isolated from clinical specimens. All shades of yellow pigment are observed, ranging from pale to bright yellow and orange. Most of the strains are catalase positive, but catalase-negative strains might be observed. Some microbacteria grow under anaerobic conditions but only weakly. Some microbacteria are nitrate reductase negative, which distinguishes them from the phenotypically closely related genus *Cellulomonas*, all presently defined species of which are nitrate reductase positive (Table 5). Microbacteria may ferment mannitol but not xylose, whereas the lack of xylose fermentation has not been observed for *Cellulomonas* strains described so far.

Species identification is almost impossible since for many defined *Microbacterium* species the type strain is the only representative, preventing the creation of a comprehensive database. Final identification to the species level is best achieved by chemotaxonomic (interpeptide bridges) and molecular genetic (e.g., 16S rRNA gene sequencing) investigations. The most frequently isolated, validated microbacteria in clinical specimens include *M. oxydans, M. paraoxydans, M. aurum,* and *M. lacticum* (66, 82).

Microbacteria are usually susceptible to vancomycin (except *M. resistens* [50]), but susceptibility to other antimicrobial agents is unpredictable (in particular, resistance to aminoglycosides has been observed [42]), and therefore every individual clinically significant strain must be tested.

Genus *Curtobacterium*

Curtobacteria are very infrequently isolated yellow- or yellow-orange-pigmented oxidative coryneform bacteria. In contrast to most microbacteria, they produce acid from carbohydrates very slowly (within 4 to 7 days) (38). Curtobacteria are usually nitrate reductase negative but strongly hydrolyze esculin (Table 5). *C. pusillum* and related strains have a most unusual CFA composition that is not observed in any other coryneform bacteria, with a ω-cyclohexyl fatty acid identified as feature 7 ($C_{18:1\omega7c/\omega9c/\omega12t}$) representing more than 50% of all CFAs (38). Again, the differentiation of curtobacteria is very difficult and should be performed in a reference laboratory. The MICs of macrolides and rifampin for curtobacteria are very low.

Genus *Leifsonia*

Leifsonia aquatica is very rarely encountered in clinical specimens. It is always motile, does not hydrolyze either gelatin or casein, and has a stronger DNase activity than most microbacteria (65). *L. aquatica* is the only species within the genus *Leifsonia* which is able to grow in broth enriched with 5% NaCl (34). Its yellow pigment develops relatively slowly within 3 to 4 days. The MICs of vancomycin for some *L. aquatica* strains were shown to be elevated (8 μg/ml) (65), but the precise mechanism of this resistance is not known.

Genera *Janibacter, Pseudoclavibacter/ Zimmermannella,* and *Knoellia*

Strains belonging to genera *Janibacter, Pseudoclavibacter/ Zimmermannella,* and *Knoellia* have been derived from the environment, foods, or animals but have also been recovered recently from clinical materials and characterized using 16S rRNA gene sequencing. These taxa were described as having white, creamy, or yellowish pigment, and all were nonmotile. *Janibacter, Pseudoclavibacter/Zimmermannella,* and *Knoellia* were oxidative, and *Brachybacterium* strains were fermentative. All strains tested so far were susceptible to vancomycin. Since no comprehensive biochemical data based on a large number of strains are available at present, these genera were not included in Table 5.

Genus *Arcanobacterium*

The genus *Arcanobacterium* comprises six species, all of which except *A. pluranimalium* exhibit beta-hemolysis on SBA. The three medically relevant species, *A. haemolyticum*, *A. pyogenes*, and *A. bernardiae,* are all catalase negative. All species show a fermentative glucose metabolism, with succinic and lactic acids as their major end products. All arcanobacteria grow and express hemolysis best in a CO_2-enriched atmosphere.

The colonies of the type species, *A. haemolyticum*, are 0.5 mm in diameter after 48 h of incubation at 37°C, and two morphotypes have been described: one rough type isolated mainly from the respiratory tract and one smooth type isolated mainly from wounds. The biochemical reactions of *A. haemolyticum* are given in Table 5. Of major value for the identification of *A. haemolyticum* is the so-called CAMP inhibition test (see the description of the CAMP inhibition test in the section on *C. pseudotuberculosis*) (Fig. 2). The protein responsible for this phenomenon is a phospholipase D excreted by *A. haemolyticum*, and this protein is genetically and functionally similar to the ones expressed by *C. ulcerans* and *C. pseudotuberculosis*. *A. haemolyticum* and the two other medically relevant arcanobacteria are correctly identified by the API Coryne system (60).

A. pyogenes colonies are the largest of all arcanobacterium colonies, with diameters of up to 1 mm after 48 h of incubation. Of all the arcanobacteria, this species also shows the sharpest zone of beta-hemolysis on SBA. The protein responsible for hemolysis, named pyolysin, is also an important virulence factor in vivo. Gram stains may show some branching rods. *A. pyogenes* is the only *Arcanobacterium* species of medical relevance that expresses β-glucuronidase activity and that is capable of fermenting xylose.

A. bernardiae (57, 105) shows glassy, whitish colonies of <0.5 mm in diameter after 48 h. Some colonies have a creamy consistency, whereas others are sticky. Gram staining shows relatively short rods without branching. Most *A. bernardiae* strains belong to the very few coryneform bacteria that are able to ferment glycogen. Another peculiar feature of *A. bernardiae* strains is their ability to produce acid faster from maltose than from glucose.

The MICs of all β-lactams, rifampin, and tetracycline for arcanobacteria are very low, whereas aminoglycosides and quinolones have reduced activities against arcanobacteria (Funke, unpublished). Macrolides also exhibit excellent activities against arcanobacteria and are an alternative to β-lactam antibiotics for the treatment of infections, since treatment failures due to β-lactam antibiotics because of the inability of β-lactam antibiotics to act intracellularly have been reported.

ANTIMICROBIAL SUSCEPTIBILITIES

The susceptibility patterns for each taxon were given with the descriptions of each taxon (see above). Since the antimicrobial susceptibility of coryneform bacteria is not predictable in every case, susceptibility testing should always be performed with clinically significant isolates (see "Clinical Significance"). Due to the emergence of vancomycin-resistant gram-positive organisms, it has become inappropriate to recommend glycopeptides as first-line drugs for the treatment of infections caused by coryneform bacteria. It is also noteworthy that some coryneform bacteria (e.g., *Microbacterium resistens*) are intrinsically vancomycin resistant.

The Clinical and Laboratory Standards Institute has recently published testing conditions and interpretive criteria for susceptibility testing of coryneform bacteria by use of a broth microdilution method (13). Direct colony suspensions equivalent to a 0.5 McFarland standard are prepared, and strains are incubated in cation-adjusted Mueller-Hinton broth with 2 to 5% (vol/vol) lysed horse blood at 35°C in ambient air for up to 48 h. Interpretive categories for the MICs obtained are presently available for 16 antimicrobial agents including newer drugs like quinupristin-dalfopristin (63) or linezolid (51). Very few studies comparing broth microdilution and disk diffusion results for susceptibility testing of coryneform bacteria (149) have been performed.

In the past, MICs have been determined by either the E test (AB Biodisk, Solna, Sweden) or the agar dilution or broth microdilution method. The results of the E test have been shown to correlate well with those of both the broth microdilution and the agar dilution methods for *Corynebacterium* spp. (92, 152). The E test should be carried out on Mueller-Hinton agar supplemented with 5% sheep blood. The same medium is used for the agar dilution method (59), but this method is not applicable in the routine laboratory; rather, it should be used in studies with individual antimicrobial agents.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

The guidelines related to when coryneform bacteria should be identified to the species level (see "Clinical Significance") are also applicable for evaluating and interpreting culture results; i.e., whenever coryneform bacteria are identified to the species level, the results should be reported.

In the rare case of microscopically suspected *C. diphtheriae* (i.e., a positive Neisser staining result), the physician in charge of the patient should be notified immediately, although culture results and toxin testing results become available only later.

It is evident that repeated isolation of a predominant strain of a coryneform bacterium or a coryneform bacterium growing in pure culture suggests an etiological relationship to the patient's disease. If coryneform bacteria are present in blood cultures, the physician in charge should be notified immediately, and it should be emphasized when reporting this result that the clinical significance of the coryneform bacteria must be carefully examined by cooperation between the microbiology laboratory and the physician. In our experience, one positive blood culture out of two or three aerobically and anaerobically incubated pairs of blood cultures is hardly ever clinically significant (except in cases of treated endocarditis). Care must be taken in the interpretation of the results for those patients for whom half or more of the blood specimens taken for culture become positive for coryneform bacteria, in particular when lipophilic corynebacteria are cultured, since not all blood samples taken from patients with endocarditis due to lipophilic corynebacteria may eventually become positive.

On the other hand, coryneform bacteria should be reported as "normal flora" when they are grown in equal or smaller numbers from nonsterile sites together with other resident flora. It is suggested that the primary isolation plates be retained for at least 72 h before they are discarded in order to have the opportunity to assess the bacterial population retrospectively.

APPENDIX 1

Genus *Gardnerella*

The genus *Gardnerella* does not have a particular phylogenetic relationship to any of the established genera described in this chapter. It is remotely related to the genus *Bifidobacterium* (94), and these genera share some important features, such as production of acetic and lactic acids as fermentation products. A 16S rRNA gene PCR product selected for detection of a *G. vaginalis*-specific sequence was found to have some less-than-specific homology with several *Bifidobacterium* species (100). *G. vaginalis* is the only species belonging to the genus *Gardnerella*. Studies on the ultrastructure of the cell wall of *G. vaginalis* have demonstrated that it has a cell wall similar to but much thinner than the cell walls of other gram-positive bacteria (i.e., there is a smaller peptidoglycan layer) (124). Lysine is the diamino acid of the cell wall, and CFAs are similar to those detected in *Actinomyces* spp., *Arcanobacterium* spp., and *Corynebacterium* spp., with $C_{16:0}$ and $C_{18:1\omega9c}$ predominating. The G+C content of 42 to 44 mol% is lower than that of every other genus described in this chapter.

Gram stains show thin gram-variable rods or coccobacilli (Fig. 1f). Catalase is not produced, and cells are nonmotile and have a slow fermentative metabolism.

Natural habitat

G. vaginalis can be found in the anorectal flora of healthy adults of both sexes as well as in that of children (10). It is also part of the endogenous vaginal flora in women of reproductive age. The optimal pH for the growth of *G. vaginalis* is between 6 and 7. The organism can also be recovered from the urethras of the male partners of women with bacterial vaginosis (BV) (10).

Clinical significance

G. vaginalis is associated with BV; its causative role in the syndrome is controversial (131, 132). Recurrent BV is due to reinfection rather than to relapse (i.e., overgrowth of the previously colonizing biotype). In pregnant women, BV may lead to preterm birth, premature rupture of membranes, and chorioamnionitis (10). *G. vaginalis* may also be recovered from cultures of blood from patients with postpartum or postabortal fevers and may also cause infections in newborns. Although it might be recovered from the urethras of males, its association with disease in males is questionable. Serious infections in sites other than those associated with the genital tract or obstetrics are rare but have been reported.

Collection, transport, and storage of specimens

Vaginal and extravaginal specimens can be collected with cotton-tipped swabs. It is best to take one swab for direct examination and to take another swab for culture if necessary, such as for epidemiologic studies. If culture media cannot be directly inoculated, then the swab should be placed in a transport medium (e.g., Amies) and culture should be done within 24 h. It is noteworthy that *G. vaginalis* is susceptible to sodium polyanethol sulfonate (SPS), so an SPS-free medium (or an SPS medium supplemented with gelatin) should be used in order to achieve optimal recovery of *G. vaginalis* from blood culture systems whenever *G. vaginalis* is suspected.

Isolation and identification

The gold standard for the diagnosis of BV is direct examination of vaginal secretions and not the culture of *G. vaginalis,* since *G. vaginalis* can also be recovered from healthy women. A bedside test for BV is examination of the vaginal discharge to detect the typical "fishy" trimethylamine odor, which is enhanced after alkalinization with 10% KOH. The typical smear of vaginal discharge from BV patients shows "clue cells" (bacteria covering epithelial cell margins) together with mixed flora consisting of large numbers of small gram-negative (predominantly *Prevotella* and *Porphyromonas* spp.) and gram-variable *(G. vaginalis)* rods and coccobacilli, whereas lactobacilli are almost always absent. It is recommended that a standardized Gram staining interpretative scheme be used in order to improve the reproducibility of this method (102, 132). Detection of *G. vaginalis* in vaginal specimens processed using a DNA probe-based system, Affirm VPIII (Becton Dickinson), has been reported to be useful as a surrogate for wet mount cell examination (6). Although not recommended for routine laboratory procedures, the isolation of *G. vaginalis* can support the diagnosis of BV. Vaginal swabs are cultured on Vaginalis agar (see chapter 20 for the preparation) and should be semiquantitatively streaked out with a loop. Incubation is at 35 to 37°C in a 5% CO_2-enriched atmosphere or in a candle jar. Beta-hemolysis is observed on human or rabbit blood-containing media but not on SBA. Plates may be checked for the growth of diffuse beta-hemolytic colonies of <0.5 mm in diameter after 24 h, but very often *G. vaginalis* is best observed after 48 h. Gram staining of the suspected colonies confirms the diagnosis of *G. vaginalis.*

Eight *G. vaginalis* biotypes had been proposed on the basis of the reactions for lipase, β-galactosidase, and hippurate hydrolysis (107), with biotypes 1, 5, and 6 being most common. The diagnostic value of these biotypes is questionable since they have not been demonstrated to be associated with certain diseases or certain forms of disease. They may have some value for longitudinal studies, but molecular genetic typing methods are likely to be superior to biotyping. *G. vaginalis* strains are consistently α-glucosidase and starch hydrolysis positive, but only 90% of all *G. vaginalis* strains hydrolyze hippurate. The API Coryne system identifies *G. vaginalis* well (60). Confirmation of the identification of *G. vaginalis* can also be achieved by antimicrobial agent disk inhibition tests with 50 μg of metronidazole (inhibition present), 5 μg of trimethoprim (inhibition present), and 1 mg of sulfonamide (inhibition absent).

"*G. vaginalis*-like" organisms recovered from patients with BV have been demonstrated to represent *Actinomyces turicensis* strains (141). *G. vaginalis* can be differentiated from these organisms in that it has acetic acid as the main end product of glucose fermentation and is unable to produce acid from xylose, whereas *A. turicensis* strains have succinic acid as the end product and produce acid from xylose.

Antimicrobial susceptibilities

Metronidazole is the drug of choice both for local therapy of BV and for systemic therapy of extravaginal infections caused by BV-associated flora. Systemic infections due to *G. vaginalis* alone can be treated with ampicillin or amoxicillin, since β-lactamase-producing *G. vaginalis* strains have not been observed so far. Susceptibility testing for *G. vaginalis* is not recommended.

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Nocardia, Rhodococcus, Gordonia, Actinomadura, Streptomyces, and Other Aerobic Actinomycetes*

PATRICIA S. CONVILLE AND FRANK G. WITEBSKY

35

TAXONOMY

The word "actinomycete" is derived from two Greek roots (actino- and -mycete) meaning "ray" (and hence also "rod") and "fungus," respectively. The anaerobic organisms now in the genus *Actinomyces* and the aerobic organisms grouped together as the "aerobic actinomycetes" were previously presumed to be related to one another on the basis of shared features of organismal and colonial morphology. The aerobic actinomycetes, a group for which no agreed-upon operational definition currently exists, are now known to be an evolutionarily heterogeneous assemblage of genera. At some stage they all form gram-positive rods, and most of the more commonly isolated species exhibit at least rudimentary branching under certain growth conditions; all grow better under aerobic than anaerobic conditions, a feature distinguishing them from organisms in the genus *Actinomyces*. The organisms containing mycolic acids in their cell walls (included in the genera *Dietzia, Gordonia, Nocardia, Rhodococcus,* and *Tsukamurella*) are rather closely related on the basis of molecular genetic studies (51, 221); these mycolic-acid-containing genera appear phylogenetically more closely related to the genera *Corynebacterium* and *Mycobacterium* (both of which are sometimes considered to be aerobic actinomycetes) than to the other non-mycolic-acid-containing genera usually also included with the aerobic actinomycetes. According to a recent classification scheme, the various genera are placed in several different suborders, emphasizing the lack of close evolutionary relationships among some of them (221). Regardless of their actual evolutionary relationships, several genera (such as *Gordonia* and *Rhodococcus*) exhibit similar colonial and microscopic morphologic features.

The number of recognized pathogenic species of aerobic actinomycetes has been rising rapidly. Only genera containing species of clinical significance are dealt with in this chapter; the genus *Tropheryma* is discussed in a separate chapter. Ultimately the increasingly fine discrimination of species may result in delineation of important species-specific differences in geographic distribution, pathogenic and other biological mechanisms, disease associations, and antimicrobial susceptibility patterns. However, the enormous proliferation of distinct species for which association with human disease has been claimed presents several problems for the clinical microbiologist. First, phenotypic testing has been rendered virtually useless for accurate discrimination among species. Particularly for the aerobic actinomycetes, the number of phenotypic tests available in the clinical laboratory (and in most research laboratories) is far too small for differentiation among so many species, and often information on the percent positivity for a specific reaction in a given species is not known. Furthermore, precisely the same biochemical testing format has generally not been employed with isolates of all the species being compared, making the usefulness of multistudy comparisons uncertain. Second, some types of testing, such as analysis of cell wall constituents and of whole-cell sugars, are available in only a few research settings and are rarely of use for species level identification. Some general information regarding these features is provided herein, but the references should be consulted for performance procedures and additional details. Third, only nucleic acid-based techniques are currently adequately discriminatory, reproducible, and sufficiently widely available to be useful for precise species identifications in clinical laboratories. Fourth, because of the growing problems with accurate species determinations, the clinical literature is rife with erroneous identifications; nowhere is this problem more apparent than with organisms in the genus *Nocardia*. Additionally, as some species have been described on the basis of only a single isolate, and for others only a handful of reports exist, little if any meaningful clinical information can be associated with many species names. Particularly when molecular methods are not available, precise identification of isolates may be impossible—and is also frequently not of immediate clinical utility.

There are a few terminological issues that, while they pertain to all bacteria, seem to cause confusion particularly frequently with regard to the aerobic actinomycetes. First, for every bacterial species, there is only one "type strain," the strain on which the original description of the species was based. Other strains that are thought to belong to the same species as a given type strain could be referred to as "reference strains," but they are not type strains. Second, the term "sensu stricto" means "in the strict sense." The term, when used with a species name, should be restricted to mean organisms belonging to that particular species, as determined by the best available methods. So, to refer to isolates

* This chapter contains information presented in chapter 35 by June M. Brown and Michael M. McNeil in the eighth edition of this Manual.

that are "*Nocardia asteroides* sensu stricto" should mean isolates that by molecular methods are identical to the type strain of *N. asteroides* (ATCC 19247^T).

DESCRIPTION OF THE GENERA

Actinomadura

The microscopic and colonial morphology of organisms in the genus *Actinomadura,* which contains approximately 57 validly named species, is very similar to those in the genus *Streptomyces*. Microscopically, they are thin, short, branching gram-positive rods (175) (see Fig. 2A) that are modified acid-fast stain negative. Colonies are wrinkled, some aerial mycelium may be present, and the color of the colonies may vary considerably among different isolates (Fig. 1A). Cell walls contain the sugar madurose, a feature shared only with organisms in the genus *Dermatophilus*.

Amycolata and *Amycolatopsis*

The genera *Amycolata* and *Amycolatopsis* were proposed by Lechevalier et al. for nocardia-like organisms that were gram positive (Fig. 2B) and modified acid-fast negative (152). Aerial hyphae are frequently produced (Fig. 1F). Among the species transferred to the genus *Amycolatopsis* was *Nocardia orientalis,* two isolates of which were reported from clinical specimens (99). A study based on 16S rRNA sequences recommended combining the genera *Amycolata* and *Pseudonocardia* into an emended genus, *Pseudonocardia* (252). Currently there are 4 validly named species in the genus *Amycolata* and 26 validly named species in the genus *Amycolatopsis*.

Dermatophilus

The two species currently making up the genus *Dermatophilus* are probably not closely related to most of the other organisms considered in this chapter; by molecular methods this genus was placed in the suborder *Micrococcineae*, whereas the majority of the aerobic actinomycete human pathogens were placed in the suborder *Corynebacterineae* (221). *Dermatophilus congolensis* colonies are somewhat heaped, are opaque, show β-hemolysis on blood agar, and may develop a yellow to orange color with age (275). Aerial mycelium may be produced if the organism is grown in an atmosphere of increased CO_2; the organisms are facultatively anaerobic (95). The microscopic morphology is unusual and striking; this branching organism, with hyphae 0.6 to 1.0 μm in diameter, develops both longitudinal and transverse septa (Fig. 2D). The resulting chains of coccoid cells may occur in as many as eight parallel rows (275). The organisms are gram positive but modified acid-fast negative. The coccoid cells may develop into motile zoospores under favorable environmental conditions.

Dietzia

The species *Dietzia maris* was removed from the genus *Rhodococcus* because of chemotaxonomic and 16S rRNA sequence differences from *Rhodococcus* species (203). Two additional species have since been included in the genus. These organisms apparently rarely, if ever, branch, but like organisms in the genus *Rhodococcus*, they may show coccal and rod forms and are modified acid-fast positive (89).

Gordonia

The biology of the genus *Gordonia* has been recently reviewed (7). Colonies are typically orange and usually wrinkled (Fig. 1I). On Gram stain, *Gordonia* species appear coryneform and show no significant branching; these organisms could easily be mistaken for a component of normal oral flora in sputum specimens (154) (Fig. 2C). The genus "*Gordona*" (now *Gordonia*), originally described by Tsukamura, was revived in 1988 by Stackebrandt et al. (222) to contain species with mycolic acids of approximately 46 to 66 carbon atoms in length (90) and a predominant menaquinone of nine isoprene units. *Gordonia* and *Rhodococcus* were also found to be separable on the basis of their 16S rRNA sequences. There are approximately 19 validly named species in the genus.

Nocardia

The most commonly isolated aerobic actinomycete human pathogens belong to the genus *Nocardia*. In direct Gram smears, organisms generally appear as very long, obviously branching, thin (0.5 to 1.0 μm in diameter), and finely beaded gram-positive rods (Fig. 2E, F, and N). Unlike the individual cells composing a streptococcal chain, the beads generally do not abut one another. Occasionally, chains, or what appear to be clumps, of beads are the only clue to the presence of a *Nocardia*-like organism (Fig. 2F). Organisms may not be detectable with a Gram stain, and a modified acid-fast stain should be done on direct patient material when a nocardial infection is suspected. While nearly all isolates of *Nocardia* are modified acid-fast positive, not all organisms, or even the entirety of a given organism, will necessarily retain the acid-fast stain (Fig. 2H and O) (see "Microscopic Morphology" below). Particularly when prepared from cultures, smears may show streptococcus-like chains or small branching filaments, probably as a result of the fragile nocardial mycelium breaking during smear preparation (189, 190).

Colonial morphology varies from species to species and frequently varies from isolate to isolate within a species. Colonies can be white, gray, buff, salmon, or orange (Fig. 1D, E, G, and L). Colony color may best be seen on the reverse when colonies are grown on translucent media, as color may become obscured on the surface by powdery aerial hyphae typically produced. Such aerial hyphae are formed to at least some extent by nearly all nocardial isolates, but they are also found with isolates in other aerobic actinomycete genera.

Of the nearly 70 species of *Nocardia* currently recognized, over 25 have been reported as human isolates, and several others have been reported as pathogens of other animals. Importantly, isolates in many reports of infection, even in the recent literature, are incorrectly identified because of failure to use optimal identification methods.

A brief account of the nomenclatural history of the designation "*Nocardia asteroides*" is necessary to clarify a confusing and widely unappreciated taxonomic issue. In 1888 Edmond Nocard obtained an isolate of an organism thought to be the causative agent of bovine farcy; this organism was given the name *Nocardia farcinica* by Trevisan in 1889, and thereupon it became the type strain for both the genus and species. Gordon and Mihm (98) found, using their battery of phenotypic tests, that *N. farcinica* could not be distinguished from isolates to which the name *Nocardia asteroides* had been applied. Because of some uncertainty relating to the isolate obtained by Nocard and what was presumed to be the conspecificity of the organisms then known as *N. farcinica* and *N. asteroides*, an appeal was made to the Judicial Commission to have the type species of the genus changed to *N. asteroides,* with strain ATCC 19247^T selected as the type strain of the species. The appeal was accepted, but the isolate

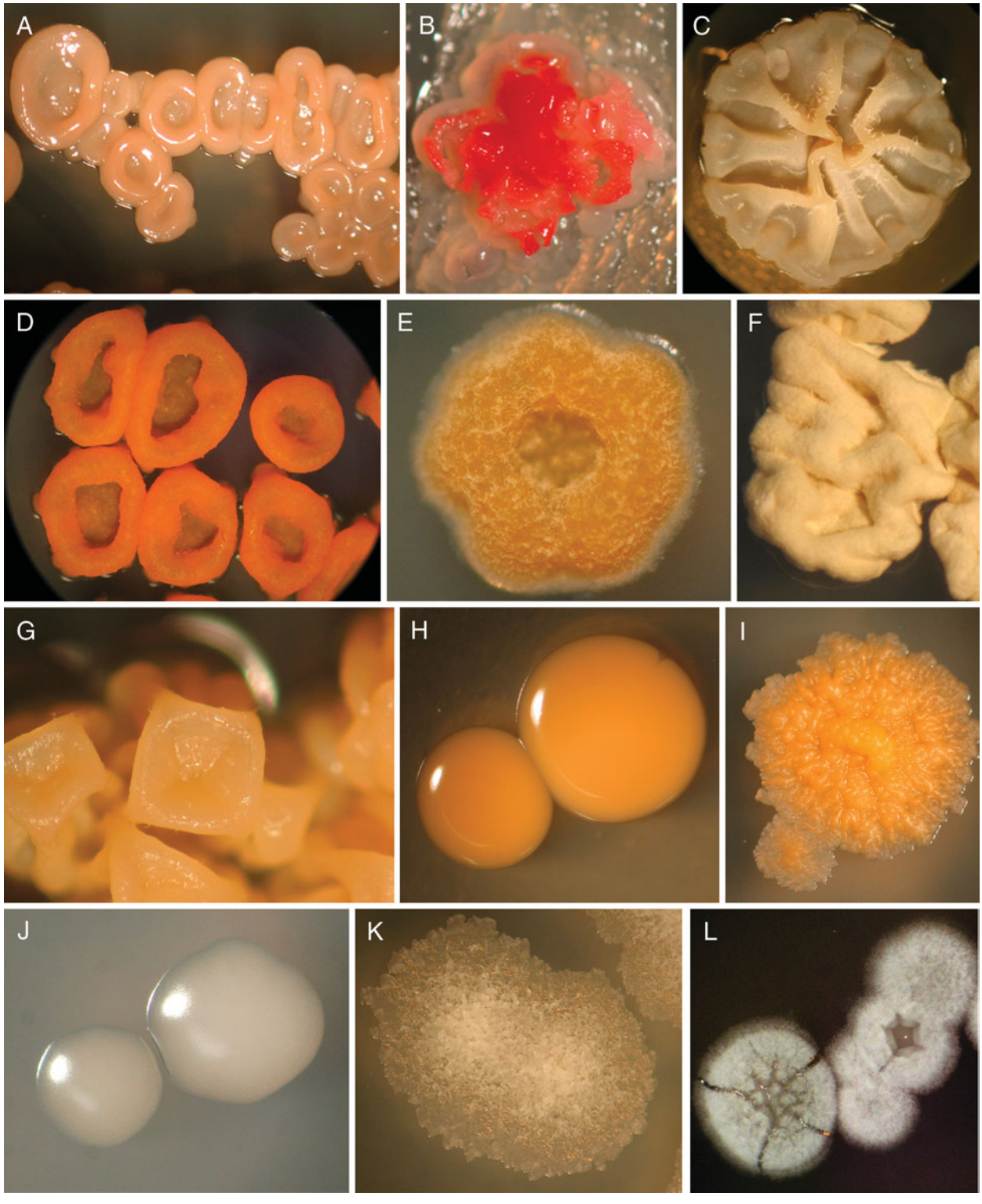
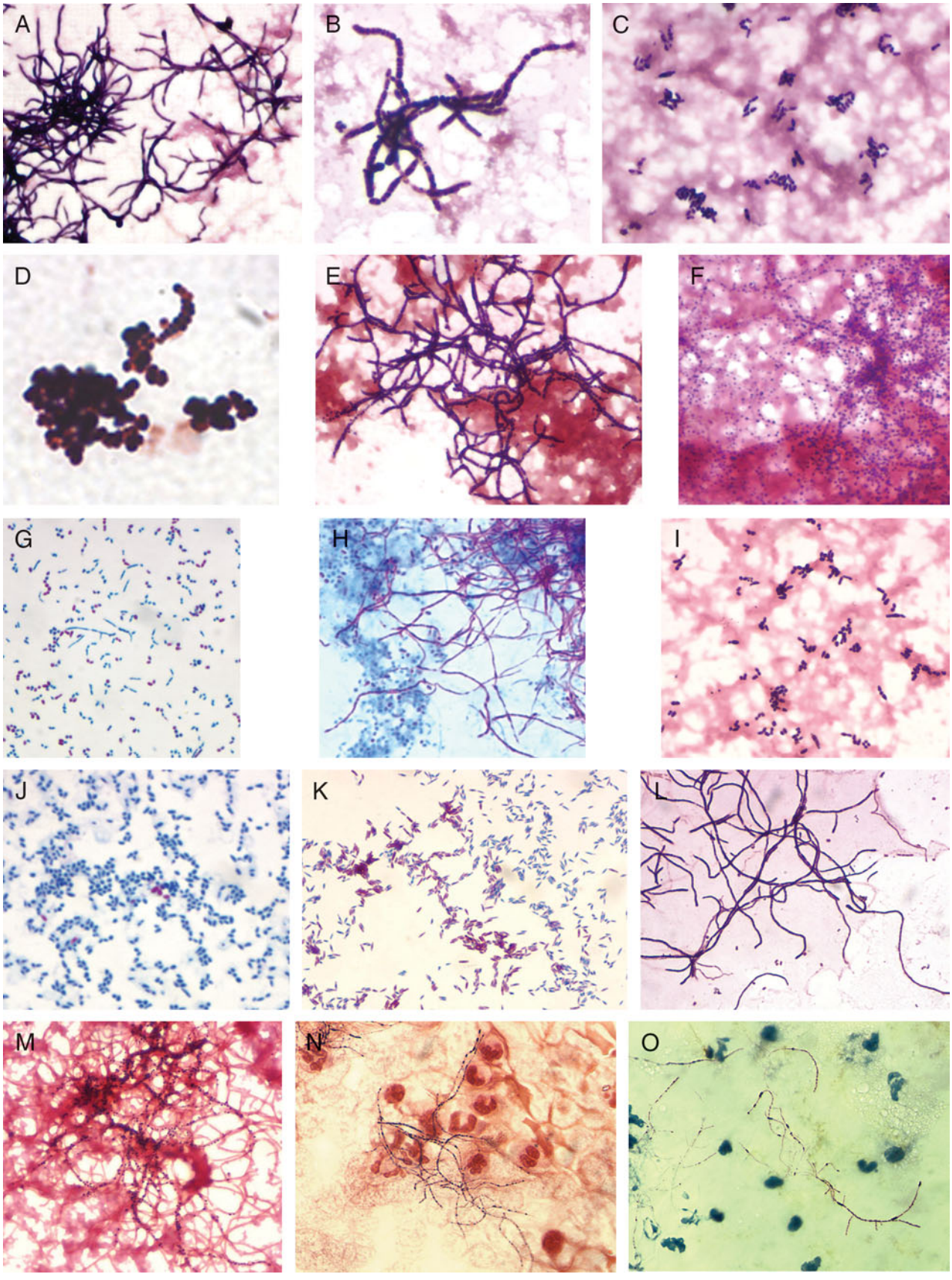


FIGURE 1 Colonial morphology of type strains of various aerobic actinomycetes grown on Sabouraud dextrose agar (Emmons modification). While appearances are typical for the species illustrated, the extent of possible colonial variation within a given species is not known. (A) *Actinomadura latina* at 21 days; smooth umbilicate colonies with no aerial hyphae; (B) *Actinomadura pelletieri* at 10 days; ropy but smooth-surfaced colony with no aerial hyphae; (C) *Streptomyces griseus* at 10 days; rugose colony with some aerial hyphae; (D) *Nocardia brasiliensis* at 10 days; umbilicate colonies; sparse aerial hyphae present, but visible only on microscopic inspection of colonies; (E) *Nocardia carnea* at 25 days; aerial hyphae present; (F) *Amycolatopsis orientalis* at 21 days; aerial hyphae present; (G) *Nocardia otitidiscaviarum* at 7 days; rugose colony; sparse aerial hyphae present, visible only on careful microscopic inspection; (H) *Rhodococcus equi* at 10 days; smooth colonies with no aerial hyphae; (I) *Gordonia bronchialis* at 10 days; irregular but smooth surface with no aerial hyphae; (J) *Tsukamurella pulmonis* at 10 days; smooth colonies with no aerial hyphae; (K) *Tsukamurella strandjordii* at 10 days; rough colonies with no aerial hyphae; (L) *Nocardia cyriacigeorgica* at 10 days; dense powdery aerial hyphae cover most or all the surface of the colonies.


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of *N. farcinica* was retained as the type strain for that particular species, as not all were convinced that the two species were truly identical.

Nocardiopsis

The genus *Nocardiopsis* was originally described by Meyer to accommodate an organism that at the time was called *Actinomadura dassonvillei* but differed chemotaxonomically and in certain colonial morphologic features from other organisms in the genus *Actinomadura*. The cell wall of this organism does not contain madurose. The substrate mycelium fragments into coccal forms, and the aerial hyphae fragment into variable-sized spores. Colonies are described as wrinkled or folded; the aerial mycelium varies from sparse to abundant (178). Currently, there are approximately 27 validly named species in the genus, and PCR primers specific for the genus *Nocardiopsis* have been described (209).

Pseudonocardia

Currently, there are approximately 24 validly published species names in the genus *Pseudonocardia*. See "*Amycolata*" above.

Rhodococcus

Species in the genus *Rhodococcus* tend to form smooth to mucoid colonies that may become increasingly pigmented and appear rougher with age. Colony color may vary from essentially colorless through yellow to red (Fig. 1H). At 37°C colonies may be only about 1 mm in diameter after 24 h of incubation. An aerial mycelium is generally not macroscopically visible but may occasionally be seen microscopically (89). The microscopic morphology of rhodococci can range from coccoid to bacillary depending on species and specimen type and on the stage of growth of the organism; cells may be observed intracellularly in macrophages (64). The organisms exhibit a rod-coccus growth cycle; rod forms are best visualized by growing isolates in a liquid medium, and under such conditions some branching of individual cells may be found. Generally, however, the organisms appear as gram-positive, beaded to solidly staining coccobacilli (Fig. 2I). Rhodococci are aerobic, catalase positive, and nonmotile; they are modified acid-fast positive, but the modified acid-fast stain needs to be performed and interpreted with particular care when dealing with isolates that may belong to this genus, as only a tiny fraction of the cells may retain the stain (Fig. 2J). Acid-fast smears prepared from isolates growing on trypticase soy agar with 5% sheep blood or chocolate agar may appear to be acid-fast negative (78). An important caveat regarding *Rhodococcus* species and *Gordonia* species is that they can easily be dismissed as "diphtheroids" because of their Gram stain morphology (Fig. 2C and I) and their frequent failure to develop obvious pigmentation during the first few days of growth. There are chemotaxonomic and 16S rRNA sequence differences between the two genera (see Table 5) (90). The genus and the species often included within it have been the subject of several recent reviews (19, 92, 103). Some data suggest that it may be justifiable to separate the species into several additional genera (103). There are approximately 34 validly named species in the genus.

Streptomyces

Species in the genus *Streptomyces* form branching, gram-positive rods (from 0.5 to 2.0 μm in diameter) that may appear more solidly gram positive than is typical for *Nocardia* species. Filaments may be fragmented (166), and beading may be seen (Fig. 2L and M). Members of the genus are modified acid-fast negative. The colonial morphology of these organisms may be similar to that of *Nocardia* species; an aerial mycelium is generally formed (Fig. 1C). The genus and species level taxonomy of this huge group, which contains approximately 517 validly named species, remains problematic (5, 172). Many of these species have been patented because of the commercially useful products they synthesize (5).

Tsukamurella

The genus *Tsukamurella* (named to honor the microbiologist Michio Tsukamura) was created to accommodate organisms with mycolic acids with chains of 62 to 78 carbon atoms in length (90); menaquinones of the MK-9 type are predominant (90). *Tsukamurella* species generally appear as long, straight to slightly curved, thin rods with no apparent branching; they are gram positive and modified acid-fast positive (Fig. 2K). Colonies tend to be small and can be smooth to rough but do not produce an aerial mycelium; the color varies from whitish to orange (Fig. 1J and K). The type species of the genus, *T. paurometabola* (corrected from *T. paurometabolum*), was previously known as *Corynebacterium paurometabolum*. There are approximately eight validly named species in the genus.

FIGURE 2 Microscopic morphology of various aerobic actinomycetes. (A) *Actinomadura latina;* Gram stain from Tween-albumin broth (TAB) at 4 days; long, branching, relatively solidly staining rods. (B) *Amycolata autotrophica;* Gram stain from TAB at 4 days; long, beaded rods with some branching. (C) *Gordonia bronchialis;* Gram stain from TAB at 4 days; coryneform rods with no obvious branching. (D) *Dermatophilus congolensis;* Gram stain from SAB at 14 days in CO_2; dense aggregates of cells of varying sizes; note chains of longitudinally and transversely dividing cells. (E) *Nocardia abscessus;* Gram stain from TAB at 4 days; long, branching rods; this is a rather solidly staining example, but some beading can be seen. (F) *Nocardia abscessus;* Gram stain from TAB at 10 days; long, beaded, branching rods; note that the beads generally do not abut one another. (G) *Nocardia farcinica;* modified acid-fast stain from horse blood agar at 2 to 3 days; many coccal forms are present; it is mostly these that are staining modified acid-fast positive. (H) *Nocardia veterana;* modified acid-fast stain from TAB at 25 days; some long, branching forms are staining modified acid-fast positive. (I) *Rhodococcus equi;* Gram stain from TAB at 4 days; coccobacilli and short coryneform rods without obvious branching. (J) *Rhodococcus equi;* modifed acid-fast stain from charcoal yeast extract agar at 10 days; only a small percentage of the cells stain positive. (K) *Tsukamurella pulmonis;* modified acid-fast stain from Lowenstein-Jensen medium at 4 days; thin rods, many of which stain positive. (L) *Streptomyces griseus;* Gram stain from SAB at 4 days; long, branching, fairly uniformly staining rods with little beading evident. (M) *Streptomyces candidus;* Gram stain from TAB at 10 days; long, thin, branching rods not distinguishable from *Nocardia* species. (N) Direct Gram stain of sputum which grew a *Nocardia* species; note lacey network of long, thin, branching, beaded rods. (Courtesy Daniel P. Fedorko.) (O) Direct modified acid-fast stain of *Nocardia* sp. in the same specimen as in panel N; the beads are purplish, but the intervening areas of the organism stain positive. (Courtesy Daniel P. Fedorko.)

NATURAL HABITATS

While the aerobic actinomycetes are widely distributed in the environment, the extent to which particular species are geographically restricted is not well known. Their primary ecological niche is probably the decomposition of plant material (172).

Epidemiology and Transmission

The majority of infections caused by aerobic actinomycetes stem from environmental sources, and even most nosocomial infections appear attributable to an environmental source such as dust from construction work (23). There has not yet been a documented case of direct patient-to-patient spread of an aerobic actinomycete infection without the intermediation of another human agent or of environmental contamination.

Most of the reports of outbreaks and pseudo-outbreaks of infection caused by aerobic actinomycetes have been attributed to *Nocardia* species. A 1979 outbreak in a renal intensive care unit involved at least seven patients. A patient on the unit was found to have been excreting, for 7 months from a nephrostomy, what was eventually identified as *N. asteroides*. An investigation resulted in the isolation of the organism from air and dust in the unit. The environmental contamination probably arose more from aerosols produced during disposal of the nephrostomy urine (115, 160, 227). Reports from other institutions involving *Nocardia* species also suggested nosocomial transmission and involved renal transplant recipients (12, 109), heart transplant patients (79), and patients with postoperative wound infections (23, 259).

A seven-patient outbreak of *Gordonia bronchialis* sternal wound infections was traced to a nurse from whose hands and other body sites the organism was isolated; it was also isolated from two of the nurse's dogs (204).

Two pseudo-outbreaks of infection with *N. asteroides* associated with the use of the BACTEC 460 TB system and inadequate needle sterilization have been reported (159, 193).

An outbreak of pseudoinfection attributed to *T. paurometabola*, involving specimens from 10 different patients and attributed to a common source somewhere in the laboratory, has also been reported (10).

CLINICAL SIGNIFICANCE

Table 1 lists the majority of species of aerobic actinomycetes, other than those in the genus *Nocardia,* that have been isolated from human specimens to date. Table 2 contains a listing of species of *Nocardia* that have been reported as human isolates to date. More commonly isolated species of aerobic actinomycetes of particular significance are dealt with in the sections below. For many of the newly described species there is a paucity of data to establish that the organisms were indeed agents of disease or to establish any clinically significant differences, such as in geographic distribution or susceptibility pattern, among the species. The source of bacterial names used is J. P. Euzéby (*List of Bacterial Names with Standing in Nomenclature;* http://www.bacterio.net). Representative examples of colonial and microscopic morphologies are shown in Fig. 1 and 2.

Actinomadura

A useful clue to the identification of organisms in the genus *Actinomadura* is the nature of the lesion from which an isolate originates. Most commonly this organism causes a mycetoma, a chronic, invasive, slowly progressive infection usually occurring in the foot ("Madura foot") and nearby anatomic structures, and nearly always resulting from traumatic implantation of the organism. Draining sinuses are typically present in a mycetoma; macroscopically visible grains (organism aggregates or microcolonies) may be visible in the discharge from the lesions. There are not adequate data available to allow using grain color for species or even genus level discrimination of the etiologic agent. The infection occurs most frequently in tropical regions, where people are more likely to walk barefoot. The word "mycetoma" is used only to describe the clinical nature of the infection, not the etiologic agent. Actinomycotic mycetomas are caused by aerobic actinomycetes; eumycotic mycetomas are caused by true fungi. Organisms in this genus have very rarely been implicated in other types of infection. Only three species have been reported as pathogens: *Actinomadura latina, A. madurae*, and *A. pelletieri*, with *A. madurae* being most commonly isolated. A set of 21 phenotypic tests was found useful for species level identification of isolates causing mycetoma (234). The same study also suggested that additional species other than the few commonly said to be etiologically involved remained to be described. In many reports mentioning the species causing mycetomas, identification has been made solely on the basis of the histological appearance of the grains, on the basis of a small number of phenotypic tests, or in ways not specified in any detail (45, 137, 163, 164). As with virtually all the aerobic actinomycetes, molecular methods are the only practical procedures for clinical or even reference laboratories to use for definitive species level identification of organisms in this genus.

Amycolata and *Amycolatopsis*

There are no recent reports documenting human infection caused by species in either of the genera *Amycolata* and *Amycolatopsis*. A newly described species, *Amycolatopsis palatopharyngis*, was isolated from what was described as "the infected palatopharyngeal mucosa" of an elderly male, but no further clinical information was provided (116). Three recently described species and several other aerobic actinomycete species may be causative agents of equine placental infection and abortion (145).

Dermatophilus

D. congolensis causes dermatitis in a wide variety of animals worldwide, including cattle, horses, goats, and sheep, but has only rarely been noted as a cause of human infection (233). In humans, the organism has been reported to cause a variety of cutaneous manifestations, including scaling and exudative lesions, pustules, pitted keratolysis (86), and "hairy" leukoplakia of the tongue (37). Filamentous and coccoid forms of the organism may be visualized directly in tissue specimens. Optimal therapy has not been defined, but infections may be self-limiting. *Dermatophilus chelonae* has been reported to cause disease in several species of animals (168, 257).

Dietzia

Only *Dietzia maris* has been associated with human infection. In one reported case the organism was isolated from blood and from an intravascular catheter; biochemical and chemotaxonomic studies of the isolate were performed (20), but the isolate was noted to be modified Kinyoun-stain negative, although *D. maris* would be expected to be modified acid-fast positive. The other reported case involved infection of a hip prosthesis; a variety of identification techniques were employed, including 16S rRNA

TABLE 1 Species of aerobic actinomycetes in genera other than *Nocardia* reported as human isolates[a]

Genus and species	Site(s) of isolation	Approximate no. of patients	Reference(s)
Actinomadura latina	Mycetoma	3	Euzéby[b]
A. madurae	Mycetoma	Many	158, 235
	Peritonitis	1	263
	Pneumonia and bacteremia	1	175
	Sputum	24	174
	Various sites	Few	172, 174
A. pelletieri	Mycetoma	Many	158, 235
Amycolatopsis orientalis (reported as *Nocardia orientalis*)	Cerebrospinal fluid	1	99
	Unspecified	1	99
A. palatopharyngis	Pharynx	1	Euzéby
Dermatophilus congolensis	Skin lesions	Few	86, 106, 233
	Tongue leukoplakia	1	37
Dietzia maris	Catheter-related bacteremia	1	20
	Hip bone	1	197
Gordonia bronchialis	Sternal wound infection	7	204
	Bacteremia	1	217
G. polyisoprenivorans	Catheter-related bacteremia	1	135
G. otitidis	Ear drainage	1	Euzéby
	Pleural fluid	1	Euzéby
G. rubripertincta	Pulmonary infection	1	107
G. sputi	Mediastinitis	1	144
G. terrae	CNS infection	2	72, 73
	Catheter-related bacteremia	5	196
	Hand mycetoma after trauma	1	13
	Mastitis after nipple piercing	1	274
Gordonia species	Bacteremia	1	205
	Catheter-related bacteremia	2	36
	Catheter-related bacteremia and endocarditis	1	154
Nocardiopsis dassonvillei	Mycetoma	1	2
	Bacteremia	1	17
	Pulmonary infection	2	References in reference 17
	Conjunctivitis	1	References in reference 17
	Cutaneous infection	Few	References in reference 17
N. synnemataformans	Sputum	1	270
Rhodococcus aurantiacus (see text—probable *T. inchonensis*)	Abscesses	1	237
	Peritonitis	1	43
	Pulmonary	1	238
	Meningitis	1	202
R. equi	Pulmonary infection, bacteremia, many other body sites	Many	8, 48, 64, 134, 185, 201, 256
R. erythropolis	Disseminated skin infection	1	243
	Endophthalmitis	1	245
R. fascians (reported as *R. luteus*)	Endophthalmitis	1	245
R. globerulus	Keratitis	1	66
R. gordoniae	Bacteremia	1	124
R. rhodochrous	Corneal ulcer	1	94
Rhodococcus species	Peritonitis	1	31
	Skin lesion	1	167
	Meningitis	1	69
	Pneumonia and bacteremia	1	219
	Various sites	Few	172, 219
Streptomyces albus	Mycetoma	1	5
S. bikiniensis	Bacteremia	1	180
S. griseus	Blood, brain, wound, other	Few	172, 174
S. thermovulgaris	Bacteremia	1	77
S. somaliensis	Mycetoma	Many	14, 172

(Continued on next page)

TABLE 1 Species of aerobic actinomycetes in genera other than *Nocardia* reported as human isolates[a] *(Continued)*

Genus and species	Site(s) of isolation	Approximate no. of patients	Reference(s)
Streptomyces species[c]	Pneumonia, various sites	Few	40, 74, 172
Tsukamurella inchonensis	Bacteremia	1	Euzéby
	Lung	1	46, 268; Euzéby
T. paurometabola	Catheter-related bacteremia	4	125, 214; Euzéby
(see text—probable *T. inchonensis*)	Skin infection	1	101
T. pulmonis	Sputum	1	Euzéby
	Catheter-related bacteremia	4	162, 211
	Conjunctivitis	1	261
T. strandjordii	Catheter-related bacteremia	1	133, 211; Euzéby
T. tyrosinosolvans	Bacteremia	2	Euzéby
	Pneumonia	2	Euzéby
	Catheter-related bacteremia	2	211, 215
	Conjunctivitis	2	261
Tsukamurella species	Knee prosthesis	1	148
	Pneumonia	1	3
	Peritonitis	1	212
	Implanted cardioverter-defibrillator	1	4

[a] Data in this table are based largely on the English-language literature accessible through web-based search engines; the species listing provided should not be regarded as complete. Several of the review articles refer to reports that we have not reviewed ourselves. In some cases, insufficient data are presented to determine whether or not the organism isolated was in fact a cause of disease. Many of the isolates were identified only phenotypically; the application of modern molecular identification techniques might have resulted in a different identification from that reported.

[b] The reference for the isolation of this species is the publication initially describing the organism as listed in J. P. Euzéby, *List of Bacterial Names with Standing in Nomenclature*; http://www.bacterio.net.

[c] The review articles mention several additional *Streptomyces* species that have been reported as human isolates.

sequencing, which showed 98% similarity with the *D. maris* type strain (197). The patient responded to treatment with teicoplanin.

Gordonia

There have been relatively few reports of infections attributed to species in the genus *Gordonia* (Table 1). One of the very few clusters of infection attributable to any aerobic actinomycete involved seven patients who developed sternal wound infections following coronary artery bypass surgery (204). The organism involved, *Gordonia bronchialis* (then called *Rhodococcus bronchialis*), was identified by biochemical testing and cell wall mycolic acid analysis. There have also been case reports of bacteremia, central nervous system (CNS) infections, pulmonary disease, and soft tissue infection caused by various *Gordonia* species. An immunocompromised state and the presence of foreign bodies appear to have been contributing factors for some of the patients. The techniques and interpretive criteria used to identify the organisms involved differ significantly among the various reports. For Table 1, when an isolate was not assigned to a particular species, or when such identification details as were presented made the identification problematic, cases were attributed to *Gordonia* species (Table 1).

Nocardia

Nocardia infections generally result either from trauma-related introduction of the organism or, particularly in immunocompromised patients, from inhalation and the resulting establishment of a pulmonary focus. It should be noted that the brain is one of the most common secondary sites of infection (190). A great advance in the clinically useful categorization of pathogenic nocardial isolates was provided by Wallace and his coworkers (249). They divided organisms phenotypically resembling *N. asteroides* into six different drug pattern types and one additional miscellaneous group. With more recent work, numerous different species have been described within this set of organisms, which came to be known as the *Nocardia asteroides* complex, and included *N. farcinica* (drug pattern type V), *Nocardia nova* (drug pattern type III), and isolates of drug pattern types I, II, IV, and VI. Subsequent work has resulted in the description of at least one separate species in almost all these drug pattern types. Undoubtedly, by using current species definition criteria, numerous species of *Nocardia* remain to be described. Roth et al., in their examination of the nearly complete 16S rRNA gene sequence of 74 *Nocardia* strains representing 25 established species, noted 10 taxa that appeared to be substantially different from each one's most closely related species. These 10 taxa probably represent undescribed species (207).

Given the current state of the literature, it is possible to make only the most tentative statements regarding geographic distribution and disease correlates of different species. Fortunately, with the use of a few selected phenotypic tests (see below), it is possible to assign some isolates in this genus to certain species or species groups with moderate confidence and thus to make some predictions regarding their likely antimicrobial susceptibility patterns. Nonetheless, susceptibility testing of all clinically significant isolates is recommended.

Nocardia abscessus

Nocardia abscessus was formally named in 2000 by Yassin et al. (271). ATCC strain 23824, the reference strain of *N. asteroides* drug pattern type I (207, 226), was one of the

TABLE 2 *Nocardia* species reported as human pathogens

Species[a]	Role as pathogen[b]	Site(s) of isolation	Approximate no. of patients
N. abscessus	Definite	See text	See text
N. africana	Unclear	Sputum	8
N. anaemiae	Unclear	Unknown	1
N. aobensis	Unclear	Unknown	5
N. araoensis	Possible	Pulmonary	1
N. arthritidis	Unclear	Sputum	1
N. asiatica	Unclear	Pulmonary, granuloma	6
N. asteroides	See text	See text	See text
N. beijingensis	Probable	Mostly pulmonary	19
N. brasiliensis	Definite	See text	See text
N. brevicatena complex	Definite	See text	See text
N. carnea[c]	Unclear	Eye, pleural fluid	3
N. concava	Probable	Skin	2
N. cyriacigeorgica	Definite	See text	See text
N. elegans	Possible	Sputum	1
N. farcinica	Definite	See text	See text
N. higoensis[d]	Unclear	Pulmonary	1
N. inohanensis	Unclear	Unknown	1
N. kruczakiae	Definite	Pulmonary	2
N. mexicana	Probable	Mycetoma	3
N. niigatensis	Probable	Skin abscesses	4
N. nova	Definite	See text	See text
N. orientalis	See under *Amycolatopsis*		
N. otitidiscaviarum	Definite	See text	See text
N. paucivorans	Definite	See text	See text
N. pneumoniae	Unclear	Sputum	1
N. pseudobrasiliensis	Definite	See text	See text
N. senatus	Unclear	Sputum	1
N. testacea	Unclear	Sputum	1
N. transvalensis	Definite	See text	See text
N. veterana	Definite	See text	See text
N. vinacea[e]	Unclear	Unknown	1
N. yamanashiensis	Unclear	Skin abscess	1

[a] Unless otherwise noted, the reference for the isolation of each of these species is the publication initially describing the organism as listed in J. P. Euzéby, *List of Bacterial Names with Standing in Nomenclature*. http://www.bacterio.net.

[b] The role of an organism was considered unclear if the site of isolation was unknown, if it was isolated from only one patient without substantial corroborating information that the isolate was a pathogen, or if it was obtained only once from respiratory specimens or other unspecified sites from more than one patient, without information establishing the pathogenicity for any isolate. The role was considered possible if it was isolated more than once from one patient, but without other significant information provided. The role was considered probable if it was isolated from more than one patient from a mycetoma or skin abscess. The role was considered definite if a substantial amount of corroborating data was provided.

[c] See reference 99.

[d] See reference 129.

[e] See reference 130.

isolates found to be *N. abscessus* on the basis of 16S rRNA sequencing and DNA-DNA hybridization. Several of the strains in the report naming the species were from abscesses; in a subsequent report from Japan, several isolates from pulmonary sources and one from a brain abscess were reported (128). Two isolates have been reported from Germany, one from pericardial fluid (258) and the other from a posttraumatic wound (114). In their description in 1988 of the antimicrobial susceptibility patterns of 78 clinical isolates of *Nocardia* from various sources, Wallace et al. noted that 20% of the isolates had this drug pattern type. By the testing procedures and interpretive criteria employed, the organisms were susceptible to amikacin, ampicillin, carbenicillin, broad-spectrum cephalosporins, minocycline, and sulfamethoxazole; half of the isolates had high MICs to imipenem (249).

Nocardia asteroides and the "*N. asteroides* Complex"

There are innumerable reports of human infection attributed to *N. asteroides;* at least until recently, it has probably been considered to be the most commonly isolated human pathogenic *Nocardia* species. Notably, molecular analyses of the pathogenic isolates attributed to this species that have been conducted thus far have indicated that all belong to some other named or as-yet-unnamed species.

Infection has been reported to occur in patients immunocompromised as a result of human immunodeficiency virus (HIV) infection (198); systemic lupus erythematosis (100); chronic granulomatous disease (71); malignancies of various types (230); and organ transplantation including bone marrow (47), heart (194), kidney (160), liver (81), and lung (119). Trauma (218) and the presence of intravenous

catheters (161) also predispose patients to infection. In immunocompromised patients the lung appears to be the most common initial site of infection, presumably acquired by inhalation from an environmental source. Infection may become disseminated, and the organism has been isolated from almost every conceivable body site, including blood (141), natural and prosthetic cardiac valves (68, 253), testes (156), thyroid (41), and peritonsillar abscess (1). Ocular involvement can occur either as a metastatic lesion (138) or as a result of ocular trauma (117). The brain is a relatively frequent site of metastatic infection (218). In a report on 21 patients with nocardial meningitis, 43% were noted to have a concomitant brain abscess (29).

The term "*N. asteroides* complex" has been used for organisms phenotypically resembling the *N. asteroides* type strain. However, several distinct species have now been described within that complex, and precisely what the complex is intended to designate is often no longer clear. The designation should be used, if at all, only if the species intended to be included are clearly stated.

Nocardia brasiliensis

N. brasiliensis appears to be the most common cause of actinomycotic mycetoma (see "*Actinomadura*" above) in the Western Hemisphere, especially in Mexico (44, 45). The organism probably occurs worldwide; there are reports of infection from Australia (85), West Bengal (164), and Europe (165) and many from North America (216). A variety of cutaneous manifestations in addition to mycetoma have been reported, including cellulites, abscesses, and lymphocutaneous infection. Nearly all cases are a result of trauma, including that caused by thorns (165), cat scratch (27), and insect bite (191). Most of the trauma-related infections have occurred in immunocompetent individuals. Disseminated infection, usually originating from a pulmonary focus, has also been reported (216); such infections are more likely to occur in immunocompromised patients (140). Some cases, such as one of a brain abscess resulting from dissemination from a pulmonary focus (184), do occur in patients who appear to be immunocompetent. However, most of the cases of invasive disease, as well as some of the cases of cutaneous infection, attributed to *N. brasiliensis* prior to 1995 may have been due to *N. pseudobrasiliensis*. In that year Wallace et al. (247) reported that a subset of organisms that had been identified as *N. brasiliensis* but were predominantly associated with noncutaneous invasive disease appeared to belong to another taxon, formally named *N. pseudobrasiliensis* the following year (208). Unlike true *N. brasiliensis* isolates, most *N. pseudobrasiliensis* isolates hydrolyze adenine, are susceptible to ciprofloxacin and clarithromycin, and are resistant to minocycline. The two species also differed in their mycolic acid patterns, 16S rRNA gene sequences, and restriction fragment length polymorphism (RFLP) patterns for both the 16S rRNA and 65-kDa heat shock protein genes.

Nocardia brevicatena Complex

Brown et al. suggested in 1997 that *Nocardia* isolates sharing both an unusual drug susceptibility pattern and one or another of three different RFLP patterns of an amplified portion of the 65-kDa heat shock protein gene should be considered to form the *Nocardia brevicatena* complex (B. A. Brown, R. W. Wilson, V. A. Steingrube, Z. Blacklock, and R. J. Wallace, Jr., *Abstr. 97th Gen. Meet. Am. Soc. Microbiol.* 1997, abstr. C-65, p. 131, 1997). These organisms included 19 clinical isolates from the United States, 10 clinical isolates from Australia, and three ATCC reference strains of *N. brevicatena*. The 19 U.S. clinical isolates had the type II *N. asteroides* drug pattern (249), being sensitive to ampicillin, carbenicillin, broad-spectrum cephalosporins, minocycline, sulfamethoxazole, and kanamycin. All 32 isolates studied were also susceptible to amikacin and tobramycin but resistant to gentamicin. There have been no subsequent reports of clinical isolates or taxonomic studies of these organisms. Given the different RFLP patterns involved, several different species may be included in the complex. Some workers include *N. paucivorans* in this complex. The 16S rRNA sequences of the type strains of the two species are 99.5% similar, but DNA-DNA hybridization results indicate that *N. brevicatena* and *N. paucivorans* are separate species (269).

Nocardia cyriacigeorgica

N. cyriacigeorgica (spelling corrected from the original *N. cyriacigeorgici*) was described on the basis of an isolate obtained from the sputum of a patient with chronic bronchitis (272). Subsequent isolates were obtained from brain abscesses in an immunocompromised patient (84) and from a patient with pneumonia following a near-drowning incident, from whom *N. farcinica* and several other pathogens were also isolated (240). Isolates of this species were reported to constitute 13 of 96 (14%) clinical isolates of *Nocardia* from Thailand (199) and 13 of 86 (15%) of such isolates from Belgium (254). It should be noted that the 16S rRNA sequence of the type strain of this species (1,400 bp) is identical to that of the reference strain of "*N. asteroides* drug pattern type VI" (ATCC 14759) (207, 226). As there are no published results of DNA-DNA hybridization studies of these two strains, it is remotely possible that they may in fact belong to different species. In the original work describing the different *N. asteroides* drug pattern types, type VI was the most commonly isolated variety (35%) (249), so should DNA-DNA hybridization prove them to be the same, *N. cyriacigeorgica* may be the most frequent human nocardial pathogen, at least in areas where actinomycotic mycetomas are relatively rare. Note that if one speaks of "*N. asteroides* drug pattern type VI sensu stricto," this should mean isolates corresponding (using the best available methods) to the isolate deposited as the "reference" strain of this drug pattern type. However, the designation "*N. asteroides* drug pattern type VI" is not a valid species name. This drug pattern type was initially characterized by resistance to penicillin and susceptibility to broad-spectrum cephalosporins (249).

Nocardia farcinica

The organisms initially described by Wallace et al. as belonging to the group "*N. asteroides* drug pattern type V" (249) were subsequently found to belong to *N. farcinica* (250). Isolates of this species are perhaps the most resistant of all *Nocardia* isolates; they are typically resistant to cefotaxime, cefamandole, ampicillin, gentamicin, and tobramycin and susceptible to amikacin, sulfamethoxazole, and usually also to ciprofloxacin and imipenem (249). Isolates that showed in vitro resistance to trimethoprim-sulfamethoxazole have been reported to respond to meropenem alone (112) and to the combination of linezolid and minocycline (155). This species may have a particular propensity for causing disseminated disease; Wallace et al. reported that among 30 patients for which disease extent was known, 57% had disseminated disease and one-third had CNS involvement (250). Most patients infected by this species, especially those with disseminated disease, have some type of immunocompromise (231, 250), but cutaneous and other infections have been reported to occur in the apparently immunocompetent as well (210). The lung is a common site of involvement, affecting 43% of

patients in one review, while 30% had brain or meningeal involvement (231). The organism has been isolated from blood from a number of patients (50) and from many other sites in addition to the lungs and the CNS; more unusual sites have included the eye of a patient who washed her contact lenses in basin-stored water (75), the site of a cochlear implant (147), and the larynx of an immunocompromised patient for whom no other site of involvement was detected (56). Isolates of this species were also reported to make up 34 of 96 (35%) clinical isolates of *Nocardia* from Thailand (199) and 38 of 86 (44%) of such isolates from Belgium (254). The complete genomic sequence of a clinical isolate (*N. farcinica* IFM 10152) has been determined (121). The genome was found to consist of a single circular chromosome; two circular plasmids were also present. Among the notable findings were the presence of three copies of the rRNA operon and a number of genes that were thought to contribute to the virulence and drug resistance of the organism.

Nocardia nova

N. nova was described by Tsukamura in 1982 and was distinguished from *N. asteroides* by several phenotypic tests (236) and confirmed to be a separate and distinct species by DNA-DNA hybridization (266). Wallace et al. found that 18% of 78 clinical isolates fell into their type III pattern, characterized by susceptibility to ampicillin and erythromycin and resistance to carbenicillin (249). In a subsequent study of 223 clinical isolates, employing both biochemical and susceptibility testing procedures, 17% of the isolates, as well as the type strain of *N. nova*, had similar characteristics, including the type III drug pattern; these isolates were all then considered members of *N. nova* (248). Of the patients for whom clinical information was available, 35% were thought to have disseminated disease; organisms were obtained from many sites, including blood, lung, CNS, skin and soft tissue, joints, and cornea. An upper extremity sporotrichoid form of nocardiosis attributed to *N. nova* in an HIV-positive patient who sustained a thumb injury while working in a field has been reported (120). More recent investigations have revealed that the organisms identified as *N. nova* by phenotypic testing, as well as by the RFLP patterns obtainable from the *hsp*65 gene, actually may belong to other species in addition to *N. nova*, including *N. africana*, *N. kruczakiae*, and *N. veterana* (59). These species can be distinguished from one another and from *N. nova* sensu stricto only by detailed molecular analysis. Isolates identified only by phenotypic testing as belonging to one or another of these species are probably best reported as members of the "*N. nova* complex."

Nocardia otitidiscaviarum

The initial isolate of the species *N. otitidiscaviarum*, described by Snijders in 1924, was obtained from the infected middle ear of a guinea pig; for a time this species was known as *N. caviae* (97). This species is a relatively infrequent cause of human infection. Clark et al. reviewed 28 cases of cutaneous infection, including several of mycetoma, attributed to this species; many of those for which information was available were considered trauma related (52). There are a few reports of infection at other sites, including brain abscess (110), pyothorax (273), catheter-related infection (153), and disseminated infection (195). This species is relatively reliably identified on the basis of its decomposition reactions; it decomposes xanthine and hypoxanthine but not casein or tyrosine. In a recent molecular study of numerous clinical isolates, several different sequences were obtained from nine different strains that had been phenotypically identified as *N. otitidiscaviarum* (192). These results were interpreted as suggesting the presence of several different species within this group, but some of the results also suggested that individual isolates might contain two or more differing copies of the 16S rRNA gene.

Nocardia paucivorans

The species *N. paucivorans* was described by Yassin et al. in 2000 on the basis of a respiratory tract isolate from a patient with chronic lung disease (269). A subsequent isolate was reported from a patient with cerebral nocardiosis; by the susceptibility testing procedure used, the isolate was susceptible to nearly every drug tested (76). Another isolate from a brain abscess attributed to this species that showed 98.4% 16S rRNA gene similarity to a "reference" strain has also been reported; this organism was susceptible by the procedure employed to all drugs tested (258). An isolate from a mitral valve has been reported (28), and 2 of 86 (2.3%) clinical isolates from Belgium were identified as belonging to this species (254). The sensitivity pattern of two of the reported isolates is similar to that of drug pattern type II (see "*Nocardia brevicatena*" above), but both appeared susceptible to gentamicin.

Nocardia pseudobrasiliensis

N. pseudobrasiliensis is more often a cause of noncutaneous invasive infection, although it can cause cutaneous infection as well. Most infections have occurred in immunocompromised patients; cases have been reported from North and South America, Japan, and Australia (30, 127, 247) (see "*Nocardia brasiliensis*" above).

Nocardia transvalensis

N. transvalensis was originally described in 1927 on the basis of an isolate from an African patient with a mycetoma. In 1992 McNeil et al. reported on 16 patients from whom isolates attributed to this species had been obtained (173). The isolates from 10 of the patients were considered clinically significant; the other isolates were considered colonizers or of uncertain significance. A variety of sites of infection were involved; some of the infections were disseminated, and several patients were known to be immunocompromised. Wilson et al. subsequently distinguished four taxa that they proposed be grouped together as the "*N. transvalensis* complex" (260); included among these was the type strain of *N. transvalensis*. All isolates were considered resistant to at least one of the aminoglycosides tested, and the majority were resistant to amikacin, suggesting that they might all be grouped with organisms exhibiting drug pattern type IV, originally defined as resistance to all aminoglycosides and susceptibility to ciprofloxacin (249). There are a few other case reports of disseminated infection attributed to this species, including one in a liver transplant patient (255) and one in a heart transplant patient (157). The isolate from the heart transplant patient developed sulfonamide resistance during the course of treatment (157). A total of 11 clinical isolates assigned to this species have been reported from Japan and Thailand (131, 199).

Nocardia veterana

N. veterana was first described on the basis of an isolate obtained from the bronchial lavage fluid of a patient with pulmonary lesions, but that isolate was thought not to be clinically significant (104). Subsequently, there appeared several case reports that indicated that this species could cause human disease; these included reports of a patient with a mycetoma (132), three patients with pulmonary disease (200), and ascitic fluid infection in an HIV patient

(87). In a subsequent report of three additional clinical isolates, two of which were shown to have been causative agents of pulmonary disease, it was noted that the isolates had an antimicrobial susceptibility pattern essentially identical to the patterns of *N. africana* and *N. nova* (60), demonstrating that species identification could not be definitively established by susceptibility testing alone. It was also noted that the isolates of this species that were studied showed 16S rRNA gene similarities to the type strains of *N. africana* and *N. nova* of 99.0 and 97.7%, respectively. In a collection of 101 isolates initially identified as *N. nova*, further analysis using molecular procedures indicated that at least 15 were most probably isolates of *N. veterana* (R. W. Wilson, P. S. Conville, L. Mann, B. A. Brown-Elliott, C. Crist, F. G. Witebsky, and R. J. Wallace, Jr. *Abstr. 104th Gen. Meet. Am. Soc. Microbiol.* 2004, abstr. U-075, p. 636).

Nocardiopsis

Nearly all the very few infections attributed to organisms in the genus *Nocardiopsis* have been attributed to *N. dassonvillei*. In a letter to the editor regarding a case of actinomycetoma attributed to this species, it was stated that *N. dassonvillei* was "regularly encountered" at the Centers for Disease Control and Prevention, with 21 isolates having been identified from 1981 through 1986; no clinical details were provided (2). There are also a few case reports attributing other types of infection to this species (Table 1). One other species in the genus has been reported as isolated from a human specimen (Table 1).

Rhodococcus

The most commonly isolated pathogen in the genus *Rhodococcus* is *Rhodococcus equi*. While other species have occasionally been reported to cause disease (Table 1), it is impossible to obtain reliable species level identifications without resorting to molecular methods (19, 92). *R. equi* itself may be split into several different species (170). Infections caused by *R. equi* have been the subject of several reviews (8, 63, 134, 232). The organism has long been known as a significant pulmonary pathogen in horses (hence the species name) (201). The initial report of human disease caused by the organism (reported as *Corynebacterium equi*) involved a patient on high-dose steroids with a pulmonary abscess; he had worked briefly in stockyards contaminated with animal feces shortly before the onset of his illness (88). Herbivore manure provides an ideal growth medium for the organism, and inhalation of the organism is presumed to be the major mode of infection in horses (201) and is probably also the principal mechanism for human infections. Direct inoculation and oral ingestion are other possible routes of infection (256). Most patients who develop *R. equi* infection are immunocompromised, and at least until recently approximately two-thirds of infected patients were also HIV infected (256). Essentially any body site can be involved, but the lung is a site of involvement in approximately 80% of immunocompromised patients and at least 40% of immunocompetent patients infected with this species. Bacteremia has been reported in >80% of immunocompromised patients and approximately 30% of immunocompetent patients (256). Pulmonary cavitation is a frequent finding. Malacoplakia, an aggregation of histiocytes containing concentrically layered basophilic structures known as Michaelis-Gutmann bodies, has been noted as a histopathologic finding in several studies (102, 232). Two virulence-associated antigens (VapA and VapB) encoded by plasmids have been identified; isolates producing the VapA antigen are the predominant, possibly the sole, cause of disease in horses, but isolates producing the VapA antigen, the VapB antigen, or neither antigen have been isolated from human cases of infection (194). A combination of antimicrobials is generally used for the treatment of infections. Agents used include aminoglycosides, erythromycin, imipenem, quinolones, rifampin, and vancomycin; linezolid may also be efficacious (256). Some rifampin-resistant strains with *rpoB* gene mutations have been reported (9).

Streptomyces

The most common type of infection attributed to species in the genus *Streptomyces* is mycetoma (see "*Actinomadura*" above), and the most commonly mentioned etiologic agent is *Streptomyces somaliensis*, although in many reports the identification procedures employed could not have ensured that other species were not involved. There are a few reports implicating other species in the genus as occasional pathogens. In case reports of bacteremias attributed to *Streptomyces bikiniensis* (180) and *Streptomyces thermovulgaris* (77), molecular methods were used in identifying the isolates, and such methods were described in detail in a case report of a mycetoma attributed to *Streptomyces albus* (166). While the majority of isolates from nonmycetomatous lesions probably represent either contamination or colonization, these organisms are capable of occasionally causing disease.

Tsukamurella

There are case reports attributing a variety of infections to different species in the genus *Tsukamurella* (Table 1). In the older literature there are a few reports of infection attributed to *Rhodococcus aurantiacus*, such as pulmonary infection, meningitis, peritonitis, and subcutaneous abscesses (43, 237). Recently, *R. aurantiacus* ATCC 25938 (the initial type strain of the species), which has had a convoluted taxonomic history (58), has been placed in *T. inchonensis* (133, 268). Organisms isolated from clinical material and thought to belong to this genus have generally been found to resemble strain ATCC 25938. The organisms reported to cause an outbreak of pseudoinfection (10) (and quite possibly most or all of the organisms involved in clinical infections and identified as *T. paurometabola*) were in fact almost certainly what is now called *Tsukamurella inchonensis*.

Other Genera

Some other genera of aerobic actinomycetes do have roles in human diseases but are unlikely to be encountered in the clinical laboratory. Organisms in three genera of thermophilic aerobic actinomycetes, *Saccharomonospora*, *Saccharopolyspora*, and *Thermoactinomyces*, have been implicated in the etiology of hypersensitivity pneumonitis. Spores of these organisms may be encountered when performing air sampling; molecular methods are helpful for species level identification when needed (108, 264). There are currently approximately 7, 10, and 8 validly published species names in the genera *Saccharomonospora*, *Saccharopolyspora*, and *Thermoactinomyces*, respectively. *Crossiella equi* is an aerobic actinomycete that can cause equine placentitis (70), but there are no reports of human infection attributed to this organism.

COLLECTION, TRANSPORT, AND STORAGE

In temperate climates the respiratory tract is the most frequent portal of entry for the aerobic actinomycetes and therefore the primary site of nocardial infections in the immunocompromised host. Sputum is the most easily obtained pulmonary

specimen, and examination of several fresh early-morning samples collected on separate days (90, 251) may maximize the chances of organism recovery; recovery of an aerobic actinomycete from multiple samples may help to establish the clinical significance of the isolated organism (90, 142). *Nocardia* species may, however, be difficult to recover from sputum even in documented cases of pulmonary infection (190), either because of low numbers of organisms present in the sample or because contaminating bacteria in the sample may overgrow the more slowly growing aerobic actinomycetes. More invasive procedures, such as bronchoalveolar lavage or fine needle or open lung biopsy, may be required to obtain a definitive diagnosis (190). These more invasive procedures may be necessary for diagnosis of as many as 44% of primary pulmonary infections (85); macrophage-rich samples may be necessary to maximize recovery of organisms such as rhodococci, which tend to localize within these cells (64).

Exudates from abscesses or mycetomas should be delivered to the laboratory in a sterile container for examination for characteristic granules and for smear and culture. In the case of disseminated cutaneous lesions or small lesions secondary to trauma, a skin biopsy can be useful. The use of swabs is not recommended, as fibers can make smear interpretation difficult (251). To optimize isolation of *Dermatophilus*, scabs or crusty lesions should be removed and soaked in sterile distilled water, and the fluid should be inoculated onto blood agar plates (275).

In immunocompromised patients the aerobic actinomycetes, especially *Nocardia*, can disseminate to almost any organ. A biopsy sample or aspirate, when obtainable, may be the best specimen to evaluate for the presence of such organisms. Normally sterile body fluids should be collected in a sterile container and sent immediately to the laboratory. *Nocardia* isolates are infrequently recovered from cerebrospinal fluid, even if numerous brain lesions are present, because the organisms may be confined to the brain abscess itself. Blood should be inoculated directly into blood culture media; many commercially available blood culture or lysis centrifugation systems have been shown to support the growth of *Nocardia* species. Various aerobic actinomycetes have been implicated as the cause of catheter-related bacteremia; potentially infected catheter tips should be transported to the laboratory in a sterile container and cultured by appropriate methods.

In all cases where infection with an aerobic actinomycete is suspected, it is of utmost importance that the laboratory be notified of the suspected diagnosis. This will ensure that samples will receive appropriate handling, that the correct direct smears will be prepared, and that the sample will be inoculated onto the appropriate media and incubated for an extended period of time at the appropriate temperature.

All samples should be transported promptly to the laboratory following the specimen collection and handling procedures outlined in chapters 5 and 20.

DIRECT EXAMINATION

Careful microscopic examination of clinical specimens suspected of containing aerobic actinomycetes is extremely important. The observation of organisms characteristic of *Nocardia* and other aerobic actinomycetes should alert laboratory personnel to inoculate appropriate media and to extend incubation at the appropriate temperature. In addition, the detection of organisms directly in the patient specimen may assist in the interpretation of culture results.

Smears can be made directly from sputum, drainages, and aspirates; however, liquid specimens such as bronchoalveolar lavage or normally sterile body fluids that are not excessively cellular should be concentrated before smear preparation and media inoculation. For tissue samples, smears can be made from ground material and from touch preps. Two important stains that should be used in the clinical laboratory for direct samples are the Gram stain and the modified acid-fast stain. Histopathologic examination of fixed tissue by use of special stains may also reveal the presence of organisms (262).

The modified acid-fast stain used on direct specimens may more accurately reflect the true partially acid-fast nature of the organisms than do modified acid-fast stains prepared from colonial growth (Fig. 2O). The modified acid-fast stain uses a weaker decolorizer (1% H_2SO_4) than does the mycobacterial stain (3% HCl) (see chapters 21 and 36 for details on reagent preparation and staining methodology). Because of the difficulty of standardizing this technique, it is imperative that positive and negative controls be run simultaneously with patient smears. The control slides can be made from growing suspensions of *Streptomyces* species (negative control) and *Nocardia* species (positive control). Smears should be evaluated by experienced laboratory personnel, and the quality of the stain itself should be evaluated before results are reported. *Gordonia, Nocardia, Rhodococcus,* and *Tsukamurella* (and possibly *Dietzia*) are known to be partially acid fast with this stain.

Careful attention should be paid to the cellular material present in the sample. Phagocytized gram-positive or acid-fast organisms can sometimes be seen within macrophages and mononuclear cells; in the modified acid-fast smear these may appear as beaded cells with strongly acid-fast granules within non-acid-fast or weakly acid-fast rods (189).

In cases of suspected actinomycetoma, aspirated material should be examined grossly for the presence of granules by spreading the sample in a sterile petri dish. Granules found should be washed and crushed. Smears should be prepared from the crushed material and appropriate media should be inoculated.

Recently, Couble et al. reported on a method for direct detection of *Nocardia* isolates from clinical samples by using PCR along with DNA probes directed to a portion of the 16S rRNA gene. Analysis of 18 patient samples that were culture positive for various *Nocardia* species gave positive results for all samples; specimens containing *Mycobacterium tuberculosis* gave negative results (65). Further assessment of this technique will be needed to assess its clinical utility.

ISOLATION

Blood agar, chocolate agar, brain heart infusion agar, Sabouraud dextrose agar, and Lowenstein-Jensen medium will support the growth of aerobic actinomycetes. *Dermatophilus congolensis* may not grow on Sabouraud dextrose agar or Lowenstein-Jensen medium (275). Buffered charcoal yeast extract agar (BCYE) is particularly useful for the recovery of *Nocardia* species. Specimens from sterile sites or concentrated sterile body fluids can be inoculated directly onto these media. Specimens from respiratory sites, skin, and other potentially contaminated sites, such as mycetomas, should additionally be inoculated onto selective media, such as modified Thayer Martin agar (183) and selective BCYE (containing polymyxin B, anisomycin, and either vancomycin or cefamandole) (244). Sabouraud agar with added chloramphenicol may not be useful, as it may also suppress the growth of some *Nocardia* species (105). A specialized medium for the recovery of *R. equi,* using a Mueller-Hinton agar-based

medium with added ceftazidime and novobiocin, has been described (246).

Cultures for aerobic actinomycetes should be handled as fungal cultures, thus ensuring that the cultures will be held and regularly examined over an extended period. Two BCYE plates (for samples from sterile sites) or selective BCYE plates (for respiratory or other potentially contaminated sites) should be inoculated. One BCYE or selective BCYE should be incubated at 30°C and the other at 35°C, both in ambient air. It should be noted, however, that *Streptomyces* species may show best growth at 25°C and that *D. congolensis* grows better and shows enhanced production of aerial hyphae in increased CO_2 (275). For all cultures, plates should be held for a minimum of 2 weeks, preferably for up to 3 weeks, and should be sealed to prevent dehydration.

A low-pH decontamination procedure has been successfully used for pretreatment of heavily contaminated specimens suspected of harboring *Nocardia* species. The sample is diluted 1:10 in 0.2 M HCl–0.2 M KCl at pH 2.2, mixed, and allowed to stand for 3 to 5 min, after which it is inoculated onto selective and nonselective media (26, 244). Murray et al. report a drop in viability of *Nocardia* species after 30-min exposures to *N*-acetyl-L-cysteine (NALC), NaOH-NALC, or Zephiran-TSP (182). The key to improved recovery of *Nocardia* from NaOH-NALC-treated specimens may be a shorter exposure to the decontaminating reagents (15 min), as is in fact also recommended for the recovery of mycobacteria (136).

Aerobic actinomycetes have been recovered from blood by using a variety of commercially available blood culture systems, including conventional two-bottle systems, biphasic bottles, systems using radiometric and nonradiometric detection, and lysis-centrifugation systems. By use of various blood culture systems, aerobic actinomycetes have been recovered after 3 to 19 days of incubation, which in some cases included the incubation times of terminal subcultures (180, 242). Studies employing newer blood culture systems have resulted in a recommendation that extended incubation is no longer necessary (15); however, such studies have not established that such shorter incubation would generally be adequate for the isolation of aerobic actinomycetes. If the possibility of bacteremia with a member of one of these genera is anticipated, it would be advisable to request fungal blood cultures or to perform terminal subcultures if the incubation period of routine blood cultures cannot be extended.

Cultures from all sources should be examined daily for the first week of incubation and then weekly thereafter, preferably using a dissecting microscope, which will allow detection of tiny colonies. Such microscopic examination is particularly important for specimens that contain contaminating flora, as an aerobic actinomycete can be quickly overgrown by more rapidly growing organisms. Care should also be exercised when examining Lowenstein-Jensen or Middlebrook media for mycobacteria or when examining BCYE plates for *Legionella* species, as *Nocardia* and other aerobic actinomycetes will grow on these media.

IDENTIFICATION

Microscopic Morphology

Gram-stained and modified acid-fast-stained smears should be prepared as the initial step in organism identification. Numerous fields should be reviewed to determine the most prevalent morphology and to detect the sometimes rare branching forms. Care should be taken not to confuse perpendicular aligning of the organisms with true branching (Fig. 2).

A properly prepared and carefully interpreted modified acid-fast smear can assist in the preliminary identification of the organism (Fig. 2G, H, J, and K). Quality control slides should be stained along with stains of colonies from cultures. With the modified acid-fast stain, the background should be blue; slides that have a pink background may be inadequately decolorized and should be repeated. The smear should be scanned for areas where individual cells can be seen or areas where single layers of cells allow clear differentiation of cell borders. The acid-fast reaction of tightly packed clumps of organisms may not represent the true partially acid-fast nature of the cells; be wary of large clumps of cells which all appear to be acid-fast positive. Acid-fast cells will be clearly red; cells that stain purple or light pink may or may not be truly acid fast. A stain that shows an unambiguous acid-fast positive reaction may frequently show only a few clearly red cells, with a majority of blue cells. Frequently, only the "beads" will appear acid-fast positive. If modified acid-fast stain results are ambiguous, transfer of the organism to a lipid-rich medium, such as Lowenstein-Jensen medium or Middlebrook 7H11 agar, and repeat staining may give a more clear-cut stain result. Similar enhancements of the acid-fast reaction have been noted after growth in glycerol nutrient broth, litmus milk, or media containing certain amino acids (142, 239). Acid fastness may become more evident as colonies age; the acid-fast reaction has been reported to be most reliable when performed from colonies after 1 to 4 weeks of growth (239). Occasionally, coccoid forms of *Streptomyces* may appear partially acid fast; hyphae, however, are acid-fast negative. Because of the difficulties of interpretation of the modified acid-fast smear, results of this stain should be considered preliminary and must be used only in conjunction with results from other tests.

Slide Cultures

Slide cultures may be used to evaluate the microscopic morphology of actively growing cultures. Small blocks of a minimal agar (such as tap water agar) are inoculated on the side with the organism of interest, and a coverslip is placed on the top of the block. The slide culture is incubated in a humid environment at 25°C for 2 to 3 weeks and examined regularly for the characteristic features of the various genera. Vegetative mycelia (also called substrate hyphae) grow beneath the surface of the agar and show various degrees of branching. *Gordonia* and *Tsukamurella* show rudimentary to no branching, while the branching of *Actinomadura*, *Nocardia*, and *Streptomyces* is generally extensive. Vegetative hyphae of *Amycolata*, *Amycolatopsis,* and *Nocardia* fragment into bacillary, coccal, or square forms; the vegetative hyphae of *Actinomadura* and *Streptomyces* show little to no fragmentation. The substrate growth of *Rhodococcus* initially appears as short rods, which either remain unchanged in shape or evolve into filaments with side projections or into filaments with elementary to extensively branched hyphae. These forms all rapidly fragment to a coccal or short rod form (113). The rhodococcal colony itself is a mass of these coccoid or coccobacillary forms. The vegetative hyphae of *D. congolensis* are characteristic for the genus and show long, tapering filaments which branch laterally at right angles, with septa forming in transverse and longitudinal planes (113).

Colonial Morphology

The colonial appearance of members of the aerobic actinomycetes is extremely variable among genera and even between isolates of the same species (Fig. 1). Environmental factors such as growth medium, incubation temperature, air

circulation, presence of CO_2, and age of culture can affect the size and consistency of the colonies and the production of aerial hyphae (21, 142). Some species produce a diffusible pigment that can vary from strain to strain (142) (see genus and species descriptions above for information on specific colonial morphologies).

Aerial Hyphae

Aerial hyphae project away from the surface of the colony into the air but may not be apparent until 7 to 14 days for most species that form such hyphae. *Nocardia* and *Streptomyces* usually produce abundant aerial hyphae that give the colonies their characteristic powdery or velvety appearance (Fig. 1E and L); rare strains of *Nocardia* produce sparse or no aerial hyphae (Fig. 1D and G). *Actinomadura, Amycolata*, and *Amycolatopsis* show variable production of aerial hyphae. *Rhodococcus* may rarely form rudimentary aerial hyphae (113). Of the genera that are partially acid fast, only *Nocardia* regularly produces aerial hyphae. The presence of spores and their relative number and arrangement on the aerial hyphae can also give some clue to the genus identification (113).

Phenotypic Identification

Limitations

Given the increasing number of species of aerobic actinomycetes, the use of only a limited number of phenotypic tests to obtain exact species or even genus level identification has become impossible. When a precise identification is required for a significant patient isolate, referral to a reference laboratory for molecular testing is strongly recommended.

Genus Assignment

To determine the genus of an unknown isolate, observation of colonial morphology, specifically the presence of aerial hyphae, is especially important. *Nocardia* and *Streptomyces* generally produce aerial hyphae, with other less commonly isolated genera also showing this morphologic trait (see Table 5). Results of a carefully prepared and carefully interpreted modified acid-fast smear will help to distinguish *Nocardia* from the other genera.

Nocardia and *Tsukamurella* (both modified acid-fast positive organisms) are resistant to lysozyme and show good growth in lysozyme broth; of these, only *Nocardia* species show aerial hyphae. *Gordonia* shows variable growth in lysozyme broth but does not show aerial hyphae. Lysozyme catalyzes the hydrolysis of certain polysaccharides in the cell wall, resulting in a weakening of the cell wall. Berd (21) recommends the use of a very small inoculum into glycerol broth with 0.005% lysozyme and a second similarly inoculated glycerol broth without lysozyme as a control. After 4 weeks of incubation, results are interpreted by comparing organism growth in the broths with and without lysozyme. All *Nocardia* isolates tested in one study (Brown et al., *Abstr. 97th Gen. Meet. Am. Soc. Microbiol.* 1997) were resistant to lysozyme, except for 10 lysozyme-susceptible isolates that belonged to the *N. brevicatena* complex. Lysozyme broth is commercially available from Remel (Lenexa, Kans.) and BD Diagnostics (Sparks, Md.).

Species Assignment

Biochemicals

Some useful phenotypic tests are not commercially available. Tables 3 and 4 present antibiotic susceptibility patterns and basic biochemical results that may provide preliminary identifications of frequently isolated *Nocardia* species or complexes obtained from clinical specimens. Most (but not all) of the isolates of a given species show the results listed in Table 4. When performing biochemical tests, it is extremely important to include appropriate positive and negative controls to ensure that tests are inoculated, incubated, and interpreted correctly. See "Evaluation, Interpretation, and Reporting" below for recommendations on reporting preliminary identifications obtained using these methods.

TABLE 3 Typical in vitro antimicrobial susceptibility patterns of various *Nocardia* species[a,b,c]

Drug	*N. abscessus*	*N. brasiliensis*	*N. cyriacigeorgica*	*N. farcinica*	*N. nova* complex[d]	*N. otitidiscaviarum*	*N. pseudobrasiliensis*	*N. transvalensis* complex[e]
AMC[f]	S	S	R	—	R	R	R	—
Amikacin	S	—	—	S	—	S	—	R
Ampicillin	S	R	R	R	S	R	R	—
Carbenicillin	S	S	—	—	R	R	S	—
Cefamandole	—	R	—	R	—	—	R	—
Ceftriaxone	S	—	S	R	—	R	—	—
Ciprofloxacin	R	R	R	S	—	S	S	S
Clarithromycin	R	R	R	R	S	—	S	R
Erythromycin	R	—	—	R	S	—	—	R
Gentamicin	—	—	—	R	—	S	—	R
Imipenem	R	—	—	S	—	R	—	S
Kanamycin	—	R	—	—	—	S	R	R
Linezolid	S	S	S	S	S	S	S	S
Minocycline	—	S	—	—	—	—	R	—
Sulfamethoxazole	S	S	—	S	—	S	S	—

[a] Abbreviations: S, susceptible; R, resistant; —, no result.
[b] From references 35 and 249.
[c] Based on interpretation of MICs, using CLSI breakpoints.
[d] Includes *N. nova* sensu stricto, *N. africana, N. kruczakiae,* and *N. veterana.*
[e] Includes *N. transvalensis* sensu stricto and *N. asteroides* drug pattern type IV.
[f] AMC, amoxicillin-clavulanic acid.

TABLE 4 Phenotypic characteristics of selected *Nocardia* spp. known to cause human disease[a,b]

Species	Growth at 45°C	Production of:			Hydrolysis of:						Utilization of:			
		Arylsulfatase (14 days)	Nitrate reductase	Urease	Adenine	Casein	Esculin	Hypoxanthine	Tyrosine	Xanthine	Acetamide[c]	Citrate[d]	L-Rhamnose[d]	D-Sorbitol[d]
N. abscessus	−	−	+	+	−	−	−	−	−	−	−	+	−	−
N. africana	+	+	−	−	−	+	−	−	−	−	NA	−	−	−
N. aobensis	−	NA	NA	+	−	−	NA	−	−	−	NA	−	−	−
N. asiatica	−	−	+	V	−	−	−	−	−	−	NA	+	+	−
N. beijingensis	−	NA	NA	+	−	−	−	−	−	+	NA	+	+	+
N. brasiliensis	−	−	+	+	−	+	+	+	+	−	−	+	−	−
N. brevicatena	−	−	−	−	−	−	+	−	−	−	−	−	−	−
N. carnea	−	NA	+	−	−	−	+	−	−	−	NA	−	−	+
N. cyriacigeorgica	+	NA	+	−	−	−	−	−	−	−	+	−	−	−
N. farcinica	+	−	−	+	−	−	+	−	−	−	+	−	+	−
N. kruczakiae	+[e]	+	NA	NA	−	−	+	−	−	−	−	+	−	−
N. mexicana	−	−	NA	+	+	−	NA	+	−/W	−	NA	NA	+	+
N. niigatensis	−	NA	NA	V	−	−	NA	W	−	−	NA	−	−	−
N. nova	−	+	+	+	−	−	−	−	−	−	−	−	−	−
N. otitidiscaviarum	V	NA	+	+	−	−	+	+	−	+	−	−	−	−
N. paucivorans	+	NA	−	+	−	−	+	−	−	−	−	−	−	−
N. pseudobrasiliensis	−	−	−	+	+	+	+	+	+	−	NA	+	−	−
N. transvalensis complex	V	−	+	+	−	−	+	+	−	−	−	+	−	V
N. veterana	+	−	−	+	−	−	−	−	−	−	−	−	—[f]	−

[a] Symbols and abbreviations: −, negative; +, positive; NA, not available; V, variable; W, weak.
[b] From reference 34.
[c] Utilization as sole source of carbon and nitrogen.
[d] Utilization as sole source of carbon.
[e] Optimal growth.
[f] Results differ in separate publications (60, 103).

Acetamide Hydrolysis

The enzyme acylamidase hydrolyzes acetamide with the production of ammonia after incubation for 7 days at 35°C. The appropriate color change in the medium must be seen, as acetamide may be assimilated but not hydrolyzed (Remel Technical Information TI #60012-A). A positive result is a distinguishing characteristic for *N. farcinica* (250).

Antibiotic Susceptibility Testing

While various species of *Nocardia* are known to have typical antibiotic susceptibility patterns (Table 3), the use of these patterns alone for species identification should be avoided. Species belonging to "complexes" (for example, the members of the *N. nova* complex) may have identical antibiograms (59).

Arylsulfatase

The enzyme arylsulfatase hydrolyzes the bond between the sulfate group and the aromatic ring of potassium phenolphthalein disulfate incorporated in the medium. With the addition of an alkali (sodium carbonate), free phenolphthalein is released, resulting in a change of color to red in the medium (136). The CDC Actinomycete Laboratory uses a modified methodology of the Kubica method, increasing the phenolphthalein concentration from 0.001 M to 0.008 M. The test can be read at 3 days and again at 2 weeks, with most strains of *N. nova* showing positive results by 14 days. Arylsulfatase medium is available commercially, but the concentration of phenolphthalein in the product being used must be considered when comparing results obtained with results reported in the literature.

Acid Production with Carbohydrates

The "Gordon" method utilizes basal medium with individual carbohydrates; the pH indicator bromcresol purple is used (96). Oxidation of carbohydrates results in a change in medium color from purple to yellow (acid) after 3 weeks of incubation at 25 or 35°C (21). The media are not available from commercial sources.

Utilization of Carbohydrates as a Sole Carbon Source

The "Goodfellow" method tests the ability of organisms to utilize various carbohydrates as a sole source of carbon. Basal medium with a single carbon source added is inoculated with an organism suspension; positive results are noted as heavier growth on medium containing the carbohydrate than is observed on basal medium without carbohydrates, after 3 weeks of incubation at 25 or 35°C (91). The media are not available commercially.

When preparing media for both the Gordon and Goodfellow tests, some substrates must be filter sterilized rather than autoclaved.

Other Compounds as a Sole Source of Carbon

The ability of organisms to utilize compounds such as sodium citrate and gluconate as a sole carbon source is also frequently used in identification schemes.

Esculin Hydrolysis

The esculin hydrolysis test determines the ability of an organism to hydrolyze the glycoside esculin through the action of the enzyme esculinase. This test is commercially available in broth or agar form.

Gelatin Hydrolysis

The ability of an organism to produce the proteolytic gelatinases (which liquefy or hydrolyze gelatin) helps to differentiate *N. brasiliensis* and *N. pseudobrasiliensis* from the other *Nocardia* species (139). This medium is commercially available from several sources. After inoculation the test is incubated at 35°C for up to 7 days; tubes must be placed at 4°C for 15 min prior to checking for hydrolysis (139).

Middlebrook Opacification

Flores and Desmond (80) describe the ability of isolates of *N. farcinica* to opacify Middlebrook 7H10 or 7H11 agar within 2 to 10 days of incubation at 28 or 35°C. Opacification was not seen with isolates of *N. asteroides*, although in a study of a limited number of organisms, rare isolates of *N. otitidiscaviarum* and *Rhodococcus terrae* were positive for this characteristic (42).

Nitrate Reduction

This procedure tests the ability of an organism to reduce nitrate to nitrites or free nitrogen gas through the action of nitrate reductase (169). The medium is available from commercial sources.

Substrate Decomposition

The differential abilities of organisms to degrade or hydrolyze the amino acid tyrosine, the purine adenine, the purine derivatives xanthine and hypoxanthine, and the milk protein casein have been shown to be useful for differentiation of some members of *Nocardia*, *Rhodococcus*, and *Tsukamurella* (139). Typically, heavy inocula of the test organisms are placed on each of the substrate-containing agars; positive reactions are seen as clearing of the medium surrounding the colonial growth. All tests should be evaluated weekly; casein results should be recorded after 14 days' total incubation, xanthine after 3 weeks of incubation, and adenine, hypoxanthine, and tyrosine after 4 weeks' total incubation. Not all of these substrates are commercially available.

Temperature Studies

Growth at various temperatures can help differentiate among *Tsukamurella* species and among the thermophilic actinomycetes. Replicates of the same medium (usually sheep blood agar or Sabouraud dextrose agar) are inoculated with a standardized inoculum and incubated at 35 and 45°C; after 3 days of incubation (139) the amounts of growth on the two tubes or plates are compared to determine temperature tolerances. If the presence of a thermotolerant species is suspected, additional media should be incubated at 50°C.

Urease

Urease hydrolyzes urea with the production of ammonia. Wauters et al. recommend the use of a 1% urea broth with cresol red as an indicator (254). Commercially prepared urease test media are also available.

Test Systems

Several studies investigating the use of commercial panels for the identification of *Nocardia* and other aerobic actinomycetes have been published. Test systems studied include the API Zym panels (bioMérieux, Durham, N.C.) (25), Rapid Anaerobe ID and Haemophilus/Neisseria ID panels (Dade Behring, Deerfield, Ill.) (22), the API 32C Yeast panel (bioMérieux) (181), the API (Rapid) Coryne panel (with an extended database) (bioMérieux), and the RapID CB Plus System (Remel, Lenexa, Kans.) (82, 83). To date, there is insufficient evidence to establish the utility of any of these tests for the identification of the increasing number of described species.

Identification schemes proposed by Kiska et al. (139) and Wauters et al. (254) utilize phenotypic and/or enzymatic tests and have been shown to be useful for the identification of several of the commonly isolated *Nocardia* species. Neither of these algorithms has been extensively evaluated by other investigators, nor have studies been done with recently described species.

Cell Wall and Cell Membrane

Some characteristics of the cell wall and cell membranes of genera of the aerobic actinomycetes are given in Table 5. Analysis of whole-cell hydrolysates using paper and thin-layer chromatography has been used to identify various forms of diaminopimelic acid and sugars present in the peptidoglycan layer of the cell walls; the type of sugars present, combined with the type of diaminopimelic acid, allowed classification of the various genera into cell wall types (67, 224).

Since members of the aerobic actinomycetes and related genera are known to contain elevated levels of cell wall lipids, analysis of the variations in chain length, differences in the degree of saturation, and the presence of various functional groups on cell wall lipids has been evaluated for identification to the genus or species level (241). McNabb et al. used high-performance liquid chromatography and the Microbial Identification System (MIDI, Inc., Newark, Del.) to analyze the fatty acids of aerobic actinomycetes. Their results showed distinct clustering of genera into two groups based on the presence or absence of branching of the cell wall fatty acids. Results also showed significant heterogeneity among species subsequently found to be heterogeneous by molecular methods (171).

Mycolic acids, high-molecular-weight fatty acids with an α-branched, β-hydroxy structure, are thought to contribute to the staining characteristics and pathogenicity of these organisms (16). The corynebacteria and mycobacteria (which also contain mycolic acids) and those aerobic actinomycetes containing mycolic acids can be differentiated based on the number of carbon atoms of their constituent mycolic acids (Table 5). It should be noted that the number of carbon atoms making up the mycolic acids of some genera varies from one report to another.

A variety of methods have been used to evaluate the cell wall lipids and mycolic acids of the aerobic actinomycetes, including thin-layer chromatography (179), gas chromatography with mass spectrometry (11), and high-performance liquid chromatography (38). By using these methods, differences have been noted among nocardiae belonging to the *N. asteroides* complex, among *Rhodococcus* species, and among the genera *Dietzia*, *Gordonia,* and *Rhodococcus* (39, 187, 188).

Isoprenoid quinones are present in the cell membranes of most bacteria; they function in electron transport, oxidative phosphorylation, and perhaps active transport (241). Menaquinones, the type of isoprenoid quinone present in the cell membranes of the aerobic actinomycetes, vary in the number of isoprene units and in the degree of unsaturation of the C_3 isoprenyl side chain. These variations have been

TABLE 5 Morphologic and chemotaxonomic characteristics of genera of aerobic actinomycetes[a]

Genus	Aerial hyphae	Acid fast	Cell wall type[b]	Mycolic acids	No. of carbon atoms in mycolic acid	Growth in lysozyme	Menaquinone
Actinomadura	V	Neg	III	Neg	NA	NF	MK-9(H4H6H8)
Amycolatopsis	V	Neg	IV	Neg	NA	Neg	MK-9(H_2H_4)
Corynebacterium[c]	Neg	Neg	IV	Pos	22–38	NT	MK-8(H_2) or MK-9(H_2)
Dermatophilus	Pos	Neg	III	Neg	NA	NF	NF
Dietzia	Neg	Neg[d]	IV	Pos	34–38	NT	MK-8(H_2)
Gordonia	Neg	W	IV	Pos	46–66	V	MK-9(H_2)
Mycobacterium[c]	V	Pos	IV	Pos	60–90	Pos	MK-9(H_2)
Nocardia	Pos[e]	W	IV	Pos	44–64	Pos	MK-8(H_4) or MK-9(H_2)
Nocardiopsis	Pos	Neg	III	Neg	NA	NF	MK-10($H_2H_4H_6$)
Pseudonocardia	V	Neg	IV	Neg	NA	NF	MK-9(H_4)
Rhodococcus	Neg[f]	W	IV	Pos	34–52	Neg	MK-8(H_2) or MK-9(H_2)
Saccharomonospora	Pos	Neg	IV	Neg	NA	Neg	MK-9(H_4)
Saccharopolyspora	Pos	Neg	IV	Neg	NA	Neg	MK-9(H_4)
Streptomyces	Pos	Neg	I	Neg	NA	V	MK-9(H_6H_8)
Thermoactinomyces	V	NF	III	NF	NF	Pos	NF
Tsukamurella	Neg	W	IV	Pos	62–78	Pos	MK-9

[a] From references 5, 34, 57, 90, 93, 95, 111, 143, and 152. Abbreviations: V, variable; Neg, negative; NA, not applicable; Pos; positive; NT, not tested; NF, test result not found; W, weak positive.
[b] Cell wall types: I, L-DAP, no sugars; III, meso-DAP, madurose, or no sugars; IV, *meso*-DAP, arabinose, and galactose.
[c] Included for completeness; some consider *Corynebacterium* and *Mycobacterium* to be aerobic actinomycetes.
[d] Original description was Pos.
[e] Occasionally no aerial hyphae produced.
[f] Occasional rudimentary aerial hyphae seen.

found to be helpful in the differentiation of members of the aerobic actinomycetes to the genus level (57, 267). Menaquinone analysis has been particularly useful for distinguishing *Nocardia* species from other mycolic acid-containing aerobic actinomycetes (Table 5).

The equipment required for modern chromatographic procedures is generally available only in research or reference laboratories. In addition, these techniques are not applicable for identification to the species level.

Molecular Identification

Molecular techniques are currently the only methods that can provide definitive identification of most isolates of aerobic actinomycetes. These methods have the added benefit of providing identifications in a fraction of the time needed for biochemical tests.

PCR and REA

The use of PCR enables the identification of aerobic actinomycetes that have unique gene regions (chapter 16). This method has been applied to various genes such as the 16S rRNA gene (for genus level identification of *Nocardia* and *Nocardiopsis* species and species level identification of *R. equi*), the *choE* gene (*R. equi*), and a putative nonribosomal peptide synthetase gene (*N. farcinica*) (18, 32, 146, 151, 209).

PCR paired with restriction endonuclease analysis (REA) is useful for the identification of commonly isolated *Nocardia* species. The procedure takes advantage of the presence of sites within the variable regions of an organism's gene sequence (chapter 16). With REA of a portion of the heat shock protein gene (HSP), Steingrube et al. were able to differentiate among eight species of *Nocardia* and the six drug pattern types of *N. asteroides* described by Wallace (225, 229, 249). Refinement of this technique with a different restriction endonuclease set eliminated the intraspecies variability seen with the initial method and allowed recognition of *A. madurae*, *R. equi*, and *T. paurometabola* (226). In 2000, an REA technique utilizing a portion of the 16S rRNA gene which allowed differentiation of 10 species of *Nocardia* was described. Comparison of identifications obtained by REA of the 16S rRNA gene and by REA of the HSP gene showed good correlation between the two methods for most *Nocardia* isolates. Some isolates, however, gave discrepant or unknown RFLP patterns with either or both of the two genes. Subsequent 16S rRNA gene sequencing showed these isolates to be different from species already described (61). By using REA of the two gene regions, reliable identifications can be obtained for the species listed in Table 6. Species not listed are not identifiable by using the 16S rRNA gene and the listed combinations of restriction endonucleases. Two or more species, including numerous newly described species, may give identical RFLP patterns with REA of the 16S rRNA gene as determined experimentally or deduced from the 16S rRNA sequences of the organisms as listed in GenBank. RFLP patterns from REA of the HSP gene for some recently described species are not determinable, due to the lack of HSP gene sequence data. As the number of recognized species increases, the applicability of the REA method will be diminished.

In addition to its use for the identification of *Nocardia* species, REA has been used for differentiation among numerous genera of aerobic actinomycetes and for identification of some species of thermophilic actinomycetes (63, 108).

Other Molecular Methods

Ribotyping (chapter 16) has been used to identify isolates of *N. farcinica* (176) and to discriminate among four members of the *N. asteroides* complex (150). Ribotyping of *Rhodococcus* species has shown specific patterns for all rhodococcal type

TABLE 6 RFLP patterns of the type strains of selected *Nocardia* species; results of PCR-REA of the 16S rRNA gene (999 bp) and of the 65-kDA HSP gene (441 bp)[a]

Species[b]	16S rRNA RFLP					HSP RFLP		
	Expected sizes of fragments (bp)[c] after digestion with:					Expected sizes of fragments (bp)[c,d] after digestion with:		
	HinPI 1	DpnII	BstEII	SphI	HindI	MspI	HinfI	BsaHI
N. abscessus	420-350-225	700-200-95	Uncut	Uncut	—[g]	180-130-120[e]	Uncut	—
N. cyriacigeorgica	420-350-225	455-250-200-95	—	—	565-435	180-(145-130)-(120-115)	Uncut	(320-270)-75-65
N. brasiliensis	420-350-225	700-200-95	730-270	835-165	—	180-(145-130)-(120-115)	315-125	185-80-70-65
N. nova	420-350-225	640-200-95-60	—	—	—	130-110-75	Uncut	—
N. otitidiscaviarum	420-350-150-75	455-250-200-95	—	—	—	180-(145-130)-(120-115) or 305-145	315-125	(270-188)-100-80
N. pseudobrasiliensis	420-350-225	525-200-175-95	—	—	—	180-130	225-190	—
N. transvalensis complex[f]	645-350	705-200-95	—	—	—	265-180 or 265-110-70	260-190	—

[a] From references 61 and 226.
[b] Only the species listed can be discriminated by these methods; see the text.
[c] Rounded to the nearest 5 bp.
[d] Fragment sizes in parentheses indicate the range of possible fragment sizes.
[e] The HSP gene RFLP pattern for *N. abscessus* was derived from the HSP gene sequence for that organism.
[f] Includes *N. transvalensis* sensu stricto and *N. asteroides* drug pattern type IV; the 16S rRNA gene RFLP pattern for *N. asteroides* drug pattern type IV was determined experimentally.
[g] —, not needed for identification.

strains and heterogeneous patterns for members of *R. equi* and *R. rhodochrous* (126, 149).

The use of the PCR-randomly amplified polymorphic DNA (RAPD) technique (chapter 16) has shown species-specific patterns for some species of *Nocardia;* Isik et al. report that *N. nova, N. pseudobrasiliensis, N. transvalensis,* and isolates identified as *N. asteroides* show a variety of patterns (122); it is not clear if the variations reported by these authors represent the presence of additional unrecognized species, within-species variation, or both.

The use of DNA probes has been evaluated for *Streptomyces* species (223), and Brownell reported that six different probes were needed to hybridize with isolates identified as "*N. asteroides*" (33).

Gene Sequencing

Gene sequencing has become a powerful tool for the identification of this complex group of organisms. Sequence analysis of the 16S rRNA gene has been used for the species level identification of members of nearly all genera of the aerobic actinomycetes (7, 17, 116, 133, 166, 185, 203, 207, 264).

The presence of a variable region, located near the 5′ terminus of the gene (61), has made partial 16S rRNA sequencing of a 500-bp region of the gene a useful method for the presumptive identification of many *Nocardia* species. Cloud et al. (54) compared identifications obtained with the MicroSeq 500 System (Applied BioSystems, Foster City, Calif.) with results obtained using phenotypic identifications and with sequencing results from a 999-bp region of the same gene. The 500-bp-based identification of 94 clinical isolates representing 10 species showed 72% agreement with identification obtained by phenotypic methods and 90% agreement with those obtained by sequencing a larger portion of the gene. Sequencing a larger region of the gene is necessary for the separation of members of some species pairs which have identical or nearly identical sequences within the 500-bp region (for example, *N. abscessus* and *N. asiatica,* which differ by 1 bp, and *N. elegans* and *N. veterana,* which have no base pair differences in this region). Patel et al. used the MicroSeq 500 system to determine the 16S rRNA gene sequences of 28 reference strains and 71 clinical isolates and found significant sequence heterogeneity among isolates identified as *N. asteroides* drug pattern type II, *N. nova, N. otitidiscaviarum,* and *N. transvalensis*. This method was also shown to be useful for the identification of *Gordonia, Rhodococcus, Streptomyces,* and *Tsukamurella* species to the genus level (192). At present, the MicroSeq 500 System is capable of distinguishing among the more commonly isolated *Nocardia* species. However, as the number of described species increases, the number of species that can be unambiguously identified using 500-bp sequences will probably decrease.

Sequence analysis of a 441-bp fragment of the 65-kDa heat shock protein gene has been shown to have sufficient variation to allow identification of 42 type or reference strains of *Nocardia* (206) and may be more discriminatory than sequence analysis of the 16S rRNA gene.

The use of an alternative gene, the *secA1* gene, has been reported (P. S. Conville, A. M. Zelazny, and F. G. Witebsky. *Abstr. 105th Gen. Meet. Am. Soc. Microbiol.* 2005, abstr. C-152, p. 134). Analysis of the *secA1* gene sequences of 31 species of *Nocardia* has shown this gene to be more variable than the 16S rRNA gene; it therefore provides greater discrimination among *Nocardia* species. In addition, in the case of genes that code for essential proteins, translation of the gene sequence into an amino acid sequence may be useful to eliminate some within-species microheterogeneities.

For some genera, such as *Nocardia*, sequence analysis of more than one gene may be useful for the recognition of new species.

There is no consensus on how similar sequences must be to consider isolates to belong to the same species. This is a very real problem with isolates of *Nocardia,* as some species possess 16S rRNA gene sequences that are between 99.5 and 99.8% similar to those of another species, yet are distinct species by DNA-DNA hybridization (59, 269). In addition, users of gene sequence data must be careful to consider gene similarity data in relation to the size of the region sequenced; some sequence differences may not be significant if a larger portion of the gene is analyzed (53).

The presence of multiple 16S rRNA genes in *N. nova* isolates has recently been reported (62). It is unclear how the presence of multiple genes with significant sequence differences will affect the ability to identify these isolates.

Species described based on characteristics of a single isolate present problems for laboratories attempting to make identification decisions; the phenotypic characteristics described may not reflect the typical reactions of the species that would be determined if more isolates were analyzed (123). In addition, for single isolates there is no information available concerning sequence variability (49) (see also chapter 19).

Users of any sequence database used for species identification should be aware of the limitations inherent in that database. The GenBank database includes the most extensive collection of sequences, and an informed user can utilize it to make identification decisions. The proprietary database used with the MicroSeq System (Applied BioSystems) for the identification of *Nocardia* species is limited in the species represented and in the number of sequences of each species it contains. Cloud et al. expanded this database with additional entries to obtain better matches for query organisms (54). The Ribosomal Differentiation of Medical Microorganisms (RIDOM) is a quality-controlled database (177), but the portion pertaining to *Nocardia* is not available for public use. Ideally, the isolate from which the top-choice sequence was obtained should be the type strain of the species, and because of method improvements, it should have been submitted after 1995. Any top choice that appears to be out of place with other closely related sequences should be further examined to ensure that it has not appeared as a result of errors in its sequence or its identification.

DNA-DNA Hybridization

Despite the lack of a single standardized DNA-DNA hybridization procedure (220), the use of the procedure may be required for definitive species level discrimination of some isolates which have a high degree of sequence similarity (59).

TYPING SYSTEMS

In a study of *N. farcinica* isolates recovered from heart transplant recipients, Exmelin et al. compared the use of RAPD and ribotyping techniques for strain typing. They found RAPD better able than ribotyping to differentiate outbreak strains from unrelated strains (79). For strain typing with RAPD, isolates initially must be proven to belong to the same species; in this case chemotaxonomic and phenotypic methods identified the outbreak strains as *N. farcinica* (79). Blümel et al. used pulsed-field gel electrophoresis to demonstrate the relatedness of 18 isolates of *N. farcinica* from postoperative wound infections in a surgical ward over an 11-year period; these isolates were unrelated to isolates recovered from other environmental locations (23).

Recently, Yamamura used repetitive sequence-based PCR (rep-PCR) to distinguish strains identified as *N. asteroides* from unrelated clinical isolates and from different ecologic sources (265). This study showed each isolate to have a distinct banding pattern.

SEROLOGIC METHODS

Serologic methods for the detection of antibody response to nocardial infections have been shown to be lacking in sensitivity and specificity. A variety of methodologies and antigens have been evaluated, including immunodiffusion, complement fixation, enzyme-linked immunosorbent assay, and immunoblot techniques. Cell-free culture filtrates, cell homogenates, and specific high-molecular-weight proteins have been studied as sources of antigens. Most methods show considerable cross-reactivity with antibodies produced against *M. tuberculosis*. Such cross-reactivity is problematic, as tuberculosis usually is included in the differential diagnosis of patients with suspected pulmonary or CNS nocardiosis. In addition, serologic assays may not be sensitive enough to detect antibodies in patients who have diminished immunologic responses, and these are the very ones who are most prone to nocardial infections (6, 24, 118, 213, 227, 228).

SUSCEPTIBILITY TESTING

The Clinical Laboratory Standards Institute (CLSI, formerly NCCLS) has published an approved standard for susceptibility testing of both mycobacteria and aerobic actinomycetes (186). The recommended procedure for the aerobic actinomycetes is broth microdilution (55). The applicability of the procedure to the genera other than *Nocardia,* such as *Rhodococcus,* is currently under discussion. Incubation of plates is at 37°C in ambient air for 3 to 5 days, the length of time depending on the particular species being tested. Both an MIC value and an interpretation of the MIC result should be provided. Currently the interpretive breakpoints for three drugs, amikacin, minocycline, and sulfamethoxazole, differ from the breakpoints recommended for rapidly growing aerobic bacteria (55).

Susceptibility testing should be performed on all isolates of aerobic actinomycetes thought to be of possible clinical significance and especially for isolates from patients for whom a sulfonamide cannot be used. It is recommended that laboratories not performing such testing on a regular basis send isolates to an experienced reference laboratory. Antimicrobial agents recommended for primary susceptibility testing are amikacin, amoxicillin-clavulanic acid, ceftriaxone, ciprofloxacin, clarithromycin, imipenem, linezolid, minocycline, sulfamethoxazole or trimethoprim-sulfamethoxazole, and tobramycin. Agents to be considered for secondary testing include cefepime, cefotaxime, doxycycline, or moxifloxacin, and gentamicin. A few drugs with trailing endpoints, such as the sulfonamides and linezolid, may present interpretive problems. Most isolates of *Nocardia* species are susceptible to trimethoprim-sulfamethoxazole; one should be careful not to assume too readily that an isolate is resistant to this combination of drugs. Additionally, most, if not all, isolates of *Nocardia* species are susceptible in vitro to linezolid (35).

Table 3 shows the typical susceptibility patterns for the different *Nocardia* species for which adequate data are available.

EVALUATION, INTERPRETATION, AND REPORTING

The aerobic actinomycetes are widespread in the environment. Therefore, particularly when an aerobic actinomycete is isolated in culture without having been visualized in the direct patient specimen, it may be impossible for the laboratory to know if the isolate is the result of specimen or laboratory contamination, patient colonization, or actual infection. Some indication of the quantity of organism present should be given for any isolate of an aerobic actinomycete. Discussion between the clinical and laboratory staff is extremely useful for selection of additional patient specimens and laboratory procedures for determination of the clinical significance of an isolate from a given patient. Identification of an organism to the genus level may help determine its significance. However, even for accurate genus assignment, molecular methods may need to be employed.

When an isolate is reported to the species level, the laboratory should be certain that clinicians are aware of the reliability of the identification method used. It is possible to identify the more commonly encountered aerobic actinomycetes, particularly *Nocardia* species, with moderate accuracy to the species level by using phenotypic methods (Table 4) (254), but some will inevitably be misidentified by such procedures. Precise identification can be achieved only by molecular methods. For accurate determination of both identification and susceptibility pattern, referral to a laboratory with expertise in working with these organisms may be necessary.

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Mycobacterium: General Characteristics, Laboratory Detection, and Staining Procedures*

GABY E. PFYFFER

36

Many species within the genus *Mycobacterium* are prominent pathogens, e.g., the members of the *Mycobacterium tuberculosis* complex as well as *M. leprae* and *M. ulcerans.* In addition, numerous species of environmental mycobacteria called nontuberculous mycobacteria (NTM) (formerly "atypical mycobacteria" or "mycobacteria other than tubercle bacilli") are responsible for various kinds of mycobacterioses.

Tuberculosis remains a major global public health problem (35). The actual global prevalence of *M. tuberculosis* infection is 32%, corresponding to approximately 1.9 billion people (51). According to the World Health Organization (229), there were 8.8 million estimated new cases (case rate, 140/100,000) of pulmonary tuberculosis, including 3.9 million smear-positive (and hence highly infectious) cases, in 2003. An estimated 1.9 million people died of tuberculosis, including patients coinfected with human immunodeficiency virus (HIV; 229,000). Analysis of epidemiological trends suggests that numbers of new cases are rising globally, but more people are being cured and death rates are falling (229). Reducing the burden of tuberculosis depends largely on how rapidly DOTS (directly observed therapy, short course) programs can be implemented. Among the prime obstacles for DOTS program expansion are shortages of trained staff, lack of political commitment, weak laboratory services, and the global impact of multidrug resistance (54), together with often inadequate management of multidrug resistance cases as well as people infected with HIV. In the Western Hemisphere, the number of reported cases is steadily decreasing (approximate case rate, 6.8/100,000), reflecting the effectiveness of prevention strategies and control measures implemented by the health authorities, among them the use of more rapid and efficient laboratory algorithms to detect *M. tuberculosis* and susceptibility testing with antituberculosis drugs. In this context, the clinical mycobacteriology laboratory plays an important role (55, 71).

With the advent of new laboratory techniques, new NTM species are being discovered, some of which are sources of diseases in humans (153). Rapid and reliable identification of NTM is thus mandatory but is becoming increasingly complex. The level of service and the choice of methods should, finally, be determined by the patient population served by the laboratory and by the resources available.

TAXONOMY AND DESCRIPTION OF THE GENUS

The genus *Mycobacterium* is the only genus in the family *Mycobacteriaceae* (216) and is related to other mycolic acid-containing genera. The high G+C contents of the DNA of *Mycobacterium* species (61 to 71 mol%, except that of *M. leprae* [55 mol%]) (144) are within the range of those of members of the other mycolic acid-containing genera, *Gordonia* (63 to 69 mol%), *Tsukamurella* (68 to 74 mol%), *Nocardia* (64 to 72 mol%), and *Rhodococcus* (63 to 73 mol%) (38).

Mycobacteria are aerobic (though some species are able to grow under a reduced-O_2 atmosphere), non-spore-forming, nonmotile, slightly curved or straight rods, 0.2 to 0.6 μm by 1.0 to 10 μm, which may branch. Colony morphology varies among the species, ranging from smooth to rough and from nonpigmented (nonphotochromogens) to pigmented. Colonies of the latter type are regularly or variably yellow, orange, or, rarely, pink, usually due to carotenoid pigments. Some species require light to form pigment (photochromogens); other species form pigment in either the light or the dark (scotochromogens). Aerial filaments are very rarely formed and never visible without magnification. Filamentous or mycelium-like growth may sometimes occur but, on slight disturbance, easily fragments into rods or coccoid elements (144).

The cell wall peptidoglycolipid contains *meso*-diaminopimelic acid, alanine, glutamic acid, glucosamine, muramic acid, arabinose, and galactose. Mycolic acids (number of carbon atoms ranging from 70 to 90), together with free lipids (e.g., trehalose-6,6′-dimycolate), provide for a hydrophobic permeability barrier (106, 112). Other important fatty acids are waxes, phospholipids, and mycoserosic and phthienoic acids. Various patterns of cellular fatty acids (number of carbon atoms ranging from 10 to 20) are found as well, among which is tuberculostearic (10-*R*-methyloctadecanoic) acid, a unique cell component for a number of members of the *Actinomycetales* (106).

The high content of complex lipids of the cell wall prevents access of common aniline dyes. Although not readily stained by Gram's method, mycobacteria are usually considered gram-positive. Once stained with special procedures, however, mycobacteria are not easily decolorized, even with

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acid-alcohol; i.e., they are acid fast. However, acid fastness can be partly or completely lost at some stage of growth by a proportion of the cells of some species, particularly the rapidly growing ones.

A natural division exists between slowly and rapidly growing species of mycobacteria. Slow growers require more than 7 days to produce colonies on solid media from a dilute inoculum under ideal culture conditions. Rapid growers, by definition, require less than 7 days when subcultured on Löwenstein-Jensen (L-J) medium but may take several weeks to appear upon primary culture from clinical specimens.

NUTRITIONAL REQUIREMENTS AND GROWTH

Most species adapt readily to growth on relatively simple substrates, using ammonia or amino acids as nitrogen sources and glycerol as a carbon source in the presence of mineral salts. A few species (e.g., *M. haemophilum* and *M. genavense*) are fastidious and require supplements such as mycobactin, hemin, or other iron compounds. To date, *M. leprae* has not been cultured outside living cells. Growth of mycobacteria is stimulated by carbon dioxide and by fatty acids which may be provided in the form of egg yolk or oleic acid, even though the latter is toxic in higher concentrations (≥1%) and has to be neutralized by albumin.

With the genome of *M. tuberculosis* deciphered some years ago, functional genomics have provided new insights into its physiological and metabolic regulation and relation to virulence. For instance, genes involved in pathways of carbon metabolism (e.g., *fadD28*) and in electron transport (*nhA* and *cydA*) and genes encoding glycan biosynthesis (e.g., *Fbp*) have been described previously (219).

Optimum temperatures for growth vary widely among species (from <30 to 45°C). Compared to that of other bacteria, the growth of most mycobacterial species is slow, with generation times of up to approximately 20 h on commonly used media. Depending upon the species, visible colonies may appear after a few days to 6 weeks of incubation under optimum conditions.

SUSCEPTIBILITY TO PHYSICAL AND CHEMICAL AGENTS

Mycobacteria are able to survive for weeks to months on inanimate objects if protected from sunlight. Members of the *M. tuberculosis* complex, for instance, survive for several months on surfaces or in soil or cow dung, from which other animals may be infected (123). Mycobacteria are easily killed by heat (>65°C for at least 30 min) and by UV (sun) light but not by freezing or desiccation. They are more resistant to acids, alkalies, and some chemical disinfectants than most other nonspore-forming bacteria. Quaternary ammonium compounds, hexachlorophene, and chlorhexidine are bacteriostatic at best. The concentration of malachite green in egg-based media (e.g., L-J medium) was selected to maximize the growth of mycobacteria while inhibiting that of other microorganisms. Other commonly used sterilants, such as ethylene oxide and formaldehyde vapor, as well as disinfectants such as chlorine compounds, 70% ethanol, 2% alkaline glutaraldehyde, peracetic acid, and stabilized hydrogen peroxide, are effective in killing *M. tuberculosis*. However, agents that are inactivated in the presence of organic matter (e.g., alcohols) cannot be relied upon to disinfect sputum and other protein-containing materials. With iodophors, the bactericidal effect depends on the content of available iodine as well as on the presence of organic matter (14).

HABITATS

The genus *Mycobacterium* includes obligate pathogens, opportunistic pathogens, and saprophytes. The major ecological niche for the *M. tuberculosis* complex and *M. leprae*, incapable of replication in the inanimate environment, is tissues of humans and warm-blooded animals. In contrast, the NTM are free-living mycobacteria and are usually found in association with watery habitats such as lakes, rivers, and wet soil, etc. Some human pathogenic NTM species, however, have yet to be recovered from soil or water, e.g., *M. ulcerans, M. haemophilum, M. asiaticum, M. shimoidei,* and *M. szulgai* (5). Some other NTM species have rarely been recovered from natural waters or soils but are readily recovered from tap water in settings where disease caused by NTM is frequent or cultures from clinical specimens are often positive. Examples are *M. kansasii, M. xenopi, M. malmoense*, and *M. simiae* (5). The *M. avium* complex (MAC), *M. gordonae,* and rapidly growing mycobacteria have been recovered from tap water as well and also occur in association with nosocomial disease and pseudooutbreaks (5, 83, 208). Although not components of the normal bacterial flora of humans or animals, NTM may be isolated from the skin, upper respiratory tract, and intestinal and genital tracts of asymptomatic individuals (57). Due to their ubiquitous nature, the question of their clinical significance is important but not always easy to answer (5).

A well-known source for positive cultures is bronchoscopes and related devices. Organisms isolated were *M. tuberculosis* (170), *M. xenopi* (12), and *M. chelonae* (66), as well as other NTM.

CLINICAL SIGNIFICANCE AND DESCRIPTION OF SPECIES

With the advent of molecular techniques for appropriate identification, close to 200 mycobacterial species have now been described. This chapter will focus on the slowly growing mycobacteria; rapidly growing mycobacteria are described in chapter 38.

M. tuberculosis Complex

The *M. tuberculosis* complex taxon includes *M. tuberculosis, M. bovis* (*M. bovis* subsp. *bovis, M. bovis* subsp. *caprae,* and *M. bovis* BCG), *M. africanum, M. microti, M. canettii,* and *M. pinnipedii,* which form a tight, discrete group of organisms and display >95% DNA-DNA homology. Recent comparative genomics using the complete DNA sequence of *M. tuberculosis* have provided information on regions of the genome that are deleted in other members of the complex (4, 67). Identification to the species level is justified for epidemiologic, public health, and therapeutic reasons.

M. tuberculosis

M. tuberculosis has an extremely low level of genetic variation, suggesting that the entire population of *M. tuberculosis* organisms resulted from clonal expansion after an evolutionary bottleneck some 35,000 years ago (C. Gutierrez, S. Brisse, R. Brosch, M. Fabre, B. Omais, M. Marmiesse, P. Supply, and V. Vincent, *Abstr. 26th Annu. Congr. Eur. Soc. Mycobacteriol.*, abstr. L-3, 2005).

In today's industrialized world, a higher prevalence of tuberculosis occurs among the medically underserved ethnic minorities, the urban poor, homeless persons, prison inmates, alcoholics, intravenous drug users, the elderly in general, foreign-born persons from areas of high prevalence, and contacts of persons with active tuberculosis. Today, HIV infection is the greatest known risk factor for progression of

latent infection to active tuberculosis. Combined HIV and tuberculosis infections, especially in combination with drug resistance, have caused outbreaks with extremely high mortality rates (30, 31). Groups with a higher likelihood of progression also include individuals with underlying medical conditions, persons who have been infected within the past 2 years, children ≤4 years old, and persons with fibrotic and cancerous lesions detected on chest radiographs.

M. tuberculosis is carried in airborne particles (droplet nuclei) generated when patients with pulmonary tuberculosis cough. These particles, 1 to 5 μm in diameter, are kept suspended by normal air currents. Infection occurs when a susceptible person inhales the droplet nuclei. Once in the alveoli, the organisms are engulfed by alveolar macrophages. Usually, the host cell-mediated immune response limits the multiplication and spread of *M. tuberculosis*. However, some bacilli can remain viable but dormant for many years after the initial infection. Patients latently infected with *M. tuberculosis* usually have a positive purified protein derivative (PPD) skin test but are asymptomatic and not infectious. In general, persons with a latent infection have a 10% risk during their lifetime for development of active tuberculosis. By contrast, patients with HIV infection have a 10 to 15% risk per year for progression to manifest disease (2).

Tuberculosis in adults is a slowly progressive process characterized by chronic inflammation and caseation and formation of cavities. These foci may rupture into the bronchi, allowing very large numbers of organisms to spread to other areas of the lungs and to be aerosolized by coughing, hence infecting other persons. The clinical features of pulmonary tuberculosis are cough, weight loss, night sweats, low-grade fever, dyspnea, and chest pain. Extrapulmonary manifestations of *M. tuberculosis* infection include cervical lymphadenitis, pleuritis, pericarditis, synovitis, meningitis, and infections of the skin, joints, bones, and internal organs (85). Relative to the ordinary clinical picture of tuberculosis, pulmonary disease in AIDS patients often differs in radiologic findings, is usually rapidly progressive, and disseminates more frequently, sometimes even without the formation of granulomas.

In culture, colonies of *M. tuberculosis* are off-white and rough on solid media (Fig. 1), although on moist media they may tend to be smoother. In addition to the classical

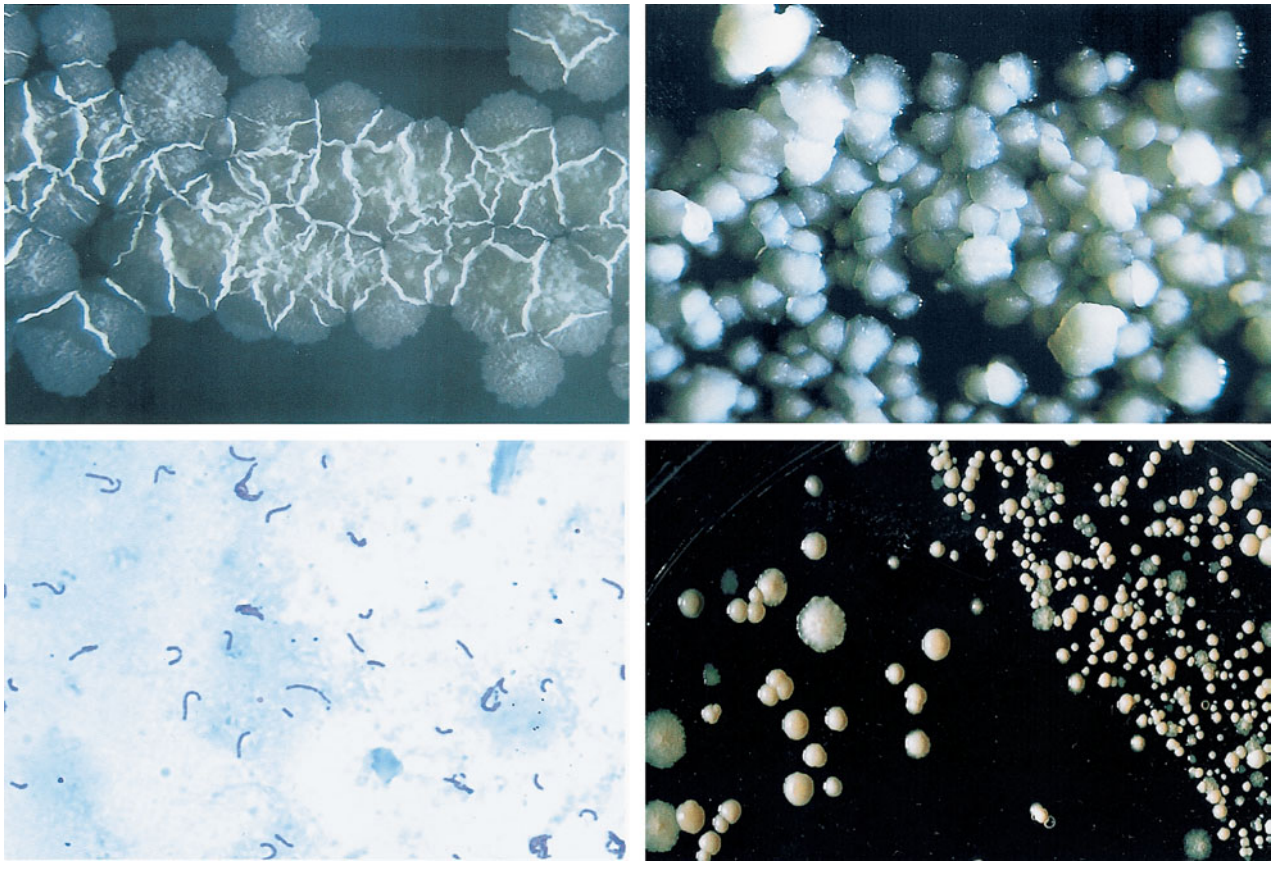


FIGURE 1 (top left) *Mycobacterium tuberculosis* on Middlebrook 7H10 agar. Note the dry and rough colonies, sometimes with a nodular or wrinkled surface.

FIGURE 2 (top right) *Mycobacterium bovis* BCG on Middlebrook 7H10 agar. Colonies may be flat as well as round with irregular edges.

FIGURE 3 (bottom left) Acid-fast staining (Ziehl-Neelsen) of *Mycobacterium microti*. Note the characteristic curved ("croissant-like") microscopic morphology. (Photograph kindly provided by D. van Soolingen, RIVM, Bilthoven, The Netherlands.)

FIGURE 4 (bottom right) *Mycobacterium canettii* on Middlebrook 7H10 agar. Note the heterogeneous colony morphology consisting of some flat and smooth but predominantly domed and glossy colonies. (Photograph kindly provided by D. van Soolingen.)

M. tuberculosis, there exists an Asian variant which is susceptible to thiophene-2-carboxylic acid hydrazide. In contrast to *M. tuberculosis*, Asian variant strains often contain few to no IS*6110* elements in their genomes (see chapter 37).

M. bovis

M. bovis causes tuberculosis in warm-blooded animals such as cattle, dogs, cats, pigs, parrots, badgers, and some birds of prey but also in primates and humans. Human disease is very similar to that caused by *M. tuberculosis* and is treated accordingly, except that pyrazinamide is inefficient due to the inherent resistance of most *M. bovis* strains. The organism grows poorly on L-J medium, but growth is stimulated if glycerol is replaced by pyruvate. In contrast to most members of the *M. tuberculosis* complex, *M. bovis* is able to grow in a reduced-O_2 atmosphere. Colonies on egg-based media are small and rounded, with irregular edges and a granular surface, and on agar media colonies are small and flat (216).

M. bovis subsp. *caprae* (6, 53), easily recognized by its susceptibility to pyrazinamide, is not only seen in cattle but accounts for 31% of human *M. bovis*-associated tuberculosis cases, mostly as pulmonary manifestations, in Germany (107). *M. bovis* subsp. *caprae* thus adds to the agents of human tuberculosis contracted from animals.

M. bovis BCG

In many parts of the world, *M. bovis* bacillus Calmette-Guérin (BCG) is still used for vaccine purposes. It was distributed by Calmette in 1924 to laboratories around the world and has been maintained in vitro by serial passages. Today, there exists a genetically heterogeneous conglomerate of BCG strains (Fig. 2) (11, 183) which predominantly conform to the properties described for *M. bovis* except that they are more attenuated in virulence. In rare instances, BCG may disseminate as a complication of intravesical BCG immunostimulation against bladder cancer (1).

M. africanum

M. africanum is a cause of human tuberculosis in tropical Africa. Colonies of *M. africanum* resemble those of *M. tuberculosis*, and physiological and biochemical properties position the organism between *M. tuberculosis* and *M. bovis*. Prior to molecular genetics methods, the definition of *M. africanum* was difficult and its validity was questioned by some authors. Recent genotypic analyses based on variable-number tandem repeats and other molecular characteristics have set *M. africanum* clearly apart from other members of the complex (64, 206). Likewise, Mostowy et al. (126) and Niemann et al. (133) consider *M. africanum* a unique species within the *M. tuberculosis* complex. The distribution of deleted sequences suggests that *M. africanum* subtype II isolates are situated among strains of "modern" *M. tuberculosis* while subtype I isolates are heterogenous and constitute two distinct evolutionary branches within the *M. tuberculosis* complex.

M. microti

Originally isolated from rodents such as voles and shrews, *M. microti* causes naturally acquired tuberculosis in guinea pigs, rabbits, llamas, cats, and other warm-blooded animals. It has been identified as a causative agent of tuberculosis in both immunocompetent and immunosuppressed humans (204). Usually revealing a characteristic, "croissant"-like morphology in stained smears (Fig. 3), the organism normally fails to grow in culture. At least the vole type of *M. microti* can easily be recognized upon spacer oligotyping (see chapter 37) since it contains an exceptionally short genomic direct repeat region resulting in identical two-spacer sequence reactions (204).

M. canettii

M. canettii was first collected by Georges Canetti back in 1969. van Soolingen et al. (205) and Pfyffer et al. (147) reported *M. canettii* causing lymphadenitis in a child and generalized tuberculosis in an HIV-positive patient, respectively. Its natural reservoir was unknown. The facts that both patients were exposed in Africa and that more cases of cervical lymphadenitis have been reported from Djibouti (56) support the hypothesis that *M. canettii* may be more abundant on the African continent. In 2002, pulmonary manifestation of *M. canettii* infection was reported (122).

With its smooth, round, and glossy colonies (Fig. 4), it differs considerably from all other members of the *M. tuberculosis* complex and can even be mistaken for an NTM. Studies by Brosch et al. (20) and Marmiesse et al. (113) of 20 regions where insertion-deletion events took place in the genome of *M. tuberculosis* suggest that *M. canettii* diverged first from the rest of the *M. tuberculosis* complex. Based on variable-number tandem repeat genotyping and analysis of *hsp65* gene polymorphism in 44 strains of *M. canettii,* Fabre et al. (56) confirmed that *M. canettii* is the most probable source species of the *M. tuberculosis* complex rather than just another branch of the taxon. In fact, it is assumed that *M. canettii* appeared some 2.8 million years ago and may, therefore, be the ancestor of all members of the *M. tuberculosis* complex. Moreover, the amount of synonymous nucleotide variation in housekeeping genes suggests that early tubercle bacilli were contemporaneous with the early hominids in East Africa and have thus been coevolving with their human hosts much longer than previously thought (Gutierrez et al., *Abstr. 26th Annu. Congr. Eur. Soc. Mycobacteriol.*).

M. pinnipedii sp. nov.

On the basis of host preference and phenotypic and genotypic characteristics, the new member of the *M. tuberculosis* complex *M. pinnipedii* has been defined by Cousins et al. (37). Pinnipeds appear to be the natural host, but the organism is also pathogenic for guinea pigs, rabbits, Brazilian tapirs (*Tapirus terrestris*), possibly cattle, and humans. Infections with the "seal bacillus" manifest with granulomatous lesions in the lymph nodes, lungs, pleura, and spleen and are able to disseminate.

M. leprae

Estimates from the World Health Organization put the global prevalence of leprosy (Hansen's disease) at 11 million people, with the majority of cases occurring in south and southeast Asia, Africa, and Latin America (202). Since multidrug therapy consisting of dapsone, rifampin, and clofazimine (230) has been introduced, the prevalence rate has dropped by 85% and leprosy has been eliminated in 98 countries. The number of new cases (about 600,000 to 700,000 per year) has, however, shown no substantial decline despite multidrug therapy (228). In the past centuries, leprosy occurred on a large scale also in Europe, in particular in Norway. Even though the disease has remained endemic in small pockets in the United States (Texas, California, Louisiana, Hawaii, and Puerto Rico) (114), the majority of infections now seen in North America have been acquired abroad. These imported cases appear to present a negligible risk for transmission within the United States.

Leprosy is a chronic, granulomatous, and debilitating disease (77). Its principal manifestations include anesthetic

skin lesions and peripheral neuropathy with nerve thickening. The term leprosy encompasses a continuous spectrum of disease, from a form with very few demonstrable bacilli (tuberculoid leprosy) to a progressive, widespread form with massive numbers of organisms due to the absence of cell-mediated immunity (lepromatous leprosy). The majority of leprosy patients show manifestations between these two polar forms and are clinically unstable. Medical complications arise from nerve damage and immune reactions (77).

Shedding from the nose, rather than from skin lesions, is important for transmission, which results most likely from prolonged and intimate contact with a person with multibacillary disease. The natural reservoir for *M. leprae* is not well established, but naturally occurring infections in the nine-banded armadillo *(Dasypus novemcinctus)* have been documented in Texas and Louisiana.

M. leprae differs from all other mycobacteria in that it cannot be cultured in vitro. There is, to date, one single report in which *M. leprae* was claimed to show sparse growth in a special medium at 32°C (135). By tradition, the diagnosis of leprosy is essentially a clinical one, based on finding one or more signs of disease which are supported by the presence of acid-fast bacilli (AFB) on slit skin smears or in skin biopsy specimens. In the case of lepromatous disease, nodules and plaques are the preferred sites for biopsies, which will reveal numerous AFB. Conversely, in patients with tuberculoid leprosy, the rims of lesions should be biopsied and usually only a few or no AFB are found. A number of PCR assays have been established to conclusively identify the organism (140, 142).

Nontuberculous Mycobacteria Frequently Involved in Human Disease

Slowly Growing Species

M. avium Complex

MAC organisms have been isolated from water, soil, plants, and other environmental sources. They are important pathogens of poultry and swine but were not recognized as a cause of human disease until the 1940s. Generally, these organisms are of low pathogenicity. Single positive specimens with low numbers of AFB as colonizers are not infrequently observed in individuals without apparent disease. This complicates the interpretation of culture results, particularly from specimens of the respiratory tract (5, 57). However, as a result of the AIDS pandemic, the MAC has become the most common environmental NTM causing disease in humans.

Before the advent of AIDS, the most common presentation of MAC infection was pulmonary disease showing several different clinical patterns, i.e., tuberculosis-like infiltrates, nodular bronchiectasis, solitary nodules, and diffuse infiltrates in immunocompromised patients (209). Tuberculosis-like upper lobe fibrocavitary disease due to the MAC occurs typically in white men 45 to 60 years of age who are heavy smokers, many of whom abuse alcohol, and some of whom have preexisting lung disease. The clinical presentation is similar to that of tuberculosis. Nodular bronchiectasis occurs usually in elderly, nonsmoking women with no predisposing disorders of the lungs or immune system other than associated bronchiectasis ("Lady Windermere syndrome") (46). These patients usually present with persistent cough only. The MAC is also isolated from up to 20% of young adults with cystic fibrosis, particularly in the southeastern United States, but its contribution to the disease process has not been established (100). Further, it is also the leading cause of localized mycobacterial lymphadenitis in children, especially in those 1 to 5 years of age (223). The lymphadenitis is usually unilateral and involves lymph nodes in the submandibular, submaxillary, or periauricular area. Less frequent are thoracic infections in otherwise healthy children (59).

Although disseminated MAC infections in non-AIDS patients are extremely rare (87), patients with AIDS may present with disseminated or focal infections (86). Disseminated infection occurs mostly when the CD4 count is below 100 cells/mm^3. Bacteremia occurs in almost all such patients, its magnitude ranging from <1 to 10^2 CFU/ml. The organism is found almost exclusively in circulating monocytes. Almost any organ (e.g., lungs and intestines) may be involved, with levels of mycobacteria as high as 10^{10} CFU/g of tissue. Focal infections commonly involve the lungs or the gastrointestinal tract and occasionally also peripheral lymph nodes (89).

MAC organisms are well-known for their heterogeneous colony morphology. Glossy, whitish colonies may often occur together with smaller, translucent colonies. A third, less frequent morphology resembles the dry and flat colonies of *M. tuberculosis*. Some MAC strains may develop a yellowish pigment with age. The MAC comprises a heterogeneous group of AFB that are divided into the two species *M. avium* and *M. intracellulare*. Serovars 1 to 6, 8 to 11, and 21 are *M. avium*, and serovars 7, 12 to 20, and 22 to 28 are *M. intracellulare*. Inclusion of *M. scrofulaceum* (serovars 41 to 43) in the group as the *M. avium-M. intracellulare-M. scrofulaceum* complex is no longer appropriate, given the advances in mycobacterial systematics (214, 215). *M. avium* and *M. intracellulare* are easily distinguishable by genetic methods, such as the use of DNA probes, 16S rRNA gene sequencing, and analysis of the restriction enzyme patterns of the 65-kDa heat shock protein gene *(hsp)* (185). There is evidence for a third species within the MAC which is generally referred to as the MAC-X (215). Those strains are recognized by the commercial DNA MAC AccuProbe (Gen-Probe, San Diego, Calif.) but not by the *M. avium* and *M. intracellulare* probes (Gen-Probe). More than 90% of MAC organisms isolated from AIDS patients are *M. avium* (as are most of the pathogenic isolates from pigs and cattle). In these patients, distinct serovars of *M. avium* have been found to account for the majority of infections (serovars 4, 6, 8, 9, and 11 [95] and serovars 1, 4, and 8 [165]).

Based on phenotypic and genetic characteristics, three subspecies of *M. avium* have been proposed: *M. avium* subsp. *avium, M. avium* subsp. *paratuberculosis* (187), and *M. avium* subsp. *silvaticum.* The latter is an obligate pathogen of ruminants (Johne's disease). In humans *M. avium* subsp. *paratuberculosis* is suspected to be involved in Crohn's disease. With the many new genetic tools available, taxonomy of this group becomes more difficult. Results of comparative DNA sequencing (49) are consistent with the hypothesis that *M. avium* subsp. *paratuberculosis* type I strains are an evolutionary intermediate between *M. avium* subsp. *avium* and *M. avium* subsp. *paratuberculosis* type II isolates or share a subset of *M. avium* subsp. *avium* type-specific loci through horizontal transfer. Recently, a new subspecies of *M. avium, M. avium* subsp. *hominissuis*, has been proposed by Mijs et al. (121).

M. genavense

M. genavense is a slowly growing NTM that was isolated in 1991 from the blood of an AIDS patient in Geneva,

Switzerland (19), and subsequently in the United States and in several European countries (39, 57, 195). It has been associated with enteritis, genital and soft tissue infections, and lymphadenitis in HIV-positive and in HIV-negative immunocompromised individuals. Clinically, disseminated disease in HIV-positive patients is similar to that caused by the MAC. However, stool specimens are more often smear-positive in *M. genavense* infections (136). *M. genavense* is also the most common cause of mycobacterial disease in a variety of pet birds, including parrots and parakeets (84, 152).

Analysis of the 16S rRNA gene sequences indicates that this species is most closely related to *M. simiae*. The organism was first isolated in BACTEC 13A medium, but only after extended incubation (6 to 8 weeks). It fails to grow on L-J medium, 7H11 agar, or other media commonly used for the isolation of mycobacteria. Middlebrook 7H11 agar supplemented with mycobactin J (Allied Monitor, Fayette, Mo.), however, supports the growth of *M. genavense* (39), as do microaerophilic conditions (156), the radiometric BACTEC 7H12 PZA test medium (197), and the addition of blood and charcoal to acidified Middlebrook agar (155).

M. haemophilum

M. haemophilum was first isolated in 1978 from a subcutaneous lesion in a patient with Hodgkin's disease (169). Approximately 50% of infections have been in patients with AIDS, with a relatively large number reported from New York City (25). The other cases have been in other immunosuppressed individuals (169, 210) but also in immunocompetent pediatric patients with localized cervical lymphadenopathy (168) or with a pulmonary nodule (220). The classical clinical presentation is that of multiple skin nodules appearing in clusters or without a definite pattern, commonly involving the extremities, and occasionally associated with abscesses, draining fistulas, cellulitis, and osteomyelitis (57).

M. haemophilum infections may be underrecognized because of the organism's predilection for a low incubation temperature (30°C) and its unique requirement for ferric ammonium citrate or hemin for growth. If *M. haemophilum* is suspected in a clinical specimen but culture remains negative, the organism may be recovered by using a chocolate agar plate.

M. kansasii

In the United States and many other countries, *M. kansasii* is second only to the MAC as a cause of NTM lung disease (5, 57). The organism has been cultured from tap water in municipalities around the world where clinical disease occurs. *M. kansasii* lung disease is common in mine workers in both the United Kingdom and South Africa (35) and differs from that due to the MAC in that the response to chemotherapy is much better (5).

Chronic pulmonary disease resembling classical tuberculosis is the most common manifestation of *M. kansasii* infection; it often involves the upper lobes. Extrapulmonary infections are uncommon and include cervical lymphadenitis in children (232), cutaneous and soft tissue infections, and musculoskeletal disease (13). *M. kansasii* infection rarely disseminates, except in patients with severely impaired cellular immunity (e.g., due to organ transplants) or in patients with AIDS (5).

M. kansasii is a photochromogenic species. Studies of the base sequences of the 16S rRNA gene suggest that phylogenetically it is closely related to the slowly growing, nonpigmented species *M. gastri*. Molecular studies have defined five genotypes of *M. kansasii*, all of which are able to cause human disease (148).

M. malmoense

The species name *M. malmoense* is derived from the city of Malmö in Sweden, where the first strains were isolated from patients in 1977. Disease due to this organism was later found in other European countries (5, 81, 194). It remains rare in the United States (21), Canada (3), and other areas of the world. However, in these countries, *M. malmoense* infection may be more common than suspected, because it may require 8 to 12 weeks to isolate some strains, which is longer than many laboratories in North America hold mycobacterial cultures.

Patients with *M. malmoense* infection are usually young children with cervical lymphadenitis or adults with chronic pulmonary disease (5, 57), mostly middle-aged men with previously documented pneumoconiosis. Extrapulmonary and disseminated infections have rarely been reported. The species has been recovered from soil and water (5).

M. marinum

M. marinum causes cutaneous infections as a result of trauma to the skin and subsequent exposure to contaminated freshwater fish tanks ("fish tank granuloma") or salt water (109). The disease occurs worldwide. In the United States, it is most common in southern coastal states (92). The most typical presentation is a single papulonodular lesion confined to one extremity, usually the elbow, knee, foot, toe, or finger. It appears 2 to 3 weeks after inoculation and, with time, may become verrucous or ulcerated (57). A second type resembles cutaneous sporotrichosis, in which the primary inoculation is followed by spread along the lymphatics. More severe complications include tenosynovitis, arthritis, bursitis, and osteomyelitis. Disseminated infections, including infections in patients with AIDS or persons undergoing systemic steroid therapy, have been rare.

M. marinum is photochromogenic and requires incubation at 28 to 30°C upon primary isolation. Recently, Israeli *M. marinum* isolates from humans and fish were compared by direct sequencing of the 16S rRNA and *hsp65* genes, restriction mapping, and amplified fragment length polymorphism analysis. Surprisingly, significant molecular differences separated all clinical isolates from the piscine isolates (201).

M. simiae

M. simiae was first isolated in 1965 from monkeys. Clinical isolates have come from a few geographic areas, including the southwestern United States (5, 164), Israel (88), and the Caribbean (5). The organism has been recovered from tap water in some of these areas, and most positive cultures from humans have come from single positive specimens with low colony counts, suggesting that most positive cultures represent environmental contamination. Clinical manifestations such as osteomyelitis and chronic pulmonary and disseminated disease are rare (for references, see reference 57). Cases of *M. simiae* infection mimicking MAC infection in AIDS patients have been reported (88). *M. simiae* is one of the very few NTM synthesizing niacin. Unless tested for pigment production under the influence of light, it may be misidentified as *M. tuberculosis* by inexperienced observers.

M. szulgai

M. szulgai was first described as a distinct species in 1972. Its distribution appears to be worldwide, but the natural reservoir is unknown. Patients are almost exclusively middle-aged men presenting with chronic pulmonary disease

indistinguishable from tuberculosis (196). The remaining presentations include rare cases of bursitis, cervical adenitis, tenosynovitis, cutaneous infections, and osteomyelitis (5, 57). Cases of *M. szulgai* infection in AIDS patients and disseminated disease in an immunocompetent patient have been reported as well.

Although *M. szulgai* is closely related to *M. malmoense* based on the 16S rRNA gene sequences, phenotypic distinction between the two species is easy. *M. szulgai* is scotochromogenic at 37°C and photochromogenic at 25°C (97).

M. ulcerans

The frequency of *M. ulcerans* infection has long been underestimated due to difficulties in isolating the pathogen. Today, *M. ulcerans* infection is the third most frequent mycobacterial disease in humans after tuberculosis and leprosy. In Africa the disease is known as Buruli ulcer, and in Australia it is known as Bairnsdale ulcer (5, 57, 68). Closely associated with tropical wetlands, *M. ulcerans* most likely proliferates in mud beneath stagnant waters. Disease likely occurs via direct contact with a contaminated environment or via water-dwelling fauna such as insects. It typically begins as a painless lump under the skin at the site of previous trauma on the lower extremities. After a few weeks, a shallow ulcer develops at the site of the lump. *M. ulcerans* produces a toxin that causes necrosis (203). The type of disease ranges from a localized nodule or ulcer to widespread ulcerative or nonulcerative disease including osteomyelitis. If untreated, severe limb deformities with contractures and scarring are common. There is growing evidence that *M. ulcerans* also produces disease in wild animals such as lizards, opossums, koala bears, armadillos, rats, mice, and cattle (151).

Failure to cultivate this organism in the past was due to its fastidious, heat-sensitive nature (optimum temperature, 30°C) as well as to a long generation time relative to that of other environmental mycobacteria. Many conventional decontamination protocols used in mycobacteriology interfere with the viability of *M. ulcerans*. Treatment with mild hydrochloric acid (final concentration, 0.03 N) (138) or oxalic acid (233) as a decontamination agent provides the best results. A mixture of polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin (PANTA) may control secondary contamination. Good recovery of *M. ulcerans* is obtained on L-J medium with glycerol (137, 233). Reduced oxygen tension enhances the rate of recovery in the BACTEC 460TB system (137). Molecular techniques have been developed which may help to lead to more rapid diagnosis (162).

M. xenopi

M. xenopi was first isolated in 1957 from skin lesions on an African toad *(Xenopus laevis),* but it was not recognized as a human pathogen until 1965. By 1979, 50 cases of *M. xenopi* infections in humans had been reported, primarily from the United Kingdom, France, Denmark, Australia, and the United States (5, 57). In some areas such as Canada and southeast England, it is second only to the MAC as an NTM clinical isolate (5). A recent case report of *M. xenopi* spondylodiscitis in an AIDS patient not only highlights its potential pathogenic role but also points to the uncertainties in therapeutic management (119). Increased isolation of *M. xenopi* from clinical specimens may also be due to improved laboratory techniques (50). The optimum growth temperature for the species is 45°C, and it seems to occur frequently in hot-water systems. Nosocomial infection and pseudo-infection via water storage tanks in hospitals have been described previously (176).

Pulmonary disease is the most common manifestation of infection (5, 93), usually occurring in male adult patients with underlying lung disease such as chronic obstructive pulmonary disease or bronchiectasis. Extrapulmonary infections such as septic arthritis (96), spondylitis (42), and dissemination (98) in immunocompromised individuals have also been described.

Nontuberculous Slowly Growing Mycobacteria Rarely Recovered or Rarely Causing Human Disease

Several species of slowly growing mycobacteria including *M. gordonae*, *M. scrofulaceum*, and the *M. terrae* complex are not infrequently recovered but are rarely associated with human disease. Some of the case reports of infections attributable to these mycobacteria, especially from the era before the introduction of molecular laboratory techniques, lack sufficient documentation of identification or disease association. Other species (such as *M. asiaticum* and *M. shimoidei*) are so rarely recovered that most laboratories will never see them.

M. asiaticum

M. asiaticum was not recognized as a distinct species until 1971, although it had previously been isolated from monkeys. The first published report of human disease described five patients in Australia with pulmonary disease (17). The organism has since infrequently been isolated from patients with respiratory disease in the United States and elsewhere (184). Cases of bursitis and tenosynovitis have been described. *M. asiaticum* is similar to *M. gordonae*, differing primarily in that *M. gordonae* is scotochromogenic whereas *M. asiaticum* is photochromogenic (214).

M. celatum

First described in 1993 (23), *M. celatum* has been isolated from diverse geographic areas, e.g., throughout the United States, Finland, and Somalia, and mostly from respiratory tract specimens but also from stool and blood. In one study, 32% of the patients from whom it was isolated were infected with HIV (23). *M. celatum* has also been isolated from immunocompetent patients, e.g., from a child suffering from lymphadenitis and from an elderly female patient with a fatal pulmonary infection.

M. celatum shares morphological and biochemical characteristics with the MAC, *M. malmoense*, and *M. shimoidei* and can, thus, not be identified with conventional tests. Within the bacterial chromosome, *M. celatum* has two copies of the 16S rRNA gene (157). Several subtypes (1 to 3) have been identified (23), with the use of 16S rRNA gene sequencing or restriction fragment length polymorphism analysis of the gene encoding the 65-kDa heat shock protein (149). Due to high similarities in the 16S rRNA gene sequence with that of *M. tuberculosis,* a few strains have been misidentified as members of the *M. tuberculosis* complex by using a commercially available DNA probe (189) or as *M. xenopi* on account of similar biochemical and cultural features. Unlike *M. xenopi*, *M. celatum* usually shows resistance to even high concentrations of rifampin (23).

M. gordonae

M. gordonae is the most commonly encountered nonpathogenic species in clinical mycobacteriology laboratories. This scotochromogenic species is widely distributed in soil and water. A pseudo-outbreak associated with drinking water

in a French hospital underlined the necessity for proper maintenance of water supply equipment (108). In only 1 of 38 published reports of *M. gordonae* infection was there convincing evidence that the organism played a role in disease (214). There are also reports of peritonitis in patients undergoing continuous ambulatory peritoneal dialysis (75). Eckburg et al. (52) have reviewed clinical and chest radiographic findings among persons with sputum cultures positive for *M. gordonae* and concluded that it is a nonpathogenic colonizing organism, even among persons with local or general immune suppression and abnormal chest X-ray findings.

M. scrofulaceum

The name *M. scrofulaceum* was derived from scrofula, a historical term used to describe mycobacterial infections of the cervical lymph glands. Until the 1980s, *M. scrofulaceum* was the most common cause of mycobacterial cervical lymphadenitis in children. Since then it has been replaced primarily by the MAC (223). Other types of clinical disease are rare. They include pulmonary disease, conjunctivitis, osteomyelitis, meningitis, granulomatous hepatitis, and disseminated disease (for references, see reference 57). *M. scrofulaceum* accounted for 14% of the isolates tested in respiratory specimens collected from South African miners (34).

M. shimoidei

M. shimoidei was first described in 1988 in a Japanese patient with chronic cavitary lung disease. Only a few clinical cases have been reported since (115). It is a thermophilic organism growing well at 45°C. Biochemically, the organism is similar to the *M. terrae* complex but can be separated by catalase and β-galactosidase tests. The unique sequences of the 16S rRNA gene and the 16S-23S rRNA gene spacer region allow unambiguous identification of the organism (104).

M. terrae Complex

The *M. terrae* complex consists of three species, *M. terrae, M. nonchromogenicum,* and *M. triviale*. Clinical disease due to *M. terrae* is generally limited to pulmonary disease and tenosynovitis of the hand following local trauma (173). *M. nonchromogenicum*, ubiquitous in the aquatic environment, was the cause of bacteremia in an AIDS patient (116). Separation of the members of the complex, especially *M. terrae* from *M. nonchromogenicum*, requires molecular methods.

New Species of Nontuberculous Mycobacteria

Most new NTM species have been described within the last 5 to 10 years (192). Much less is known about these species than about those mentioned previously, and clinical cases of infection are limited to a very few.

M. bohemicum

M. bohemicum is a bright yellow-pigmented species originally described in 1998 after being isolated from a patient with Down's syndrome and tuberculosis (158). Rare cases of cervical lymphadenitis in children (193) have been reported, as well as a fatal case of an elderly female with generalized eczema, lesions, and scattered nodules that spread from her hands (191). Isolates have been recovered from veterinary (goat) and environmental (freshwater) sources (191). For identification, analysis of the 16S rRNA gene sequence is required (158, 193).

M. branderi

M. branderi was first described in 1992 after characterization of 14 respiratory isolates collected in Finland. Since then, *M. branderi* has also been isolated from an infected hand wound (222). Identification of the organism requires 16S rRNA gene sequence analysis (103, 222), which shows that the species is most closely related to *M. celatum, M. cookii,* and *M. xenopi*.

M. conspicuum

M. conspicuum was first isolated in 1995 from two AIDS patients with disseminated disease (180). The organism grew at 22 to 31°C and produced pale yellow colonies on solid media. Unusually, the strains do not grow at 37°C except in BACTEC 12B medium. Based on its 16S rRNA gene sequence, it is most closely related to *M. asiaticum* and *M. gordonae*. To date, only two strains have been isolated. Since the organism prefers a rather low temperature for growth, it may, however, be missed in the laboratory.

M. heckeshornense

M. heckeshornense was described in 2000 as a scotochromogenic species (159, 163) recovered in multiple cultures from the lung of an immunocompetent patient with severe bilateral cavitary disease. On the basis of its unique 16S rRNA and 16S-23S spacer gene sequences, it appears to be a new species (159, 163). *M. heckeshornense* is reported to grow poorly on Middlebrook agar and egg-based media. Colonies resemble those of *M. xenopi.*

M. heidelbergense

The nonphotochromogenic organism *M. heidelbergense* was described in 1997 as an isolate from a child with recurrent cervical lymphadenitis (70). An *M. heidelbergense* infection was also found to mimic a lung tumor in a previously healthy woman. Biochemically, *M. heidelbergense* is indistinguishable from *M. malmoense* but unable to grow under microaerophilic conditions. Sequencing of the 16S rRNA gene, however, positions the organism on a branch separate from *M. malmoense* but within a group of slowly growing mycobacteria with a high degree of similarity to *M. simiae* (70).

M. interjectum

M. interjectum was recovered from children with chronic lymphadenitis (45) and from an AIDS patient with diarrhea (69). Colonies are smooth and scotochromogenic. Visible growth is produced within 21 to 28 days. Fatty acid patterns revealed by gas-liquid chromatography are similar to those of *M. scrofulaceum*. By 16S rRNA gene sequencing, the organism is shown to be most closely related to *M. simiae*.

M. intermedium

The photochromogenic species *M. intermedium* was repeatedly isolated from a patient with chronic pulmonary disease and can be easily confused with *M. asiaticum,* although differences exist in colony morphologies (118).

M. kubicae

Described in 2000 (63), most strains of the scotochromogenic organism *M. kubicae* have been isolated from respiratory sources. Its pathogenic potential remains, however, unknown. Based on sequence analysis of the 16S rRNA gene, it clusters with the group of *M. simiae*-related slow growers.

M. lacus

The new species *M. lacus* was cultured from the synovia of a 68-year-old female presenting with bursitis of the elbow following a local trauma. Granulomas were detected in the bursa upon its excision (200).

M. lentiflavum

M. lentiflavum was first described in 1996 as a cluster of slowly growing pigmented organisms. The majority of the isolates were from respiratory specimens. The organism has been reported as an etiological agent of cervical lymphadenitis and of disseminated infection in an HIV-positive patient. It does not grow at 45°C (181). The organism has a bright yellow pigment and distinct patterns of mycolic acids and cellular fatty acids.

M. palustre

The only strain of *M. palustre* isolated so far originated from a 4-year-old girl with lymphadenitis. Gene sequencing indicates a close relationship with *M. kubicae* and *M. simiae*-like organisms (190).

M. parmense

Closely related to *M. lentiflavum* and *M. simiae*, *M. parmense* has been isolated from a cervical lymph node of a 3-year-old child (58).

M. sherrisii

Five strains (some of which were of clinical origin), previously considered to be *M. simiae* serotype 2, have recently been characterized as a novel group of mycobacteria. Qualitative whole-genomic DNA hybridization analysis confirmed that this group is genetically distinct from *M. simiae* and *M. triplex* (171).

M. triplex

M. triplex was characterized in 1996 as an unusual cluster of slowly growing mycobacteria that strongly resembled the MAC (62) but were DNA MAC probe negative. The majority of organisms were from either lymph nodes or respiratory samples. *M. triplex* has caused disseminated mycobacteriosis in an HIV-positive patient during antiretroviral therapy and in a liver transplant patient. According to 16S rRNA gene sequencing, the organism is most closely related to *M. genavense* and *M. simiae*. It differs from *M. simiae* in not producing any pigment and from *M. genavense* in growing easily on solid media (62).

M. tusciae

The yellow pigmented organism *M. tusciae*, first described in 1999, has been found in tap water but also in a few clinical specimens, i.e., in a lymph node of an immunocompromised child and in a respiratory specimen from a patient with cystic fibrosis (198).

SAFETY AND TRANSPORT ISSUES

Laboratory Safety Procedures

Nosocomial transmission of *M. tuberculosis* from patients or specimens is of major concern to health care workers (24). Because of the low infective dose of *M. tuberculosis* for humans (50% infective dose, <10 bacilli), specimens from suspected or known cases of tuberculosis must be considered potentially infectious and handled with appropriate precautions (32). Control of aerosols and other forms of mycobacterial contamination is achieved in the laboratory by the use of properly functioning biological safety cabinets (BSC), centrifuges with safety carriers, and meticulous processing techniques (see also chapter 8).

Classification of mycobacteriology laboratory practices should be based on risk assessment (i.e., volume of tests, types of testing, prevalence of tuberculosis, and frequency of multidrug-resistant *M. tuberculosis*). Biosafety level 2 practices and facilities are required for laboratory work assessed as low risk. Aerosol-generating manipulations should be conducted in a class I or II BSC. More rigorous biosafety level 3 practices and level 3 facilities are required for laboratories associated with higher risk. These laboratories process specimens for mycobacterial culture and propagate and manipulate cultures of *M. tuberculosis* (e.g., perform identification and susceptibility testing). Such practices require that laboratory access be restricted, that directional airflow be used to maintain the laboratory under negative pressure, and that workers wear special laboratory clothing and gloves. Biosafety level 3 practices and facilities are required for those laboratories growing *M. tuberculosis* to high volumes, working with large numbers of resistant isolates, or performing tests with unknown risk. For a detailed description of safety requirements in the mycobacteriology laboratory, refer to the Centers for Disease Control and Prevention and National Institutes of Health biosafety guide for laboratories (32).

All respiratory protective devices (respirators) used in the workplace should be certified by the National Institute for Occupational Safety and Health (NIOSH) (130). Respirators that contain a NIOSH-certified N-series filter with a 95% efficiency (N-95) rating are appropriate for use. They meet the recommendations from the Centers for Disease Control and Prevention for selection of respirators for protection against *M. tuberculosis*, i.e., (i) the unloaded filter must filter particles 0.3 μm in diameter with an efficiency of 95% at flow rates of up to 50 liters/min; (ii) the respirator must be qualitatively or quantitatively fit tested to obtain a face seal leakage rate of no more than 10%; (iii) it must fit different facial sizes and characteristics, which is accomplished by making the respirators available in at least three sizes; and (iv) it must be checked for face piece fit by the person wearing the respirator each time it is worn in accordance with Occupational Safety and Health Administration (OSHA) standards. Surgical masks are not NIOSH-certified respirators and must not be worn to provide respiratory protection.

The determination of when and if to use respiratory protective devices in the laboratory should be based on risk assessment. A respirator program should be implemented by the laboratory and should include a written protocol describing situations when respirator use is necessary and procedures addressing (i) selection of the appropriate respirator, (ii) conducting of fit testing, and (iii) training of personnel in the use, fit checking, and storage of the respirator.

All work involving specimens or cultures, such as making smears, inoculating media, adding reagents to biochemical tests, opening centrifuge cups, and performing sonication, must be carried out in a BSC. The handling of all specimens suspected of containing mycobacteria (including specimens processed for other microorganisms), with the exception of centrifugation for concentration purposes, must be done within the BSC. Specimens that are to be taken out of the BSC should be covered before transport. All work surfaces, including bench tops and the inside of the BSC, should be cleaned with an appropriate disinfectant before and after work. Effective disinfectants include Amphyl (Reckitt Benckiser North America, Wayne, N.J.) and other phenol-soap mixtures and 0.05 to 0.5% sodium hypochlorite (concentration varies according to the nature of the contaminated surface). Five-percent phenol is no longer recommended as a surface disinfectant due to the documented toxicity of this compound to personnel. UV light is a useful adjunct for surface decontamination and may be used to radiate the work area when it is not in use. Centrifuges should be

used with aerosol-free safety carriers to contain debris in the event that tubes break. Use of electric incinerators rather than open flames is recommended. The excess inoculum from inoculating loops, wire, or spades may be removed by dipping the tool into a container of 95% ethanol in washed sand prior to insertion into an incinerator. Disposable inoculating loops are recommended, as are syringes with permanently attached needles if needles are required. An autoclave should be available in an easily accessible area and used to decontaminate infectious waste before removal to disposal areas.

Personnel should be regularly monitored with the Mantoux PPD skin test (annually and more often if a conversion in the laboratory or the institution has been documented) to demonstrate conversions. Those with positive skin tests should be evaluated for active tuberculosis with a chest X ray and clinical evaluation. Physical examinations should be performed when necessary. New converters should be referred to the employee health and infection control departments for epidemiological evaluation. Laboratories should have written protocols describing procedures for handling laboratory accidents. In case of a laboratory accident with possible formation of aerosols, personnel should hold their breath as much as possible, make sure that BSC are on and centrifuges are turned off, and then leave the room with the door closed for at least 30 min (the amount of time depends on the type of accident and the amount of risk). Using appropriate respiratory protection devices, personnel can return to the accident area to clean the spill. PPD-negative personnel should be skin tested at 3 and 6 months after the accident. The QuantiFERON-TB Gold (Cellestis, Valencia, Calif.) cellular immune response assay may have a role in such testing in the near future (116a). Persons who are immunocompromised should be discouraged from working in the mycobacteriology laboratory (32).

Transportation and Transfer of Biological Agents

Mycobacteria are on the list of infectious agents being regulated for shipping and transfer. Recent, more stringent regulations for the transportation of biological agents have been enacted in the United States to ensure that the public and workers in the transportation chain are protected from exposure to any infectious agents (for other countries, please consult the specific regulations). Protection is achieved through (i) the requirements for rigorous packaging that will withstand rough handling and contain all liquid material within the package without leakage to the outside, (ii) appropriate labeling of the package with the biohazard symbol and other labels to alert the workers in the transportation chain to the hazardous contents of the package, (iii) documentation of hazardous contents of the package should such information be necessary in an emergency situation, and (iv) training of workers in the transportation chain to familiarize them with the hazardous contents in order to be able to respond to emergency situations.

The reader is referred to the following regulatory documents for further information.

Public Health Service. 42 CFR Part 72. *Interstate Transportation of Etiologic Agents.* This regulation is in revision to harmonize it with the other U.S. and international regulations. A copy of the current regulation may be obtained from the Internet at http://www.cdc.gov/od/ohs/biosfty/shipregs.htm.

Department of Transportation. 49 CFR Parts 171–178. *Hazardous Materials Regulations.* Applies to the shipment of both biological agents and clinical specimens. Information may be obtained from the Internet at http://www.access.gpo.gov/cgi-bin/cfrassemble.cgi?title=199849.

U.S. Postal Service. 39 CFR Part 111. *Mailability of Etiologic Agents.* Codified in the *Domestic Mail Manual 124:38: Etiologic Agents Preparations.* A copy of the *Domestic Mail Manual* may be obtained from the Government Printing Office by calling 1-202-512-1800 or from the Internet at http://www.access.gpo.gov/cgi.bin/cfrassemble.cgi?title=199839.

OSHA. 29 CFR Part 1910.1030. *Occupational Exposure to Bloodborne Pathogens.* Provides minimal packaging and labeling requirements for transport of blood and body fluids. Information may be obtained from the local OSHA office or from the Internet at http://www.osha.gov/pls/oshaweb/owadisp.show_document?p_table=STANDARDS&p_id=10051.

International Air Transport Association. *Dangerous Goods Regulations.* These regulations provide packaging and labeling requirements for infectious substances and materials, as well as for clinical specimens that have a low probability of containing an infectious substance. A copy of the *Dangerous Goods Regulations* may be obtained by calling 1-800-716-6326 or through the Internet at http://www.iata.org/ps/publications/9065.htm.

There is also a Saf-T-Pak CD including updates available at http://www.saftpak.com/cdupdatedetails.htm.

Permits *must* be obtained for importation and exportation of biological and infectious agents. These are obtained by contacting the Centers for Disease Control and Prevention, Atlanta, Ga. (http://www.cdc.gov).

COLLECTION AND STORAGE OF SPECIMENS

General Rules

Many different types of clinical specimens may be collected for mycobacteriological analyses (90). The majority originate from the respiratory tract (sputum, tracheal and bronchial aspirates, and bronchoalveolar lavage specimens), but urine, gastric aspirates, tissues, biopsy specimens, normally sterile body fluids such as cerebrospinal fluid, and pleural and pericardial aspirates are other commonly submitted specimens. Blood and fecal specimens are usually submitted from immunocompromised patients only.

Specimens should always be collected and submitted in sterile, leak-proof, disposable, appropriately labeled laboratory-approved containers without any fixatives. Waxed containers must not be used because they may yield false-positive AFB smear results (97). Generally, transport media or preservatives are not necessary owing to the robust nature of mycobacteria. Minute biopsy material (e.g., fine-needle aspirates) may be immersed in a small amount of sterile physiological saline. Collection should bypass areas of possible contamination as much as possible, e.g., tap water, since the presence of environmental mycobacteria may result in false-positive smear and/or culture results (208). In general, swabs are not optimal for the recovery of AFB since they provide limited material and the hydrophobicity of the mycobacterial cell envelope often compromises a transfer from swabs onto solid or into broth media. If transport to the laboratory is delayed more than 1 h, specimens (except blood) should be refrigerated at 4°C. Likewise, upon arrival in the laboratory, specimens should be refrigerated until processed (see chapters 5 and 20 for additional information on specimen collection).

Sputum

Sputum, expectorated or induced, is the principal specimen obtained for the diagnosis of pulmonary tuberculosis. An early-morning specimen should be collected on three consecutive days. Pooled sputum specimens are unacceptable for mycobacterial processing because of increased contamination (97). Since the yield of culture from smear-positive specimens is high, two AFB smear-positive specimens are considered enough for the initial evaluation of pulmonary tuberculosis (182). Nelson et al. (131) demonstrated that the majority of culture-proven pulmonary tuberculosis cases are diagnosed from the first or second sputum specimen and that only rarely is a third specimen of diagnostic value. Another study, however, still concluded that three respiratory specimens are needed despite highly sensitive culture techniques (76). Follow-up cultures should be considered because it is culture (and not smears) that yields a definite answer of whether chemotherapy has been effective (120). Children may have difficulties producing sputum. In this age group, a gastric aspirate is usually the specimen of choice for the diagnosis of pulmonary tuberculosis.

Bronchial Aspirates, Bronchoalveolar Lavage Specimens, Fine-Needle Aspirates, and Lung Biopsy Specimens

In some patients unable to produce sputum, invasive collection techniques such as bronchoscopy may be necessary to diagnose pulmonary tuberculosis or mycobacteriosis. Special care is imperative for cleansing the bronchoscope to avoid cross-contamination with AFB from a preceding patient who underwent bronchoscopy (12, 66, 170). Also, the bronchoscope should not be in contact with tap water, which may contain environmental mycobacteria. Specimens collected by other invasive techniques such as fine-needle aspiration and open-lung biopsy may be submitted in difficult-to-diagnose cases.

Gastric Lavage Fluids

Aspiration of swallowed sputum from the stomach by gastric lavage may be necessary for infants, young children, and the obtunded. Early-morning specimens from fasting patients are recommended in order to obtain sputum swallowed during sleep. Samples of 5 to 10 ml, adjusted to neutral pH, should be collected on three consecutive days. If they cannot be processed within 4 h, the laboratory should provide sterile disposable containers with 100 mg of sodium carbonate for collection. Nonneutralized specimens are not acceptable because long-term exposure to acid is detrimental to mycobacteria.

Urine

The first morning urine specimen should be collected on three consecutive days at midstream (by clean catch) into a sterile container. The first morning specimen provides the best results because organisms accumulate in the bladder overnight. A minimum of 40 ml of urine is usually required for culture. Twenty-four-hour pooled specimens and small-volume specimens (unless a larger volume is not obtainable) are unacceptable. Catheterization should be used only if a midstream sample cannot be obtained.

Body Fluids

As much body fluid as possible (e.g., cerebrospinal, pleural, peritoneal, pericardial, or synovial fluid) is aseptically collected by aspiration or during surgical procedures. Bloody specimens may be anticoagulated with sodium polyanethol sulfonate. Because certain body fluids (such as cerebrospinal fluid and peritoneal dialysis effluent) may contain very small numbers of mycobacteria, it is advisable to submit larger specimen volumes (e.g., >5 ml for cerebrospinal fluid) than are required for other specimen types to increase culture yields and the chance to detect mycobacterial organisms.

Tissues (Lymph Node, Skin, and Other Biopsy Material)

At least 1 g of tissue, if possible, should be aseptically collected into a sterile container. It must not be immersed in saline or other liquid or wrapped in gauze. For cutaneous ulcers, biopsy material should be collected from the periphery of the lesion and incubated at both 30 and 37°C. Specimens submitted in formalin are unacceptable for smear and culture. Minute biopsy material may be moistened with a small amount of sterile saline.

Abscess Contents, Aspirated Pus, and Wound Specimens

As much material as possible should be aspirated aseptically. In cutaneous lesions, material is aspirated from beneath the margin of the lesion. Also, for this type of specimen, a second set of cultures has to be incubated at 30°C since one of the organisms with a lower optimum temperature (e.g., *M. haemophilum, M. marinum,* or *M. ulcerans*) may be the infectious agent.

Blood

The majority of disseminated mycobacterial infections are due to the MAC; therefore, if this organism is isolated from blood, it is always associated with clinical disease (78). If blood has to be transported before inoculation of the medium, sodium polyanethylene sulfate, heparin, or citrate may be used as an anticoagulant; blood collected in EDTA and coagulated blood are not acceptable. Direct inoculation of blood onto a solid medium is not recommended. The MAC survives for prolonged periods in Isolator lysis-centrifugation tubes (Wampole Laboratories, Cranbury, N.J.). Therefore, processing of Isolator tubes can be delayed for 24 h if absolutely necessary (79). If blood cannot be immediately processed by the laboratory, it should be stored at room temperature. The use of Isolator blood sediments to inoculate BACTEC 12B medium is contraindicated because one of the components, polypropylene glycol, is inhibitory to mycobacteria in the BACTEC 12B system (213). An identical effect on the growth of mycobacteria has also been reported for the Septi-Chek AFB liquid medium and the Mycobacteria Growth Indicator Tube (MGIT) broth (65), but this is controversial (73).

For many years, the Isolator system and the radiometric BACTEC 13A blood culture bottle (BD Diagnostics, Sparks, Md.) were the only reliable and, therefore, recommended systems for mycobacterial blood cultures (7). Very recently, the BACTEC 13A medium was, however, discontinued. Therefore, cultures from blood and bone marrow specimens have to be obtained by using alternative media, e.g., the MYCO/F LYTIC bottles (BD) (7, 207) or the MB/BacT ALERT blood medium (bioMérieux, Marcy-L'Etoile, France) (74). A recent study demonstrated that the continuously monitored systems (BACTEC MYCO/F LYTIC and MB/BacT ALERT) were as sensitive as and faster for the detection of MAC bacteremia than the heretofore standard manual Isolator system and the radiometric BACTEC 13A medium (41).

Stool Specimens

Stool specimens (>1 g) have been used for detection of the MAC from the gastrointestinal tracts of patients with AIDS, in conjunction with specimens from other sites. Past recommendations have been that stool be cultured for mycobacteria only if the direct smear of unprocessed stool is positive for AFB. The sensitivity of the stool smear, however, is only 32 to 34% (124), suggesting that its results should not determine whether a culture for mycobacteria be performed. Screening with smears is, therefore, not an effective way to identify patients at risk for developing disseminated MAC infection (78, 79).

Inadequate Specimens

Processing of inappropriate clinical specimens for mycobacteria is a waste of both financial and personnel resources. There are quite a few reasons why a specimen should not be accepted (and the clinician should be notified), e.g., (i) too small an amount is submitted (ii) specimens consist of saliva, (iii) dried swabs are submitted (biopsy specimens are preferable), (iv) pooled sputum or urine is submitted, (v) sample containers are broken, and (vi) the interval between specimen collection and processing was too long (>7 days) (139). Clinical staff must be properly trained to prevent submission of unacceptable specimens.

ISOLATION PROCEDURES

Because mycobacteria are usually growing slowly and require long incubation times, a variety of microorganisms other than mycobacteria can overgrow cultures of specimens obtained from nonsterile sites. Appropriate pretreatment and processing procedures (homogenization, decontamination, and concentration [90, 97]), culture media, and conditions of incubation must be selected to facilitate optimum recovery of mycobacteria (see also chapter 37). In particular, pretreatment of specimens has to be done carefully, i.e., by eliminating contaminants as much as possible while not seriously affecting the viability of mycobacteria.

Processing of Specimens

Decontamination of a specimen should be attempted only if it is thought to be contaminated. Tissues or body fluids collected aseptically usually do not require pretreatment. If the need to decontaminate a specimen is not clear, the specimen may be refrigerated until routine bacteriologic cultures are checked the next day. It may, however, be easier to initially inoculate a chocolate agar plate to check for sterility before a sample is processed for mycobacteria.

Normally Sterile Specimens

Normally sterile tissue samples may be ground in sterile 0.85% saline or 0.2% bovine albumin and then inoculated directly to the media. Because body fluids commonly contain only small numbers of mycobacteria, they should be concentrated to maximize the yield of mycobacteria before inoculation of media, i.e., centrifuged at $\geq$3,000 $\times$ *g* for 15 min prior to inoculation of the sediment. If the volume of fluid submitted for culture is small and fluid cannot be obtained again, it may be added directly to liquid media.

Contaminated Specimens

The majority of specimens submitted for mycobacterial culture consist of a complex organic matrix contaminated with a variety of organisms. Mucin may trap mycobacterial cells and protect contaminating bacteria from the action of decontaminating agents. Thus, mycobacteria are recovered optimally from clinical specimens through the use of procedures which reduce or eliminate contaminating bacteria while releasing mycobacteria trapped in mucin and cells. Liquefaction of certain specimens, particularly sputum, is often necessary. Mycobacteria are then concentrated to enhance detection in stained smears and culture.

Digestion and Decontamination Methods

Sodium hydroxide, the most commonly used decontaminant, also serves as a mucolytic agent but must be used cautiously because it is only somewhat less harmful to tubercle bacilli than to the contaminating organisms. The stronger the alkali, the higher its temperature during the time it acts on the specimen, and the longer it is allowed to act, the greater will be the killing action on both contaminants and mycobacteria (105). Harsh decontamination can kill 20 to 90% of the mycobacteria in a clinical specimen (97). Homogenization should occur by centrifugal swirling, and this swirling should not be vigorous enough to allow material to rise to the cap. After agitation, there should be at least a 15-min delay before opening the tube to allow any fine aerosol droplets formed during the mixing to settle. All such procedures should be carried out in a class II BSC.

Most commonly, a combination liquefaction-decontamination mixture is used. *N*-acetyl-L-cysteine (NALC), dithiothreitol, and several enzymes effectively liquefy sputum. These agents have no direct inhibitory effect on bacterial cells; however, their use permits treatment with lower concentrations of sodium hydroxide, thereby indirectly improving the recovery of mycobacteria. Addition of cetylpyridinium chloride (CPC; see Appendix 1) to specimens mailed from remote collection stations to a central processing station has yielded good recovery of *M. tuberculosis* without overgrowth by contaminating bacteria (175), but based on our experience, this agent seriously compromises culture in the BACTEC 460TB system.

For rural areas, the use of sodium carbonate, CPC, and sodium borate has been recommended to allow *M. tuberculosis* to remain viable for 5 to 18 days (18). Under field conditions, liquefaction and concentration of sputum for acid-fast staining may be conducted by treating the specimens with equal volumes of 5% sodium hypochlorite solution (undiluted household bleach) and waiting 15 min before centrifugation (97, 166). Such a treated specimen can, however, not be cultured because the chemical seriously affects the viability of AFB. The major limitation is, therefore, that a second specimen must be collected for culture. The method is very useful, however, for rapid smear preparation and interpretation in laboratories that do not process specimens for culture or that do not have a BSC.

No one method of digestion and decontamination is ideal for all clinical specimens, for all laboratories, and for all circumstances. The laboratorian must be aware of the inherent limitations of the various methods used. Even under the best of conditions, all currently available procedures are toxic for mycobacteria to some extent. Thus, the best yield of mycobacteria may be expected to result from the use of the mildest decontamination procedure that sufficiently controls contaminants. Strict adherence to specimen processing protocols is mandatory to ensure survival of the maximal number of mycobacteria. Most laboratories process specimens in batches; current recommendations suggest that specimen batches should be processed daily (186). The most widely used digestion-decontamination method is the

NALC-2% NaOH method (97) (see Appendix 1), which is compatible with the radiometric BACTEC 460TB system and other commercially available newer broth culture systems. Pretreatment of clinical specimens with sodium dodecyl (lauryl) sulfate (SDS)-NaOH is, by contrast, not suitable for the MGIT cultivation method (145) since it results in poor recovery of mycobacteria and a delayed mean time to detection of AFB. Sodium hydroxide, oxalic acid, and to a lesser extent, mild HCl have a detrimental impact on the viability of *M. ulcerans* (138). A novel procedure for processing respiratory specimens utilizing C_{18}-carboxypropylbetaine has also been described previously (188). Although culture and smear sensitivity significantly improved compared to that with the NALC-NaOH procedure, the contamination rate was extremely high (20.8%).

Commonly used digestion-decontamination methods are described with step-by-step instructions in the guide by Kent and Kubica (97), the *Clinical Microbiology Procedures Handbook* (90), and Appendix 1 of this chapter. In general, the specimen is diluted with an equal volume of digestant and allowed to incubate for some time. A neutralizing buffer is added, and the specimen is centrifuged in order to sediment any AFB present. Centrifugation should be carried out at $\geq 3{,}000 \times g$ for 15 min to get maximum recovery. The sediment is then inoculated to the appropriate liquid and solid media.

Whatever method is used, care must be taken to prevent laboratory cross-contamination of patient specimens during processing due to aerosols (8, 22, 172). A single false-positive culture for *M. tuberculosis* could easily be the basis of a diagnosis of tuberculosis, with profound consequences for clinical management, epidemiologic investigations, and public health control measures (see chapter 2).

Optimization of Decontamination Procedures

While no contamination or very low rates of contamination indicate that the pretreatment conditions were too harsh and eliminated not only bacteria and fungi but also mycobacteria, a rate exceeding 5% of all digested and decontaminated specimens cultured is generally defined as excessive contamination. A high contamination rate suggests either too weak decontamination or incomplete digestion. One or a combination of several of the following measures may be used to help decrease the contamination rate.

1. Cautiously and slightly increase the strength of the alkali treatment. Be aware that 4% NaOH will in time probably kill most tubercle bacilli.
2. Use a selective medium (one containing antibiotics) in addition to a nonselective primary culture medium to inhibit the growth of bacterial and fungal contaminants. Selective 7H11 agar (Mitchison medium), Mycobactosel agar (BD), or the Gruft modification of L-J medium should be considered. The most useful media for recovering the MAC from stool specimens have been Mitchison's selective 7H11 agar and Mycobactosel L-J medium (231).
3. Make sure specimens are completely digested; partially digested specimens may not be completely decontaminated. Increase the NALC concentration to digest thick, mucoid specimens.
4. Use an alternative digestion-decontamination procedure for problem specimen types. Respiratory secretions from patients with cystic fibrosis, often overgrown with pseudomonads, can successfully be decontaminated with NALC-NaOH followed by the addition of 5% oxalic acid to the concentrated sediment.

To determine the decontaminating capabilities of each new batch of reagents, the laboratory may wish to inoculate blood agar plates with four to six decontaminated sputum specimens in addition to inoculating mycobacterial media. Numbers of contaminants that grow after 48 h of incubation at 35°C should be minimal to none (97).

Acid-Fast Staining Procedures

Smear microscopy is still one of the most rapid and most inexpensive ways to diagnose tuberculosis. In parallel, it is a rapid means to identify the most contagious patients. Normally, its predictive value for *M. tuberculosis* in expectorated sputum is >90% (111).

The common Gram stain is not suitable for mycobacteria. They may be Gram stain invisible, may appear as clear zones or "ghosts," or may appear as beaded gram-positive rods, particularly rapidly growing mycobacteria (199). Special acid-fast staining procedures are necessary to promote the uptake of dyes. Although the exact nature of the acid-fast staining reaction is not completely understood, phenol allows penetration of the stain, which is facilitated by higher temperatures as applied, for instance, with Ziehl-Neelsen staining. Mycobacteria are able to form stable complexes with certain arylmethane dyes such as fuchsin and auramine O. The cell wall mycolic acid residues retain the primary stain even after exposure to acid-alcohol or strong mineral acids. This resistance to decolorization is required for an organism to be termed acid fast. Certain staining protocols include a counterstain to highlight the stained organisms for easier microscopic recognition. Information about specific staining procedures (those for carbol fuchsin and fluorochrome) is given in chapter 21.

Because acid-fast artifacts may be present in a smear, it is necessary to view the cell morphology carefully. AFB are approximately 1 to 10 μm long and typically are slender rods, 0.2 to 0.6 μm wide, that may appear curved or bent. Individual bacilli may display heavily stained areas and areas of alternating stain, producing a beaded appearance. Assessing AFB morphology for presumptive identification of mycobacterial species has to be done with caution and requires ample training and experience of the laboratory personnel. In liquid medium, *M. tuberculosis* often exhibits serpentine cording, but cords are also seen with some NTM species (125). NTM may appear pleomorphic, showing as long filaments or coccoid forms, with uniform staining properties. *M. kansasii* organisms can often be suspected in stained sputum smears because of their large size and cross-banding appearance (9). Cells of rapidly growing mycobacteria may be <10% acid fast and may not stain with the fluorochrome stain (94). If the presence of a rapid grower is suspected and acid-fast stains, in particular fluorochrome stains, are negative, it may be worthwhile to stain the smear with carbol fuchsin and use a weaker decolorizing process. Organisms that are truly acid fast are difficult to overdecolorize. The laboratory must be aware that there are nonmycobacterial organisms with various degrees of acid fastness such as *Rhodococcus* species, *Nocardia* species, and *Legionella micdadei*, as well as the cysts of *Cryptosporidium, Isospora, Cyclospora*, and *Microsporidium* spores. Based on a recent study, Kinyoun's cold carbol fuchsin method is inferior to both the Ziehl-Neelsen and fluorochrome methods (179).

Each slide made from a clinical specimen should be thoroughly examined for the presence of AFB. When a carbol fuchsin-stained smear is read, a minimum of 300 fields should be examined (magnification of ×1,000) before the smear is reported as negative (90, 97). A fluorochrome

stained smear is read at a lower power (×250) than a carbol fuchsin stained smear; therefore, more material can be examined in a given period. At the lower magnification, a minimum of 30 fields of view should be examined. This requires as little as 90 s. This ease of detection of AFB with the fluorochrome stain makes it the preferred staining method for clinical specimens, although an inexperienced observer may misinterpret fluorescent debris as bacilli.

All smears in which no AFB have been seen should be reported as negative. Conversely, when acid-fast organisms are detected on a smear, the smear should be reported as AFB positive and the staining method should be specified. It is best to confirm positive smears by having them reviewed by another experienced reader. Ideally, all positive fluorochrome-stained smears should be confirmed by a carbol fuchsin-based staining method, e.g., Ziehl-Neelsen, and slides should be stored for future reference (97). The widely accepted practice of confirming positive fluorochrome stains may be challenged in the future. In a recent study, Murray et al. (127) demonstrated that applying a stain to a liquefied (dithiothreitol), concentrated sample and examining the sample before the decontamination process (NaOH) is the most effective method for the detection of AFB.

Information about the quantity of AFB observed on the smear should be provided. The recommended interpretations and reporting of smear results are given in Table 1. If only one or two organisms are seen on an entire smear, this result should be noted but not reported. Confirmation of this finding should be attempted by preparation of additional smears from the same specimen or, if possible, smears from a new specimen. Observations made with the fluorochrome smears should be converted into a format that equates these observations with those made with a 100× oil immersion objective.

However, the reliability of smear microscopy is highly dependent not only on the experience of the laboratory technician but also on the number of AFB present in the specimen. While 10^6 AFB/ml of specimen usually result in a positive smear, only 60% of the smears are positive if 10^4 AFB/ml are present (55).The overall sensitivity of the smear has been reported to range from 22 to 80% (111). An important factor influencing sensitivity is the minimum amount of sputum submitted to the laboratory. In a long-term study, the sensitivity of a concentrated smear from >5 ml of sputum was significantly greater than the sensitivity of a smear processed regardless of volume (211). Other factors influencing smear sensitivity include the type of specimens examined, staining techniques, the experience of the reader, the patient population being evaluated, and pretreatment or lack of pretreatment of the specimens (indirect versus direct smears). Respiratory specimens yield the highest smear positivity rate (111). In practice, the fluorochrome stain is more sensitive than the carbol fuchsin stain, even when smears are read at lower magnification, probably because the fluorochrome-stained smears are easier to read.

The specificity of the smear for the detection of mycobacteria is very high. Prolonged or very harsh specimen decontamination and short incubation of cultures may account for smear-positive, but culture-negative, results. Patients with pulmonary tuberculosis may have positive smears with negative cultures (for 2 to 10 weeks on average) during a course of appropriate treatment (101).

Cytocentrifugation of sputum has resulted in controversial results concerning the sensitivity of smear microscopy (166, 224). Concentration of sputum by centrifugation after liquefaction with 5% sodium hypochlorite is a possible means of increasing smear sensitivity, particularly in developing countries.

The diagnostic yield of acid-fast staining of body fluids is less than that of respiratory specimens because the number of mycobacteria is usually lower. A variety of techniques have been used to concentrate mycobacteria from cerebrospinal fluid and other body fluids, but comparative data are lacking. Centrifugation is not an effective way to concentrate mycobacteria in body fluids since mycobacteria have a buoyant density of approximately 1 and, therefore, many organisms remain in the supernatant (102). Sequential layering of several drops of uncentrifuged fluid onto a slide or polycarbonate membrane filtration is probably the most effective means of concentrating mycobacteria for microscopy (174).

With each new batch of staining reagents, good laboratory practice includes the preparation of a positive and a negative smear for internal quality assessment. Smears containing *M. tuberculosis* or an NTM (positive control) and a gram-positive organism, preferentially a *Nocardia* sp. strain which is not totally acid fast (negative control), may be prepared in advance. Cross-contamination of slides during the staining process and the use of water contaminated with NTM during staining procedures are potential sources of false-positive results (48, 208). Staining jars or dishes should not be used. Transfer of AFB in the oil used for microscopy may also occur. Troubleshooting protocols to prevent false-positive and false-negative smear results have been

TABLE 1 Acid-fast smear evaluation and reporting[a]

Report	No. of AFB seen by staining method and magnification		
	Fuchsin stain	Fluorochrome stain	
	×1,000	×250	×450
No AFB seen	0	0	0
Doubtful; repeat	1–2/300 F[b] (3 sweeps)[c]	1–2/30 F (1 sweep)	1–2/70 F (1.5 sweeps)
1+	1–9/100 F (1 sweep)	1–9/10 F	2–18/50 F (1 sweep)
2+	1–9/10 F	1–9/F	4–36/10 F
3+	1–9/F	10–90/F	4–36/F
4+	>9/F	>90/F	>36/F

[a] Adapted from reference 97.
[b] F, microscope fields.
[c] In all cases, one full sweep refers to scanning the full length (2 cm) of a smear 1 cm wide by 2 cm long.

established by the Association of State and Territorial Public Health Laboratory Directors and the Centers for Disease Control and Prevention (8).

Culture

In detecting as few as 10^1 to 10^2 viable organisms/ml of specimen, culture is more effective than smears. Media available for the recovery of mycobacteria (97) include nonselective and selective ones, the latter containing one or more antibiotics to prevent overgrowth by contaminating bacteria or fungi. Broth media are preferred for rapid initial isolation of mycobacteria.

Solid Media

Egg-Based Media

Egg-based media contain whole eggs or egg yolk, potato flour, salts, and glycerol and are solidified by inspissation. These media have a good buffer capacity and a long shelf life (several months when refrigerated) and support good growth of most mycobacteria. Also, materials in the inoculum or medium toxic to mycobacteria are neutralized. Disadvantages of these media include variations from batch to batch depending on the quality of the eggs used, difficulties in distinguishing colonies from debris, and the inability to achieve accurate and consistent drug concentrations for susceptibility testing. When egg-based media become contaminated, they may liquefy.

Of the egg-based media, L-J medium is most commonly used in clinical laboratories. In general, it recovers *M. tuberculosis* well but is not as reliable for the recovery of other species. Petragnani medium contains about twice as much malachite green as does L-J medium and is most commonly used for recovery of mycobacteria from heavily contaminated specimens. American Trudeau Society medium contains a lower concentration of malachite green than L-J medium and is, therefore, more easily overgrown by contaminants, but the growth of mycobacteria is less inhibited, resulting in earlier growth of larger colonies.

Agar-Based Media

Agar-based media are chemically better defined than egg-containing media. Agar-based media are transparent and provide a ready means for detecting early growth of microscopic colonies easily distinguished from inoculum debris. Colonies may be observed in 10 to 12 days, in contrast to 18 to 24 days with egg-based media. Microscopic examination can be performed by simply turning over the plate and examining it by focusing on the agar surface through the bottom of the plate at a magnification of ×10 to ×100. This type of examination may provide both earlier detection of growth than unaided visual examination and presumptive identification of the species of mycobacteria present. The use of plates with thinly poured 7H11 agar (10 by 90 mm; Remel, Lenexa, Kans.) facilitates this process as microcolonies are visible after 11 days (217). This method is an alternative to broth cultures for some laboratories. Agar-based media can be used for susceptibility testing. They do not readily support the growth of contaminants (97); however, the plates are expensive to prepare, and their shelf life is relatively short (1 month in the refrigerator). Care should be exercised in preparation, incubation, and storage of the media, because excessive heat or light exposure may result in deterioration and in the release of formaldehyde, which is toxic to mycobacteria.

Middlebrook medium contains 2% glycerol, which enhances the growth of the MAC. Nonantibiotic supplements may be helpful for recovery of other mycobacteria and for use in special situations. The addition of 0.2% pyruvic acid is recommended if the presence of *M. bovis* is suspected (47), and 0.25% L-asparagine or 0.1% potassium aspartate added to 7H10 agar maximizes production of niacin (99). The addition of 0.1% enzymatic hydrolysate of casein to the Middlebrook 7H11 formulation (the only difference from 7H10) improves the recovery of isoniazid-resistant strains of *M. tuberculosis*.

Selective Media

The addition of antimicrobial agents may be helpful in eliminating the growth of contaminating organisms. If a selective medium is used for a particular specimen, it should not be used alone but in conjunction with a nonselective agar- or egg-based medium. Egg-based selective media include L-J Gruft with penicillin and nalidixic acid and Mycobactosel L-J medium with cycloheximide, lincomycin, and nalidixic acid. Mitchison selective 7H11 (7H11S) medium and its modifications contain carbenicillin (especially useful for inhibiting pseudomonads), polymyxin B, trimethoprim lactate, and amphotericin B.

Heme-Containing Medium for the Growth of *M. haemophilum*

M. haemophilum will grow on egg- or agar-based media only if they are supplemented with hemin, hemoglobin, or ferric ammonium citrate (169). Thus, specimens from skin lesions, joints, or bone should be inoculated not only onto chocolate agar but also onto Middlebrook 7H10 agar with hemolyzed sheep erythrocytes, hemin, or a factor X disk or onto L-J medium containing 1% ferric ammonium citrate to enhance recovery of this organism. Broth media should be similarly supplemented. *M. haemophilum* can also be isolated from radiometric BACTEC 12B medium as well as from MB Redox broth (168).

Biphasic Media

The Septi-Chek system (BD) is a mycobacterial culture system consisting of a capped bottle containing 20 ml of modified 7H9 broth in an enhanced (20%) CO_2 atmosphere and a paddle containing three types of solid media, i.e., modified L-J medium, Middlebrook 7H11 agar, and chocolate agar, encased in a plastic tube. Bacterial contamination is detected on the chocolate agar. Cultures are inoculated by removing the bottle cap, adding the processed specimen, and then attaching the paddle to the bottle. Solid media are inoculated after 24 h of incubation in an upright position by inverting the bottles. A supplement containing glucose, glycerol, oleic acid, pyridoxal HCl, catalase, albumin, and antibiotics (PANTA) is added to the culture bottle before inoculation. During the incubation period, the bottles are periodically tipped to reinoculate the solid media as cultures are being read. The sensitivity of this system is comparable to that of the BACTEC 460TB system (91). Although the average time to detection of growth is longer than that with the radiometric BACTEC, it is shorter than that with conventional media.

Liquid Media

Broth media may be used for both primary isolation and subculturing of mycobacteria. Cultures based on liquid media

yield significantly more rapid results than solid medium-based cultures. Also, isolation rates for mycobacteria are higher. Middlebrook 7H9 and Dubos Tween albumin broths are commonly used for subculturing stock strains of mycobacteria and preparing the inoculum for drug susceptibility tests and other in vitro tests. 7H9 broth is used as the basal medium for several biochemical tests. Tween 80 can be added to liquid media and acts as a surfactant which allows the dispersal of clumps of mycobacteria, resulting in more homogeneous growth.

At present, a number of elaborate commercially available culture systems marketed for the isolation of mycobacteria range from simple bottles and tubes such as the MGIT (BD) and MB Redox (Heipha Diagnostica Biotest, Heidelberg, Germany) to semiautomated systems (the BACTEC 460TB system; BD) and fully automated systems (e.g., the BACTEC 9000 MB and BACTEC MGIT 960 [BD]; the ESP Culture System II [Trek Diagnostic Systems, Westlake, Ohio]; and the MB/BacT ALERT 3D system [bioMérieux]).

MB Redox

MB Redox (Heipha Diagnostica Biotest) is a nonradiometric medium based on a modified Kirchner medium enriched with growth-promoting additives, antibiotic compounds, and a colorless tetrazolium salt as a redox indicator which is reduced to colored formazan by actively growing mycobacteria. With the naked eye, AFB are detected in the medium as pink to purple pinhead-sized particles. Recovery rates are similar to those observed with other liquid systems (178). Overall, it is a cost-efficient alternative with the disadvantage that it requires much handling during visual reading.

MGIT

The MGIT (BD) contains a modified Middlebrook 7H9 broth in conjunction with a fluorescence quenching-based oxygen sensor (silicon rubber impregnated with a ruthenium pentahydrate) to detect the growth of mycobacteria. The large amount of oxygen initially present in the medium quenches the fluorescence of the sensor. The growth of mycobacteria or other microorganisms in the broth depletes the oxygen, and the indicator fluoresces brightly when the tubes are illuminated with UV light at 365 nm. For the manual version, a Wood's lamp or a transilluminator can be used as the UV light source, and in the automated BACTEC MGIT 960 system (see below), tubes are continuously monitored by the instrument. Prior to use, the 7H9 broth is supplemented with oleic acid-albumin-dextrose to promote the growth of mycobacteria and with PANTA to suppress the growth of contaminants.

Overall, the sensitivity and time to growth detection of the MGIT system are similar to those of the BACTEC 460TB system and have been superior to those of solid media in clinical evaluations (36, 146). However, contamination rates for the MGIT system are currently higher than those for the BACTEC 460TB system, probably owing to the enrichments added to the MGIT broth, which enhance the growth of both mycobacteria and nonmycobacterial organisms.

The principal advantages of the manual MGIT system over the BACTEC 460TB system include reduced opportunity for cross-contamination of cultures, no need for needle inoculation, no radioisotopes, and no need for special instrumentation other than the UV light source. Its limitations include higher contamination rates, masking of fluorescence by blood or grossly bloody specimens, and possible lack of compatibility with some methods of digestion and decontamination of specimens (145). For susceptibility testing of primary drugs, the MGIT is an equivalent replacement for the BACTEC 460TB system, in both the manual and automated versions (see chapter 77).

BACTEC 460TB System

^{14}C-labeled palmitic acid as a carbon source in the medium is metabolized by microorganisms into $^{14}CO_2$, which is monitored by the instrument. The amount of $^{14}CO_2$ and the rate at which the gas is produced are directly proportional to the growth rate of the organism in the medium.

An antimicrobial mixture serving as a growth-promoting supplement (PANTA; see above) is added to BACTEC 12B medium inoculated with decontaminated specimens in order to suppress residual contaminants. To potentially sterile specimens, polyoxyethylene stearate is added to enhance mycobacterial growth. For pretreatment of nonsterile specimens, the NALC-NaOH protocol is the method of choice, although some other procedures such as the SDS-NaOH method are compatible with the BACTEC 460TB system as well (167). Specimens processed by the Zephiran-trisodium phosphate, benzalkonium chloride, or CPC method can, however, not be used with the BACTEC 460TB system because residual quantities of these substances in the inoculum inhibit mycobacterial growth.

The use of the BACTEC 460TB method has significantly improved recovery rates and times of mycobacterial isolation from respiratory secretions and other specimens (161). Organisms in smear-positive specimens usually grow within a few days. The average detection time is 9 to 14 days for *M. tuberculosis* and <7 days for NTM. This short detection time is also obvious with smear-negative specimens and specimens from treated patients. BACTEC 13A medium, traditionally used for blood and bone marrow aspirate specimens, was recently discontinued. Other limitations of the BACTEC 460TB system include the inability to observe colony morphology, difficulty in recognizing mixed cultures, overgrowth by contaminants, cost, radioisotope disposal, and extensive use of syringes with its potential for needle punctures among laboratory technicians. Since it is only semiautomated, vials have to be transferred to the incubator once the growth index (GI) has been read by the instrument.

The BACTEC 460TB system allows efficient antimicrobial susceptibility testing as well (see chapter 77). Generally, the initial positive vial can be used directly for identification and drug susceptibility testing. It is good laboratory practice to confirm acid fastness and to subculture positive BACTEC vials to a chocolate agar to check for potential contaminants or, if suspected, for mixed cultures.

Automated, Continuously Monitoring Systems

Several automated, continuously monitoring systems have recently been developed for the growth and detection of mycobacteria, i.e., the BACTEC 9000 MB (BD), the BACTEC MGIT 960 (BD), the ESP Culture System II (Trek Diagnostic Systems), and the MB/BacT ALERT 3D (bioMérieux). All have in common that they are no longer based on the use of radioisotopes. The BACTEC 9000 MB system uses the same fluorescence quenching-based oxygen sensor as the MGIT system to detect growth. The technology used in the ESP Culture System II is based on the detection of pressure changes in the headspace above the broth medium in a sealed bottle resulting from gas production or

consumption due to the growth of microorganisms. The MB/BacT ALERT 3D system, finally, employs a colorimetric carbon dioxide sensor in each bottle to detect the growth of mycobacteria. Each of the systems includes a broth similar to 7H9 supplemented with a variety of growth factors and antimicrobial agents.

These systems have similar performance and operational characteristics. In clinical evaluations, recovery rates were similar to those of the BACTEC 460TB system and superior to those of conventional solid media (BACTEC 9000 MB [143], BACTEC MGIT 960 [72, 221], ESP Culture System II [225]; and MB/BacT ALERT 3D [150, 221]). Time to detection of mycobacteria is almost the same as in the radiometric BACTEC 460TB technique. Throughout, contamination rates reported have been higher with these new systems than with the BACTEC 460TB system. All share the advantages over the radiometric broth system of having no potential for cross-contamination by the instrument, being less labor-intensive, having continuous monitoring, using no radioisotopes, addressing safety more appropriately, and offering electronic data management. Since these systems are monitoring continuously, bottles are incubated in the instruments for their entire life in the laboratory. As a consequence, these systems are both instrument and space intensive. Some automated systems also lack the versatility of the BACTEC 460TB system in that direct inoculation of blood is, so far, not possible. The same holds for the incubation of cultures harboring mycobacteria with a lower optimum temperature such as *M. haemophilum*, *M. marinum,* and *M. ulcerans*. Except in the case of the BACTEC 9000 MB system, susceptibility testing applications for the primary antituberculosis drugs, including pyrazinamide (in some systems), are available (see chapter 77).

Medium Selection

Medium selection for the isolation of mycobacteria and culture reading schedules are usually based on personal preferences and/or laboratory tradition. Both should be optimized for the most rapid detection of positive cultures and identification of mycobacterial isolates. The variety of media and methods available today is sufficient to permit laboratories to develop an algorithm that is optimal for their patient population and administrative needs. Workload, financial resources, and in particular, the limited amounts of processed sediments are, however, restraining factors in working with too many different types of media. Thus, cultivation of mycobacteria always involves a compromise.

Today, it is generally accepted that the use of a liquid medium in combination with at least one solid medium is essential for good laboratory practice in the isolation of mycobacteria. The addition of a solid medium is advantageous for those strains which occasionally do not grow in liquid medium, aids in the detection of mixed mycobacterial infections, and can serve as a backup for broth with its higher contamination rate. All positive cultures, even if identified directly from the broth, must be subcultured to solid media to detect mixed cultures and to correlate direct identification results with colony morphology. The Septi-Chek system can be used as a stand-alone system. In contrast, the radiometric BACTEC 460TB system and the new, nonradiometric growth systems such as the BACTEC 9000, BACTEC MGIT 960, ESP Culture System II, and MB/BacT ALERT 3D cannot serve as stand-alone culture systems for mycobacteria for reasons stated above. In a recent meta-analysis of 10 published studies encompassing 1,381 strains from 14,745 clinical specimens, the BACTEC MGIT 960 and BACTEC 460TB systems revealed sensitivities and specificities in detecting mycobacteria of 81.5 and 99.6% and 85.8 and 99.9%, respectively. Combined with solid media, the sensitivities of the two systems increased to 87.7 and 89.7%, respectively (40).

Detection of colonies on solid medium certainly offers several advantages over detection of growth in broth, because colonial morphology can provide clues for identification and facilitate the selection of confirmatory tests, including DNA probe tests. However, smears from broth-based systems can sometimes provide microscopic clues such as cord formation (see above), and it is possible to use the sediment from such cultures for confirmation by gene probes before growth is detected on solid media. The criterion of cord formation for presumptive identification of *M. tuberculosis* should be applied with caution since the phenomenon is also being observed with some NTM species (9, 125).

Incubation

Temperature

The optimum incubation temperature for most cultures is 35 to 37°C. Exceptions to this rule include cultures obtained from skin and soft tissue suspected to contain *M. marinum, M. ulcerans, M. chelonae*, or *M. haemophilum,* which has a lower optimum temperature. For such specimens, a second set of media should be inoculated and incubated at 25 to 33°C. BACTEC 460TB vials should be incubated at 36 to 38°C because optimum metabolism of the radiolabeled substrate occurs at 37 to 37.5°C for most species. Lower temperatures increase detection time. The newer liquid medium-based culture automated systems do not offer the possibility to incubate at temperatures lower than 36 ± 1°C.

Atmosphere

Five to 10% CO_2 in air stimulates the growth of mycobacteria in primary isolation cultures using conventional media (10). Middlebrook agar requires a CO_2 atmosphere to ensure growth, while it is necessary to incubate egg media under CO_2 for only the first 7 to 10 days after inoculation, i.e., the log phase of growth. Subsequently, L-J cultures can be removed to ambient-air incubators if space is limited. In the absence of CO_2 incubators, plates may be incubated in commercially available bags with CO_2-generating tablets. Candle extinction jars are unacceptable for use in the mycobacteriology laboratory because the oxygen tension is less than that required for the growth of mycobacteria. Broth systems usually do not require incubation at increased CO_2 concentrations.

Time

Mycobacterial cultures on solid and in liquid media are generally held for 6 to 8 weeks before being discarded as negative. Specimens with positive smears that are culture negative should be held for an additional 4 weeks. The same is true for culture-negative specimens which were positive for mycobacteria by one of the nucleic acid-based amplification assays or for cases with a persisting suspicion of tuberculosis. Plates should be incubated with the medium side down until the entire inoculum has been absorbed. Once this has happened, media should be incubated inverted in CO_2-permeable polyethylene bags or sealed with CO_2-permeable shrink-seal or cellulose bands to prevent them from drying

up during the incubation period. Tubed media should be incubated in a slanted position with the screw caps loose for at least a week until the inoculum has been absorbed; they can then be incubated upright if space is at a premium. Caps on the tubes should be tightened at 2 to 3 weeks to prevent desiccation of the media. Specimens from skin lesions should be incubated for 8 to 12 weeks if the presence of *M. ulcerans* is suspected.

Reading Schedule

Mycobacteria are relatively slowly growing organisms, and thus cultures can be examined less frequently than routine bacteriologic cultures. All solid media should be examined within 3 to 5 days after inoculation to permit early detection of rapidly growing mycobacteria and to enable prompt removal of contaminated cultures. Young cultures (up to 4 weeks of age) should be examined twice a week, whereas older cultures may be examined at weekly intervals. Use of a hand lens for opaque media and a microscope for agar media will facilitate early detection of microcolonies.

Septi-Chek and the manual MGIT may be inspected for growth several times per week or daily for the first 1 to 2 weeks; Septi-Chek bottles should be inverted for reinoculation of the agar medium if growth is not observed. Afterwards, these systems are inspected twice weekly or weekly for growth.

For BACTEC 460TB vials, the reading schedule varies according to the laboratory workload. Low-volume laboratories may read cultures three times a week for the first 2 or 3 weeks and weekly thereafter for a total of 6 weeks, and high-volume laboratories may read cultures twice a week for the first 2 weeks and weekly thereafter. Some laboratories prioritize smear-positive specimens by separating them from smear-negative specimens and test the former more frequently. In addition, separation of "probable positive" cultures from negative ones will decrease the possibility of vial cross-contamination by the instrument. With more frequent testing of all specimens, however, earlier detection of positive cultures is expected. Readings of negative cultures in 12B medium usually remain below a GI of 10; a GI of 10 or more is considered presumptively positive. At this point, the vials should be separated and tested daily. Acid-fast staining is performed when the GI is >50 to determine whether the culture contains mycobacteria. The morphology of mycobacteria seen in smears from 12B medium may be used by experienced laboratory personnel to presumptively identify the *M. tuberculosis* complex and to decide how to proceed with identification methods (9). In addition, a smear of the broth from the vial may be Gram stained and/or the broth may be subcultured onto a sheep blood agar or chocolate agar plate to determine whether contamination is present. When the GI is 500 or more, BACTEC 460TB antimicrobial susceptibility testing can be performed (see below).

When using one of the new continuously monitoring systems (BACTEC 9000, BACTEC MGIT 960, ESP Culture System II, and MB/BacT ALERT 3D), technicians are automatically alerted by the instrument if a specimen turns positive. Irrespective of the system used, the acid fastness of the organism has to be confirmed by smear staining. Also, it is highly advisable to subculture the broth on a sheep blood or chocolate agar plate to rule out contaminants. Once the growth of AFB is detected, susceptibility testing can be performed, always following the instructions specified by the manufacturers.

IMMUNODIAGNOSTIC TESTS FOR TUBERCULOSIS

A variety of immunodiagnostic tests for tuberculosis based on the recognition of specific host responses to the infecting organism have been described previously. Historically, the first immunodiagnostic test was the tuberculin skin test. The shortcomings of this test include the inability to distinguish active disease from past sensitization and unknown predictive values (177).

Much effort has been devoted to the development of serological tests for tuberculosis, but no test has found widespread clinical use (43). The specificity of serological tests with crude antigen preparations is too low for clinical application. Specificity can be increased by using purified antigens, but since not all patients respond to the same antigens, the increased specificity often results in decreased sensitivity (33, 43). Sensitivity and specificity increase if enzyme-linked immunosorbent assay (44) results obtained with a set of purified antigens are combined. Antigens tested in serological assays are, for instance, the 38-kDa antigen, lipoarabinomannan, antigen 60, the antigen 85 complex, and glycolipids including phenolic glycolipid Tb1, 2,3-diacyltrehalose, and lipooligosaccharide. Weldingh et al. (218) have recently assessed the serodiagnostic potential of 35 *M. tuberculosis* proteins and identified four novel serological antigens.

A number of antigen capture assays based on enzyme-linked immunosorbent assays or radioimmunoassays or agglutination of antibody-coated latex particles have been described previously. The sensitivities of immunoassays for the detection of mycobacterial antigens in cerebrospinal fluid ranged from 65.8 to 100% and the specificities ranged from 95 to 100% in six major studies (43). Experience with sputum and other specimens is limited. The use of antigen tests cannot be recommended at this time.

Although broad experience is not yet available, the recently developed, commercially available whole-blood gamma interferon assays are promising candidates to improve the current level of diagnostic accuracy for tuberculosis infection, in particular if skin tests are equivocal. Guidelines for using the QuantiFERON-TB test (Cellestis Ltd.) (132) for diagnosing latent *M. tuberculosis* infection are available (117). Ferrara et al. (60) have demonstrated that the QuantiFERON-TB Gold test (Cellestis Ltd.) has higher specificity than the tuberculin skin test when used for selected populations. The QuantiFERON-TB Gold test is (i) less affected by BCG for vaccination than the common skin tests, (ii) able to discriminate responses due to NTM, and (iii) less prone to variability and subjectivity associated with placing and reading of the tuberculin skin test. Another approach is based on the overlapping *M. tuberculosis* antigens ESAT-6 and CFP-10 and similarly offers increased specificity over the PPD skin test when used in an ex vivo enzyme-linked immunospot (CTL Laboratory, Cleveland, Ohio) assay for gamma interferon detection for the diagnosis of *M. tuberculosis* infection from recent exposure (82, 110).

CROSS-CONTAMINATION

With the advent of molecular techniques designed for molecular epidemiology, cross-contamination linked either to laboratory procedures or, more rarely, to contaminated bronchoscopes can easily be proven (12, 66, 170). False-positive results may be generated at any step from specimen collection to reading of cultures (8, 61). Laboratory personnel should be alerted for a possible laboratory error if (i) the

culture result is not compatible with the clinical picture, (ii) there is a late-appearing cluster of cultures which have scanty growth (<10 colonies on solid medium) or a significant delay in recovering mycobacteria from a liquid system, (iii) there is a large number of isolates of a particular species that is usually rare in the laboratory or of an organism that is normally considered an environmental contaminant, or (iv) there is only one positive culture from multiple specimens submitted from a single patient. Practices which can lead to false-positive culture results are numerous and include inadequate sterilization of instruments or equipment (such as bronchoscopes), use of contaminated water for specimen collection or for laboratory procedures, transfer of organisms from one specimen to another through direct contact or via common reagents or equipment, mix-up of testing samples or lids of specimen containers, and failure to take precautions which minimize the production of aerosols, etc. Laboratory aspects of cross-contamination are addressed in more detail in the following section.

QUALITY ASSURANCE

General Aspects

Much of the information in this section was obtained from recent publications (5, 8, 90, 97, 128, 129). In addition to the specific recommendations listed here, standard components of laboratory quality assurance, such as personnel competency, procedure manuals, proficiency testing, and quality control (QC) of media, tests, and reagents, should be in place.

The Public Health Service introduced the levels of service concept for mycobacteriology laboratories in 1967. In this scheme, laboratories define the level of service which best fits the needs of the patient population they serve, the experience of their personnel, their laboratory facilities, and the number of specimens they receive. The concept of levels of service is supported by the Centers for Disease Control and Prevention and the American Thoracic Society (80). The College of American Pathologists proposed extents of service for participation in mycobacterial interlaboratory comparison surveys. American Thoracic Society levels I, II, and III correlate with College of American Pathologists extents 2, 3, and 4, respectively. Five types of mycobacteriology laboratories have been defined by the Clinical Laboratory Improvement Amendments (CLIA). These are modified from the extents described by the College of American Pathologists, but the definitions are awaiting consensus and thus may be modified in the future (226). All specimens submitted for mycobacterial examination should have cultures as well as smears performed. The American Thoracic Society and Centers for Disease Control and Prevention recommend that laboratories examine a minimum of 10 to 15 smears per week to maintain proficiency in performance and interpretation and that they process and culture 20 specimens per week to maintain proficiency in the culture and identification of *M. tuberculosis*. The Association of State and Territorial Public Health Laboratory Directors proposed that only two levels of service be designated: (i) specimen collection, specimen transport, and (optional) microscopy of at least 20 smears per week and (ii) complete mycobacteriology service from microscopy to complete species identification and drug susceptibility testing (212).

Personnel working in the clinical mycobacteriology laboratory must have proper training and certification in the specific functions that they perform. All laboratories performing mycobacteriology testing must be enrolled in a proficiency program which monitors a laboratory's performance by using external samples which are sent for testing. These programs must follow the specifications outlined by the CLIA.

Multiple test parameters are monitored by adherence to the quality assurance guidelines described in the recent NCCLS (now the Clinical and Laboratory Standards Institute) standard document (128). Acceptable results derived from testing QC reference strains do not guarantee accurate results with all clinical isolates. If inconsistent results are seen with clinical isolates, the test should be repeated in an attempt to ensure accuracy. Each laboratory should put its own policies into effect regarding the verification of atypical test results.

QC is vital for monitoring a laboratory's effectiveness in detecting and isolating mycobacteria. The CLIA and accreditation programs represent the minimum acceptable standards of practice (226), and laboratories performing mycobacterial testing should follow QC recommendations in the scientific literature and in ad hoc publications (8, 90, 128).

The laboratory must maintain a collection of well-characterized mycobacterial strains that are used for QC of test systems. These controls may be obtained from the American Type Culture Collection and proficiency testing programs. Frequently used stock cultures can be maintained on L-J slants or in 7H9 broth at 37°C or at room temperature if subcultured monthly. Cultures on L-J slants may be held for up to 1 year if stored at 4°C. Such maintenance is not recommended for strains with drug resistance. Freezing of organisms suspended in skim milk or broth medium and storage at −20 to −70°C is the best option for long-term maintenance of stock cultures.

Routine QC tests are recommended with new lots of media used with commercial systems (90). Laboratories that prepare their own media must also document the performance characteristics of each new lot.

QC of Smear, Culture, and Molecular Tests

Ideally, positive control slides should be prepared from a concentrated sputum sample obtained from a patient with active tuberculosis. In practice, many laboratories use suspensions of stock cultures or seeded negative sputa as positive controls for acid-fast staining procedures. The *Clinical Microbiology Procedures Handbook* (90) describes a method for preparing control slides. Control slides are also commercially available. An increase in the percentage of smear-positive but culture-negative specimens of >2% that cannot be attributed to a response to mycobacterial therapy or the presence of AFB in the negative controls suggests that water or reagents used in the pretreatment or staining procedures were contaminated with NTM (97). *M. gordonae* or the *M. terrae* complex is most often involved. A procedure for detection of AFB in working solutions and reagents is described in the Association of State and Territorial Public Health Laboratory Directors and Centers for Disease Control and Prevention document (8). AFB may also be carried over from one slide to another if slides are not set properly apart from each other during the staining process. AFB may also be found in the oil used with the immersion lens after a positive slide is examined. The sensitivity of the AFB smear is directly related to the relative centrifugal force (RCF, or *g* force) attained during centrifugation. Thus, laboratories should calculate the RCF of their centrifuge and periodically monitor and document that they are reaching sufficient RCF by checking the revolutions per minute with a tachometer (97).

Laboratories should also monitor contamination rates (percentages of specimens producing contaminating growth on culture media) for decontaminated specimens. Contamination rates of 3 to 5% are generally considered acceptable. Lower rates usually indicate that the decontamination procedure is too harsh and the procedure needs to be modified to minimize the lethal effect on mycobacteria. Contamination rates above 5% often indicate a too-weak decontamination which could compromise mycobacterial cultures due to overgrowth of contaminants. It should be emphasized that the widespread use of liquid media increases the generation of aerosols; as a consequence, the risk of contamination between samples also increases. Laboratories that handle large numbers of isolates of the MAC, *M. abscessus*, or specimens from patients with cystic fibrosis will probably have much higher contamination rates due to the high incidence of colonization of the sputum with gram-negative bacteria, especially *Pseudomonas aeruginosa*.

Culturing of mycobacteria is naturally prone to errors because of the multiple steps involved in processing of cultures, the viability of mycobacteria for long periods in the laboratory environment, and the large number of mycobacteria present in some specimens (22). False-positive cultures may result from mislabeling, specimen switching during handling, specimen carryover (including proficiency testing specimens), contaminated reagents, or cross-contamination between culture tubes or vials (22, 29). Inclusion of a positive control (e.g., a suspension of *M. tuberculosis*) in the processing of patient specimens is discouraged due to the risk of cross-contamination. Cross-contamination of culture vials in the BACTEC 460 TB system due to inadequately sterilized sample needles has been documented (15). Standardized laboratory procedures that minimize the potential for errors leading to false-positive cultures should be followed, and mechanisms should be in place to rapidly recognize the occurrence of false-positive cultures. Transfers or inoculation of cultures must be accomplished by using individual transfer pipettes, single-delivery diluent tubes, or disposable labware. The order in which specimens are processed and media are inoculated should be recorded. Processing of a negative control specimen following processing of patient specimens with the same digestion or decontamination solution can be used for detecting possible specimen contamination of the solutions (8). Alternatively, processing solutions may be planted directly. Laboratories should prospectively track positivity rates and establish a threshold which, when exceeded, will prompt an investigation (172). The significance of an isolate may be determined by reviewing the order in which specimens were handled for all manipulations (e.g., initial processing, liquid medium readings, and subculturing), the direct-smear results, the time to positivity, and the clinical history. Cross-contamination in the BACTEC 460TB system or other automated systems is probably rare if the manufacturer's recommendations for operation and maintenance are closely followed.

Since the introduction of molecular fingerprinting of *M. tuberculosis* strains, false-positive cultures have been demonstrated to occur more frequently than previously assumed, accounting for from 1 to more than 10% of positive cultures (154, 172). The deleterious impact of these undesirable events may be minimized if the evidence of false positivity is established in a timely manner and a rapid molecular method of fingerprinting is available. It has been suggested that single positive cultures of *M. tuberculosis* strains grown from AFB smear-negative specimens should be analyzed by a PCR-derived typing technique to rule out laboratory contamination (154). Cultures of strains with identical fingerprints isolated within a 1-week period from different patients should be considered probably falsely positive (172).

The Centers for Disease Control and Prevention and others have recommended that AFB smear results be available and that positive results be reported within 24 h of specimen receipt (8, 186). The time required for identification and susceptibility testing of *M. tuberculosis* should average 14 to 21 days and 15 to 30 days from the time of specimen receipt, respectively (8, 26, 227). Recently, Pascopella et al. (141) have evaluated laboratory reporting of tuberculosis test results and patient treatment initiation in California.

Nucleic acid amplification (NAA)-based assays require several levels of controls (e.g., to detect amplification inhibition as well as contamination between specimens) in addition to positive or negative controls (129). When used as approved by the Food and Drug Administration, NAA tests for *M. tuberculosis* infection diagnosis do not replace any previously recommended tests (28). Laboratories that test patient specimens by using research or home brew methods or commercially available NAA assays for nonapproved or off-label indications and report their results must validate the assays and establish their performance characteristics prior to diagnostic use. Available information is often insufficient to guide test interpretation. Approved guidelines for molecular diagnostic methods in clinical microbiology are available from the Clinical and Laboratory Standards Institute; in these documents, the development, validation, quality assurance, and routine use of NAA assays are addressed in detail (129). However, basing the identification of *M. tuberculosis* on a sole positive home brew PCR result is not recommended because the results of such assays vary considerably (8, 27).

Potential probes and/or primers must be selected for sensitivity by using multiple clinical and reference strains of the target organism. Additionally, specificity must be evaluated by testing for cross-hybridization with other organisms which may be present in patient samples (129).

Several types of validation tests are used to evaluate the presence of target nucleic acid in the sample and to determine that it was isolated in a manner in which the target has not been introduced. Testing to assess amplification should include positive and negative controls and controls for detection of the presence of inhibitors, such as endogenous nucleic acid. Other QC measures include those referring to assays of restriction enzymes and reagents, inspection of equipment, and laboratory design (i.e., separate areas for processing, amplification, and detection steps) (129). Excellent proficiency testing schemes to assess laboratory performance of NAA tests are currently available both in the United States (160) and in Europe (134).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Adequate funding and focused training are critical in maintaining state-of-the-art mycobacteriology laboratories (16, 128, 129, 226). Laboratories play a pivotal role in the diagnosis and control of tuberculosis, and every effort should be made to implement sensitive and rapid methods for the detection, identification, and susceptibility testing of the *M. tuberculosis* complex as well as other mycobacterial species. Specifically, these include (i) the use of fluorochrome staining for mycobacteria in smears, (ii) the use of a broth-based or microcolony method for culture, (iii) the use of rapid identification methods (e.g., gene probes and line probe

assays), and (iv) direct susceptibility testing of smear-positive specimen concentrates.

The 24-h turnaround time for AFB smear results presents a challenge for most laboratories. The daily processing of specimens required to meet this goal adds considerable expense to the laboratory budget. Turnaround time goals for AFB smear results should be established for each institution after consultation with infection control practitioners and infectious-disease specialists.

NAA assays offer the promise of same-day detection and identification of *M. tuberculosis*. Implementation of these new technologies presents several new challenges. Although the performance characteristics of many of these assays are quite good for smear-positive respiratory specimens, limited information exists on the use of these tests for the diagnosis of paucibacillary pulmonary or extrapulmonary disease. The new technology will supplement rather than replace culture. Culture will still be required to obtain organisms for susceptibility testing and detect mycobacteria other than *M. tuberculosis*.

The significance of the isolation of NTM may be difficult to assess since many species are opportunistic pathogens, and the reader is referred to the criteria suggested by the American Thoracic Society for the evaluation (5). In addition to these criteria, accurate identification of NTM will prevent rarely encountered pathogens from being mistaken for nonpathogenic species.

Thus, accurate and timely reporting of the results of AFB microscopy, culture, identification, and drug susceptibility testing is essential to the effective management of individual patients and to the appropriate implementation of public health and infection control measures.

APPENDIX 1
Commonly Used Digestion-Decontamination Methods

Refer to references 90 and 97 for details.

NALC-NaOH method

Reagents

Digestant: For each 100 ml, combine 50 ml of sterile 0.1 M (2.94%) trisodium citrate with 50 ml of 4% NaOH. The NaOH and citrate mixtures can be mixed, sterilized, and stored for future use. To this solution, add 0.5 g of powdered NALC just before use. Use within 24 h of addition of the NALC because the mucolytic action of NALC is inactivated on exposure to air.

Phosphate buffer: The buffer is 0.067 M and pH 6.8. Mix 50 ml of solution A (0.067 M Na_2HPO_4; 9.47 g of anhydrous Na_2HPO_4 in 1 liter of distilled water) and 50 ml of solution B (0.067 M KH_2PO_4; 9.07 g of KH_2PO_4 in 1 liter of distilled water). If the final buffer requires pH adjustment, add solution A to raise the pH or solution B to lower it.

BSA (optional): Use sterile 0.2% bovine serum albumin (BSA) fraction V (pH 6.8).

Procedure

1. Transfer up to 10 ml of specimen to a sterile, graduated, 50-ml plastic centrifuge tube labeled with appropriate identification. The tube should have a leakproof, aerosol-free screw cap. Add an equal volume of the NALC-NaOH solution. The final concentration of NaOH in the tube is 1%.

2. Tighten the cap completely. Invert the tube so that the NALC-NaOH solution contacts all the inside surfaces of the tube and cap, and then mix the contents for approximately 20 s on a Vortex mixer. If liquefaction is not complete during this time, agitate the solution at intervals during the following decontamination period.

3. Allow the mixture to stand for 15 min at room temperature with occasional gentle shaking by hand. Avoid movement that causes aeration of the specimen. A small pinch of crystalline NALC may be added to viscous specimens for better liquefaction. Specimens should remain in contact with the decontaminating agent for only 15 min, since overprocessing results in reduced recovery of mycobacteria. If more active decontamination is needed, slightly increase the concentration of NaOH.

4. Add phosphate buffer (pH 6.8) up to the 50-ml mark on the tube.

5. Centrifuge the solution for at least 15 min at $\geq 3{,}000 \times g$.

6. Decant the supernatant fluid into a splashproof discard container containing a suitable disinfectant. Do not touch the lip of the tube to the discard container. Wipe the lip of each tube with disinfectant-soaked gauze (separate piece for each tube) to absorb drips, and recap.

7. Using a separate sterile pipette for each tube, add to the sediment 1 to 2 ml of sterile, 0.2% BSA fraction V (pH 6.8) or 1 to 2 ml of phosphate buffer (pH 6.8), and resuspend the sediment with the pipette or by shaking the tube gently by hand. BSA may have a buffering and detoxifying effect on the sediment and increases the adhesion of the specimen to solid media. However, BSA may lengthen detection times (for instance, in the BACTEC 460TB system).

8. Inoculate the specimens onto appropriate solid culture media and into broth media. Use a separate disposable capillary pipette for each specimen to deliver 3 drops to solid medium.

9. Prepare a smear for acid-fast staining. Use a sterile disposable pipette to place 1 drop of the sediment onto a clean, properly labeled microscope slide, covering an area approximately 1 by 2 cm. Place the smears on an electric slide warmer at 65 to 75°C for 2 h to dry and fix them. Alternatively, air dry the smears and fix them by passing the slide three or four times through the blue cone of a flame (heat fixing does not always kill mycobacteria, and the slides are potentially infectious).

10. Refrigerate the remaining sediment for later use if needed (direct susceptibility testing, further treatment if the specimen is contaminated, etc.).

The NALC-NaOH method can be used to process gastric lavage specimens, tissues, stool, urine, and other body fluids. For neutralized gastric lavage specimens and other body fluids ($\geq$10 ml), centrifuge at $\geq 3{,}000 \times g$ for 30 min in sterile screw-cap 50-ml centrifuge tubes, decant the supernatants, resuspend the sediments in 2 to 5 ml of sterile distilled water, and proceed as for sputum. If a gastric lavage specimen is mucopurulent, add 50 mg of NALC powder per 50 ml of lavage fluid and vortex before centrifugation. Tissue that is not collected aseptically can be ground, placed in a tube, homogenized by vortexing, and processed as for sputum. For stool specimens, place approximately 1 g of a formed specimen or 1 to 5 ml of a liquid specimen in a total volume of 10 ml of 7H9 broth, sterile water, or sterile saline; vortex vigorously for 30 s; and then allow large particles to settle to the bottom of the tube for 15 min. Remove 7 to 8 ml of supernatant, place into a 50-ml centrifuge tube, and process as for sputum.

Sodium hydroxide method

Reagents

Digestant: NaOH solution (2 to 4%). Sterilize by autoclaving.

2 N HCl: Dilute 33 ml of concentrated HCl to 200 ml with water. Sterilize by autoclaving.

Phenol red indicator: Combine 20 ml of phenol red solution (0.4% in 4% NaOH) and 85 ml of concentrated HCl with distilled water to make 1,000 ml.

Phosphate buffer: The buffer is 0.067 M and pH 6.8. See the NALC-NaOH procedure for buffer preparation.

Procedure

Follow the steps described for the NALC-NaOH method, substituting 2% NaOH for the NALC-alkali digestant.

1. Transfer a maximum volume of 10 ml of specimen to a sterile 50-ml screw-cap plastic centrifuge tube. Add an equal volume of NaOH.

2. With the cap tightened, invert the tube and then agitate the mixture vigorously for 15 min on a mechanical mixer, or vortex

vigorously and let stand for exactly 15 min. If it is necessary to reduce excessive contamination, the NaOH concentration can be increased to 3 or 4%.

3. Add phosphate buffer (pH 6.8) up to the 50-ml mark on the tube. Recap the tube, and swirl by hand to mix well.

4. Centrifuge the specimen at ≥3,000 × *g* for 15 min, decant the supernatant, and add a few drops of phenol red indicator to the sediment. Neutralize the sediment with HCl. Thoroughly mix the contents of the tube. Stop acid addition when the solution is persistently yellow.

5. Resuspend the sediment in 1 to 2 ml of phosphate buffer or sterile 0.1% BSA fraction V.

6. Inoculate the resuspended sediment to appropriate culture media, and prepare a smear.

Zephiran-trisodium phosphate method

Principle

This system can be used when the laboratory cannot monitor the amount of time of exposure to the decontaminating agent, since the timing of this digestion-decontamination process is not critical. Benzalkonium chloride (Zephiran), a quaternary ammonium compound, together with trisodium phosphate selectively destroys many contaminants while having little activity on tubercle bacilli. Zephiran is bacteriostatic to mycobacteria, and so the digested, centrifuged sediment must be neutralized with buffer before being inoculated onto agar medium. The phospholipids of egg medium neutralize this compound. It is incompatible with the BACTEC 460TB system.

Reagents

Zephiran-trisodium phosphate digestant: Dissolve 1 kg of trisodium phosphate ($Na_3PO_4 \cdot 12H_2O$) in 4 liters of hot distilled water. Add 7.5 ml of Zephiran concentrate (17% benzalkonium chloride [Winthrop Laboratories, New York, N.Y.]), and mix. Store at room temperature.

Neutralizing buffer: Neutralizing buffer has a pH of 6.6. Add 37.5 ml of 0.067 M disodium phosphate solution to 62.5 ml of 0.067 M monopotassium phosphate solution (for preparation of buffer solutions, see the NALC-NaOH procedure).

Procedure

1. Transfer a maximum volume of 10 ml of specimen to a sterile, 50-ml screw-cap plastic centrifuge tube. Add an equal volume of the Zephiran-trisodium phosphate digestant.

2. Tighten the cap, invert the tube, and then agitate the mixture vigorously for 30 min on a mechanical shaker. Permit the material to stand, without shaking, for an additional 20 to 30 min at room temperature.

3. Centrifuge the specimen at ≥3,000 × *g* for 15 min, decant the supernatant, and add 20 ml of neutralizing buffer. Vortex for 30 s to thoroughly suspend the sediment in the buffer (the neutralizing buffer serves to inactivate traces of Zephiran in the sediment, which is critical if inoculation of an agar-based medium is intended).

4. Centrifuge the specimen again for 15 min.

5. Decant the supernatant, retaining some fluid to resuspend the sediment.

6. Inoculate egg-based medium, and make a smear. The phospholipids of egg medium provide neutralization for this quaternary compound.

Oxalic acid method

Principle

The oxalic acid method is superior to alkali methods for processing specimens consistently contaminated with *Pseudomonas* species and certain other contaminants. Specimens processed by this method may be used with the BACTEC 460TB system. This method can also be used to decontaminate a previously processed sediment when cultures are contaminated with *Pseudomonas*.

Reagents

5% oxalic acid
Physiological saline (0.85%)
4% NaOH
Phenol red indicator or pH paper

Procedure

1. Add an equal volume of 5% oxalic acid to 10 ml or less of specimen in a 50-ml centrifuge tube (1:1, vol/vol).

2. Vortex the solution, and then allow it to stand at room temperature for 30 min with occasional shaking.

3. Add sterile saline up to the 50-ml mark on the centrifuge tube. Recap the tube, and invert it several times to mix the contents.

4. Centrifuge for 15 min at ≥ 3,000 × *g*, decant the supernatant fluid, and add a few drops of phenol red indicator to the sediment. Alternatively, use pH paper.

5. Neutralize with 4% NaOH.

6. Resuspend the sediment, inoculate it to media, and make a smear.

CPC method

Principle

CPC, a quaternary ammonium compound, is used to decontaminate specimens, while sodium chloride effects liquefaction. CPC is bacteriostatic for mycobacteria inoculated onto agar-based media. This effect is not neutralized in the digestion process, and thus sediments from specimens treated with CPC should be inoculated only on egg-based media. This method is incompatible with the BACTEC 460TB system.

This method is a means of digesting and decontaminating specimens in transit (>24 h). Mycobacteria remain viable for 8 days in the solution.

Reagents

CPC digestant-decontaminant: Dissolve 10 g of CPC and 20 g of NaCl in 1,000 ml of distilled water. The solution is self-sterilizing and remains stable if protected from light, extreme heat, and evaporation. Dissolve with gentle heat any crystals that might form in the working solution. Other reagents used in processing include sterile water and sterile saline or 0.2% sterile BSA fraction V.

Procedure

1. Collect 10 ml or less of sputum in a 50-ml screw-cap centrifuge tube.

2. Inside a BSC, add an equal volume of CPC-NaCl, cap securely, and shake by hand until the specimen liquefies.

3. Package the specimen appropriately as specified by current postal regulations, and send it to a processing laboratory.

4. Upon receipt in the processing laboratory (allow at least 24 h for digestion-decontamination to be completed), dilute the digested-decontaminated specimen to the 50-ml mark with sterile distilled water and recap securely. Invert the tube several times to mix the contents.

5. Centrifuge at ≥3,000 × *g* for 15 min, decant the supernatant fluid, and suspend the sediment in 1 to 2 ml of sterile water, saline, or 0.2% BSA fraction V.

6. Inoculate the resuspended sediment onto egg medium, and make a smear.

Sulfuric acid method

Principle

The sulfuric acid method may be useful for urine and other body fluids that yield contaminated cultures when processed by one of the alkaline digestants.

Reagents

4% sulfuric acid
4% sodium hydroxide
Sterile distilled water
Phenol red indicator

Procedure

1. Centrifuge the entire specimen for 30 min at ≥3,000 × *g*. This may require several tubes.

2. Decant the supernatant fluids; pool the sediments if several tubes were used for a single specimen.
3. Add an equal volume of 4% sulfuric acid to the sediment.
4. Vortex, and let stand for 15 min at room temperature.
5. Fill the tube to the 50-ml mark with sterile water.
6. Centrifuge at ≥3,000 × *g* for 15 min, and decant the supernatant.
7. Add 1 drop of phenol red indicator, and neutralize with 4% NaOH until a persistent pale pink color forms.
8. Inoculate the media, and make a smear.

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Mycobacterium: Laboratory Characteristics of Slowly Growing Mycobacteria*

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37

IDENTIFICATION OF MYCOBACTERIA

Mycobacteria should always be identified to the species level if possible. They are usually preliminarily identified by traits such as growth rate and pigmentation which will direct the selection of key biochemical tests to further characterize them. Unfortunately, different species may present convergent biochemical profiles and morphological features (Table 1). Similarly, variation occurs among strains, and the strains' properties may not match those of the type strain. Traditional methods are well established, standardized, reproducible, and relatively inexpensive but limited in scope to the species of which large numbers of strains have been studied. Thus, identification errors may result, especially because no characteristic phenotype has been identified for several recently described species recognized on the basis of new 16S rRNA gene sequences.

Alternative laboratory methods for mycobacterial identification include analysis of mycolic acids by chromatography, genetic investigations using nucleic acid probes, and nucleic acid sequencing. It is now recommended to undertake mycobacterial identification with a strategy combining phenotypic and genotypic tests. For obvious reasons, laboratories should perform identification of the *Mycobacterium tuberculosis* complex (MTBC) by using a rapid method (103) to facilitate prompt identification and reporting of results to physicians.

Phenotypic Tests

Table 1 shows characteristic test results for the most commonly encountered species. Detailed descriptions of methods, procedures, and controls can be found elsewhere (40, 48, 115; see also chapter 36).

Growth Rate and Preferred Growth Temperature

Growth rate refers to the length of time required to form mature, isolated colonies visible without magnification on solid media. Mycobacteria forming colonies within 7 days are termed rapid growers, while those requiring longer periods are termed slow growers. Genome analyses support this separation, since slowly growing mycobacteria have been shown to have only one copy of the genes encoding rRNA whereas rapidly growing mycobacteria, except for *M. chelonae* and *M. abscessus*, have two sets of those genes (6). Moreover, comparative 16S rRNA gene sequencing data clearly separate the rapidly growing mycobacteria from the slowly growing species (95).

Isolated colonies are observed after media are inoculated with 0.1 ml of a 10^{-4} dilution of a standard culture suspension (SCS) prepared at an optical density at 580 nm of 0.25 by using a tube with a 2-cm diameter; this roughly corresponds to a suspension of 1 mg (wet weight) of bacilli per ml (115). The cultures are incubated at 35 to 37°C. Some species have special nutrient or temperature requirements for growth (see the previous chapter for details). Cultures are observed at 5 to 7 days and weekly thereafter for visible colonies. Growth in relation to temperature can usually be adequately determined by observing cultures at 37 and 30°C. When more definitive identification is needed, isolates should be incubated at 28, 30, 35 to 37, and 42 or 45°C.

Pigmentation and Photoreactivity

Mycobacteria are classified into three groups based on the production of pigments. Photochromogens produce nonpigmented colonies when grown in the dark and pigmented colonies only after exposure to light. Scotochromogens produce deep-yellow- to orange-pigmented colonies when grown in either light or darkness (some strains show increased pigment production upon continuous exposure to light). Nonchromogens are nonpigmented in both the light and the dark or have only a pale yellow, buff, or tan pigment that does not intensify after light exposure. These responses to light exposure were originally delineated to aid in the identification of nontuberculous mycobacteria (NTM). Members of the MTBC, however, are considered nonchromogens, and pigmented mycobacteria may be preliminarily reported as NTM.

Testing for pigment production should be done on isolated colonies from young cultures. Three tubes of media are inoculated with an SCS diluted to yield isolated colonies as described above. Two tubes are wrapped to be shielded from light, and the third is left uncovered. When growth is detected in the unshielded tube, one of the wrapped tubes should be examined. If colonies are not pigmented, the newly unshielded tube with its cap loosened is exposed to light (100-W tungsten bulb or fluorescent equivalent, placed

* This chapter contains information presented in chapter 37 by Véronique Vincent, Barbara A. Brown-Elliott, Kenneth C. Jost, Jr., and Richard J. Wallace, Jr., in the eigth edition of this Manual.

TABLE 1 Distinctive properties of cultivable, slowly growing mycobacterial species encountered in clinical specimens[a]

Descriptive term	Species	Optimal temp (°C)	Usual colony morphology[b]	Pigmentation[c]	Niacin	Growth on T2H (10 μg/ml)	Nitrate reduction	Semiquantitative catalase (mm of bubbles)	68°C catalase	Tween hydrolysis	Tellurite reduction	Tolerance to 5% NaCl	Arylsulfatase, 3 days	Urease	Pyrazinamidase, 4 days	16S rRNA gene ref. no.	Nucleic acid probes available
TB complex	*M. tuberculosis*	37	R	N (100)	+ (95)	+	+ (97)	<45 (89)	− (1)	± (68)	−/+ (36)	− (0)	− (0)	± (64)	+	X58890	+[d]
	M. bovis	37	Rt	N (100)	−	−	− (9)	<45 (69)	− (2)	− (21)	ND	− (0)	− (0)	± (50)	−	IDEM	+[d]
	M. bovis BCG	37	R	N (100)	−	−	−	<45	−	+/−	ND	−	−	+	−	IDEM	+[d]
	M. africanum	37	R	N (100)	V	V	V	<45	−	−	−	−	−	+	+	IDEM	+[d]
	M. canettii	37	Sm	N (100)	−	+	+	<45	−	−	ND	ND	−	ND	+	IDEM	+[d]
	M. microti	37	Sm	N (100)	+	−	V	<45	−	ND	ND	ND	ND	+	+	IDEM	+[d]
	M. caprae	37	Sm	N (100)	−	−	−	<45	−	+/−	ND	−	−	ND	ND	IDEM	+[d]
	M. pinnipedii	37	R	N (100)	V	ND	−	<45	−	−	ND	ND	ND	ND	ND	IDEM	+[d]
Nonchromogens	*M. avium*	35–37	Smt/R	N	−	+	−	<45	±	−	+	−	−	−	+	X52198	+[i]
	M. intracellulare	35–37	Smt/R	N	−	+	−	<45	±	−	+	−	−	−	+	X52927	+[i]
	M. haemophilum[e]	30	R	N	ND	+	−	<45	−	−	−	−	−	−	+	X88923	−
	M. malmoense	30	Sm	N (88)	− (0)	+	− (1)	<45 (99)	−/+	+ (99)	+ (74)	− (0)	− (0)	− (9)	+	X52930	−
	M. shimoidei	37	R	N	−	+	−	<45	−	+	ND	−	−	−	+	AJ005005	−
	M. genavense	37	Smt	N	−	+	−	>45	+	−	ND	ND	−	+	+	X60070	−
	M. celatum	35	Sm/Smt	N (100)	−	+	− (0)	<45 (100)	+ (100)	− (0)	+ (100)	− (0)	+ (100)	− (0)	+ (100)	L08170, L08169	−
	M. ulcerans	30	R	N	−	+	−	<45	+	−	ND	−	−	V	−	X58954	−
	M. terrae complex	35	Sm/R	N (93)	− (1)	+	± (67)	>45 (93)	+ (92)	+ (99)	−/+ (46)	− (2)	− (2)	− (13)	V	X52925 (*M. terrae*)	−
	M. triviale	37	R	N (100)	− (0)	+	+ (89)	>45 (100)	+ (100)	+ (100)	− (25)	+ (100)	± (56)	−/+ (33)	V	X88924	−
	M. gastri	35	Sm/SR/R	N (100)	− (0)	+	− (0)	<45 (100)	− (11)	+ (100)	± (50)	− (0)	− (0)	−/+ (44)	−	X52919	−
	M. branderi	35	Sm	N (100)	−	ND	−	−	−	−	ND	ND	−[f]	−	+	X82234	−
	M. heidelbergense	35	Sm	N (100)	−	ND	−	−	+	+	−	−	−	+	+	X70960	−
	M. triplex	35	Sm	N (100)	− (0)	+ (100)	+ (100)	>45 (100)	+ (100)	− (0)	ND	− (0)	− (0), + (50)[f]	+ (100)	+	U57632	−
	M. sherrisii	37	Sm	N	+/− (60)	ND	− (0)	−/+ (40)	+ (100)	− (100)	ND	ND	− (100)	+ (100)	−/+ (40)	AY353699	−
	M. lacus	37–42	SR	N	−	ND	+	<45	−	Weak	+	−	Weak	+	+	AF406783	−
Chromogens	*M. kansasii*	35	Sm/SR/R	P (96)	− (4)	+	+ (99)	>45 (93)	+ (91)	+ (99)	−/+ (31)	− (0)	− (0)	−/+ (49)	−	X15916	+
	M. marinum	30	Sm/SR/R	P (100)	−/+ (21)	+	− (0)	<45	− (30)	+ (97)	−/+ (39)	− (0)	−/+ (41)[f]	+ (83)	+	X52920	−
	M. avium	35–37	Sm/R	S	−	+	−	<45	±	−	+	−	−	−	+	X52198	+
	M. intracellulare	35–37	Sm/R	S	−	+	−	<45	±	−	+	−	−	−	+	X52927	+
	M. simiae	37	Sm	P (90)	± (63)	+	− (28)	>45 (93)	+ (95)	− (9)	+ (82)	− (0)	− (0)	+	+	X52931	−
	M. asiaticum	37	Sm	P (86)	− (0)	+	− (5)	>45 (95)	+ (95)	+ (95)	− (20)	− (0)	− (0)	− (10)	−	X55604	−
	M. xenopi	42	Sm	N/S[g]	−	+	−	<45	+/−	−	ND	− (0)	+	−	ND	X52929	−
	M. gordonae	37	Sm	S (99)	− (0)	+	− (1)	>45 (90)	+ (96)	+ (100)	− (29)	− (0)	V	V (31)	−/+	X52923	+

M. scrofulaceum	37	Sm	S (97)	− (0)	+	− (5)	>45 (84)	+ (94)	− (2)	± (64)	− (0)	V	+	±	X52924	−
M. szulgai	37	Sm or R	S/P (93)	− (0)	+	+ (100)	>45 (98)	+ (93)	−/+ (49)	± (53)	− (0)	−	+ (72)	+	X52926	−
M. flavescens	37	Sm	S (100)	− (0)	+	+ (92)	>45 (94)	+ (100)	+ (100)	−/+ (44)	± (62)	− (0)	+ (72)	+	X52932	−
M. parmense	37	Sm	S	−	+	−	<45	+	+	−	−	−	+	ND	AF466821	−
M. kubicae	37	Sm	S	ND	ND	−	>45	ND	−	ND	ND	− (20)	−	ND	AF133902	−
M. pulustre	37–42	Sm	S/P	ND	ND	V (54)	<45	ND	+ (100)	ND	ND	V (46)	+ (100)	+ (100)	AJ308603	−
M. intermedium	35	Sm	P	−	ND	−	ND	+	+	ND	ND	+[h]	+/− (V)	−	X67847	−
M. lentiflavum	35	Sm	S	−	ND	−	± (V)	± (V)	−	ND	ND	−	−	± (V)	AF317658	−
M. interjectum	35	Sm	S	−	ND	−	V	+	−	ND	ND	V	+	+	X70961	−
M. bohemicum	37–40	Sm	S	−	ND	−	<45	+	−	ND	−	−	Weak	−	U84502	−
M. conspicuum	30	Sm	S		+	−	<45	− (100)	+	−	−	+[h]	−	−	X88922	−
M. tusciae	30	R	S	−	+	+	−		+ (10 days)	ND	−	−[f]	+	ND	AF058299	−
M. heckeshornense	35	Sm	S	−	ND	−	−	+	−	ND	−	−	−	−	AF174290	−

[a] Modified from references 27, 29, 40, 48, 87, 105, 106, 109, 120, and 121. Plus and minus signs indicate the presence and absence, respectively, of the feature: V, variable; ±, usually present; −/+, usually absent; ND, not determined; ref. no., reference number. The percentage of strains positive in each test is given in parentheses, and the test result is based on these percentages.

[b] R, rough; Sm, smooth; SR, intermediate in roughness; Smt, smooth and transparent; Rt, rough and thin or transparent.

[c] P, photochromogenic; S, scotochromogenic; N, nonchromogenic (*M. szulgai* is scotochromogenic at 37°C and photochromogenic at 24°C; *M. palustre* is scotochromogenic at 37°C, and 85% of isolates are photochromogenic at 42°C).

[d] Probe identifies the MTBC.

[e] Requires hemin as growth factor.

[f] Arylsulfatase reaction at 14 days is positive.

[g] Young cultures may be nonchromogenic or possess only pale pigment that may intensify with age.

[h] Results for 3-day arylsulfatase reaction not available; 10-day arylsulfatase reaction positive.

[i] A MAC nucleic acid probe that recognizes *M. avium*, *M. intracellulare*, and the "X" strains is commercially available.

20 cm from the culture) for 1 to 5 h. Maximal oxygenation of the culture (loose cap, isolated colonies) is necessary for induction of the pigment, which is controlled by an oxygen-dependent, photoinducible enzyme. The tube is then reshielded and reincubated, and the colonies in the light-exposed tube are compared with those in the shielded tube after 24 h. Variations within species occur.

Colony Morphology

Colony morphology of mycobacteria can be evaluated, according to the scheme developed by Runyon (84a), by microscopically observing young (5- to 14-day-old), isolated colonies on plates inverted under the 10× power objective of a stereomicroscope with transmitted light. The best medium is a clear solid one like Middlebrook 7H10 or 7H11 agar. The large numbers of NTM species have made it increasingly difficult to provide a tentative identification of an NTM species by this method, and so the information gained by this technique is often used to direct the diagnostic procedure to other, more specific tests. The morphology of *M. tuberculosis* usually allows for a tentative identification of this species. Examination of the morphologies of colonies is also important for the detection of mixed cultures. Figure 1 shows the colony types of some frequently isolated species.

Arylsulfatase

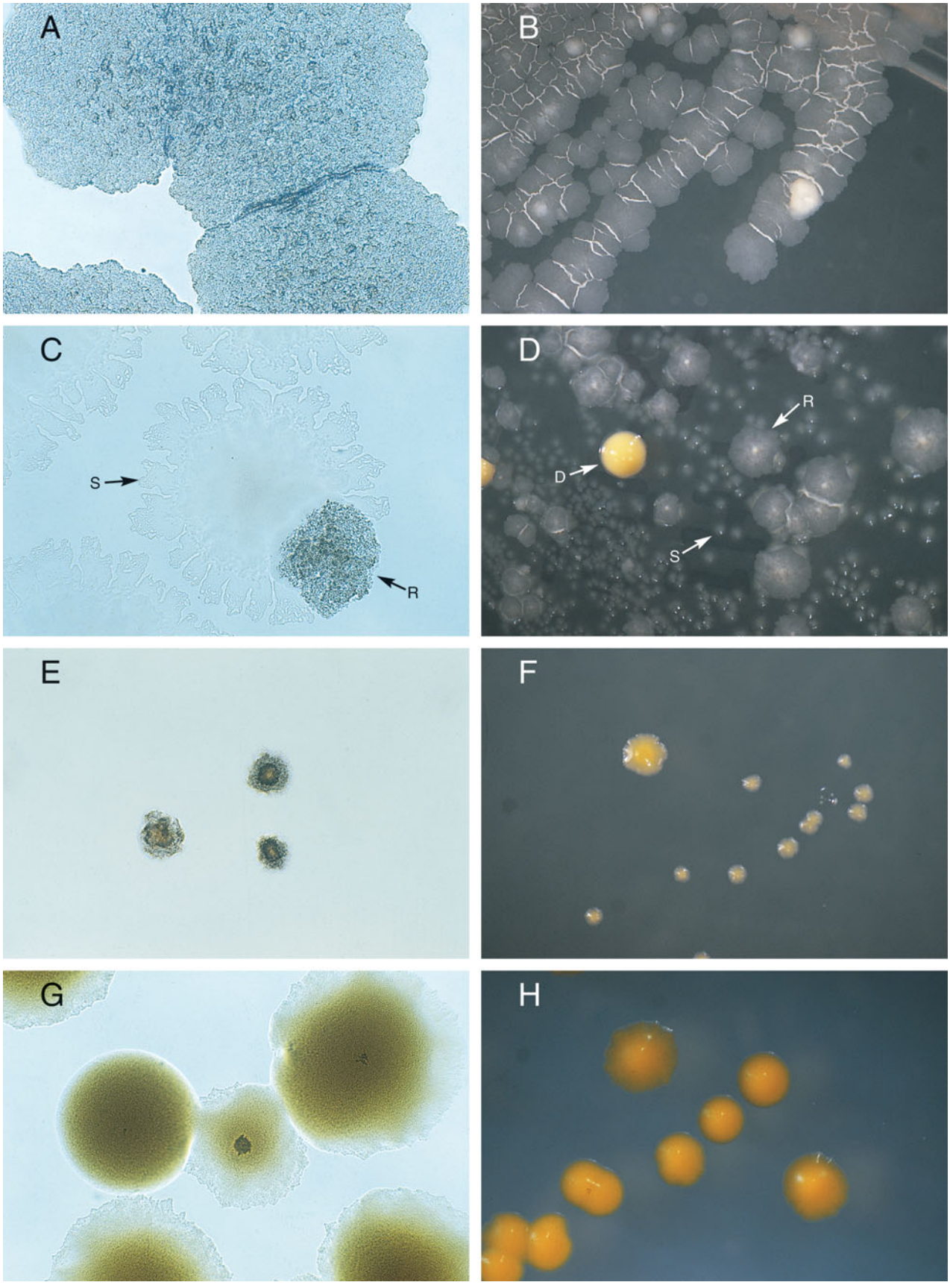
Arylsulfatase hydrolyzes the bond between the sulfate group and the aromatic ring of tripotassium phenolphthalein disulfate to form free phenolphthalein, which is easily detected by a red appearance when alkali is added. Arylsulfatase activity can be detected in all mycobacteria after prolonged incubation. Performing the test after only 3 days helps in identifying several slowly growing mycobacteria such as *M. xenopi* and *M. celatum*.

Cultures in Dubos liquid medium (2 ml) containing 0.08 M phenolphthalein disulfate (tripotassium salt) are tested after 3 days of incubation by adding 0.3 ml of 1 M Na_2CO_3. The development of pink indicates a positive reaction. *M. xenopi* and *M. avium* can be used as positive and negative controls, respectively.

Catalase

Catalase is an intracellular, soluble enzyme capable of degrading hydrogen peroxide into water and oxygen. Two tests are used to detect catalase activity, a semiquantitative test that reflects differences in enzyme kinetics and a heat tolerance test. The enzyme is detected by adding H_2O_2 to a culture and observing for the formation of bubbles in the reaction mixture.

The semiquantitative test divides the mycobacteria into two groups, those producing low catalase activity and those producing high catalase activity, based on the sizes of the columns of bubbles produced (less or more than 45 mm). A butt (not a slant) of a Löwenstein-Jensen (L-J) medium tube (16 by 150 mm) is inoculated with 0.2 ml of an SCS prepared as described above for growth rate determination. Tubes are incubated for 2 weeks at 35°C with the caps loosened. Then 1 ml of a reagent consisting of a 1:1 mixture of 10% Tween 80 and 30% H_2O_2 is added. Be sure to loosen the caps and place the tubes on an adsorbent surface in case bubbles overflow. The column of bubbles yielded is measured (in millimeters) after the tube has stood upright for 5 min at room temperature. *M. tuberculosis* H37Ra and *M. kansasii* can be used as controls for low and high catalase activity, respectively.



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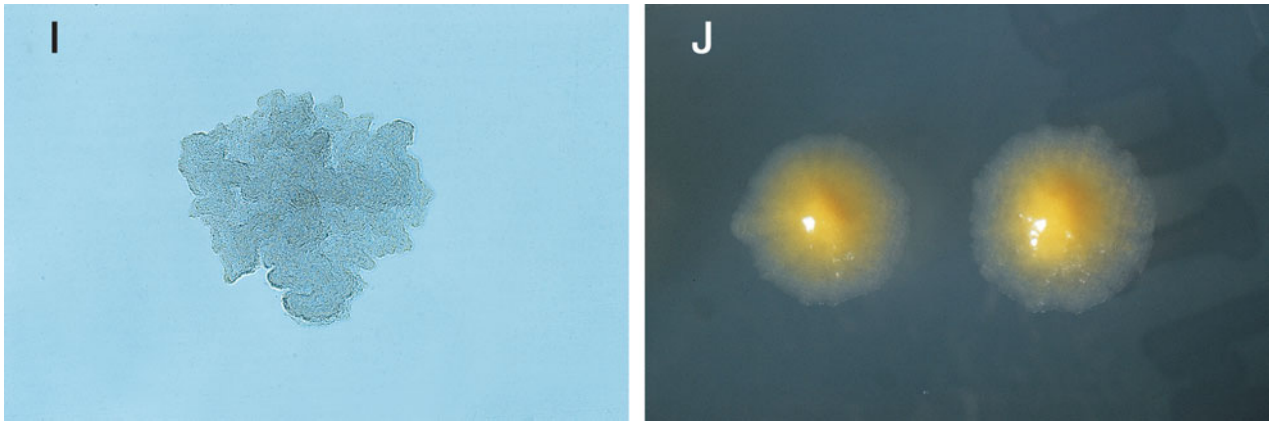


FIGURE 1 (A) *M. tuberculosis* growth after 15 days. Thin, nonpigmented, rough colonies are seen on 7H11 agar; cording is apparent. (B) *M. tuberculosis* growth after 10 days. Dry, buff, wrinkled colonies are visible on 7H11 agar. (C) *M. avium* growth after 10 days. A flat, nonpigmented, smooth (S) colony with an irregular edge and a compact, nonpigmented, rough (R) colony are seen on 7H11 agar. (D) *M. avium* growth after 10 days. Nonpigmented colony variants are visible on 7H11 agar; smooth-flat (S), dome (D), and rough (R) variants are indicated. (E) *M. xenopi* growth after 15 days. Nonpigmented, compact, rough colonies with irregular peripheries are seen on 7H11 agar; the colonies resemble a bird's nest. (F) *M. xenopi* growth after 15 days. Smooth, dome-shaped, and slightly yellow colonies are visible on 7H11 agar. (G) *M. gordonae* growth after 10 days. Orange, smooth, opaque entire colonies and orange, smooth, opaque colonies with irregular edge tints are seen on 7H11 agar. (H) *M. gordonae* growth after 10 days. Smooth, orange, hemispheric colonies are seen on 7H11 agar. (I) *M. kansasii* growth after 10 days in the dark. Nonpigmented, rough colonies are seen on 7H11 agar; stranding of bacilli is seen (similar to cording). (J) *M. kansasii* growth after 15 days. A smooth colony with the center elevated and thickened and with a thin, rough periphery is seen on 7H11 agar; it is orange after light exposure. (Photographs courtesy of Daniel Fedorko and Yvonne Shea, Department of Laboratory Medicine, Microbiology Service, National Institutes of Health, 2002.)

Niacin Accumulation Test

Niacin (nicotinic acid) functions as a precursor in the biosynthesis of coenzymes NAD and NADP. Although all mycobacteria produce nicotinic acid, some have a block in the NAD scavenging pathway and excrete niacin. The niacin accumulated in the culture medium is then detected by its reaction with a cyanogen halide in the presence of a primary amine. Niacin-negative *M. tuberculosis* isolates are extremely rare. A positive niacin test result should not be used alone to identify *M. tuberculosis*, however, because some strains of *M. simiae* and other mycobacteria, although infrequently encountered, also accumulate niacin. Performance of the supportive tests for nitrate reduction and 68°C catalase are necessary for confirming the identification of *M. tuberculosis*.

A niacin paper strip version is available commercially (BD, Sparks, Md., and Remel Inc., Lenexa, Kans.). A heavily grown L-J culture medium is covered with 1 ml of distilled water, and the tube is placed horizontally to allow extraction for 20 min. Then 0.5 ml of the liquid is transferred to a tube. The strip is inserted, and the tube is sealed immediately. After 15 min at room temperature, yellow coloration of the liquid (not the strip) indicates a positive test result. *M. tuberculosis* H37Ra and *M. avium* can be used as positive and negative controls, respectively.

Nitrate Reduction

Mycobacteria differ quantitatively in their abilities to reduce nitrate to nitrite. The nitrate reduction test is performed by adding 2 ml of $NaNO_3$ substrate to a bacterial heavy suspension prepared with two loopfuls of bacteria emulsified in 0.2 ml of distilled water. The tube is shaken manually and incubated upright for 2 h in a 37°C water bath. After the tube is removed from the bath, 1 drop of reagent 1 (50 ml of HCl in 50 ml of H_2O), 2 drops of reagent 2 (0.2 g of sulfanilamide in 100 ml of H_2O), and 2 drops of reagent 3 (0.1 g of *N*-napthylthylenediamine dihydrochloride in 100 ml of H_2O) are added to the SCS. Immediate development of a pink tone is considered to indicate positivity. *M. tuberculosis* H37Ra and *M. avium* can be used as positive and negative controls, respectively.

Pyrazinamidase

The enzyme pyrazinamidase hydrolyzes pyrazinamide (PZA) into ammonia and pyrazinoic acid, which can be detected by the addition of ferric ammonium sulfate. This test is most useful in separating *M. marinum* from *M. kansasii* and *M. bovis* from *M. tuberculosis*. In addition, one mechanism of PZA resistance of *M. tuberculosis* appears to be the inability of the organism to produce pyrazinoic acid, which is assumed to be the active component of the drug PZA. A pyrazinamidase-negative *M. tuberculosis* isolate is assumed to be PZA resistant as well.

The test medium consists of Dubos broth base containing 0.1 g of PZA, 2.0 g of pyruvic acid, and 15.0 g of agar per liter. The medium is dispensed in 5-ml amounts, autoclaved, and solidified in a tube in an upright position. The agar medium is heavily inoculated with growth from the culture so that the inoculum should be visible. After incubation at 37°C for 4 days, 1 ml of 1% ferrous ammonium sulfate is added. The preparation is observed for up to 4 h for a pink band in the agar, which indicates a positive test result. *M. avium* and uninoculated medium are used as positive and negative controls, respectively.

Sodium Chloride Tolerance

M. triviale is the only slowly growing mycobacterium able to grow in the presence of, or tolerate, 5% sodium chloride.

L-J medium containing 5% NaCl or lacking salt is inoculated with 0.2 ml of an SCS and incubated at 30 or 35°C. Growth or no growth is scored at 4 weeks. *M. triviale* and *M. tuberculosis* H37Ra can be used as positive and negative controls, respectively.

Inhibition by Thiophene-2-Carboxylic Acid Hydrazide

Inhibition by thiophene-2-carboxylic acid hydrazide (T2H) is used to distinguish niacin-positive *M. bovis* from *M. tuberculosis* and other nonchromogenic slowly growing mycobacteria. Most *M. bovis* isolates are susceptible to T2H, whereas *M. tuberculosis* and most other slowly growing mycobacteria are resistant.

Middlebrook 7H11 medium containing 10 μg of T2H (Aldrich Chemical Co., Milwaukee, Wis.) per ml is dispensed in 5-ml amounts onto slants. Tubes with and without T2H are inoculated with 0.2 ml of the 10^{-2} and 10^{-4} SCS dilutions. When growth is visible on the control tubes, the colonies are counted. The organism is recorded as resistant if growth on the T2H medium is >1% of the growth on the control. *M. tuberculosis* H37Ra and *M. bovis* can be used as positive and negative controls, respectively.

Tellurite Reduction

Tellurite reductase reduces colorless potassium tellurite into a black metallic tellurium precipitate. The tellurite reduction test is used to separate *M. avium* and *M. intracellulare* from most other nonchromogens. Some rapid growers can similarly produce a positive tellurite test result.

Two drops of a 0.2% aqueous solution of potassium tellurite are added to 5-ml cultures (7 days old) in Middlebrook 7H9. Cultures are incubated and examined daily for 4 days or more. A positive test result is shown by a jet black precipitate. *M. avium* and *M. kansasii* can be used as positive and negative controls, respectively.

Tween 80 Hydrolysis

Lipases produced by some mycobacterial species hydrolyze the detergent polyoxyethylene sorbitan monooleate (Tween 80) into oleic acid and polyoxyethylene sorbitol. Neutral red in the pH 7 test medium is bound by Tween 80 and has an amber color at a neutral pH. If Tween 80 is hydrolyzed, however, neutral red is no longer bound and reverts to its usual red color at pH 7. The Tween 80 hydrolysis test allows for differentiation among the slowly growing NTM species.

The substrate solution consists of 0.5 ml of Tween 80 in 100 ml of 0.067 M phosphate buffer (pH 7.0) to which 2 ml of a 1% aqueous solution of neutral red is added. The solution is dispensed in screw-cap tubes in 4-ml amounts and autoclaved. A loopful of bacteria is suspended in a tube and incubated at 37°C. A change in color from amber to pink or red is recorded after 24 h and 5 and 10 days of incubation as a positive reaction. *M. kansasii* and *M. avium* can be used as positive and negative controls, respectively.

Urease

The ability of an isolate to hydrolyze urea into ammonia and CO_2 is useful in identifying both scotochromogens and nonchromogens. *M. scrofulaceum* is urease positive, whereas *M. avium* and *M. intracellulare* organisms are urease negative. The urease test is particularly helpful in the recognition of pigmented strains of *M. avium*.

The test medium is prepared by mixing 1 part of urea agar base concentrate with 9 parts of distilled water and is dispensed in 4-ml amounts into tubes. A loopful of bacteria is emulsified in a test tube and incubated at 37°C for 3 days. A positive reaction is indicated by a pink to red color. *M. scrofulaceum* and *M. gordonae* can be used as positive and negative controls, respectively.

Mycolic Acid Analysis

Mycolic acid analysis has been recommended as one of several minimal criteria for the description of new mycobacterial species (115). Mycolic acids are high-molecular-weight (20 to 90 carbon atoms) alpha-substituted, beta-hydroxy fatty acids found in the cell walls of members of several other bacterial genera: *Corynebacterium, Rhodococcus, Gordonia, Dietzia, Nocardia*, and *Tsukamurella* (see also chapter 35).

High-pressure liquid chromatography (HPLC) of mycolic acid esters has been demonstrated to be a rapid and reliable method for identification of many *Mycobacterium* species. A standardized method that includes sample preparation and chromatographic analysis has been described previously (14). One to two loopfuls of cells grown on solid medium are suspended in a methanolic potassium hydroxide solution and saponified by heating. After acidification and extraction with chloroform, free mycolic acids are derivatized to para-bromophenacyl esters. Internal-standard molecular weight markers are added, and the sample is injected. The mycolic acid esters are separated on a reversed-phase C_{18} column by a methanol-methylene chloride gradient elution and detected by UV spectrophotometry (UV-HPLC). An extract prepared from *M. intracelluare* ATCC 13950 is used as a positive control and provides an external standard peak naming reference. The high biomass requirement of UV-HPLC can be reduced at least 200-fold by derivatizing mycolic acids to 6,7-dimethoxy-4-coumarinyl-methyl esters, which are measured by fluorescence detection HPLC (FL-HPLC). The increased analytical sensitivity of FL-HPLC allows the identification of acid-fast bacilli from smear-positive clinical specimens, liquid medium cultures, and minute amounts of biomass from solid medium (42). MTBC identification by FL-HPLC has been reported to achieve a sensitivity of 99% with BACTEC 12B medium with a growth index of ≥50 (42).

HPLC patterns can be identified to the species or group level by visual or mathematical means. A pattern atlas derived from a multicenter study of more than 350 strains, representing 23 species, that illustrates species patterns is available on the Centers for Disease Control and Prevention website at http://www.cdc.gov/ncidod/dastlr/TB/TB_HPLC.htm. Additionally, pattern overlays of closely related species have been presented along with pattern variations produced by strains of a single species. However, *M. tuberculosis* and *M. bovis* produce indistinguishable patterns. The standardized method (14) recommends a visual comparison of a sample HPLC pattern to an atlas of reference strain patterns in combination with the use of peak height ratios. This approach was reported to achieve an accuracy of 96.1% (104). Libraries that report both an identification and the quality of a match are commercially available (Pirouette and INSTEP software [Infometrix Inc., Woodinville, Wash.] and the Sherlock mycobacterium identification system [MIDI, Inc., Newark, Del.]).

Although HPLC initial equipment costs are high (approximately $50,000) and considerable expertise is required to operate nonautomated systems, material costs per test are economical compared with those for commercial molecular probes. Sample preparation is simple, and many mycobacterial

species or groups can be identified in a single analysis. However, mycolic acid analysis does not provide the specificity or the sensitivity of molecular approaches as described below.

Mycobacterial Genomes

The complete genome sequence of *M. tuberculosis* H37Rv comprises 4,411,529 bp and contains approximately 4,000 genes (20). *M. tuberculosis* has an extremely clonal population structure, with genomic variation caused largely by insertion sequence movement rather than point mutation (10, 46, 94). A recent study showed that, similar to *Yersinia pestis* (2) and *Salmonella enterica* serotype Typhi (49), the MTBC consists of a successful clonal population that recently emerged from a much more ancient and larger bacterial species group encompassing *M. canettii* and additional genetic groups of smooth strains (36).

Additional genome sequences are available or are being determined for several mycobacteria. More information can be found at the websites http://genolist.pasteur.fr/TubercuList/index.html for *M. tuberculosis* H37Rv, http://www.tigr.org/tigr-scripts/CMR2/GenomePage3.spl?database = gmt for *M. tuberculosis* CDC1551, http://www.sanger.ac.uk/Projects/M_bovis for *M. bovis*, and http://genolist.pasteur.fr/Leproma/index.html for *M. leprae.*

Genotypic Identification of Mycobacterial Strains

PCR-REA

In 1993, Telenti and colleagues proposed a method for rapid identification of mycobacteria to the species level based on PCR amplification of a 439-bp fragment of the gene encoding the 65-kDa heat shock protein *(hsp65)* followed by restriction enzyme digestion using BstEII and HaeIII (102). The method has been extensively used for the identification of slowly growing mycobacteria (77). *M. tuberculosis* is easily differentiated from the NTM by a characteristic band with HaeIII restriction endonuclease digestion. However, members of the MTBC are not discriminated by PCR-restriction enzyme analysis (PCR-REA). By contrast, most NTM can be recognized by their PCR-REA patterns. Several alleles have been identified in *M. gordonae* and *M. kansasii* yielding several distinct PCR-REA patterns for a single species (77). Some other gene sequences (including those of *rpoB, dnaJ*, and the 16S-23S rRNA gene spacer) have been tentatively used for PCR-REA, but none have been studied as extensively as that of *hsp65*.

The advantages of PCR-REA are that equipment is not very expensive and the method is relatively rapid and identifies most mycobacterial species, including some not identified by phenotypic methods and/or HPLC. The disadvantages are that it is a relatively complex procedure and, due to the increase of newly described species, it may not allow unambiguous species identification but may assign to groups of mycobacterial species. A multicenter evaluation of the method showed that differences in gel running conditions and lack of training in interpretation of patterns contributed to low accuracy (58). Furthermore, it is not commercialized or Food and Drug Administration (FDA) approved and it requires a significant amount of in-house validation.

Commercially Available Identification Probes

AccuProbe

Acridinium ester-labeled DNA probes based on the detection of rRNA (Gen-Probe Inc., San Diego, Calif.) specific for the MTBC, the *M. avium* complex (MAC), *M. kansasii*, and *M. gordonae* (as well as separate probes for *M. avium* and *M. intracellulare*) are FDA approved and commercially available. The current total test time for the AccuProbe assay is less than 2 h (17, 59). Briefly, target 16S rRNA is released from the organism by sonication. The labeled DNA probe combines with the organism's rRNA to form a DNA-rRNA hybrid. The labeled product is detected in a luminometer.

Tests with DNA probes can be performed using isolates from solid media or from broth cultures. Combining the probes with a broth culture system has the advantage of optimizing rapid detection and identification of mycobacteria present in clinical samples (17, 43). Procedural modifications are necessary for testing isolates recovered from liquid medium. An aliquot of the broth culture is concentrated by centrifugation, and the pellet is resuspended in culture identification reagent. Testing then follows the procedure for testing colonies from solid media (as described in the AccuProbe technical insert). To eliminate high nonspecific chemiluminescence, BACTEC (BD) 13A broth medium containing blood is pretreated with 100 μl of 10% sodium dodecyl sulfate–50 mM EDTA (pH 7.2) before microcentrifugation to allow lysis of the erythrocytes and sorbitolization of the membranes. Appropriate positive and negative control organisms should be included in each assay run.

It has been shown that specificity is 100% for testing of mycobacterial colonies. Sensitivity, however, varies with the species or species complexes: 95.2 to 97.2% for the MAC, 100% for the MTBC, 100% for *M. gordonae*, and 97.4 to 100% for *M. kansasii* (33, 59, 80, 108). Later studies using AccuProbe on more than 11,000 positive BACTEC cultures also showed 100% specificity and >85 to 100% sensitivity for all species tested (17, 79).

Advantages of this test include the simplicity and rapidity (within 2 h) with which mycobacteria can be identified. The use of a nonradioactive procedure and the extended shelf life of the chemiluminescent probes offer the potential for widespread application in most clinical laboratory settings (33, 59).

A few limitations of using AccuProbe have been described. These include misidentification of *M. celatum* as MTBC due to the similarity of the 16S rRNA genes of these two species in the probe region (92). The specificity of the MTBC probe has been increased by extending the length of the selection reagent incubation step to 10 min, and a temperature of 60 to 61°C is recommended to eliminate cross-reactivity with other species, including *M. terrae* and *M. celatum* (92). Greater biomass in the test suspensions may also result in decreased specificity of the test (92). It has been shown that the use of a higher cutoff (at 80,000 relative light units instead of 30,000 relative light units) may prevent false-positive results when the MAC probe is tested with broth cultures (19). As with all laboratory tests, the user is reminded that the probe should be repeated or the results should be confirmed by an alternate method if results do not correlate with clinical or cultural observations. The MTBC probe does not differentiate among members of the MTBC.

Strip Tests

Two line probe assays have been developed, one targeting the 16S-23S rRNA internal transcribed spacer region (INNO-LiPA Mycobacteria version 2; Innogenetics, Ghent, Belgium) and the other one targeting the 23S rRNA gene (GenoType Mycobacteria; Hain Lifescience, Nehren, Germany).

Strip assays are based on the reverse hybridization of biotinylated PCR products to their complementary probes immobilized as parallel lines on a membrane strip. Kits may be applied to strains subcultured on solid or in liquid media. Lines on the strips include probes for the identification of the

Mycobacterium genus and probes for the identification of various frequently encountered or clinically relevant mycobacterial species, 16 species in the INNO-LiPA Mycobacteria version 2 and 13 species in the GenoType Mycobacteria. The overall concordance with other identification methods (AccuProbe and PCR-REA of *hsp65* or 16S sequences) has been good when tested with reference strains and mycobacterial cultures from clinical specimens (60, 81, 86, 107).

The main advantage of the kits is that a range of several species can be identified by a single PCR assay, and unlike the AccuProbe, they do not require a tentative selection of the adequate probe. The kits are commercially available in Europe at the present time and available only for research in the United States. Tests are performed in 6 h, including the preliminary PCR amplification. They require several time-consuming washes. An automated machine, Auto-LiPA (Innogenetics), which runs the washes and ensures the gentle shaking necessary for several steps of the procedure, greatly contributes to time saving and ease of application in clinical laboratories. However, the cost of this apparatus may hamper its introduction into many laboratories.

DNA Sequencing

The availability of DNA sequencing technologies constitutes a great benefit for mycobacterial identification, owing to the peculiar slow growth of mycobacterial organisms. Recent improvements in automation of target amplification and sequence analysis have led to practical implementation of DNA sequencing in the clinical laboratory. Moreover, the cost has decreased dramatically (106). Sequencing of the entire 16S rRNA gene (*rrs* gene, approximately 1,500 bp) or the *hsp65* gene (approximately 4,400 bp) cannot be done in a routine clinical laboratory. However, identification of species-specific signatures within variable regions of these highly conserved genes allows the design of simple PCR protocols followed by the direct sequencing of the PCR-amplified products. Catalogues of sequences of mycobacterial species may be retrieved from databases (GenBank/EMBL website at http://www.ncbi.nlm.nih.gov) and conveniently imported and stored in a file in the laboratory. Liquid cultures or colonies from solid medium may be used for DNA extraction by boiling the sample without any further purification. The polymorphism of several conserved genes has been investigated for identifying mycobacterial species, such as the gene encoding the 32-kDa protein (91), the *dnaJ* gene (101), the *sod* gene encoding the superoxide dismutase (126), the *gyrB* gene coding for the gyrase subunit B (45), the *rpoB* gene coding for the RNA polymerase (51), the internal transcribed spacer 16S-23S sequence (67, 84), and the *secA1* gene coding for a key component of the major pathway of protein secretion across the cytoplasmic membrane (124). The most widely used targets are 16S rRNA (*rrs* gene) and the *hsp65* gene.

The strategy for sequencing and identification of the signature sequences of the 16S rRNA gene has been extensively described (52). Specific primers for mycobacteria (Table 2) have been designed for the amplification of the 16S rRNA gene to avoid contamination and enhance specificity. Amplification of a 1,030-bp region encompassing the 16S rRNA gene sequence is performed with primers 285 and 264. The sequencing reaction is performed with primer 244 by using sequencing kits for automated DNA sequencers to characterize the hypervariable region A, located on the 5′ side of the *rrs* gene, corresponding to *Escherichia coli* 16S rRNA gene positions 129 to 267. 16S rRNA genes reflect a limited conserved region of the entire genome, and the molecular clock of the marker is rather slow. Species of recent divergence thus may contain 16S rRNA gene sequences that are highly similar. Moreover, no single 16S rRNA gene interstrain nucleotide sequence difference value that unequivocally defines species boundaries has been established for the genus *Mycobacterium* (119). For instance, *M. szulgai* and *M. malmoense* present a 2-nucleotide difference only in the 1,384-nucleotide segment examined whereas some *M. intracellulare* serotypes reveal microheterogeneity with 1 to 7 different nucleotides in a 782-nucleotide segment (119). For routine identification, strains should be identified according to a 16S rRNA hypervariable region A matching the type strain sequence. Additional investigation using another molecular marker (e.g., *hsp65*) may be required for accurate identification since hypervariable region A cannot be used to discriminate between some species encountered in clinical specimens, i.e., *M. kansasii* and *M. gastri, M. ulcerans* and *M. marinum*, and *M. shimoidei* and *M. triviale* (52). In addition, no polymorphisms are present among the different species in the MTBC.

TABLE 2 Oligonucleotides used for mycobacterial identification by DNA sequencing

Target	Primer[a]	Sequence	Position[b]
rrs	285	5′ GAGAGTTTGATCCTGGCTCAG 3′	9–29
rrs	264	5′ TGCACACAGGCCACAAGGGA 3′	1046–1027
rrs	244	5′ CCCACTGCTGCCTCCCGTAG 3′	361–342
hsp65	Tb11	5′ ACCAACGATGGTGTGTCCAT 3′	396–415
hsp65	Tb12	5′ CTTGTCGAACCGCATACCCT 3′	836–817

[a] Primers are described in references 44 and 52.

[b] "Position" refers to the *E. coli* 16S rRNA gene numbering for the *rrs* gene and to the *M. tuberculosis* numbering for the *hsp65* gene.

Partial sequencing of the *hsp65* gene is performed using primers Tb11 and Tb12 (44) for the amplification of a 441-bp region (nucleotides 396 to 836 according to the numbering of the *M. tuberculosis hsp65* gene) (88). As indicated above for the PCR-REA method, several *hsp65* alleles may be identified within a species. The polymorphism in the *hsp65* gene allows unambiguous identification of species with close 16S rRNA gene sequences such as *M. gastri* and *M. kansasii*. As with the 16S rRNA gene, all MTBC members have the same *hsp65* allele except *M. africanum* and *M. canettii*, which show a single nucleotide polymorphism within the gene (30, 36).

Genotypic Markers for Species Identification within the MTBC

Although *M. tuberculosis* is the most prevalent tubercle bacillus isolated in clinical laboratories, identification of other members of the MTBC is of the utmost epidemiological importance and may govern the management of contact-tracing investigations and/or treatment. Incomplete identification of tubercle bacilli can lead to misdiagnosis of *M. bovis* BCG infections in patients treated for bladder cancer or misdiagnosis of nosocomial BCG infections in cancer patients (97, 116, 117). Moreover, the identification of *M. bovis* allows public health investigation and tracing of epidemics possibly due to cattle-to-human transmission (37). A retrospective study (1980 to 1997) of active pediatric tuberculosis in a United States-Mexico cross-border region showed that *M. bovis* accounted for 10.8% of all tuberculosis cases and for 33.9% of cases with positive cultures (23). The main risk factor was exposure to a zoonotic source, namely, ingestion of unpasteurized dairy products (7, 23). Similarly, rare agents of tuberculosis (*M. microti, M. canettii, M. caprae,* and *M. pinnipedii)* have to be properly identified for documentation of their epidemiology at the national level (37).

The MTBC members cannot be differentiated by the AccuProbe test. Similarly, the *hsp65* gene and the PCR-REA patterns are identical for *M. tuberculosis* and *M. bovis*. However, strains may be differentiated according to conventional identification tests (Table 1) as well as host range and virulence. The sequence polymorphism of the *oxyR* and *pncA* genes led to the development of allele-specific PCR tests which allow differentiation of *M. bovis* from all other MTBC members (25). The distribution of deletions (RD sequences) among the tubercle bacilli contributes to the identification of the different members of the MTBC: this approach is based on PCR tests only (Table 3). Since many laboratories use amplification procedures, it has been proposed that this PCR-based approach be incorporated into the laboratory routine by clinical mycobacteriology laboratories (68). A commercialized strip assay relying on *gyrB* probes has been recently developed for the differentiation of the MTBC members (Hain Lifescience). An evaluation study showed that the species were unequivocally identified but *M. tuberculosis* and *M. canettii* shared a common pattern (81). Moreover, as mentioned below, spoligotyping confirms the specific identification.

Molecular tests should be carried out on *M. tuberculosis* strains with phenotypes which do not fully match that of the type strain, e.g., strains which hybridize with the MTBC probe and do not yield rough, cream, cauliflower-like colonies or do not produce niacin or nitrate reductase. Monoresistance to PZA has been shown to be of poor predictive value as an initial screening tool for *M. bovis* (24, 38). If molecular analysis is not available in the laboratory, strains should be sent to a reference laboratory.

Direct NAA Tests

Three nucleic acid amplification (NAA) kits designed to detect MTBC bacilli directly from patient specimens are commercially available. The amplified *M. tuberculosis* direct test (AMTD; Gen-Probe) consists of transcription-mediated amplification of a specific 16S rRNA target performed at constant temperature for the detection of MTBC rRNA in smear-positive and -negative respiratory samples. The Amplicor *M. tuberculosis* PCR assay (Roche Molecular Systems, Branchburg, N.J.) consists of PCR amplication of a 584-bp region of the 16S rRNA gene sequence. Incorporation of dUTP instead of dTTP prevents carrier contamination in the amplification reaction, and the use of uracil-*N*-glycosylase enzymatically cleaves any contaminating amplicons from previous reactions.

Both of these tests are FDA approved for respiratory samples, the AMTD for both smear-positive and smear-negative specimens and the Amplicor for smear-positive specimens only. Moreover, investigators have shown that, with specific modifications, the tests may also detect *M. tuberculosis* in nonrespiratory specimens (76). The overall AMTD specificity ranged from 92.1 to 100%. For respiratory specimens, the sensitivity was between 91.7 and 100% for smear-positive specimens and decreased to between 65.5 and 92.9% for smear-negative specimens. For nonrespiratory specimens, the sensitivity was between 88 and 100% for smear-positive specimens and between 63.6 and 100% for smear-negative specimens (76). Regarding the Amplicor assay, specificity was similar to the AMTD value for smear-positive specimens and ranged from 90 to 100% for respiratory specimens and from 87.5 to 100% for extrapulmonary specimens. However, sensitivity was lower for smear-negative samples, from 50 to 95.9% for respiratory specimens and from 17.2 to 70.8% for extrapulmonary specimens. Inhibition has been detected in less than 1 to 5% of clinical specimens with the AMTD and up to 20% with the Amplicor assay. The Amplicor assay showed a significantly higher inhibition rate for nonrespiratory specimens and false-positive results were related to cross-reactions with mycobacteria other than tubercle bacilli (76).

The BDProbeTec strand displacement amplification (BD) is an isothermal enzymatic process that coamplifies sequences of IS*6110* (specific to the MTBC) and the 16S rRNA gene (common to most mycobacterial species). The process is based on the nicking of the recognition sequence in double-stranded DNA by a restriction endonuclease and further extension of that site with DNA polymerase that synthesizes a new strand of DNA while displacing the existing one. The sensitivity of the test has been shown to be between 98.5 and 100% for smear-positive specimens and between 33.3 and 85.7% for smear-negative and extrapulmonary specimens, with inhibition rates between 0.3 and 14% (76).

Since tuberculosis of the central nervous system is one of the most malignant forms of human tuberculosis, the rapid and accurate laboratory diagnosis of tuberculous meningitis is of prime importance. Conventional bacteriology methods are usually inefficient (negative microscopy and late positive culture if any). Recently, a systematic review and meta-analysis documented the diagnostic accuracy of NAA tests for tuberculous meningitis. The summary estimates were as follows: sensitivity, 0.56 (95% confidence interval [CI], 0.46 to 0.66); specificity, 0.98 (95% CI, 0.97 to 0.99); positive likelihood ratio, 35.1 (95% CI, 19.0 to 64.6); and negative likelihood ratio, 0.44 (95% CI, 0.33 to 0.60). Consequently, commercial NAA tests show a potential role in confirming tuberculous meningitis diagnosis, although their overall low sensitivity precludes the use of these tests to rule out tuberculous meningitis with certainty (66).

NAA tests can be performed in as few as 6 to 8 h on processed specimens and, therefore, offer the promise of same-day reporting of results for detection and identification of *M. tuberculosis*. Used as approved by the FDA, NAA tests for *M. tuberculosis* detection do not replace any previously recommended tests and supplement rather than replace culture (16). A recent multicenter study stressed the need for external quality control (64).

TABLE 3 Genotypic characteristics of the MTBC members[a]

Strain	*oxyR*/ 285	*pncA*/ 169	RD4	RD9	RD12	RD[b] specific
M. tuberculosis	G	C	+	+	+	TbD1[c]
M. bovis	A	G	−	−	−	
M. bovis BCG	A	G	−	−	−	RD1
M. africanum	G	C	+	−	+	
M. canettii	G	C	+	+	+	RDcan[d]
M. microti	G	C	+	−	+	RDmic[e]
M. caprae	A	C	+	−	−	
M. pinnipedii	G	C	+	−	+	RDpin[f]

[a] Data from references 5, 11, 25, 31, 113, and 114. +, present; −, absent.
[b] RD locus numbering according to reference 31.
[c] TbD1 region is absent from "modern" *M. tuberculosis* only and present in all members of the MTBC (11).
[d] RDcan corresponds to a region specifically absent from *M. canettii* only that partially overlaps RD12 (11).
[e] RDmic corresponds to a region specifically absent from *M. microti* only that partially overlaps RD1 (11).
[f] RDpin corresponds to a region specifically absent from *M. pinnipedii* only that partially overlaps RD2 (11).

MYCOBACTERIAL STRAIN TYPING

Molecular Typing Methods for MTBC Strains

Historically, unusual drug susceptibility patterns and phage typing have been used for epidemiological studies of tuberculosis, but they have significant limitations. The description of the repetitive insertion sequence IS*6110* marked a major advance in the molecular epidemiology of tuberculosis (110). Since then, additional sequences of interest for *M. tuberculosis* fingerprinting have been described (32, 111). *M. tuberculosis* DNA fingerprinting has proven to be a powerful epidemiological tool for tracing of transmission groups and determination of their risk factors, for differentiation of exogenous reinfection and relapse, and for detection of laboratory cross-contamination, among other applications (15, 22, 111).

IS*6110* RFLP

The restriction fragment length polymorphism (RFLP) technique using the IS*6110* repetitive sequence as a probe was the first extensively used method for typing MTBC strains. IS*6110* is an insertion sequence present in variable copy numbers, from 0 to 26 in MTBC strains (15). A standardized protocol, based on the use of the restriction enzyme PvuII, has been proposed for IS*6110* RFLP comparison. Standardization of the procedure facilitates interlaboratory comparability of patterns (110).

The IS*6110* RFLP technique is widely applicable to *M. tuberculosis* since fewer than 0.1% of strains tested have no copies of the element (61). However, several sites of IS*6110* insertion are preferential loci for integration into the genome, contributing to reducing the potential diversity of fingerprint patterns (15). The RFLP technique requires approximatively 2 μg of high-quality DNA and hence has to be performed with large quantities of cells. A subculture of the isolate or heavy growth on the original slant and a long turnaround time are required. The technical steps are both labor-intensive and lengthy. Moreover, sophisticated computer image analysis software is required for image analysis, and the availability of well-trained users is sparse. Owing to these difficulties, the IS*6110* RFLP technique cannot be carried out in clinical laboratories and is limited to reference laboratories. PCR-based methods targeting IS*6110* have been developed to reduce the time lag for obtaining IS*6110* RFLP patterns. Although their differentiation levels are close to that of the IS*6110* RFLP technique, the methods suffer from similar drawbacks (lack of accuracy for strains with few IS*6110* copies and lack of expertise for the interpretation of the image patterns) and have not been widely applied (53, 55, 78).

Spoligotyping

The spoligotyping (which stands for "spacer oligotyping") method (Table 4) is based on the polymorphism of the direct repeat (DR) locus. This region, present in all MTBC strains in a unique locus, contains multiple, well-conserved 36-bp repeats interspersed with nonrepetitive short spacer sequences of 34 to 41 bp. Strains are tested by hybridizing their PCR-amplified DR regions to a membrane which consists of an array of 43 covalently bound oligonucleotides representing the polymorphic spacers identified in the *M. tuberculosis* H37Rv DR sequence and in the sequence of *M. bovis* BCG. The method has the clear advantage of being able to simultaneously discriminate among the members of the MTBC and differentiate the clinical isolates in one assay. Spoligotypes appear highly stable, suggesting that isolates with different spoligotypes are rarely related (111). Moreover, spoligotyping allows the identification of prevalent genotypes, especially the Beijing genotype, frequently encountered in China, in other regions of Asia, in the former USSR, and in other geographical areas (54). In the United States, the largest known epidemic associated with drug-resistant strains was due to the so-called "W" strain, an evolutionary branch of the Beijing family (8).

The discriminative power of spoligotyping is less than that of the IS*6110* RFLP technique, and the method tends to group strains in large clusters (111). Spoligotyping, however, is more discriminating for strains with no or few copies of IS*6110* and has been recommended for *M. bovis* since most *M. bovis* strains have few IS*6110* copies (111). Used for the detection of *M. tuberculosis* directly in clinical specimens, spoligotyping failed to yield reproducible patterns. Spoligotyping use in routine clinical laboratories may be difficult to implement due to the multistep hybridization procedures required by the method.

PCR Strategies Targeting Tandem Repeats

Genetic loci containing variable-number tandem repeats (VNTR) have been identified in the *M. tuberculosis* genome (32). VNTR are a source of allelic polymorphism which can be analyzed by amplification of each locus by using primers specific for flanking regions and estimation of the sizes of the PCR products, which reflect the number of VNTR copies. Results can be conveniently coded in a simple numerical format, corresponding to the number of repeated units in each locus. Initial VNTR typing based on limited sets of loci lacked discriminatory ability (55). However, a VNTR method based on 12 loci of a type of VNTR sequences called mycobacterial interspersed repetitive units (MIRUs) has been developed (89, 100). The 12 MIRU-VNTR loci present two to eight alleles which correspond to a potential of over 16 million different combinations. The method yields a discriminatory power close to that of IS*6110* RFLP typing (better for low-copy-number IS*6110* strains) and accurately clusters epidemiologically related strains (39, 56, 93, 99). The MIRU-VNTR method can be performed either in a manual format or on a fluorescence-based DNA analyzer with automation by using multiplex PCRs (99). A microfluidic labchip instrument has been used for rapid typing based on MIRU-VNTR (21). Thanks to its feasibility, portability, high reproducibility, and discriminatory power, the MIRU-VNTR technique is progressively replacing the IS*6110* RFLP method (9, 22, 53).

Whole-Genome Fingerprinting Methods

Promising results have been obtained with DNA arrays. The Affymetrix-GeneChip system has been applied to identify genomic deletions in *M. tuberculosis* isolates (46). It relies on the use of a high-density oligonucleotide microarray harboring 20 probe pairs targeted to every open reading frame and intergenic region of *M. tuberculosis* H37Rv, thus totaling 111,488 probe pairs after exclusion of noninformative probes. Hybridized DNA is detected with a confocal laser scanner. In a small-scale evaluation, the patterns of deletions detected were identical for epidemiologically related clones but differed between different clones, suggesting that the system is suitable for epidemiological studies (46). However, the system is not practical for analyzing large numbers of strains. High-density oligonucleotide microarrays will become commercially available to be used for clinical laboratory purposes in the future.

Single-nucleotide polymorphisms based on the synonymous mutations have been detected in *M. tuberculosis*. Since *M. tuberculosis* displays restricted allelic variation (94), the

TABLE 4 Characteristic spoligotypes of some MTBC members

Strain	Spoligotype[a]					
	1	9	19	29	39	43
M. tuberculosis H37Rv	■■■

increase in Crohn's disease has been reported stresses the potential exposure to aerosols carrying *M. avium* subsp. *paratuberculosis* and generated from the river (75).

M. kansasii Typing Methods

In Europe, the use of various molecular markers showed five subspecies within *M. kansasii* (3, 71). However, strains from the United States were shown to belong exclusively to subspecies I. These results demonstrate that subspecies I is predominant in the United States as it is in Europe and that this genotype I is highly clonal, with the same major genotype responsible for human infection worldwide (125).

Typing Methods for Other Slowly Growing NTM Species

For the other slowly growing species, information on the degree of polymorphism displayed by the different techniques is limited.

Molecular epidemiology of *M. intracellulare* can rely only on PFGE since the *M. avium* insertion sequences IS*1245* and IS*1311* are absent from all strains of *M. intracellulare* (35). Polymorphic PFGE patterns have been obtained for epidemiologically unrelated strains (62, 90).

IS*1395*, a specific insertion sequence, has been detected in *M. xenopi*. All *M. xenopi* strains have the element in 3 to 18 copies. Despite this high copy number, the element provides limited polymorphism, and unrelated strains were shown to share several bands in IS*1395* RFLP patterns. Comparable results were obtained with PFGE, suggesting high homogeneity of the *M. xenopi* genome (73).

Although rarely clinically significant, *M. gordonae* is a common laboratory contaminant and is frequently the cause of pseudo-outbreaks related to endoscopy, tap water, ice machines, or refrigerated fountains (118). Several molecular markers have been detected in *M. gordonae,* including the polymorphic GC-rich sequence (PGRS) and MPTR repetitive elements, also present in the MTBC and in *M. kansasii*, and two insertion sequences, IS*1511* and IS*1512*, which display high polymorphism (70). PFGE has also been successfully applied to *M. gordonae* (57).

The molecular epidemiology of *M. celatum* clearly differentiates *M. celatum* type 1 from *M. celatum* type 2 (69). A specific insertion sequence, IS*1407*, is present in *M. celatum* type 1 only. *M. celatum* type 2 displays polymorphic PFGE patterns, whereas *M. celatum* type 1 presents a single pattern.

A limited polymorphism was demonstrated within *M. haemophilum* with either a repetitive element in an RFLP study (50) or PFGE (123). Random amplification of polymorphic DNA typing also showed some polymorphism in *M. malmoense* (47). Two distinct repeated sequences, IS*2404* (83, 96) and IS*2406* (96), both present in high copy numbers (>50 copies), were identified in *M. ulcerans*. Interestingly, although comparative genetic analysis of *M. ulcerans* and *M. marinum* showed a very close phylogenetic relationship, both elements are absent from *M. marinum*. These data led to the hypothesis that *M. ulcerans* recently diverged from *M. marinum* by the acquisition of IS*2404* and IS2606, the species diversity being driven mainly by the genetic activity of the insertion sequences (96). Due to their high copy numbers, the two repeated elements are of poor value for typing *M. ulcerans* strains. Amplified fragment length polymorphism and IS*2404* RFLP analysis showed six types within *M. ulcerans* which correlated with the geographic origins of the strains (18). A novel-category VNTR allows the identification of eight genotypes within *M. ulcerans* and five genotypes within *M. marinum* (1, 98).

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Mycobacterium: Clinical and Laboratory Characteristics of Rapidly Growing Mycobacteria*

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38

TAXONOMY AND DESCRIPTION OF ORGANISMS

During the past 2 decades, there has been an increasing awareness of nontuberculous mycobacteria (NTM), including the rapidly growing mycobacteria (RGM), of which numerous species and phylogenetic groups are clearly established human pathogens. These include primarily the *Mycobacterium fortuitum* group; the *M. chelonae-M. abscessus* group, previously known collectively as the *M. fortuitum* complex; and to a lesser extent the *M. smegmatis* group. Three rare pathogens each belong to separate groups and have been associated with clinical disease: *M. mucogenicum*, *M. mageritense,* and *M. wolinskyi* (1, 4, 5, 47, 49, 64, 68).

The RGM are generally defined as nontuberculous species that grow within 7 days on laboratory media. Most clinically significant (pathogenic) species or groups are not pigmented. Only one pathogenic group (the *M. smegmatis* group) is pigmented, developing a slowly apparent or late pigment (4, 5, 68). All RGM contain long-chain fatty acids known as mycolic acids that can be quantitated using chromatographic techniques such as high-performance liquid chromatography (HPLC).

For many years, the *M. fortuitum* group comprised *M. fortuitum* (formerly *M. fortuitum* biovariant *fortuitum*), *M. peregrinum* (formerly *M. fortuitum* biovariant *peregrinum*), and the *M. fortuitum* third biovariant complex. Recent studies have shown that each of the latter two species comprise multiple additional species or "taxa" (47). *M. peregrinum* was found to contain two taxa originally designated *M. peregrinum* type I and type II (3). Taxonomic studies have now shown that isolates of *M. peregrinum* type II are human isolates of *M. senegalense* (60). In 1991, the third biovariant complex was described as containing two major subgroups (sorbitol positive and sorbitol negative) (63). In the last 7 years, seven new species have been identified from this "unnamed" complex. The sorbitol-positive subgroup currently consists of *M. houstonense, M. brisbanense,* and *M. mageritense*, and the sorbitol-negative subgroup consists of *M. porcinum, M. boenickei, M. neworleansense,* and *M. septicum* (28, 46, 47, 64, 65).

The *M. chelonae-M. abscessus* group is composed of at least three species, including *M. chelonae* (formerly *M. chelonae* subsp. *chelonae*), *M. abscessus* (formerly *M. chelonae* subsp. *abscessus*), and the newly described species *M. immunogenum* (5, 74).

The *M. smegmatis* group was initially described as composed of three species: *M. smegmatis* sensu stricto and two new species, *M. goodii* (4) and *M. wolinskyi* (4). Previously, these species were known as *M. smegmatis* types I, II, and III, respectively (68). This grouping was based on the absence of 3-day arylsulfatase activity in all three species. Current studies based on DNA sequence analysis suggest that *M. wolinskyi* is phylogenetically distinct from the other two species and likely belongs in a separate group (1).

M. mucogenicum, originally known as the *M. chelonae*-like organism, or MCLO, also constitutes a separate phylogenetic group distinct from all others, as does *M. mageritense* (1).

Unfortunately, many clinical laboratories do not take advantage of the new techniques for identifying isolates as identification of the RGM to the species level has traditionally been given low priority. This reluctance to provide identification to the species level partially reflects the lack of available simple, inexpensive, and accurate methods. Although combination analysis of results of biochemical tests, including those for carbohydrate utilization and susceptibility patterns, allows satisfactory identification of the majority of clinically significant isolates of RGM, these tests may be technically difficult to interpret and time-consuming for an inexperienced laboratory. Since no DNA probes are available in the United States for identification of the RGM and since HPLC alone cannot adequately identify most species of RGM, molecular testing by a reference laboratory is now necessary to accurately identify most of the 22 currently known pathogenic RGM to the species level.

CLINICAL SIGNIFICANCE

The RGM are opportunistic pathogens that produce disease in a variety of clinical settings. The three major clinically important species of RGM responsible for disease in humans include *M. fortuitum*, *M. chelonae,* and *M. abscessus*. Names of other potentially pathogenic and clinically significant RGM species have been included in Table 1 (4, 5, 47, 60, 64,

*This chapter contains information presented in chapter 36 by Gaby E. Pfyffer, Barbara A. Brown-Elliott, and Richard J. Wallace, Jr., and in chapter 37 by Véronique Vincent, Barbara A. Brown-Elliott, Kenneth C. Jost, Jr., and Richard J. Wallace, Jr., in the eighth edition of this Manual.

TABLE 1 Currently recognized species of RGM

Species	Characteristic				
	Established clinical significance	Pigmentation	Unique phenotype	Unique *hsp*65 REA pattern	Unique (complete) 16S sequence
Common human pathogens					
M. abscessus	Yes	No	No	Yes	Yes
M. chelonae	Yes	No	Yes	Yes	Yes
M. fortuitum	Yes	No	Yes	Yes	Yes
Less common human pathogens (>10 clinical isolates or cases)					
M. boenickei	Yes	No	No	Yes	Yes
M. goodii	Yes	Yes	No	Yes	Yes
M. houstonense	Yes	No	No	No	No[a]
M. immunogenum	Yes	No	No	Yes	Yes
M. mucogenicum	Yes	No	Yes	Yes	Yes
M. peregrinum	Yes	No	No	Yes	No[a]
M. porcinum	Yes	No	No	Yes	Yes
M. senegalense	Yes	No	No	Yes	Yes
M. smegmatis	Yes	Yes	No	Yes	Yes
Rare human pathogens (<10 cases or isolates)					
M. brisbanense	Yes	No	No	Yes	Yes
M. brumae	Yes	No	No	ND[b]	Yes
M. canariasense	Yes	No	No	Yes	Yes
M. elephantis	Yes	Yes	No	Yes	Yes
M. lacticola	Yes	Yes	Unknown	Yes	Yes
M. mageritense	Yes	No	Yes	Yes	Yes
M. neworleansense	Yes	No	No	No	Yes
M. novocastrense	Yes	Yes	No	ND	No
M. septicum	Yes	No	No	Yes	No[a]
M. wolinskyi	Yes	No	No	Yes	Yes
Unproven human pathogens					
M. agri	No	Yes	No	ND	Yes
M. aichiense	No	No	No	ND	Yes
M. alvei	No	No	No	ND	No
M. aurum	No	Yes	No	ND	Yes
M. austroafricanum	No	Yes	No	ND	Yes
M. chitae	No	No	No	ND	Yes
M. chlorophenolicum	No	Yes	No	ND	Yes
M. chubuense	No	Yes	No	ND	Yes
M. confluentis	No	No	No	ND	Yes
M. diernhoferi	No	Yes	No	ND	ND
M. duvalii	No	Yes	No	ND	Yes
M. fallax[c]	No	No	No	ND	Yes
M. flavescens	No	Yes	No	ND	Yes
M. frederiksbergense	No	Yes	No	ND	No
M. gadium	No	Yes	No	ND	Yes
M. gilvum	No	Yes	No	ND	Yes
M. hassiacum	No	Yes	No[d]	ND	Yes
M. hodleri	No	Yes	No[e]	ND	No
M. holsaticum	No	Yes	No	Yes	Yes
M. komossense	No	Yes	No	ND	Yes
M. madagascariense	No	Yes	No	ND	Yes
M. moriokaense	No	No	No	ND	Yes
M. murale	No	Yes	No	ND	Yes
M. obuense	No	Yes	No	ND	Yes
M. parafortuitum	No	Yes	No	ND	Yes
M. phlei	No	Yes	No	ND	Yes
M. psychrotolerans	No	Yes	No	ND	Yes

(Continued on next page)

TABLE 1 *(Continued)*

Species	Characteristic				
	Established clinical significance	Pigmentation	Unique phenotype	Unique *hsp*65 REA pattern	Unique (complete) 16S sequence
M. rhodesiae	No	Yes	No	ND	Yes
M. sphagni	No	Yes	No	ND	Yes
M. thermoresistibile	No	Yes	No	ND	Yes
M. tokaiense	No	Yes	No	ND	Yes
M. vaccae	No	Yes	No	ND	Yes
M. vanbaalenii	No	Yes	No	ND	Yes

[a] *M. houstonense* is 100% identical to *M. farcinogenes*, and *M. peregrinum* (type I) is 100% identical to *M. septicum*.
[b] ND, not determined.
[c] RGM at 30°C, slowly growing at 35°C.
[d] Grows at 65°C.
[e] Grows on or degrades a variety of organic substrates.

65, 73, 74). RGM are presumed to be common in the environment but have been most often identified in tap water when associated with wound infections (15). The specific reservoir for *M. abscessus* chronic lung infections has yet to be identified.

Wound Infections

The most common infection seen with the RGM is posttraumatic wound infection. Aside from the injury, the patients are generally healthy, and drug-induced immune suppression is associated with minimal increase in risk for this type of infection. The *M. fortuitum* group accounts for approximately 60% of cases of localized cutaneous infections, but any of the 22 pathogenic RGM species listed in Table 1 can cause disease (4, 5, 60, 64, 65, 68, 73).

Traumatic wound infections, especially open fractures, often involve species within the *M. fortuitum* third biovariant complex (47, 63, 65). More than 75% of the infections reported in connection with a series of 85 isolates of the *M. fortuitum* third biovariant complex from the United States and the Queensland, Australia, state laboratory were associated with skin, soft tissue, or bone infections (63). The majority of infections occurred 4 to 6 weeks following puncture wounds or open fractures. Metal puncture wounds (48%) and motor vehicle accidents (26%) were the most common antecedent injuries, and approximately 40% of the injury sites involved the foot or leg. Stepping on a nail was the most frequently related scenario. None of the isolates in this series were studied by molecular techniques that would identify them as one of the newly described species within the *M. fortuitum* third biovariant complex (i.e., *M. houstonense, M. boenickei, M. porcinum,* or *M. mageritense*).

In a 1989 report (67), approximately 80% of RGM wound isolates related to cardiac surgery were from seven southern coastal states, including Texas, Louisiana, Georgia, Maryland, Alabama, Florida, and South Carolina. A second report published in the same year showed that 92% of 37 identified cases of surgical wound infection following augmentation mammaplasty were also in patients in southern coastal states, with the majority in Texas, Florida, and North Carolina (61).

Sporadic cases of localized wound infections following medical or surgical procedures, including needle injections, can occur with *M. chelonae* but are less common than those with *M. fortuitum*. The clinical picture of posttraumatic wound infection ranges from localized cellulitis or abscesses to osteomyelitis (70). Localized or disseminated infections with *M. chelonae* most frequently occur in patients receiving long-term corticosteroid treatment and/or chemotherapy, organ transplant recipients, and patients with rheumatoid arthritis or other autoimmune disorders or those receiving immunosuppressive therapy (62). However, immune suppression in diseases such as AIDS has not been a significant risk factor for development of localized or disseminated *M. chelonae* infections.

Less frequently, *M. wolinskyi* and the *M. smegmatis* group, including *M. goodii* and *M. smegmatis* sensu stricto, have been reported in cases of infection following traumatic injury and surgical or medical procedures such as cardiac surgery, breast reduction surgery, and face lift plastic surgery. Cellulitis and localized abscesses are the most common manifestations (4).

Starting in 2000, an outbreak of furunculosis caused by *M. fortuitum* on the lower extremities occurred in 32 otherwise healthy patients who were patrons of a nail salon in California. The organism was also cultured from contaminated foot baths, and shaving the legs prior to the foot bath and pedicure was identified as a risk factor (48, 75, 76). Other species, including *M. abscessus* and *M. mageritense*, have subsequently been recovered in other cases of furunculosis.

Pulmonary Infections

Chronic lung infections can occur with RGM, most often in nonsmoking older women with bronchiectasis (sometimes associated with the *M. avium* complex [MAC] as well). The causative agent in more than 80% of cases of pulmonary disease due to RGM is *M. abscessus* (24).

Similarities exist between patients infected with the MAC and those infected with *M. abscessus* such that a common pathogenicity or host susceptibility factor may be involved (24). Multiple cultures of *M. abscessus* from respiratory samples are usually associated with significant pulmonary disease.

Patients with cystic fibrosis (CF) may also become infected with *M. abscessus* (16, 37). Recovery of *M. abscessus* in this setting is being reported more frequently than previously. *M. abscessus* is the second most common species of NTM recovered from CF patients (after the MAC) and may be the most common species associated with clinical disease in this setting (37). Patients with CF also have bronchiectasis in addition to chronic, recurrent airway and parenchymal infections that may be the primary risk factors for susceptibility to NTM disease.

Rarely, other RGM, including *M. chelonae*, the *M. smegmatis* group, and *M. fortuitum,* can be involved in pulmonary disease. *M. fortuitum* has been reported as a pathogen in half of the

cases of chronic aspiration disease secondary to underlying gastroesophageal disorders such as achalasia (24). Pulmonary disease with *M. chelonae* and *M. smegmatis* has been described in only a few cases, including one case of lipoid pneumonia (4, 5, 58, 68, 70).

Hypersensitivity pneumonitis among metal grinders working in industrial plants with contaminated metalworking fluids has been associated with a newly described species of RGM, *M. immunogenum*. This species is able to grow and remain viable in degraded metalworking fluid (74). This species has not been reported to be recovered from open lung biopsy specimens from these patients.

Disseminated Cutaneous Disease

Disseminated cutaneous disease due to members of the *M. fortuitum* group (including *M. fortuitum*) is rare even in immunocompromised patients, including those with AIDS (5, 7, 70). In the modern taxonomic era, we have seen fewer than five well-documented cases of disseminated cutaneous disease due to *M. fortuitum*.

Disseminated cutaneous disease due to *M. chelonae* is much more common. It typically presents as multiple, chronic, painful red nodules, usually involving the lower extremities. These lesions then drain spontaneously, with the drainage usually acid-fast bacillus smear positive. Almost all patients are immunosuppressed, usually from low-dose corticosteroid therapy for diseases such as rheumatoid arthritis and other autoimmune diseases or organ transplantation. Although the disease is presumably a consequence of hematogenous spread, bacteremia is rarely identified. A portal of entry for the pathogen is rarely evident (5, 62, 71). For a series of 100 clinical isolates from skin and soft tissue, it was reported that 53% were from patients with disseminated cutaneous infections (62). Approximately 75% of cases of disseminated cutaneous infection are due to *M. chelonae,* and about 20% are due to *M. abscessus* (5, 70). Disseminated disease involves other sites (blood and internal organs) only in severely immunosuppressed individuals, although immune suppression such as that occurring with AIDS has not been a significant risk factor for the development of disseminated cutaneous or extracutaneous disease.

Disseminated cutaneous disease due to *M. abscessus* occurs rarely but is serious (70). Like those of disseminated *M. chelonae* disease, most cases occur in chronically immunosuppressed patients receiving corticosteroids, and the pathogen has no apparent portal of entry. Also like those of *M. chelonae* infection, cases of *M. abscessus* disseminated cutaneous infection rarely involve detectable bacteremia and/or endocarditis, but patients usually present with multiple draining cutaneous nodules, usually in the lower extremities.

Health Care-Associated Infections

Health care-associated disease with the RGM has been reported most commonly with *M. fortuitum*, *M. chelonae*, *M. abscessus*, and *M. mucogenicum*, although any species may be involved. Most infections follow contamination with tap water harboring the infecting RGM. Types of infections include postsurgical wound infections, catheter sepsis, infections following hemodialysis (27, 61, 70, 72), postinjection abscesses, vaccine-related outbreaks (5, 11, 22, 55, 57, 61), and otitis media following tympanostomy tube replacement (21). These have been seen as both sporadic cases and localized outbreaks. Recent outbreaks have involved cosmetic procedures such as liposuction (35) and infections following nail salon procedures (23, 48, 75, 76).

Central catheter-associated infections are the most common health care-associated infections due to the RGM (5, 43, 46, 70). They are the most common cause of RGM bacteremia, but the disease may also present as local wound drainage as part of an exit site or tunnel infection (5, 28, 43). Other types of catheters, including peritoneal catheters, ventriculoperitoneal shunts, and shunts for hemodialysis, can also become infected (5).

Surgical wound infections due to RGM are a well-recognized clinical entity. In the 1970s and 1980s, these were most commonly associated with augmentation mammaplasty and coronary artery bypass surgery, and multiple disease outbreaks occurred (57). Infections following these types of surgery are now less common, and mastectomies for breast carcinoma, liposuction, and knee replacements have replaced these procedures as the most common causes of disease.

Infections following the insertion of prosthetic devices, including prosthetic heart valves, artificial knees and hips, lens implants, and metal rods inserted into the vertebrae to stabilize bones following fractures, have also been described (4, 5, 70). Again, *M. fortuitum* is the most common pathogen, but any of the pathogenic RGM, including the *M. smegmatis* group, can be associated with this type of infection (4, 5). The infection usually presents with watery drainage, wound breakdown, and low-grade fever.

In addition to true outbreaks of infection, numerous health care-associated pseudo-outbreaks have been described. Contaminated or malfunctioning bronchoscopes (5, 25, 57), automated endoscope cleaning machines (33, 57, 61, 74), and contaminated laboratory reagents and ice (27, 31) have been implicated (61).

Bone and Joint Infection

Infection with the RGM may also result in bone and joint infection. Like bacterial disease, osteomyelitis may follow open bone fractures, puncture wounds, and hematogenous spread from another source. The most common scenario is an open fracture of the femur, often followed by orthopedic surgical procedures. The most frequent pathogen recovered in this setting is the *M. fortuitum* group, including two newly described species, *M. houstonense* and *M. boenickei* (5, 47).

Vertebral osteomyelitis has also been described (40, 45). Sarria and colleagues identified 15 cases, and clinical information was available for six of the cases. Four of the six cases of RGM vertebral osteomyelitis involved *M. fortuitum,* and all of the patients had some type of underlying condition (45).

Bone involvement secondary to a puncture wound is likely the second major cause of osteomyelitis. Infections most commonly involve the *M. fortuitum* group (5, 58, 59). *M. fortuitum* infections associated with prosthetic knees and joints have also been reported.

The two newly described species, *M. goodii* and *M. wolinskyi,* have also been associated with osteomyelitis. In a recent report, 13 (36%) of 36 patients with infection caused by these two species were diagnosed with osteomyelitis (4).

Generally, osteomyelitis due to RGM is treated with surgical debridement and antimicrobial therapy for a minimum of 6 months (5, 66).

MISCELLANEOUS DISEASES

Other diseases associated with the RGM are summarized in the following paragraphs.

CNS Disease

Central nervous system (CNS) disease involving the RGM is rare, but morbidity and mortality are high. Most of the reported cases have been associated with *M. fortuitum* (5, 12). In a review by Flor et al., 52 isolates of NTM were reported that were associated with meningitis, of which 12% were identified as *M. fortuitum* (19).

Treatment of CNS infections due to RGM is difficult and often requires extended multiple-antimicrobial treatment (6 to 12 months) in addition to surgical intervention (5).

Corneal Infections (Keratitis)

The number of RGM recovered from ocular infections has been increasing over the last 20 years. A retrospective review of cases of NTM keratitis from 1982 to1997 at an eye institute in Florida showed that 19 of 24 cases were due to RGM (20).

A review of the literature involving keratitis due to the NTM from 1965 to 1992 found that 21 (55%) of 38 isolates were identified as *M. fortuitum*, 16 (42%) of 38 belonged to the *M. chelonae-M. abscessus* group, and 1 (2%) was identified only as "group IV mycobacteria." However, prior to 1978, isolates from all of the cases reported were incompletely identified as "*M. fortuitum,*" so it is not possible to ascertain modern species classifications (6).

Since the early 1990s, other descriptions of eye infections associated with the RGM have been published, including cases occurring after keratoplasty and laser in situ keratomileusis (LASIK) surgery (5).

Corneal infections have an indolent course, and often scraping or biopsy following debridement of the epithelial layer is necessary to obtain appropriate specimens for culture. Treatment of patients with keratitis due to RGM is difficult. The most widely used antimicrobial treatment involves the use of topical aminoglycosides and/or systemic amikacin, gentamicin, kanamycin, or neomycin. Other topical therapies have also included clarithromycin drops for infections due to *M. abscessus* or *M. chelonae,* tobramycin for infections due to *M. chelonae,* and ciprofloxacin for infections due to the *M. fortuitum* group. However, most of these antimicrobials have been shown to penetrate poorly through intact corneal epithelium (5, 6).

For patients who do not respond to topical treatments, surgical intervention including penetrating keratoplasty, corneal graft, lamellar keractectomy, and/or superficial debridement is recommended and was required in the majority of the previously reviewed cases (20).

Cervical Lymphadenitis

Although the most common mycobacterial cause of lymphadenitis is *M. tuberculosis* in adults and the MAC in children, RGM have also been implicated. At least 19 cases of cervical lymphadenitis due to *M. fortuitum* have been reported in the literature. Details were not available for all of the cases reviewed (10, 70). The majority of the patients responded to therapy with either complete resolution or significant decrease in the sizes of the affected lymph nodes (10).

Treatment for RGM lymphadenitis usually involves incision and drainage, followed by a combination of antimicrobials that includes an initial course of intravenous amikacin and concludes with one or more oral antibiotics. The recommended duration of therapy is at least 6 months (5, 10, 66).

Otitis Media

The most common NTM species associated with chronic otitis media is *M. abscessus*. In a 1988 outbreak of 17 cases of otitis media in two ear-nose-throat clinics, patients presented with chronic ear drainage, with a perforated tympanic membrane and prior insertion of a tympanostomy tube (32). In another series of infections, 20 of 21 cases of sporadic chronic otitis media (some with associated mastoiditis) were due to *M. abscessus* following ear tube placement. Therapy included removal of the ear tubes, surgical debridement, and antibiotic therapy including initial amikacin for susceptible isolates and either cefoxitin or imipenem for 3 to 6 weeks, followed by long-term oral therapy with erythromycin (preclarithromycin era) or clarithromycin. Approximately one-half of the isolates from these cases were also aminoglycoside resistant, resulting from the long-term use of aminoglycoside ear drops (21).

Otitis media or mastoiditis due to RGM other than *M. abscessus* has been infrequently reported (70).

IDENTIFICATION OF RGM

Collection, Transport, and Storage of Specimens

Details of standard methods for collection, transport, and storage of specimens are included in chapter 20.

Direct Examination and Isolation Procedures

Direct examination and isolation procedures are generally detailed in chapter 36. Primary isolation of RGM requires culture at 28 to 30°C rather than 35°C for wound cultures, especially for recovery of *M. chelonae*.

Biochemical Testing

As previously stated, the RGM are defined as NTM that grow within 7 days (most species within 3 to 4 days) (41, 56). Until the advent of molecular techniques, conventional laboratory identification of the RGM was based primarily upon growth rate, pigmentation, colonial morphology, and results of selected biochemical tests. These standard tests include those for arylsulfatase production, tolerance to 5% NaCl, nitrate reductase activity, and iron uptake. All members of the *M. fortuitum* group and the *M. chelonae-M. abscessus* group exhibit a strongly positive arylsulfatase reaction at 3 days (5, 41, 56). The *M. smegmatis* group (*M. smegmatis* and *M. goodii*) and *M. wolinskyi* are similar in growth rates but do not exhibit arylsulfatase activity at 3 days (4, 68). Approximately 95% of the isolates of *M. smegmatis* (sensu stricto) and 80% of *M. goodii* isolates develop a late (7 to 10 days) yellow-orange pigmentation. *M. wolinskyi* differs from the other two species in that it remains nonpigmented (4, 68).

Previously, a study by the former International Working Group on Mycobacterial Taxonomy (30) showed that growth in 5% NaCl was discriminatory for *M. abscessus* and *M. chelonae*. In this study, 100% of the isolates of *M. abscessus* grew in the presence of 5% NaCl but only 17% of the isolates of *M. chelonae* were viable in the same medium. The same study also reported that *M. abscessus* isolates were citrate negative and that 100% of *M. chelonae* isolates grew in the presence of citrate as a sole carbon source (30). These data have subsequently been corroborated by other investigators (4, 77).

Routine conventional testing has generally proven inadequate for recognition of other established species of RGM, such as *M. peregrinum,* and is definitely lacking for identification of most of the newly described species of RGM (4, 5, 47, 74).

Supplemented Biochemical Testing: Carbohydrate Utilization

The supplementation of the standard biochemical tests with a test for the utilization of carbohydrates (mannitol, inositol, sorbitol, rhamnose, and citrate) (Table 2) has allowed more complete and accurate laboratory identification of established species and discrimination of some (but not all) newly described species (5, 56, 59). It is no longer acceptable to fail to identify RGM isolates to the species level, especially isolates considered to be clinically significant. This differentiation is most critical for *M. chelonae* and *M. abscessus* (5).

One point that should be emphasized is that many of the studies involving carbohydrate utilization by the RGM have been performed with media prepared in-house. There have been few studies of currently available commercial media to ascertain if these will provide equivalent results. With this in mind, the use of commercial products will require careful in-house validation. One example of this problem was noted by Conville and Witebsky in the identification of strains of *M. mucogenicum* by using commercially manufactured carbohydrates (14).

Antimicrobial Susceptibility Tests

As discussed above, other adjunctive nonmolecular tests, including antimicrobial susceptibility tests, have also been utilized for the identification of the RGM. The polymyxin B disk diffusion method is used to distinguish between the *M. fortuitum* group and the *M. chelonae-M. abscessus* group. Generally, isolates of the *M. fortuitum* group exhibit a partial or clear zone of inhibition of ≥10 mm around the polymyxin disk whereas isolates of the *M. chelonae-M. abscessus* group show no zone of inhibition (69). Isolates of the *M. fortuitum* group are usually susceptible to a broader range of antimicrobials, including amikacin, quinolones, sulfonamides, linezolid, and imipenem. Most of the isolates of this group appear to be intrinsically resistant to the macrolides through the presence of an inducible *erm* gene (36).

Although originally considered to be two subspecies within one species, *M. chelonae* and *M. abscessus* are in fact two separate species, and infections with these species have distinctly different clinical manifestations. *M. chelonae* is most often associated with skin and soft tissue infections, especially in patients receiving chronic steroid therapy (62), and is rarely a cause of chronic lung disease (24, 70). *M. abscessus* may also cause skin and soft tissue infections but is primarily a lung pathogen, being responsible for more than 80% of the chronic lung disease cases caused by RGM (5, 24, 70). Thus, it is extremely important to differentiate these RGM to the species level and perform susceptibility testing so that appropriate antibiotic therapy can be initiated (5, 66).

M. chelonae and *M. abscessus* also have different antimicrobial susceptibility patterns. One of the major differences between the two species is in resistance to cefoxitin. In agar disk diffusion tests, *M. chelonae* shows complete resistance to cefoxitin, with no partial or complete zones of inhibition, in contrast to the partial or complete zones of inhibition seen with *M. abscessus*. MICs of cefotoxin for isolates of *M. chelonae* are ≥ 256 μg/ml. *M. abscessus* is intermediately susceptible; modal MICs for *M. abscessus* isolates are 32 μg/ml. MICs of amikacin for isolates of *M. abscessus* are also lower, and *M. abscessus* is resistant to tobramycin, whereas tobramycin is more active than amikacin against *M. chelonae*. Additionally, isolates of *M. chelonae* are more susceptible in vitro to some of the newer antibiotics, including linezolid and gatifloxacin, than are isolates of *M. abscessus* (5).

With these differences in susceptibility patterns of the rapidly growing species in mind, tentative identification of the most commonly encountered species of RGM is possible and optimal therapeutic regimens can be designed. However, as with other phenotypic tests, susceptibility testing does not provide definitive species identification, which currently requires molecular testing for almost all RGM species (5).

HPLC Identification

HPLC analysis of mycobacterial cell wall mycolic acid content is routinely used in large reference or state health department laboratories to identify isolates of NTM (9, 53) but has been problematic with species of RGM (9).

HPLC can be helpful for placing RGM into groups but is not specific enough to identify them to the species level with a high degree of accuracy, even with the adoption of standardized growth conditions. Thus, laboratory identification based on HPLC analysis of mycolic acid profiles alone is not adequate for the identification of the pathogenic species of RGM.

MOLECULAR IDENTIFICATION METHODS

Nucleic Acid Probes

The INNO LiPA multiplex probe assay (Innogenetics, Ghent, Belgium) is a molecular biology-based product available outside the United States and utilizes the principle of reverse hybridization (54, 56). Biotinylated DNA obtained by PCR amplification of the 16S-23S internal transcribed spacer region is hybridized with specific oligonucleotide probes immobilized as parallel lines on membrane strips. The addition of streptavidin labeled with alkaline phosphatase and a chromogenic substrate results in a purple-brown precipitate on the hybridized lines. This system was evaluated with multiple species of mycobacteria, including some RGM. The main advantage of this system is that a large variety of species may be identified by a single assay without the need to select an appropriate probe. One limitation of the assay is the cross-reactivity that may be detected with strains of *M. fortuitum* (54, 56). Additionally, it failed to differentiate isolates of *M. chelonae* from those of *M. abscessus*.

Sequence Analysis Identification of RGM

16S rRNA Gene Sequence Analysis

Slowly growing mycobacterial species, with some exceptions such as *M. terrae,* contain one copy of the 16S rRNA operon, whereas the RGM, except for *M. chelonae* and *M. abscessus*, have two copies (2, 18). Routine sequencing of the entire 1,500-nucleotide sequence of the 16S rRNA gene is not feasible for most clinical laboratories. Two main hypervariable domains known as region A and region B are located on the 5′ end of the 16S rRNA gene. These regions correspond to the *Escherichia coli* positions 129 to 267 and 430 to 500, respectively. Hypervariable region A, especially, contains most of the species-specific sequence variations (so-called "signature sequences") in mycobacterial species, and sequencing of this region allows taxonomic identification of most mycobacteria, including most species of RGM (26, 29).

The MicroSeq 500 system (Applied Biosystems, Foster City, Calif.) analyzes approximately 500 bp of the 16S rRNA gene at the 5′ end, including hypervariable region A. Previous studies showed that the MicroSeq assay was able to identify

TABLE 2 Laboratory phenotypic features of the 15 most clinically important species of nonpigmented RGM[a]

Species or complex	Former designation(s)	Pigment	3-Day arylsulfatase	Nitrate reduction	Iron uptake	Carbohydrate utilization				Growth in 5% NaCl
						Mannitol	Inositol	Citrate	Sorbitol	
M. chelonae-M. abscessus group										
M. abscessus	*M. chelonae* subsp. *abscessus*	−	+	−	−	−	−	−	−	+
M. chelonae	*M. borstelense*, *M. chelonei*, *M. chelonae* subsp. *chelonae*	−	+	−	−	−	−	+	−	−
M. immunogenum	*M. immunogen*	−	+	−	−	−	−	−	−	−
M. fortuitum group										
M. fortuitum	*M. ranae*, *M. fortuitum* bv. *fortuitum*	−	+	+	+	−	−	−	−	+
M. peregrinum[b]	*M. fortuitum* bv. *peregrinum* (pipemidic acid susceptible), *M. peregrinum* (type 1)	−	+	+	+	+	−	−	−	+
M. senegalense	*M. fortuitum* bv. *peregrinum* (pipemidic acid resistant), *M. peregrinum* (type 2)	−	+	+	+	+	−	−	−	+
M. fortuitum third biovariant complex										
M. houstonense	*M. fortuitum* third biovar (sorbitol positive)	−	+	+	+	+	+	−	+	+
M. brisbanense	*M. fortuitum* third biovar (sorbitol positive)	−	+	+	+	+	+	−	+	+
M. porcinum	*M. fortuitum* third biovar (sorbitol negative)	−	+	+	+	+	+	−	−	+
M. neworleansense	*M. fortuitum* third biovar (sorbitol negative)	−	+	+	+	+	+	−	−	+
M. boenickei	*M. fortuitum* third biovar (sorbitol negative)	−	+	+	+	+	+	−	−	+
M. mucogenicum	*M. chelonae*-like organism, or MCLO	−	+	±	−[c]	+	−	+	−	−
M. smegmatis group										
M. smegmatis sensu stricto	*M. smegmatis* (group 1)	±	−	+	+	+	+	±	+	+
M. wolinskyi	*M. smegmatis* (group 2)	−	−	+	+	+	+	±	+	+
M. goodii	*M. smegmatis* (group 3)	±	−	+	+	+	+	±	+	+

[a] +, ≥90%; −, <10%; ±, 11 to 89% or late.
[b] *M. peregrinum* (type 1) has the same REA pattern as the *M. fortuitum* third biovariant (sorbitol negative), whereas *M. peregrinum* (type 2) has the same REA pattern as *M. houstonense* (*M. fortuitum* third biovariant [sorbitol positive]). Biochemical testing is necessary for differentiation of these species.
[c] Tan appearance.

most isolates of RGM to the species level (13, 26, 39). Of the discordant isolates, 61% were unusual isolates that were difficult to identify by phenotypic methods and 56% could not be identified using molecular methods. MicroSeq is limited by the fact that analysis of the first 500-bp sequence cannot discriminate between several closely related RGM species, including *M. chelonae* and *M. abscessus, M. houstonense* and *M. senegalense, M. peregrinum* and *M. septicum,* and *M. houstonense* and *M. farcinogenes* (26, 60). Another major limitation of the MicroSeq system is that the database contains only one entry per species (the type strain). This is especially problematic with isolates that do not have an exact match in the database. Newly reported species are often not in the database. Therefore, the MicroSeq 500 system requires the use of additional sequence databases (13).

A reporting criterion such as (i) distinct species, (ii) related to a species, or (iii) most closely related to a species, depending upon the amount of sequence difference between the unknown isolate and the 16S rRNA gene database entries (39), has been recommended but not validated. This is important as currently there is no standard recommendation for a cutoff value used to interpret sequencing data (13, 26, 39).

Finally, the expense of the MicroSeq assay prevents its application in many clinical laboratories (26).

Sequencing of the *hsp65* Gene

Although the gene for the 65-kDa heat shock protein *(hsp*65*)* is highly conserved among species of mycobacteria, it exhibits greater interspecies and intraspecies polymorphism than the 16S rRNA gene sequence. This variability can be advantageous in the development of other strategies for the identification of genetically related species of the RGM (44, 54). Like most genes, it has both highly conserved and highly variable nucleotide regions (42). Most sequencing or restriction fragment length polymorphism analysis has utilized a 441-bp sequence identified by Telenti et al. (51) and often referred to as the Telenti fragment.

Studies based on DNA sequencing have demonstrated interspecies allelic diversity within the RGM (38). Detailed studies of several RGM species including *M. peregrinum*, *M. porcinum, M. senegalense, M. chelonae,* and *M. abscessus* have shown four to six sequence variants (sequevars) per species that differ by 2 to 6 nucleotides within the 441-bp Telenti fragment (44, 60, 65).

Additionally, unlike 16S rRNA gene sequence analysis, the *hsp*65 sequencing method was easily able to differentiate isolates of *M. abscessus* from those of *M. chelonae* (they differ by almost 30 bp in the 441-bp *hsp65* sequence compared to only 4 bp in the entire 1,500-bp 16S rRNA gene sequence). In contrast to 16S rRNA gene sequences, sequences of the *hsp65* gene allow even RGM species with a high degree of 16S rRNA gene similarity, such as *M. fortuitum, M. septicum, M. peregrinum, M. houstonense*, and *M. senegalense,* to be distinguished as distinct species (60).

A limitation of sequencing of the *hsp65* gene is that few or no sequences from more recently described RGM species are available in databases and detailed sequencing of older species (i.e., multiple strains) has not been done so only one sequence per species is generally available. This means that in-house validation is essential (34).

PCR-REA of the *hsp65* Gene

PCR-restriction enzyme analysis (PCR-REA) of the gene for the 65-kDA heat shock protein *(hsp)* has become a valuable tool used in the identification of the RGM.

The advantage of REA nonsequencing of the *hsp65* gene is that minor differences (sequevars) within the species rarely involve a restriction site, so most species have only one REA pattern. REA requires less equipment expenditure than sequencing. However, as in sequencing, the system is only as good as its database. Also, there is no commercial system for *hsp65* REA. Ten taxonomic groups of RGM were initially evaluated (a total of 129 reference and clinical isolates of RGM) with 24 restriction endonucleases, and BstEII, HaeIII, CfoI, and AciI were found to give the best species separation (50). A number of additional RGM species were described after this study, and most have shown unique or identifying restriction fragment length polymorphism patterns by this method. These species include *M. immunogenum* (74), *M. mageritense* (64), *M. porcinum* (47, 65), *M. boenickei* (47), *M. goodii* (4), *M. wolinskyi* (4), *M. septicum* (47), and *M. senegalense* (60). Species that could still not be separated include *M. houstonense* and *M. neworleansense* (47). Algorithms for identification of mycobacterial species including RGM by using PCR-restriction fragment length polymorphism analysis of the *hsp65* gene have been proposed (17).

Thus, currently the 441-bp Telenti fragment of the *hsp*65 gene remains the most useful sequence for REA identification of the RGM, although it has not been evaluated extensively in the pigmented RGM (4, 50, 51). The advantages of REA are that the method of identification does not rely upon growth rate and nutritional requirements, the equipment is relatively inexpensive, and the results for a large number of mycobacterial species can be generated rapidly. The disadvantages are that it requires knowledge of PCR and is a relatively complex procedure that requires extensive in-house validation since the method is not approved by the Food and Drug Administration.

Sequencing of Other Secondary Gene Targets

Other molecular targets for taxonomic identification, including the gene encoding the 32-kDA protein, the superoxide dismutase gene *(sod),* the *dnaJ* gene, the *rpoB* gene, the 16S-23S rRNA internal transcribed spacer, the *secA1* gene, and the *recA* gene, have been suggested for mycobacterial identification utilizing either REA or direct sequencing (1, 60, 65). However, preliminary data suggest that the sequences of these genes are more variable than that of the *hsp65* gene and to date these sequences have been less commonly utilized in the laboratory identification of the species of RGM (1, 56, 60, 65).

Molecular Typing Methods

PFGE

Pulsed-field gel electrophoresis (PFGE) is the most widely used method for molecular strain typing of the RGM. Although PFGE has never been standardized for RGM, most investigators concur that small (2- to 3-band) differences between isolates indicate that the isolates are closely related; differences of 4 to 6 bands indicate that the strains are possibly related, and ≥7-band differences indicate that the isolates are genetically different (52). Because unrelated strains of most RGM contain highly diverse PFGE patterns, this technique has been useful in epidemiological investigations. With recent modifications of the original method, it is now possible to perform PFGE on all species of RGM (79).

Clinical usage of PFGE for genetic comparison of *M. fortuitum* strains was detailed in a report of an outbreak

of respiratory tract colonization with this species in an alcoholism rehabilitation ward in Washington, D.C. (8). Since that report, several other epidemiological investigations of RGM by using PFGE have been performed (27, 33, 35, 72, 79).

RAPD-PCR

In the random amplified polymorphic DNA PCR (RAPD-PCR) using one arbitrary primer and low-stringency conditions, the primer hybridizes to both strands of template DNA, where it is matched or partially matched, resulting in strain-specific heterogeneous DNA products. Zhang and colleagues (78) applied the RAPD-PCR or arbitrarily primed PCR analysis method to comparing strains of *M. abscessus*. They were able to confirm several previous observations about prior outbreaks of nosocomial RGM infection, including a 1988 epidemic of otitis media due to aminoglycoside-resistant *M. abscessus* in children who had previously received tympanostomy tubes (32) and an outbreak associated with cardiac surgery (67).

The need for RAPD-PCR for strain typing of *M. abscessus* is now reduced as recent studies have shown the use of thiourea to prevent the DNA lysis that related to the Tris-borate-EDTA in the PFGE running buffer (79). Whether RAPD-PCR is less or more discriminatory as a typing method than PFGE for RGM has not been established.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

The major species of RGM each have different virulence levels in different clinical settings, and they have different drug susceptibilities as well. *M. mucogenicum*, e.g., is a recognized cause of catheter sepsis but, when recovered from sputum, is usually a contaminant. Thus, all clinically significant RGM should be identified to the species level. The recommended species identification methods are evolving, with declining interest in and accuracy of phenotypic testing, including HPLC, and increasing availability and accuracy of molecular methods. Currently, molecular methods are preferred and generally are the only way to identify more recently described species such as *M. goodii, M. mucogenicum,* and *M. senegalense*. Phenotypic tests are still useful (e.g., citrate utilization to separate *M. chelonae* from *M. abscessus*) but work best when combined with molecular methods. Smaller labs should consider referring RGM isolates to a large reference laboratory.

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*Neisseria**

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39

TAXONOMY OF THE GENUS *NEISSERIA*

At this time, members of the genus *Neisseria* are classified in the family *Neisseriaceae* along with the genera *Kingella*, *Eikenella*, *Simonsiella*, and *Alysiella*. This family is now placed in the β-subgroup of the Phylum *Proteobacteria*.

DESCRIPTION OF THE GENUS *NEISSERIA*

Members of the genus *Neisseria* are coccal or rod-shaped gram-negative organisms that occur in pairs or short chains. Diplococcal species have adjacent sides that are flattened, giving them a "coffee bean" appearance. Currently, *Neisseria* species (except the three *N. elongata* subspecies and *N. weaveri*) are the only true coccal members of the family *Neisseriaceae*. *N. elongata* subspecies and *N. weaveri* are medium to large, plump rods that appear in gram-stained smears as pairs or short chains (5, 51). All species in the genus *Neisseria* inhabit the mucous membrane surfaces of warm-blooded hosts. These organisms are aerobic and nonmotile and do not form spores; most species grow optimally at 35 to 37°C. Growth of *Neisseria* species is stimulated by CO_2 and humidity; some gonococcal isolates have an obligate requirement for CO_2 for initial isolation and growth of subsequent subcultures. Those *Neisseria* species that produce acid from carbohydrates do so by an oxidative pathway. All members of the genus are oxidase positive and, except for *N. elongata* subspecies *elongata* and subspecies *nitroreducens*, are catalase positive.

While most *Neisseria* species are not exacting in their nutritional requirements for growth, the pathogenic species, and *N. gonorrhoeae* in particular, are more nutritionally demanding. *N. gonorrhoeae* does not grow in the absence of cysteine and a usable energy source (i.e., glucose, pyruvate, or lactate). Some gonococcal strains display requirements for specific amino acids, pyrimidines, and purines as a result of defective or altered biosynthetic pathways. Demonstration of these growth requirements forms the basis for a typing method for gonococci called auxotyping (see below). The neisseriae can grow under anaerobic conditions if an alternative electron acceptor (e.g., nitrites) is present.

* This chapter contains information presented in chapter 38 by William M. Janda and Joan S. Knapp in the eighth edition of this Manual.

EPIDEMIOLOGY AND TRANSMISSION

N. gonorrhoeae is the causative agent of gonorrhea. In the United States, the incidence of gonorrhea increased during the 1960s and early 1970s, with the highest incidence—over 460 cases per 100,000 population—occurring in 1975. From the mid-1980s and into the 1990s, the incidence of gonorrhea steadily declined. In 1994, reported cases of *Chlamydia trachomatis* infection exceeded reported cases of gonococcal infection for the first time. The decline in the incidence of gonorrhea ceased toward the mid-1990s; from 1998 to 2002, the incidence of gonococcal infections plateaued at 128 to 130 cases per 100,000. In the United States, the incidence of gonorrhea remains high among sexually active teenagers and young adults, with the highest attack rates found among 15- to 19-year-old women (34). In regions where collected statistics include sexual orientation, rates of gonococcal infection more than tripled from 1995 to 2003 among men who have sex with other men (MSM) (30). Maintenance and transmission of gonorrhea are related to a social subset of "core transmitters" who have unprotected intercourse with multiple new partners and either are asymptomatic or choose to ignore symptoms (91). Both social risk factors (i.e., low socioeconomic status, urban residence, lack of education, poor access to health care, unmarried status, race/ethnicity, male homosexuality, prostitution, and histories of other sexually transmitted diseases) and behavioral risk factors (i.e., unprotected intercourse, multiple partners, other high-risk partners, and drug use) have been identified for targeting by outreach/intervention and sexually transmitted disease control programs.

The risk of acquiring gonorrhea is multifactorial and is related to the number and sites of exposure. For heterosexual males, the risk of acquiring urethral infection from an infected female is about 20% for a single exposure and up to 80% for four exposures. Due to anatomical considerations, the risk of infection for females from a single exposure to an infected male is about 50 to 70% (69). Anogenital sexual contact resulting in rectal infection is also efficient, and studies among MSM have demonstrated that urethral infection following fellatio with an infected partner may account for up to 26% of urethral infections diagnosed in this population (68).

N. meningitidis causes a disease spectrum ranging from occult sepsis with rapid recovery to fulminant fatal disease. The major virulence factor of disease-associated meningococci is the polysaccharide capsule. Thirteen meningococcal

capsular polysaccharide serogroups (A, B, C, D, H, I, K, L, X, Y, Z, W135, and 29E) have been described, and most infections are caused by organisms belonging to serogroups A, B, C, Y, and W135. Meningococci cause epidemic and endemic meningitis, and serogroups A, B, and C are responsible for 90% of cases globally. Humans are the only natural host for *N. meningitidis,* and the organism is spread from person to person via the respiratory route. Meningococci also may be carried asymptomatically in the oropharynx and nasopharynx. Carriage rates range from about 8 up to 20%, with older children and young adults having higher rates than young children. Carriage may be transient, intermittent, or persistent (105). Carriage strains may be encapsulated (groupable) or nonencapsulated (nongroupable) and result in the formation of serogroup-specific antibodies and broadly cross-reactive antibodies against several other outer membrane antigens. Individuals colonized with nongroupable strains also develop antibody titers against groupable strains, probably due to shared antigenic determinants. This immune response does not eliminate carriage, but it may protect the host from overt disease.

Worldwide, pathogenic clones of *N. meningitidis* composed of different serogroups, serotypes, serosubtypes, and immunotypes are responsible for both endemic and epidemic disease (95). Endemic meningococcal disease occurs at rates of 1 to 3 cases per 100,000 persons in the United States, and in 10 to 25 cases per 100,000 persons in developing countries (95, 100). In the United States, the annual incidence of meningococcal disease has been 0.8 to 1/100,000 population, with infants, the elderly, and the immunocompromised being at greatest risk. Attack rates are highest among children aged 3 months to 1 year and among older teenagers and young adults. Changes in meningococcal epidemiology in the United States during the 1990s have included increases in the incidence of community-acquired serogroup C infections and an increased incidence of disease among teenagers and young adults of high school and college age (54). Outbreaks on college campuses have been associated with bar patronage, drinking, cigarette smoking, and dormitory residence (57). Substantial amounts of meningococcal disease have also been reported among non-first-year students and students living in off-campus housing. With the licensure of the new tetravalent (serogroups A, C, Y, and W135) meningococcal conjugate vaccine (Menactra; Sanofi Pasteur, Inc., Swiftwater, Pa.) in January 2005, the Advisory Committee on Immunization Practices now recommends routine vaccination of adolescents aged 11 to 12 years, and for those past this age, routine immunization before entry into high school or college (29). With this approach, all adolescents will receive either the tetravalent polysaccharide or tetravalent conjugate meningococcal vaccine beginning at age 11 years by the year 2008.

CLINICAL SIGNIFICANCE

Members of the genus *Neisseria* that are found in humans include *N. gonorrhoeae*, *N. meningitidis*, *N. lactamica*, *N. sicca*, *N. subflava* (biovars subflava, flava, and perflava), *N. mucosa*, *N. flavescens*, *N. cinerea*, *N. polysaccharea*, and *N. elongata* subspecies *elongata*, *glycolytica*, and *nitroreducens*. *N. gonorrhoeae* subspecies *kochii*, an extremely rare isolate, has no official taxonomic standing. *N. canis* and *N. weaveri* are found as part of the normal respiratory tract flora of dogs, *N. denitrificans* is present in the respiratory tract of guinea pigs, and *N. macacae*, *N. dentiae*, and *N. iguanae* constitute part of the oral flora in rhesus monkeys, cows, and iguanid lizards, respectively. Most human *Neisseria* species are normal inhabitants of the upper respiratory tract and are not considered pathogens; occasionally these organisms may be isolated from infections, particularly in settings of underlying disease and/or immunosuppression. *N. gonorrhoeae* is always considered a pathogen, regardless of the site of isolation. *N. meningitidis* causes significant and often life-threatening disease but may colonize the nasopharynx without causing disease.

In males, *N. gonorrhoeae* causes an acute urethritis with dysuria and urethral discharge. The incubation period averages 2 to 7 days. After this time, 95 to 99% of infected males experience a purulent urethral discharge. About 2.5% of men presenting to sexually transmitted disease clinics are asymptomatic; the prevalence of asymptomatic urogenital gonorrhea in males in the general population may be as high as 5%. If left untreated, most cases of gonorrhea in men resolve spontaneously. Complications of ascending infection are uncommon and include epididymitis, penile lymphangitis, acute prostatitis, periurethral abscess, seminal vesiculitis, infections of the Tyson's and Cowper's glands, and urethral stricture.

In females, the primary gonococcal infection is present in the endocervix, with concomitant urethral infection occurring in 70 to 90% of cases. After an incubation period of 8 to 10 days, women may present with cervicovaginal discharge, abnormal or intermenstrual bleeding, and abdominal/pelvic pain. Gonococcal infection of the vaginal squamous epithelium of postpubertal women is uncommon, and in women with hysterectomies, the urethra is the most common site of infection. Symptoms of uncomplicated endocervical gonorrhea may resemble other conditions, such as cystitis or vaginal infections. Infection of Bartholin's and Skene's glands may occur in about one-third of women with genital infections, and careful manipulation of these glands can sometimes provide purulent material for direct examination and culture. Endocervical gonorrhea may complicate pregnancy and is a cofactor for spontaneous abortion, chorioamnionitis, premature rupture of membranes, premature delivery, and infant morbidity. Infants born to women with gonorrhea are at risk for developing conjunctival ("ophthalmia neonatorum") or pharyngeal gonococcal infection. Ascending infection may occur in 10 to 20% of infected women and can result in acute pelvic inflammatory disease (PID) manifested as salpingitis (infection of the fallopian tubes), endometritis, and/or tubo-ovarian abscess, all of which can result in scarring, ectopic pregnancies, sterility, and chronic pelvic pain (15). Symptoms of gonococcal PID include lower abdominal pain, abnormal cervical discharge and bleeding, pain on motion, fever, and peripheral leukocytosis. PID caused by *N. gonorrhoeae* generally occurs early in infection and often during or shortly after the onset of menstruation.

N. gonorrhoeae may also cause pharyngeal and anorectal infections. Oropharyngeal infections occur in MSM and heterosexual women who engage in orogenital sexual contact with an infected partner. Oropharyngeal gonococcal infections are usually asymptomatic and are diagnosed by culture of the organism from the throat (12). Rarely, oropharyngeal gonococcal infection may cause acute, exudative pharyngitis or tonsillitis with cervical lymphadenopathy (9). Anorectal gonococcal infection is seen in MSM as a result of unprotected anal intercourse. Women may also acquire rectal infections by this route, but most are due to perianal contamination with cervicovaginal secretions. Rectal infections are often asymptomatic, but some individuals may develop acute proctitis with anorectal pain, a mucopurulent discharge,

bleeding, tenesmus, and constipation 5 to 7 days following infection (82). Anoscopic examination of the anal canal reveals an edematous and erythematous rectal mucosa and a purulent discharge associated with the anal crypts.

In 0.5 to 3% of infected individuals, gonococci may invade the bloodstream, resulting in disseminated gonococcal infection (DGI) (71). Disseminated disease may develop following infection at genital or extragenital sites, and repeated bouts of DGI may occur in individuals with certain complement component deficiencies (i.e., C7, C8, or C9) (40). DGI is characterized by low-grade fever, chills, hemorrhagic skin lesions, tenosynovitis, migratory polyarthralgias, and frank arthritis. Skin lesions are usually painful and appear as papules that evolve into necrotic pustules on an erythematous base. Very few lesions may be present, and most are found on the extremities. In 30 to 40% of cases, organisms from the bloodstream may localize in one or more joints to cause a purulent, destructive gonococcal arthritis (11). Joint involvement is usually asymmetric and commonly involves knee, elbow, wrist, fingers, or ankle joints. Rare complications of DGI include endocarditis and meningitis. Gonococcal endocarditis usually involves the aortic valve and follows a rapid and destructive course (90). Gonococcal meningitis has features typical of meningitides caused by other organisms. Pericarditis, pericardial effusions, and adult respiratory distress syndrome may also complicate gonococcal bacteremia. DGI may also present atypically in those with underlying diseases, including human immunodeficiency virus infection and systemic lupus erythematosus (59).

In addition to gonococcal ophthalmia neonatorum, ocular infections have been reported in adults who become infected via genital secretions (55). Laboratory personnel working with cultures may also become accidentally infected if care is not taken to protect the eyes. Eye infection results in painful periorbital cellulitis, profuse purulent discharge, conjunctival injection, eyelid edema and erythema, and epithelial and stromal keratitis. *N. cinerea* has also been reported to cause conjunctival infections, so it is important to perform confirmatory identification tests on gram-negative, oxidase-positive diplococci recovered from ocular sites (37).

Gonococcal infections in children during the newborn period are the result of ocular contamination during vaginal delivery. However, gonococcal infections beyond the immediate neonatal period are indicators of sexual abuse (3). In female children, *N. gonorrhoeae* causes vaginitis with a discharge, rather than cervicitis, because the epithelium of the prepubertal vagina is composed of columnar epithelial cells, which are the cell types that *N. gonorrhoeae* preferentially infects. With the onset of puberty, these vaginal cells are replaced by stratified squamous epithelium that is not susceptible to gonococcal infection. Urethral infection in male children resembles that seen in adults, and pharyngeal and rectal gonococcal infections, as in adults, are usually asymptomatic in children.

The clinical manifestations of infection with *N. meningitidis* can be highly variable, ranging from transient bacteremia with low-grade fever to fulminant, rapidly fatal disease. In all cases, the meningococcal strain becomes established in the upper respiratory tract and enters the bloodstream to initiate systemic disease. Invasive disease occurs in those who are newly infected with a strain against which bactericidal meningococcal serogroup-specific antibodies are lacking. Concurrent viral or mycoplasmal respiratory tract infections facilitate systemic invasion by the organism. The risk of meningococcal disease is also higher among those with complement component deficiencies (e.g., C5, C6, C7, C8, and C9) (41). Other underlying conditions, such as hepatic failure, systemic lupus erythematosus, multiple myeloma, and asplenia, may also predispose to serious meningococcal disease.

The clinical spectrum of meningococcal disease includes meningoencephalitis, meningitis with or without meningococcemia, meningococcemia without meningitis, and bacteremia without septic complications. These presentations may occur discretely or can blend into one another during clinical disease progression. Classic signs of meningitis (e.g., confusion, headache, fever, and nuchal rigidity) may be seen only in about one-half of the patients, and vomiting may be part of the presentation, particularly in children. Meningococcemia and organism dissemination are heralded by the development of a pink, maculopapular eruption that becomes petechial. Rapidly progressive disease may result in purpuric or ecchymotic skin lesions that are hemorrhagic and necrotic. Fulminant shock may dominate the clinical picture of meningococcal meningitis and acute meningococcal sepsis (100). Gangrenous changes in the extremities may occur due to peripheral vasoconstriction, and death may supervene as a result of disseminated intravascular coagulation. The classic finding of acute hemorrhagic necrosis of the adrenal glands represents the hallmark of the Waterhouse-Friderichsen syndrome (1). Meningococcal meningitis with sepsis may have a mortality rate of up to 30%.

N. meningitidis may also cause acute or chronic meningococcemia without meningitis. Patients present with fever, headache, malaise, and leukocytosis, and meningococci are recovered from blood cultures. By that time, the patient is usually clinically well and no therapy or a short course of therapy is administered. Patients with chronic meningococcemia are usually symptomatic, with a presentation clinically similar to the gonococcal arthritis-dermatitis syndrome. Individuals with deficiencies in late complement components, other hypocomplementeric states (e.g., systemic lupus erythematosus), and human immunodeficiency virus infection are also at risk for serious meningococcal disease (41).

Meningococcal pneumonia occurs infrequently and presents as a community-acquired infection that is indistinguishable clinically from other acute bacterial pneumonias. Pneumonia occurs primarily in older individuals with preexisting pulmonary compromise (102). Diagnosis is complicated by the presence of the organisms in the nasopharynx, resulting in contamination of expectorated sputum specimens. Bacteremic epiglottitis and supraglottitis have also been reported in association with serogroup B, C, and Y meningococcal infections (89). Other infections associated with *N. meningitidis* that result from hematogenous dissemination include osteomyelitis, arthritis, cellulitis, endophthalmitis, and spontaneous bacterial peritonitis.

Meningococcal conjunctivitis has been described in adults, children, and neonates and may develop as a complication of systemic meningococcal disease or as a primary infection (6). Complications limited to the eye include corneal ulcers, keratitis, subconjunctival hemorrhage, endophthalmitis, and iritis. *N. meningitidis* may also be isolated from the male urethra, the female genital tract, and the anal canal. In these sites, meningococci may cause infections that are clinically indistinguishable from gonococcal infections, including acute purulent urethritis, cervicitis, salpingitis, and proctitis (74). Orogenital, anogenital, and oroanal sexual practices are believed to be responsible for the presence of meningococci in these anatomic sites (97).

NEISSERIA GONORRHOEAE

Collection, Transport, and Storage of Specimens

Details of specimen collection are given in chapters 5 and 20.

The collection of specimens for diagnosis of gonococcal infection depends on the sex and sexual practices of the patient and on the clinical presentation. In all cases, specimens from genital sites should be collected. If the patient has a history of orogenital or anogenital sexual contacts, collection of oropharyngeal or anal canal specimens is also appropriate. In cases of suspected DGI, blood cultures and specimens from genital and extragenital sites should be obtained. Appropriate sites for culture are summarized in Table 1.

Specimens should be collected with Dacron or rayon swabs. Calcium alginate may be toxic to gonococci, and some brands of cotton swabs may contain fatty acids that may be inhibitory for gonococci. These swab types should be used only if specimens are inoculated directly onto culture growth media or are transported in nonnutritive swab transport media. Some transport media contain charcoal to inactivate toxic materials present in the swab material or in the specimen itself. Instruments used to facilitate specimen collection (e.g., vaginal specula) should be lubricated with warm water or saline because water- and oil-based lubricants may also inhibit organism growth.

The role of the clinical microbiology laboratory in diagnosing gonococcal infections in children involves the proper handling of appropriately collected specimens and the accurate identification of isolated organisms. For prepubertal females, specimens should be obtained from the vagina or urethra, the oropharynx, and the rectum and inoculated onto media as described below. Vaginal specimens are collected by swabbing the vaginal wall for 10 to 15 s to absorb any secretions, or, if the hymen is intact, the specimen is collected from the vaginal orifice. Specimens for diagnosis of rectal, urethral, and oropharyngeal gonococcal infections in children are collected as for adults.

Maximal recovery of gonococci is obtained when specimens are plated directly onto growth medium after collection. However, this technique might not always be possible or practical and various transport systems are available, as described below.

Nonnutritive Swab Transport Systems

Stuart's or Amies buffered semisolid transport media are used for transport of swab specimens for *N. gonorrhoeae*. Some swab transport systems use sponges soaked with transport medium, while others use a semisolid medium with or without activated charcoal. Studies with newer swab collection devices (Copan Transystems; Copan Diagnostics, Inc., Corona, Calif., now marketed as BBL CultureSwab Plus, BD Biosciences, Sparks, Md.) suggest that semisolid Amies transport medium with or without charcoal may preserve the viability of gonococci for as long as 48 h, although the viability of many gonococcal isolates may decrease noticeably after 24 h. Semisolid transport media are superior to devices that use medium-soaked sponges. Sponge materials in some swab transport systems may contain substances (e.g., sulfur or quaternary ammonium compounds) that may inhibit or injure fastidious organisms such as gonococci. While some recent studies have shown that gonococci may survive refrigeration in some swab transport systems up to 48 h (7), other studies have demonstrated that refrigeration beyond 6 h may result in significant reductions in viable organisms, regardless of the transport system used (39, 56). In order to prevent loss of organism viability, swab specimens submitted in transport medium should not be refrigerated and should be inoculated onto growth medium within 6 h after collection.

Culture Medium Transport Systems

Transport of specimens already inoculated onto culture media presents several advantages over swab transport systems. Commercially available systems include JEMBEC plates containing various selective media (Remel, Inc., Lenexa, Kans.), the Gono-Pak (BD Biosciences), and the InTray GC system (BioMed Diagnostics, Inc., San Jose, Calif.) (16). While the Gono-Pak and JEMBEC products require refrigerated storage, the sealed InTray system permits storage of the medium at room temperature for up to 1 year. Media are inoculated with the specimen from a swab and placed in an impermeable plastic bag with a bicarbonate-citric acid pellet. Contact of the pellet with moisture via evaporation from the medium during incubation or by crushing an ampoule of water adjacent to the pellet generates a CO_2-enriched environment within the bag. Organisms remain viable in the CO_2-enriched environment

TABLE 1 Body sites or specimens and culture media for the isolation of *N. gonorrhoeae* and *N. meningitidis*

Species	Syndrome	Gender	Site(s) or specimen(s)	Media
N. gonorrhoeae	Uncomplicated	Female	Endocervix (Bartholin's glands), rectum,[a] urethra, pharynx[a]	Selective
		Male, heterosexual	Urethra	Selective
		Male, homosexual, bisexual	Urethra, rectum,[a] pharynx[a]	Selective
	PID	Female	Endocervix endometrium,[b] fallopian tubes	Selective, nonselective
	DGI	Female/male	Endocervix, (urethra), urethra (male), skin lesions	Selective, nonselective
			Joint fluid	Nonselective, selective
			Blood	Blood culture medium
	Ophthalmia	Female/male	Conjunctivae	Nonselective
N. meningitidis	Meningitis	Female/male	CSF, skin lesions	Nonselective
			Blood	Blood culture medium
			Nasopharynx	Selective, nonselective

[a] If there is a history of oral-genital or anal-genital exposure.
[b] If a laparoscopic examination is performed.

during transport to a reference laboratory, but incubation for 18 to 24 h at 35°C prior to transport allows some initial outgrowth of the organisms and enhances survival.

Direct Examination of Clinical Specimens

Microscopy

The diagnosis of gonococcal urethritis in men can be made by observing gram-negative diplococci within or closely associated with polymorphonuclear leukocytes (PMNs) on a smear prepared from the urethral discharge (Fig. 1). When properly performed, the Gram stain has a sensitivity of 90 to 95% and a specificity of 95 to 100% for diagnosing genital gonorrhea in symptomatic men. For females, Gram stains of endocervical specimens collected under direct visualization of the cervix may also be helpful in diagnosis (see "Collection, Transport, and Storage of Specimens" above). Gram-stained smears of such specimens have a sensitivity of 50 to 70%, depending on the adequacy of the specimen and the patient population. An endocervical smear showing gram-negative intracellular diplococci, particularly from a woman with other signs and symptoms of gonococcal infection, is highly predictive. For patients with symptomatic proctitis, smears collected under direct visualization through an anoscope may provide a diagnosis for 70 to 80% of such patients, as opposed to blind collection, where Gram-stained smears have a sensitivity of only 40 to 60% (Fig. 2). Because of the presence of other gram-negative coccobacilli and bipolar staining bacilli in rectal and endocervical specimens contaminated with vaginal secretions, care must be taken not to overinterpret smears obtained from these sites. Gram-stained smears have no value in the diagnosis of pharyngeal gonococcal infection. Gram-stained smears should not be relied upon for diagnosis of gonorrhea and should be used adjunctively along with more specific tests.

Smears for Gram stain should be prepared from urethral and endocervical sites and should be collected with a swab other than that used for the collection of specimen for culture. The swab is rolled gently over the surface of a glass slide in one direction only to minimize distortion of PMNs and preserve the characteristic appearance of the microorganisms. Gram-stained smears from males with urethral discharge usually show moderate amounts to many PMNs with two or more gram-negative intracellular diplococci (Fig. 1). Smears prepared from specimens submitted in transport media may be difficult to interpret due to distortion of the PMNs or to interfering substances (e.g., charcoal). Smears from normally sterile or minimally contaminated sites (e.g., joint fluid) should also be prepared.

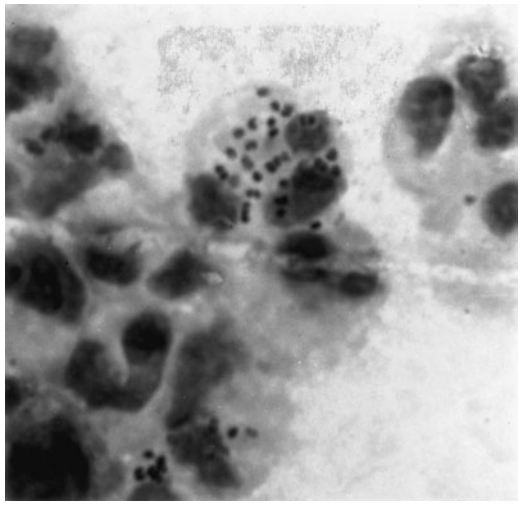


FIGURE 1 Gram stain of male urethral exudate. Some PMNs contain many diplococci; others contain none. Magnification, ×1,500.

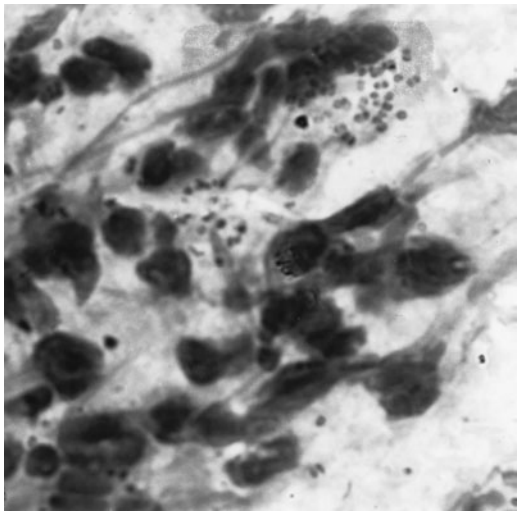


FIGURE 2 Gram stain of mucopurulent rectal exudate showing many diplococci inside PMNs. Magnification, ×1,500.

Antigen Detection

The only commercially available antigen detection test available for diagnosis of gonorrhea is the Binax NOW Gonorrhea Test (Binax, Inc., Portland, Maine). This immunochromatographic strip-based assay can be performed with male urine specimens and both vaginal and endocervical specimens. The test requires no additional materials and takes about 30 min to perform. In a comparative evaluation of the Binax NOW test performed on voided urine with conventional culture of urethral swab specimens from males, the NOW test had a sensitivity of 94.1% and a specificity of 95.8%, with corresponding positive and negative predictive values of 96.9% and 92.0%, respectively (92). The NOW test was shown to have a lower limit of detection of about 1×10^4 CFU of gonococci per ml of urine.

Nucleic Acid Detection Techniques

Nucleic acid detection techniques allow direct detection of *N. gonorrhoeae* in clinical samples and do not require viable organisms. Assays often allow the concurrent detection of *Chlamydia trachomatis*. For *N. gonorrhoeae,* these assays may be divided into three types: (i) direct probe hybridization to the target nucleic acid with direct detection of the hybrid; (ii) nucleic acid amplification tests (NAATs); and (iii) amplified-signal probe tests, which hybridize with nucleic acid and then amplify the signal of the probe. The direct probe tests only slightly increase the sensitivity of culture with a proficient specimen transport system, but the NAATs can appreciably increase sensitivity. The advantages of the tests are that specimens may be transported from geographically distant areas and stored for several days prior to testing in the laboratory. These samples can be maintained frozen prior to testing. The NAATs also allow use of noninvasive specimen types such as urine and vaginal swabs. The disadvantages of using nonculture nucleic

acid probe or amplification tests include unavailability of a viable isolate for antimicrobial susceptibility testing and the possibility of a positive test after treatment, since nucleic acids from organisms may persist for a period of time after successful therapy. One report noted that gonococcal DNA for the ligase chain reaction test remained for a mean of 1.7 days in male urine and 2.8 days in female urine, but 2.8 days in vaginal specimens (8). In contrast, chlamydia DNA may remain detectable for about 2 weeks and the recommended time for retesting is 3 weeks (43). Thus, amplification tests should not be used immediately to assess the success of therapy. Results of probe tests and NAATs may be used only to make a presumptive diagnosis of gonococcal infection and are inadmissible evidence in medicolegal cases in the United States at this time.

Direct Probe Hybridization

Two direct nucleic acid probe assays, the Gen-Probe PACE 2 and PACE 2C assays (Gen-Probe Inc., San Diego, Calif.), are approved by the Food and Drug Administration (FDA) for detecting *N. gonorrhoeae*. In the Gen-Probe tests, an acridinium ester-labeled DNA probe for a specific sequence of *N. gonorrhoeae* rRNA is allowed to hybridize with any complementary rRNA in the specimen (65). An acridinium ester hybridization protection assay detects any hybrids, and chemiluminescence generated by the acridinium ester in the hybrids is detected with a luminometer, resulting in a numerical readout. The PACE 2C test detects both *N. gonorrhoeae* and *C. trachomatis* in a single test. A probe competition assay may also be used to augment the specificity of the test. Compared to culture as the "gold standard," reported sensitivity ranged from 90.8 to 96.3% for women and 99.1 to 99.6% for men in the manufacturer's package insert, while specificity ranged from 97.5 to 100% for men and women. Evaluations of the assay in public health settings have supported the high sensitivity and specificity of the test (52).

NAATs

NAATs are designed to amplify *N. gonorrhoeae*-specific nucleic acid sequences from a particular gene target with up to a billion-fold amplification of as little as theoretically a single copy of the target nucleic acid, either DNA or RNA, depending on the specific assay. Currently, there are four commercially available NAATs for the detection of *N. gonorrhoeae:* one based on PCR, Roche AMPLICOR (Roche Molecular Diagnostics, Indianapolis, Ind.); one based on strand displacement amplification (BDProbeTec; Becton Dickinson); and two based on transcription-mediated amplification: APTIMA Combo2, which is for dual detection of gonorrhea and chlamydia, and APTIMA AGC, which is available for detection of only gonorrhea (both from GenProbe) (44, 70, 99). The AGC assay is presently available as an analyte-specific reagent.

The ligase chain reaction-based assay Abbott LCx (Abbott Laboratories Inc., Abbott Park, Ill.), which was commercially available and used by many laboratories, is no longer available due to manufacturing problems.

PCR for *N. gonorrhoeae* detects a 201-bp sequence in the cytosine methyltransferase gene. Although this assay has been used with sensitivity well above 90% for the detection of gonorrhea agents in cervical specimens, the clinical trial did not achieve a high enough sensitivity (64.8%) for the detection of gonorrhea in urine samples from women for FDA clearance, although it is highly accurate with male urine (Table 2) (70). PCR has been shown to detect gonorrhea in male urine with great accuracy with a sensitivity of 94.1% and a specificity of 99.9% in 1,291 symptomatic men, having a sensitivity which is somewhat lower (73.1%) in 721 asymptomatic men (70). It has also been used quite successfully in research settings with self-administered vaginal swabs from women, although it is not FDA cleared for use with vaginal swabs (101). Recently, this assay has been reported to have false-positive results caused by nonpathogenic *Neisseria* spp., such as *N. subflava*, *N. flavescens, N. lactamica,* and *N. cinerea* (77). These false-positive assays have been thought to be due to the interspecies genetic recombination potential that occurs among *Neisseria* spp., thus indicating the need for confirmation of positive PCR assays in extragenital specimens and those from low prevalence populations (77).

TABLE 2 Nucleic acid amplification tests for *Neisseria gonorrhoeae*[a]

Assay and specimen	Sensitivity (%)	Specificity (%)
PCR(COBAS)		
Cervical	92.4	99.5
Female urine	64.8	99.8
Male urine	94.1	99.9
Strand displacement amplification		
Cervical	96.6	98.9–99.8
Female urine	84.9	98.8–99.8
Male urine	98.1	96.8–98.7
Male urethral	98.1	96.8–98.7
Transcription-mediated amplification		
Cervical	99.2	98.7
Female urine	91.3	99.3
Male urine	97.1	99.2
Male urethral	98.8	98.2

[a] Percentages indicate comparisons with infected patient status; data from package inserts and clinical trials.

The strand displacement amplification assay (ProbeTec) is approved for detection of gonorrhea in cervical, male urethral, and female and male urine samples and has achieved widespread use in clinical laboratories throughout the United States and Europe (Table 2) (99). The BD ProbeTec ET system has very high sensitivities for gonorrhea in cervical and male urethral specimens and has demonstrated a sensitivity of 97.9% for the detection of gonorrhea in male urine in 680 patients, but it has a somewhat lower sensitivity in female urine (84.9%) (99). This assay has also been demonstrated to produce false positives with nonpathogenic *Neisseria* spp. (77).

TMA (APTIMA Combo2) has been shown to be perhaps the most sensitive assay for gonorrhea (44). It has sensitivities uniformly above 97% for cervical and male urine and urethral samples and well above 91% for female urine (Table 2). This assay has not been shown to produce false-positive results with nonpathogenic neisseriae (49).

Amplified-Signal Probe Test

The amplified-signal probe test employs hybridization of a probe with nucleic acid of the organism and then amplifies the signal of the probe. It is represented by the Digene Hybrid Capture II test (Digene, Silver Spring, Md.), which employs RNA hybridization probes which are specific for both genomic DNA and cryptic plasmid DNA sequences of *N. gonorrhoeae* and *C. trachomatis* (72). The RNA-DNA hybrids are captured in microtiter plate wells by specific antibodies, which are detected by alkaline phosphatase-labeled anti-RNA-DNA

hybrid antibodies. The signal is amplified using a chemiluminescent substrate detected by a luminometer. The test is positive if either chlamydia or gonorrhea is present, and then an organism-specific test is performed. It is approved only for cervical samples.

One study reported that the Digene assay was 92.2% sensitive and that the specificity was greater than 99% compared to culture adjudicated by direct fluorescent-antibody (DFA) staining and PCR (32). In another evaluation, compared to *N. gonorrhoeae* culture, it had a sensitivity of 93% (87/94) and a specificity of 98.5% (1,244/1,263) (87).

Additional Considerations for NAATs

Confirmation Tests for NAATs

Because of the potential for false-positive tests in low-prevalence populations, the Centers for Disease Control and Prevention (CDC) has suggested that confirmatory tests should be performed for patients testing positive from populations with a positive predictive value of <90% (26). Suggestions for confirmation include testing a second specimen with a different test using a different target; testing the original specimen with a different test that uses a different target or format; repeating the original test on the original specimen with a blocking antibody or competitive probe; or repeating the original test on the original specimen (26). The requirement for confirmatory testing for positive NAAT results is controversial, and analysis of the particular population under study will influence the decision by a laboratory as to whether to confirm these assays (86). The particular assay in use also plays a role in this decision (49, 86). At present, the only commercial assay with the capability to confirm a positive test with another gene target using the same platform is the Gen-Probe APTIMA assay, with the ACT and AGC stand-alone assays, which can be used to confirm either chlamydia or gonorrhea tests, respectively. These assays are also the only NAATs that are FDA cleared for use with vaginal swabs at present.

Noninvasive Sample Collection and Inhibitors

The ability to detect *N. gonorrhoeae* in noninvasive urine and vaginal samples, avoiding the necessity to obtain an endocervical specimen from women or a urethral swab from men, is an important advantage of NAATs over tests which require invasive specimen collection procedures. However, some specimens contain inherent inhibitors which may result in false-negative results by NAATs. The use of amplification controls in commercial NAATs can indicate when a specimen contains inhibitors by failure of the nonspecific amplification control to be amplified. When inhibition occurs, steps such as heating or dilution can be performed and the test can then be repeated. A discussion of inhibitors in amplification assays can be found on the Association of Public Health Laboratories website (http://www.aphl.org/chlamydia_lab.cfm).

Choice of NAAT and Cost Considerations

Amplified assays are more costly than culture and other detection methods. They have become widely used in clinical microbiology laboratories because of the ability to also test the sample for chlamydia and the ability to test "noninvasive" samples such as urine, as well as the convenience of transport of the sample. Factors that influence whether to choose to perform amplified testing for *N. gonorrhoeae,* as well as which test to choose, include the level of expertise in the laboratory, consideration of costs, training and equipment issues, the population being served, and whether transportation of specimens for culture is a problem. Also a consideration is whether isolates are required for susceptibility testing. The choice of which NAAT to use will also be based on sensitivity and specificity estimates, specimen types being tested (i.e., vaginal, urethral, cervical, or urine), testing that also includes chlamydia, and whether the laboratory wishes to confirm positive NAAT samples (45, 86).

Isolation Procedures

Various selective media allow recovery of *N. gonorrhoeae* from body sites harboring an endogenous bacterial flora. Enriched selective media include modified Thayer-Martin (MTM) medium, Martin-Lewis (ML) medium, GC-Lect medium (BD Biosciences), and New York City (NYC) medium. MTM, ML, and GC-Lect are chocolate agar-based media that are supplemented with additional growth factors, whereas NYC medium is a clear peptone-corn starch agar-based medium containing yeast dialysate, citrated horse plasma, and lysed horse erythrocytes. These media contain antimicrobial agents that inhibit other microorganisms and allow the selective recovery of *N. gonorrhoeae*, *N. meningitidis*, and *N. lactamica*. Vancomycin and colistin, antimicrobials present in all four formulations, inhibit gram-positive and gram-negative bacteria (including saprophytic *Neisseria* species), respectively. Trimethoprim is added to inhibit the swarming of *Proteus* spp. present in rectal and, occasionally, in cervicovaginal specimens. Nystatin (MTM medium), amphotericin B (NYC and GC-Lect media), or anisomycin (ML medium) is added to inhibit yeasts and molds. NYC medium also supports the growth of rapidly growing genital mycoplasmas and ureaplasmas. Media are available in either petri dishes or JEMBEC plates.

Media for the pathogenic *Neisseria* should be at room temperature before inoculation and should not be excessively dry or moist. Swab specimens are rolled in a "Z" pattern on selective medium and cross-streaked with a bacteriologic loop. If nonselective chocolate agar is also inoculated, these plates are streaked for isolation. Plates are incubated in a CO_2 incubator or a candle extinction jar at 35 to 37°C. The CO_2 level of the incubator should be 3 to 7%; higher CO_2 concentrations may inhibit growth of some strains. The atmosphere should be moist; with candle jars, moisture evaporating from the medium during incubation is usually sufficient for organism growth. If candle jars are used, candles should be made of white wax or beeswax; scented or colored candles release volatile products that may inhibit organism growth. CO_2 incubators that are not equipped with humidifiers can be kept moist by placing a pan of water on the lower shelf. Plates are inspected at 24, 48, and 72 h before a final report of "no growth" is issued. Suspect colonies are subcultured to chocolate agar, incubated, and used as inocula for identification procedures.

Presumptive Identification

Colony Morphology

Gonococci produce several colony types that are related to piliation of organisms in the colony. Typical colonies tend to be small (about 0.5 mm in diameter), glistening, and raised. With subculture of individual piliated colonies, the culture can be maintained in this colonial type. With nonselective subculture (i.e., a "sweep" of growth), the other colony types become evident, with all colonies eventually becoming the nonpiliated varieties. These colonies are larger (about 1 mm in diameter) and flatter and do not have the high profile and glistening highlights of piliated colony types. The presence of multiple colony types on a subculture from a primary plate may give the appearance of a mixed culture. Careful scrutiny and subculture with the use of a dissecting microscope (10×)

enable one to become familiar with these colony types. Some *N. gonorrhoeae* strains grow on commercially available sheep blood agar, although growth takes longer and is not as luxuriant as on chocolate agar. However, other strains do not grow on sheep blood agar at all.

Gram Stain and Oxidase Test

Smears prepared from suspicious colonies should be examined with the Gram stain. The Gram stain of organisms from colonial growth should show uniform, characteristic gram-negative diplococci. Some of the organisms may appear as tetrads, particularly on smears prepared from young colonies. Organisms on smears prepared from older cultures may appear swollen and variable in counterstaining intensity, while smears prepared from partially autolyzed colonies may be uninterpretable. Examination by Gram stain is essential for presumptive identification because other organisms may occasionally grow on selective media, particularly when oropharyngeal specimens are cultured (discussed below).

Oxidase test results are obtained with the tetramethyl derivative of the oxidase reagent (*N,N,N,N*-tetramethyl-1,4-phenylenediamine, 1% aqueous solution). A drop of this reagent is placed on a piece of filter paper and a portion of colonial growth is rubbed onto the reagent with a platinum loop, a cotton swab, or a wooden applicator stick. With fresh cultures, a dark purple color will appear on the filter paper within 10 s. Excellent results are obtained with the oxidase reagents that are packaged in crushable glass ampoules (e.g., BACTIDROP Oxidase; Remel, Inc.).

Superoxol Test

Superoxol (30% hydrogen peroxide) is another helpful test for rapid presumptive identification of *N. gonorrhoeae* (84). *N. gonorrhoeae* strains produce immediate, brisk bubbling when the colony material is emulsified with the reagent on a glass slide. Both *N. meningitidis* and *N. lactamica*, the other species that grow on selective media, generally produce weak, delayed bubbling, although some isolates of *N. meningitidis* may also produce immediate, brisk bubbling similar to the gonococcus in this test. Isolates of oxidase-positive, gram-negative diplococci that are recovered from urogenital sites and that grow on selective media may be presumptively identified as *N. gonorrhoeae*. The superoxol test provides an additional presumptive test for identifying these isolates. Confirmatory identification tests are recommended for all isolates and are required for identification of isolates from extragenital sites (i.e., throat, rectum, blood, joint fluid, and cerebrospinal fluid [CSF]).

Differentiation of Other Organisms on Selective Media

Presumptive and confirmatory identification of *N. gonorrhoeae* depends on the ability to differentiate this organism from others that may also grow on selective media. These organisms include *Kingella denitrificans*, *Moraxella catarrhalis*, other *Moraxella* species, *Acinetobacter* species, and *Capnocytophaga* species. *K. denitrificans* grows on MTM medium and produces colony types resembling those of *N. gonorrhoeae*. Whereas gonococci produce vigorous bubbling when colonial growth is immersed in 3% H_2O_2 or in superoxol, *K. denitrificans* produces a negative catalase reaction. *Moraxella* species are both oxidase positive and catalase positive; these organisms can be differentiated from *Neisseria* by the penicillin disk test (21). The organism is subcultured to a Trypticase-soy blood agar plate and streaked to obtain confluent growth. A penicillin susceptibility disk (10 U) is placed on the inoculum, and after overnight incubation in CO_2, a Gram stain is prepared from growth at the edge of the zone of inhibition. *Neisseria* species and *M. catarrhalis* retain their diplococcal morphology, although the cells may appear to be swollen (Fig. 3). Coccobacillary *Moraxella* species and *K. denitrificans* form filaments or spindle-shaped cells under the influence of subinhibitory concentrations of penicillin. *Acinetobacter* spp. can exhibit diplococcal morphology, but these organisms are oxidase negative. *Capnocytophaga* species appear as gram-negative, slightly curved, fusiform bacteria and are oxidase negative and catalase negative. *Capnocytophaga* spp. tend to spread over the agar surface due to gliding motility and may impede recovery of gonococci from oropharyngeal specimens incubated longer than 48 h.

Confirmatory Identification Tests for *Neisseria* Species

Confirmatory tests for *N. gonorrhoeae*, *N. meningitidis*, and other *Neisseria* species include carbohydrate acidification

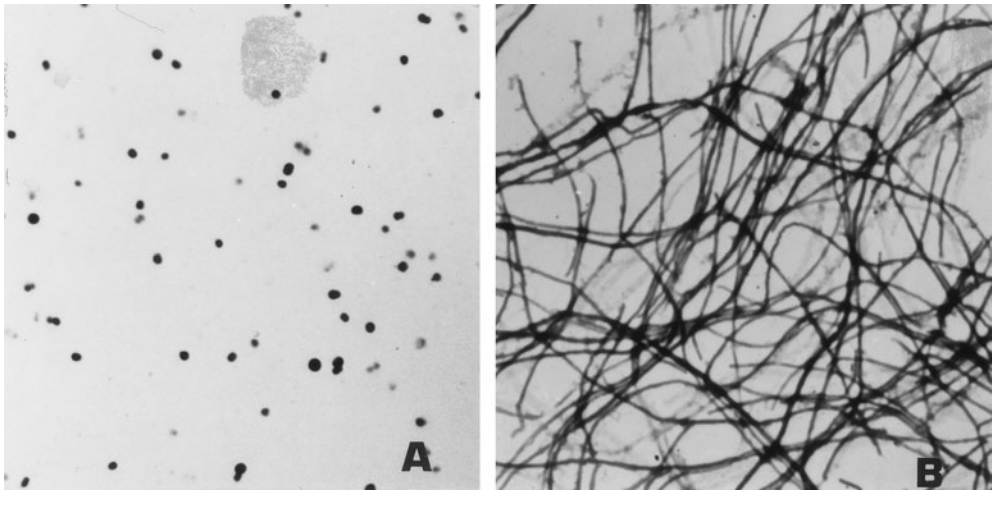


FIGURE 3 Cocci (A) and bacilli (B) exposed to subinhibitory concentrations of penicillin. Some cocci are swollen but still coccoid; bacilli form long strings. Magnification, ×1,000.

tests, chromogenic enzyme substrate tests, immunologic tests (e.g., fluorescent antibody or staphylococcal coagglutination), multitest identification systems, and DNA probe tests. Carbohydrate acidification tests and the multitest identification systems can be used to identify *N. gonorrhoeae*, *N. meningitidis*, and other *Neisseria* species (Table 3). Chromogenic substrate identification procedures are limited to those isolates that grow on selective media (i.e., *N. gonorrhoeae*, *N. meningitidis*, *N. lactamica*, and some strains of *M. catarrhalis*). Fluorescent-antibody, coagglutination, and other immunologic tests and the DNA probe culture confirmation test are available for identification of *N. gonorrhoeae* only. Most nucleic acid hybridization and NAAT procedures are approved for direct detection of *N. gonorrhoeae* in genital tract and urine specimens only.

Acid Production from Carbohydrates

Conventional CTA Carbohydrates

The traditional technique for identification of *Neisseria* species employs cystine-tryptic digest semisolid agar-base (CTA) medium containing 1% carbohydrate and a phenol red pH indicator (2). The usual test battery includes CTA-glucose, -maltose, -sucrose, and -lactose, plus a carbohydrate-free CTA control. The lactose structural analogue, *o*-nitrophenyl-β-D-galactopyranoside (ONPG), may be substituted for the lactose tube, and the addition of CTA-fructose is helpful for identifying the *N. subflava* biovars. Some CTA formulations may be supplemented with ascitic fluid to support growth of more fastidious strains. CTA media are inoculated with a dense suspension of the organism from a pure 18- to 24-h culture on chocolate agar. Either the inoculum is prepared in 0.5 ml of saline and divided among the tubes, or each tube is individually inoculated with a loopful of the organism. The inoculum is restricted to the top 0.5 in. of the agar-deep tubes. The tubes are incubated in a non-CO_2 incubator at 35°C with the caps tightened firmly. With a heavy inoculum, many isolates produce changes in the color of the phenol red indicator within 24 h. Some strains may change the indicator within 4 h, while other gonococcal strains may require 24 to 72 h to produce sufficient acid to change the indicator. Because CTA media containing 1% carbohydrate are used primarily for detection of acid by fermentative organisms, the small amounts of acid produced oxidatively by some strains of *Neisseria* species may not be detected. This method may be problematic for differentiating *N. gonorrhoeae* and *N. cinerea*. Consequently, it is no longer favored for the detection of acid production from carbohydrates. Table 3 shows the carbohydrate acidification profiles and other useful tests for the identification of *Neisseria* spp. recovered from humans.

Rapid Carbohydrate Tests

The rapid carbohydrate test is a non-growth-dependent method for detection of acid production from carbohydrates by *Neisseria* species. Small volumes (0.10 ml) of a balanced salts solution (0.04 g of K_2HPO_4 per liter, 0.01 g of KH_2PO_4 per liter, and 0.80 g of KCl per liter, pH 7.0) with phenol red indicator (0.5 ml of a 1% aqueous solution/liter) are dispensed in nonsterile tubes to which single drops of 20% filter-sterilized aqueous carbohydrates are added. A dense suspension of the organism is prepared in balanced salts solution, and 1 drop of this suspension is added to each of the carbohydrate-containing tubes. Tubes are incubated for 4 h at 35°C in a non-CO_2 incubator or a water bath. This method is economical, the reagents are easy to prepare and inoculate, and the results are clear-cut. The key to this technique is the use of reagent grade carbohydrates. Maltose from some bacteriologic media companies may produce positive or equivocal results for *N. gonorrhoeae* in this procedure, presumably due to the presence of contaminant glucose. Inocula may be obtained from the primary culture if sufficient colonies are present and if the growth is less than 24 h old. Since growth does not occur in the test medium, small numbers of contaminants do not interfere with the 4-h result. However, incubation cannot be continued overnight. The RIM-*Neisseria* Test (Rapid Identification Method-Neisseria; Remel, Inc.), the Gonobio-Test (I.A.F. Production, Inc., Laval, Quebec, Canada), and the Neisseria-Kwik test (MicroBio Logics, St. Cloud, Minn.) are commercial modifications of the rapid carbohydrate utilization test, and evaluations have reported good agreement with conventional methods, although these tests may not provide adequate differentiation between *N. gonorrhoeae* and *N. cinerea* (35, 36).

Chromogenic Enzyme Substrate Tests

Enzymatic identification systems use specific substrates that, after hydrolysis by bacterial enzymes, yield a colored end product that is detected directly or after the addition of a diazo dye-coupling reagent. These tests are used to identify species that are able to grow on selective media—*N. gonorrhoeae*, *N. meningitidis*, and *N. lactamica*. Because some *M. catarrhalis* strains grow on selective media, these systems also provide a presumptive identification of this organism as well. Chromogenic substrate identification tests should not be used to identify organisms recovered on blood and/or chocolate agar without prior subculture of the isolate to selective media. Enzymatic activities that are detected in these systems include β-galactosidase, γ-glutamylaminopeptidase, and prolyl-hydroxyprolyl aminopeptidase. β-Galactosidase and γ-glutamylaminopeptidase are specific for *N. lactamica* and *N. meningitidis*, respectively. The absence of these activities and the presence of prolyl-hydroxyprolyl aminopeptidase identify an organism as *N. gonorrhoeae*. Some *N. meningitidis* strains produce both γ-glutamyl aminopeptidase and prolyl-hydroxyprolyl aminopeptidase. *M. catarrhalis* lacks all three of these enzymatic activities. The Gonochek II (EY Labs, San Mateo, Calif.) is a commercial system that detects all three enzyme activities in a single tube (35). The BactiCard *Neisseria* (Remel, Inc.) uses filter paper pads that are impregnated with substrates for the three enzymes, plus an indoxyl butyrate substrate for identification of *M. catarrhalis* (62). The interpretive guidelines for these tests do not allow for the fact that some nongonococcal neisseria isolates (e.g., *N. subflava* bv. perflava) may be isolated on gonococcal selective media. These species are also prolyl-hydroxyprolyl aminopeptidase positive and may be misidentified as *N. gonorrhoeae* if additional tests are not performed. Recently, gonococcal strains lacking prolyl-hydroxyprolyl aminopeptidase have been isolated in Denmark and the United Kingdom (2, 42).

Immunologic Methods for Culture Confirmation

Direct Fluorescent Monoclonal Antibody Test

The DFA culture confirmation procedure uses monoclonal antibodies that recognize epitopes on the PorI (Protein I) outer membrane protein (OMP) of *N. gonorrhoeae*. The DFA test (*Neisseria gonorrhoeae* Culture Confirmation Test; Trinity Biotech, Wicklow, Ireland) is performed by preparing a smear on a DFA slide, overlaying the smear with DFA reagent, and incubating the smear for 15 min. After rinsing and mounting, the slide is examined with a fluorescence microscope. Gonococci appear as apple-green fluorescent

TABLE 3 Characteristics of *Neisseria* species of human origin[a]

Species	Colony morphology on chocolate agar	Growth on:			Acid from:					Reduction of NO_3	Polysaccharide from SUC
		MTM, ML, and NYC media (35°C)	Chocolate or blood agar (22°C)	Nutrient agar (35°C)	GLU	MAL	LAC	SUC	FRU		
N. gonorrhoeae[b]	Beige to gray-brown, translucent, smooth, 0.5–1 mm in diameter	+	0	0	+	0	0	0	0	0	0
N. meningitidis	Beige to gray-brown, translucent, smooth, 1–3 mm in diameter	+	0	V	+	+	0	0	0	0	0
N. lactamica	Beige to gray-brown, translucent, smooth, 1–2 mm in diameter	+	V	+	+	+	+	0	0	0	0
N. cinerea[c]	Beige to gray-brown to yellowish, translucent, smooth, 1–2 mm in diameter	V	0	+	0	0	0	0	0	0	0
N. polysaccharea	Beige to gray-brown to yellow, translucent, smooth, 1–2 mm in diameter	V	+	+	+	+	0	V	0	0	+
N. subflava[d]	Greenish, yellow, opaque, 1–3 mm in diameter, smooth to rough, sometimes adherent	V	+	+	+	+	0	V	V	0	V
N. sicca	White, opaque, 1–3 mm in diameter, adherent, wrinkled with age	0	+	+	+	+	0	+	+	0	+
N. mucosa	Greenish yellow, opaque, 1–3 mm in diameter	0	+	+	+	+	0	+	+	+	+
N. flavescens	Yellow, opaque, 1–2 mm in diameter	0	+	+	0	0	0	0	0	0	+
N. elongata[e]	Gray-brown, translucent, smooth, 1–2 mm in diameter, glistening, dry, claylike consistency	0	+	+	0	0	0	0	0	0	0

[a] Symbols and abbreviations: +, strains typically positive but genetic mutants may be negative; V, strain dependent; 0, negative; GLU, glucose; MAL, maltose; LAC, lactose; SUC, sucrose; FRU, fructose.

[b] *N. kochii* is considered to be a subspecies of *N. gonorrhoeae;* isolates of *N. kochii* exhibit characteristics of both *N. gonorrhoeae* and *N. meningitidis*, but are identified as *N. gonorrhoeae* by tests routinely used for identification of *Neisseria* spp.

[c] Some strains grow on selective media.

[d] Includes biovars subflava, flava, and perflava. *N. subflava* bv. perflava strains produce acid from sucrose and fructose and produce polysaccharide from sucrose; *N. subflava* bv. flava strains produce acid from fructose; *N. subflava* bv. subflava strains do not produce polysaccharide from sucrose.

[e] Rod-shaped organism. The catalase test is weakly positive or negative compared with those of other *Neisseria* spp. (catalase positive). Results in the table are for *N. elongata* subsp. *elongata*. Strains of *N. elongata* subsp. *glycolytica* may produce a weak acid reaction from D-glucose, are catalase positive, and do not reduce nitrate but do reduce nitrite. Strains of *N. elongata* subsp. *nitroreducens* (formerly CDC group M-6) may produce a weak acid reaction from D-glucose, are catalase negative, and reduce both nitrate and nitrite.

diplococci. The currently available kit is the same product that was developed and marketed by Syva in 1986. At that time, the DFA reagent was highly sensitive and specific; however, at present, many strains of *N. gonorrhoeae* fail to stain with this reagent (63). Serotyping and pulsed-field gel electrophoresis (PFGE) data indicate that gonococcal strains that are negative with the DFA reagent belong to a variety of serovars (17, 63). Since the monoclonal antibody cocktail used in this product has not been modified since 1986, package insert claims of 99.6% sensitivity and 100% specificity are no longer valid. Therefore, isolates that do not stain with the DFA reagent must be identified by another method. This limits the advantages of the DFA procedure, which included its rapidity, ability to test colonies directly from primary cultures, and the small amount of growth required for test performance. The DFA test is not intended for direct identification of organisms in smears from patient specimens.

Coagglutination Tests

Two coagglutination tests for the identification of *N. gonorrhoeae* are currently available: the Phadebact Monoclonal GC test (Boule Diagnostics AB, Huddinge, Sweden) and the GonoGen I (New Horizons Diagnostics, Columbia, Md.). The Phadebact Monoclonal GC test uses anti-PorI monoclonal antibodies bound to staphylococcal cells. Unlike the GC OMNI test previously marketed by Boule Diagnostics, this monoclonal GC test contains one reagent that reacts with serogroup WI *N. gonorrhoeae* strains and a second reagent that reacts with serogroup WII/WIII strains. Since a negative control reagent is not included, gonococcal isolates react with either the WI or the WII/WIII reagent, depending on the PorI epitopes expressed by an individual isolate. A suspension (0.5 McFarland standard) prepared in buffered saline (pH 7.2 to 7.4) is boiled and mixed with the two test reagents on a cardboard slide. Agglutination within 1 min is a positive test. Freshly subcultured serogroup WI (ATCC 19424) and serogroup WII/III (ATCC 23051) strains are recommended for quality control but are not provided with the kit. The GonoGen I coagglutination test also uses staphylococcal cells coated with anti-PorI monoclonal antibodies. This test kit contains test and control coagglutination reagents, and positive and negative test control suspensions are also included. GonoGen I also uses a boiled organism suspension (McFarland standard of 3) for testing, and agglutination with the test but not the control reagent constitutes a positive test. Attention to procedural details is necessary to prevent false-positive and false-negative results with these coagglutination tests. Some gonococci may not react with these reagents, and cross-reactions with other *Neisseria* species (i.e., *N. meningitidis*, *N. lactamica*, and *N. cinerea*) and *K. denitrificans* have been reported (2, 35, 63).

GonoGen II

GonoGen II (New Horizons Diagnostics) uses anti-PorI monoclonal antibodies conjugated to red-colored metal sol particles as the detection reagent. Colonies from agar medium are suspended in a solubilizing buffer that releases PorI-antigen-containing complexes from the cell wall. A drop of the antibody-sol particles is added, and the PorI antigen binds to the antibody-sol particles. After 5 min, 2 drops of this mixture are passed through a membrane filter that retains antigen-antibody complexes. Concentration of these complexes on the filter turns the filter red, identifying the organism as *N. gonorrhoeae*. Nongonococcal isolates do not produce these, so the entire suspension passes through the filter, resulting in the filter remaining white or pale pink. *N. gonorrhoeae* strains that do not react with the conjugate are not identified, and false-positive reactions have been noted with some *N. meningitidis* and *N. lactamica* strains (3, 63).

Multitest Identification Systems

Kit systems that identify *Neisseria* spp., *Haemophilus* spp., and other fastidious gram-negative organisms are available. These systems are the RapID NH *(Neisseria-Haemophilus)* (Remel, Inc.), the Vitek NHI (*Neisseria-Haemophilus* Identification) card (bioMerieux, Inc., Hazelwood, Mo.), the *Haemophilus-Neisseria* identification (HNID) panel (Dade Behring, Sacramento, Calif.), and the API NH (bioMerieux, Inc., La Balme-les-Grottes, France) (2, 10, 60, 61). These kits use modified conventional tests and chromogenic substrates to provide identifications within 2 to 4 h. The NHI card identifies the pathogenic *Neisseria* spp., *N. lactamica*, and *N. cinerea* (61). At present, a new NHI card is being developed for use with the Vitek-2 instrument. *N. cinerea* is not included in the database of the MicroScan HNID panel, resulting in misidentifications as *N. gonorrhoeae* or *M. catarrhalis*, and some *N. meningitidis* strains do not produce clear-cut reactions with key tests (60). RapID NH includes tests that enable identification of the pathogenic *Neisseria* and *N. cinerea* (2). The API NH system identifies gonococci, meningococci, and *N. lactamica* within 2 h; other *Neisseria* species required additional tests for correct species identification (2, 10).

DNA Probe Test for Culture Confirmation

The Accuprobe *Neisseria gonorrhoeae* Culture Confirmation Test (Gen Probe) identifies *N. gonorrhoeae* by the detection of species-specific rRNA sequences. Organisms from agar medium are lysed and mixed with a chemiluminescent acridinium ester-labeled single-stranded DNA probe that is complementary to gonococcal rRNA. DNA probe/rRNA hybrids are selected by a chemical process, and the presence of the probe is detected by hydrolysis of the acridinium ester and the consequent release of light energy. This energy is detected by a chemiluminometer and reported in relative light units. The Accuprobe test is more sensitive and specific than biochemical or immunologic culture confirmation tests and is particularly useful for confirming problem isolates (63).

NAATs

Theoretically, any of the FDA-cleared NAATs could be used to confirm the identification of an isolate from a culture. However, this particular use of the tests is not cleared by the FDA.

Typing Systems

Phenotypic and genotypic typing methods are used to differentiate between strains of *N. gonorrhoeae*. The specific characteristics chosen depend on the question(s) being asked. Antimicrobial resistance is frequently the subject of investigation or surveillance. Using the CLSI-approved method, susceptibilities to several agents are determined (i.e., penicillin, tetracycline, spectinomycin, an extended-spectrum cephalosporin [ceftriaxone or cefixime], a fluoroquinolone [ciprofloxacin or ofloxacin], and a macrolide [erythromycin or azithromycin]), and results are interpreted according to CLSI breakpoints (31, 73). Gonococcal strains are usually described by their pattern of penicillin-tetracycline susceptibilities (penicillin-tetracycline resistance phenotype) (79). Penicillin-tetracycline resistance phenotypes include penicillin resistant (PenR), tetracycline resistant (TetR), chromosomally mediated resistance to penicillin and tetracycline (CMRNG), β-lactamase-positive (PPNG), plasmid-mediated resistance to

tetracycline (TRNG), strains with plasmid-mediated resistance to both penicillin and tetracycline (PP/TR), and isolates susceptible to both penicillin and tetracycline (Susc.) (104). PPNG and TRNG isolates may exhibit chromosomally mediated resistance to tetracycline (PPNG, TetR) and penicillin (TRNG, PenR), respectively. Intermediate resistance or resistance to other antimicrobial agents is appended to the penicillin-tetracycline resistance phenotype. For example, a PPNG isolate exhibiting resistance to ciprofloxacin (CipR) would be designated PPNG, CipR.

Phenotypic characterization also includes the determination of requirements for growth using a chemically defined medium (auxotyping) and serotyping with a panel of 12 monoclonal antibodies in coagglutination tests to define serovars (85). Strains have been classified with a dual auxotype-serovar system to provide greater discrimination among strains than is possible with either typing system alone. Auxotyping involves determining growth requirements of individual strains for discrete metabolites on a chemically defined medium. For example, if a strain does not grow on a medium from which arginine has been omitted, the strain is recorded as requiring arginine. Strains with single or multiple growth requirements may be identified. Serotyping is performed with a panel of monoclonal antibody coagglutination reagents directed against epitopes on the PorI (protein I) molecule in the gonococcal outer membrane (85). Strains are divided into two major serogroups, serogroups IA and IB, based on the protein I species expressed by the strain; subdivision into serovars is then made according to the reactions observed with a panel of six protein IA- and six protein IB-specific antibody reagents. These typing methods are used to compare isolates in epidemiologic investigations, including studies on the dynamics of gonococcal strains in communities and the geographic spread of resistant strains, and for comparisons between strains for medicolegal investigations (85).

Gonococcal isolates may also be characterized by additional characteristics related to antimicrobial resistance. At least six different β-lactamase plasmids have been described in the gonococcus; these as well as two conjugative plasmids (one possessing a *tet*M determinant) have also been used to study the distribution of gonococcal strains (85). Strains possessing the *tet*M determinant may, with PCR-based tests, be assigned to one of two types: American or Dutch (58). Mutations in the *gyr*A and *par*C genes in strains exhibiting decreased susceptibilities to fluoroquinolones may also be characterized (33).

Molecular typing methods that characterize either specific genes or the entire chromosome have been used more recently to differentiate gonococcal isolates. With older methods, such as PFGE and restriction endonuclease analysis, the chromosome is digested into fragments with restriction enzymes that are resolved in polyacrylamide gels. Restriction endonuclease typing permitted differentiation among isolates belonging to the same serovar; however, restriction patterns are complex and are sometimes difficult to interpret. PFGE is tedious to perform and results in large fragments of DNA that are difficult to separate on conventional gels.

Recently developed typing methods involve amplification and restriction typing of individual genes or gene clusters. The Lip typing system permits the grouping of gonococcal isolates based on the number and sequence of pentamer amino acid repeats within the *lip* gene (94). Opa typing is based on identifying the restriction patterns of a family of 11 distinct and highly variable *opa* genes to give an *opa*-type (75). One disadvantage of the Opa typing system is that new types may evolve very rapidly, resulting in every isolate having a different type unless it is from a sexual partner or a short chain of sexual partners (75). A system for typing the variable regions of the *porB* gene using oligonucleotide probes has also been developed (93). This system can detect differences in *porB* among isolates belonging to the same serovar; however, this method requires the use of different hybridization conditions for individual probes. Real-time PCR amplification of the *porB* gene followed by pyrosequence analysis of highly polymorphic segments of the *porB1a* or *porB1b* alleles has been reported to provide a rapid, highly discriminatory approach to typing gonococcal isolates (96). *PorB* genotyping using probes or sequencing has been used along with amplification and sequence analysis of other genes (e.g., *gyr*A and *par*C) to characterize isolates with acquired resistance to antimicrobial agents used for the treatment of gonococcal infections (47). Use of molecular methods for detection of antimicrobial resistance may provide a molecular approach to surveillance whereby strain variation and dissemination of antimicrobial resistance can be monitored.

In addition, two typing methods that characterize the entire chromosome are based on amplification fragment length polymorphism analysis. These methods use different restriction enzymes to digest the DNA (76, 91). The fluorescent amplification fragment length polymorphism approach has the same discriminatory power as the Opa typing system but is more stable than the latter system (76).

Antimicrobial Susceptibilities

Resistance of *N. gonorrhoeae* to antimicrobial agents is a major problem in the control of gonorrhea. Resistance occurs both as chromosomally mediated resistance to a variety of antibiotics and plasmid-mediated resistance to penicillins (penicillinase [β-lactamase]-producing) and to tetracycline (tetracycline-resistant *N. gonorrhoeae*). The Centers for Disease Control and Prevention (CDC) recommended the use of fluoroquinolones or broad-spectrum cephalosporins for the treatment of gonorrhea (28). Now, however, because of the emergence of resistance to fluoroquinolones (primarily ciprofloxacin and ofloxacin) (66), the CDC now recommends that if symptoms persist after treatment with one of the recommended therapies, patients should be reevaluated for *N. gonorrhoeae* infection by culture and the susceptibilities of any resulting gonococcal isolate should be determined (27). Strains with clinically significant resistance to fluoroquinolones, failure to respond to 500 mg of ciprofloxacin or 400 mg of ofloxacin, or MIC of ciprofloxacin $\geq$1.0 μg/ml or MIC of ofloxacin $\geq$2.0 μg/ml are now widespread in the Far East and have been isolated from a number of cities in the United States, the United Kingdom, Australia, and Canada (23, 24, 66).

The Gonococcal Isolate Surveillance Project (GISP) in 24 to 26 sentinel sites in the United States was implemented by the CDC to monitor changes in antimicrobial susceptibilities in *N. gonorrhoeae* on a monthly basis, but this project monitors only males and only approximately 3% of gonococcal isolates (24). A similar program was established by the World Health Organization and is called GASP (Gonococcal Antimicrobial Surveillance Program) (104). The GISP tests susceptibility to penicillin, tetracycline, spectinomycin, ceftriaxone, cefixime, ciprofloxacin, and azithromycin, and the results are published annually (24). Antimicrobial resistance patterns for *N. gonorrhoeae* vary geographically within the United States, and conducting local antimicrobial susceptibility studies can provide a more accurate assessment with which to judge appropriate treatment recommendations.

Susceptibilities of gonococcal isolates can be determined by an agar dilution method, either on antibiotic-containing media, the E test, or by a disk diffusion method recommended

by the Clinical Laboratory Standards Institute (CLSI), formerly NCCLS (73). Susceptibilities to penicillin, tetracycline, spectinomycin, an extended-spectrum cephalosporin, a fluoroquinolone, and azithromycin should be determined for surveillance purposes.

Azithromycin is not presently recommended for the routine treatment of uncomplicated gonococcal infections, although it may be used for the treatment of gonorrhea in geographic areas where fluoroquinolone-resistant isolates are prevalent and if spectinomycin is not available. The 2-g dose as cleared by the FDA should be used and not the 1-g dose recommended for the primary treatment of chlamydial infections (53). However, one recent report that studied the efficacy of a 1-g dose found only one failure of the 1-g therapy among 226 gonorrhea-infected patients in the United Kingdom (50).

With increasing testing for gonorrhea being performed by NAATs, monitoring of resistance has become more difficult. However, as understanding of molecular mechanisms of resistance increases, it may be possible in the future to determine antimicrobial resistance by using molecular techniques on specimens submitted for NAATs (47, 98, 106). Since worldwide resistance to fluoroquinolones (QRNG) has increased dramatically, there is an increased need to monitor susceptibility trends in different geographical areas and for different population types; and there is a great need for additional single-dose antibiotic regimes in order to control gonorrhea (46). Resistance in *N. gonorrhoeae* and QRNG has been reviewed (46, 106). Data from California indicated that as of 2003, the rate of QRNG had risen from <1% in 1999 to 20.2% in the last half of 2003 (14). The emergence and spread of QRNG seemingly has evolved from sporadic cases to endemic transmission among heterosexuals and particularly among MSM (14).

Culture remains the only test that can be used to identify *N. gonorrhoeae* for legal purposes and for the performance of susceptibility tests. As such, maintaining the ability to perform culture in local areas is an essential component of gonorrhea control programs.

Evaluation, Interpretation, and Reporting of Results

The level of testing and the format for reporting of results should be directed by the sociodemographic characteristics (e.g., age and gender) of the patient clientele served and a knowledge of the incidence and prevalence of clinically significant disease caused by *Neisseria* species, e.g., gonorrhea, in that population. When specimens are collected from patients at high risk for gonorrhea and there are no sociologic or medicolegal implications of a diagnosis of gonorrhea, a presumptive identification of *N. gonorrhoeae* may be sufficient if the diagnosis is intended to facilitate prompt and effective treatment. However, when specimens are collected to confirm a clinical diagnosis for patients such as women and children at low risk for gonorrhea, special concerns apply to laboratory processing and retention of specimens because of the medicolegal consequences that may ensue if an organism is identified as *N. gonorrhoeae*.

Special protocols should be developed for processing specimens from alleged victims of sexual abuse and assault. Suspect gonococci isolated from children must be confirmed by at least two different methods that involve different principles (3). Tests may include carbohydrate utilization, immunologic methods, enzymatic procedures, or the DNA probe culture confirmation test (see below). Of these, tests which detect acid production from glucose and the probe confirmation test provide the least equivocal results. Nongonococcal isolates may cross-react in coagglutination tests, and gonococcal isolates may not react in the monoclonal immunofluorescence test (63). Nongonococcal isolates may produce positive prolyl-hydroxyprolyl aminopeptidase reactions in enzyme substrate tests, as do gonococcal isolates. Therefore, communication between the clinician and the laboratory is essential to ensure that specimens of medicolegal importance are processed according to these criteria.

In general, with the exception of high-risk patients for whom presumptive identifications may suffice, similar principles apply to the identification of all gram-negative, oxidase-positive diplococci. Although many tests for identification of *Neisseria* and related species are marketed as confirmatory tests, most do not provide sufficient data to accurately identify an isolate to the species level without additional tests. Some different isolates may produce identical reactions with some tests, e.g., maltose-negative *N. meningitidis* and *K. denitrificans* may give reactions identical to those of *N. gonorrhoeae* in acid production tests. In addition, problems have been identified with most tests for the identification of *N. gonorrhoeae* and related species, e.g., false-negative acid production reactions from glucose with *N. gonorrhoeae* or false-positive reactions for glucose acidification with *N. cinerea*.

Culture confirmation tests are preferred for the identification of *Neisseria* and related species because they require the isolation of an organism which can be examined by multiple tests if the results of the primary tests are equivocal. Multitest identification systems provide the most information about biochemical characteristics that may, in some cases, allow the identification of an isolate. Rapid identification tests, including serologic and nucleic acid probe or amplification tests, that provide a "yes-no" answer, i.e., an isolate either is or is not *N. gonorrhoeae*, may be adequate if it is necessary only to eliminate *N. gonorrhoeae* as the causative agent; these tests are of limited usefulness when identification to the species level is required.

Because of the consequences of misdiagnosing gonorrhea or misidentifying strains of *N. gonorrhoeae*, three levels are recommended for reporting diagnoses of gonorrhea. These levels are "suggestive," which is defined on the basis of clinical findings, "presumptive," and "definitive," with the last two levels including the results of laboratory tests. A suggestive diagnosis is defined by (i) the presence of a mucopurulent endocervical or urethral exudate on physical examination and (ii) sexual exposure to a person infected with *N. gonorrhoeae*. A presumptive diagnosis of gonorrhea is made on the basis of two of the following three criteria: (i) typical gram-negative intracellular diplococci on microscopic examination of a smear of urethral exudate (men) or endocervical secretions (women); (ii) growth of *N. gonorrhoeae* from the urethra (men) or endocervix (women) on culture media and demonstration of typical colonial morphology, positive oxidase reaction, and typical gram-negative morphology; and/or (iii) detection of *N. gonorrhoeae* by nonculture tests (e.g., antigen detection, nucleic acid probe tests, or NAATs). Definitive diagnosis requires (i) isolation of *N. gonorrhoeae* from sites of exposure (e.g., urethra, endocervix, throat, or rectum) by culture (usually on a selective medium) and demonstration of typical colonial morphology, positive oxidase reaction, and typical gram-negative morphology; and (ii) confirmation by biochemical, enzymatic, serologic, or nucleic acid tests, e.g., carbohydrate utilization, rapid enzyme substrate tests, serologic methods, or the DNA probe culture confirmation test.

For reporting purposes, the laboratory should perform species level identification and confirmation with appropriate tests in order to report a definitive result of "*N. gonorrhoeae* confirmed" for the clinician unless otherwise requested. If an

organism suspected to be *N. gonorrhoeae* is tested by rapid tests but not by additional confirmatory tests that compensate for problems associated with the primary test and is reported as "presumptive *N. gonorrhoeae*," it is important that the clinician understands that additional tests may be required to confirm this identification. Ideally, to avoid confusion, an organism should be reported only as "gram-negative, oxidase-positive diplococcus isolated" unless it has been identified to the species level with sufficient tests to ensure the accuracy of the identification.

NEISSERIA MENINGITIDIS

Collection, Transport, and Storage of Specimens

Specimens helpful in the diagnosis of meningococcal disease include CSF, blood, aspirates, biopsy specimens, and nasopharyngeal and oropharyngeal swabs (Table 1). Occasionally, meningococci may be sought in sputum and bronchoalveolar lavage specimens. Genital, rectal, and oropharyngeal isolates of *N. meningitidis* may be recovered using the collection and inoculation procedures described for *N. gonorrhoeae*. Incubation conditions for inoculated media are the same as those described for *N. gonorrhoeae*. Meningococci grow well on all selective media for the pathogenic neisseriae, and vancomycin-susceptible strains have not been described. Recovery of both gonococci and meningococci from blood cultures may be adversely affected by the anticoagulant sodium polyanethol sulfonate that is present in blood culture media. This effect may be neutralized by addition of sterile gelatin (1% final concentration) to the media or by processing the blood specimen by lysis-centrifugation (i.e., Isolator, Wampole Laboratories, Cranbury, N.J.).

Laboratory Safety

N. meningitidis is classified as a biosafety level 2 organism, which means that a biological safety cabinet should be used for the manipulation of specimens that have a substantial risk for the generation of aerosols (e.g., centrifuging, grinding, and blending). Reports of laboratory-acquired meningococcal infections suggest, however, that manipulation of cultures, rather than patient specimens, increases the risk of infection for microbiology laboratory technologists (22, 25). Such manipulations may include the preparation of heavy organism suspensions for inoculation of identification systems and for serogrouping of isolates. Use of a biological safety cabinet when manipulating cultures for these purposes helps to provide protection of the laboratorian from aerosolized organisms. Alternative measures for protection from droplet aerosols, such as the use of splash guards and masks, are currently being assessed. Education and adherence to established laboratory safety precautions should minimize the risk of meningococcal infection for workers in the clinical microbiology laboratory. Laboratory policies should also be developed for situations that may require administration of chemoprophylaxis to employees who are exposed to meningococci. Laboratories may also consider offering the quadrivalent meningococcal vaccine (which includes serogroups A, C, Y, and W135) to microbiology laboratory staff. Vaccination would decrease, but not eliminate, the potential risk of laboratory-acquired infections.

Direct Examination

Microscopy

A presumptive diagnosis of meningococcal meningitis can be made by direct examination of CSF, using the Gram stain. If sufficient (i.e., more than 1 to 2 ml) CSF is received, the specimen should be centrifuged to obtain a pellet of material for examination and culture. Cytocentrifugation of CSF specimens enhances detection of small numbers of organisms and increases the sensitivity of the Gram stain in comparison with conventionally centrifuged or uncentrifuged specimens. On Gram-stained smears prepared from clinical specimens, meningococci appear as Gram-negative diplococci both inside and outside PMNs. Organisms may display considerable size variation and tend to resist decolorization. Heavily encapsulated strains may have a distinct pink halo around the cells. Because the presence of inflammatory cells has prognostic value (e.g., with fulminant, rapidly fatal disease, many organisms and few inflammatory cells are present), the Gram stain report to the physician should include quantitation of both organisms and PMNs.

Antigen Detection

Direct tests for detection of meningococcal capsular polysaccharides in CSF, serum, and urine are also available. Direct antigen tests use antibody-sensitized latex agglutination or coagglutination to detect capsular antigens of meningococcal serogroups A, B, C, Y, and W135. The serogroup B reagent also detects the cross-reacting *Escherichia coli* K1 antigen. These reagents are available from several vendors (BD Biosciences for latex tests; Boule Diagnostics AB for coagglutination tests). A negative test does not rule out meningitis, and false-positive latex agglutination tests may occur, particularly with urine specimens. Due to the enhanced sensitivity of the Gram stain provided by specimen cytocentrifugation and the problems with the specificity of the antigen detection assays, most laboratories in the United States no longer perform these tests routinely and they are not recommended.

Nucleic Acid Detection Techniques

Molecular methods for direct detection of *N. meningitidis* in clinical specimens are not currently available.

Isolation Procedures

For recovery of *N. meningitidis*, CSF specimens should be inoculated onto nonselective chocolate agar and sheep blood agar. Specimens that may harbor other organisms (e.g., oropharyngeal and nasopharyngeal swab specimens) should be inoculated onto both selective and nonselective media. Plates are incubated in 5 to 7% CO_2 at 35°C (CO_2 incubator or candle extinction jar) and inspected after 24, 48, and 72 h before a final report of "no growth" is issued. Suspicious colonies are subcultured to blood and chocolate agar for further identification.

Identification

Colony Morphology

Colonies of *N. meningitidis* are larger than gonococcal colonies, usually attaining a diameter of about 1 mm or more after 18 to 24 h of incubation. Colonies are low and convex, with a smooth, moist entire edge and a glistening surface. On sheep blood agar, colonies are usually gray; heavily encapsulated strains may be mucoid. The medium beneath and adjacent to the colonies may exhibit a gray-green cast, particularly in areas of confluent growth. Young cultures have a smooth consistency, while older cultures become gummy due to autolysis.

Identification Procedures

N. meningitidis is identified by acid production tests or by chromogenic enzyme substrate tests. Identification procedures

for meningococci produce the best results when inoculated from fresh 18- to 24-h subcultures on chocolate or blood agar. *N. meningitidis* acidifies glucose and maltose, but not sucrose, fructose, or lactose (Table 3). Isolates recovered on selective media can be identified with chromogenic enzyme substrate tests. *N. meningitidis* strains produce γ-glutamyl aminopeptidase; some strains also produce prolyl-hydroxyprolyl aminopeptidase. Glucose-negative, maltose-negative, and asaccharolytic *N. meningitidis* strains may also be isolated occasionally. For these isolates, chromogenic substrate confirmatory tests or serogrouping should be performed.

Serogrouping and Typing

Slide agglutination is commonly used for serogrouping meningococci. A dense suspension of the organism is prepared in 0.5 to 1.0 ml of phosphate-buffered saline (pH 7.2) from a 12- to 18-h subculture on Trypticase-soy blood agar. One drop of this suspension is mixed with 1 drop of meningococcal antisera on a sectored slide, and the slide is rotated for 2 to 4 min. Groupable strains agglutinate strongly within this time. Although isolates from systemic infections usually agglutinate rapidly, those from carriers may fail to agglutinate (nongroupable strains) or may autoagglutinate in saline. Use of younger cultures from blood agar (6 to 8 h) or use of a serum-enriched medium, such as Trypticase-soy agar containing 10% decomplemented horse serum, may resolve these problems. Antisera for the major meningococcal serogroups are available from BD Biosciences. Some nongroupable strains may actually be *N. polysaccharea;* testing for production of polysaccharide from sucrose helps identify this species (see below) (18).

N. meningitidis isolates may be serotyped and subserotyped based on OMP and lipooligosaccharide antigens (95). Meningococcal isolates can be subdivided into 20 serotypes based on class 2 and 3 OMP or PorB antigens, 10 serosubtypes based on class 1 OMP (PorA) antigens, and 13 immunotypes based on lipooligosaccharide antigens. Multilocus isoenzyme electrophoresis typing and multilocus sequence typing have been used to identify genetically defined clonal groups responsible for both epidemic and sporadic, endemic disease. Meningococcal serogroup antigen gene sequencing by sialytransferase gene PCR can be used to confirm serologic grouping results and to determine the genetic grouping of serologically "nongroupable" strains (19). Other molecular methods, including DNA fingerprinting, restriction fragment length polymorphisms, PFGE, ribotyping, repetitive element-based PCR, random-amplified PCR, *por*A gene sequencing, and PCR-amplicon endonuclease analysis, have also been developed for monitoring the epidemiology of pathogenic *N. meningitidis* locally and globally (13, 80, 103).

Antimicrobial Susceptibilities

Despite the occasional recovery of *N. meningitidis* strains with decreased susceptibility to penicillin, penicillin G remains the drug of choice for treatment of meningococcal meningitis. Several broad-spectrum cephalosporins reach therapeutic levels in CSF and are also recommended treatment options. Patients may also require intensive supportive care and monitoring for detection of complications and disease progression (100). Other biological agents (e.g., antiendotoxin and anticytokine monoclonal antibodies) may also play important roles as adjunctive therapies in the management of meningococcal septic shock.

As in the gonococcus, the antimicrobial susceptibility of *N. meningitidis* strains is also evolving (83). Historically, penicillin-susceptible strains of *N. meningitidis* have penicillin MICs of ≤0.06 μg/ml. Rare β-lactamase-producing meningococcal isolates have been encountered sporadically since 1983 in Canada, South Africa, and Spain; these isolates have penicillin MICs of >256 μg/ml. Since 1987, β-lactamase-negative *N. meningitidis* strains with penicillin MICs of >0.06 μ/ml have been isolated in the United Kingdom, Europe, Greece, South America, South Africa, Asia, and the United States (83). These strains are referred to as being relatively resistant to penicillin, being moderately susceptible to penicillin, and having diminished susceptibility to penicillin. Strains with decreased susceptibility to penicillin have MICs ranging from 0.10 to 1.0 μg/ml, and resistant strains are defined as having penicillin MICs of ≥2 μg/ml. Diminished penicillin susceptibility is due to decreased binding of penicillin by altered meningococcal cell wall penicillin-binding proteins (PBP 2 and PBP 3). In the case of the PBP 2 proteins, decreased binding of penicillin to the altered binding protein results from a mutation in the nucleotide sequence of the PBP 2 gene, *pen*A. Similar low-affinity forms of PBP 2 are found in penicillin-resistant strains of *N. lactamica*, *N. flavescens*, *N. polysaccharea*, and *N. gonorrhoeae* (20). The altered PBP 2 found in these *N. meningitidis* strains apparently arose from recombinational events that resulted in replacement of nucleotide sequences in the native meningococcal *penA* gene with corresponding genetic material from the commensal *Neisseria* species.

The clinical significance of diminished penicillin susceptibility in *N. meningitidis* is unclear at present. Although both treatment failures and higher rates of complications have been observed in patients infected with relatively resistant strains, the administration of higher doses of penicillin has been clinically effective. Broad-spectrum cephalosporins, such as ceftriaxone and cefotaxime, are active against both susceptible and moderately susceptible *N. meningitidis* strains, but MICs for some agents (e.g., cefuroxime, aztreonam, and imipenem) may be significantly higher than those of susceptible strains (78).

N. meningitidis strains may also demonstrate resistance to other antimicrobial agents (64). High-level chloramphenicol resistance due to production of chloramphenicol acetyltransferase has been reported in isolates from France and Vietnam. High-level resistance to sulfonamides, including trimethoprim-sulfamethoxazole, is widespread. Rifampin resistance has also emerged, even during the administration of rifampin prophylaxis, and is due to alterations in cell membrane permeability or to mutations in the *rpoB* gene coding for the β-subunit of the meningococcal RNA polymerase. In 2000, an *N. meningitidis* strain with decreased susceptibility to ciprofloxacin (MIC of 0.25 μg/ml) was isolated from a patient with invasive meningococcal disease in Australia (88). Susceptible strains have ciprofloxacin MICs of ≤0.03 μg/ml. Finally, some *N. meningitidis* strains have acquired the *tet*M tetracycline resistance determinant. Resistance to the extended-spectrum cephalosporins that may be used for treatment in developed countries has not been described.

For antimicrobial susceptibility testing of *N. meningitidis* isolates, MIC determinations are the methods of choice. Disk diffusion tests with penicillin, ampicillin, and rifampin for *N. meningitidis* are unreliable. The CLSI recommends either broth microdilution or agar dilution MIC testing of *N. meningitidis* using cation-adjusted Mueller-Hinton broth supplemented with 2 to 5% lysed horse blood or Mueller-Hinton agar with 5% (vol/vol) defibrinated sheep blood, respectively (31). For optimal growth of some *N. meningitidis* strains, this medium may require supplementation with IsoVitalex (1%) (BD Biosciences) or GCHI enrichment (Remel, Inc.). The E test may also be valuable for determining the antimicrobial

susceptibility of individual meningococcal isolates. Since most clinical microbiology laboratories are not equipped to perform agar dilution tests, isolates from patients who are not responding to appropriate antimicrobial chemotherapy and any presumptively penicillin-resistant isolates should be tested for β-lactamase production with the chromogenic cephalosporin test (Cefinase nitrocefin disks; BD Biosciences) and forwarded to a reference laboratory for agar or broth dilution susceptibility testing.

Evaluation, Interpretation, and Reporting

Meningococci are isolated most frequently from the oro- or nasopharynges of asymptomatic carriers along with other normal flora. *N. meningitidis* isolated from throat cultures should not be reported, since reporting implies that the organism is behaving as a pathogen and requires treatment. Meningococci from oropharyngeal cultures represent carriage strains. Meningococcal carriage may be transient, intermittent, or chronic, and carriage alone is not predictive of the development of life-threatening disease (105). While chemoprophylaxis with rifampin, ciprofloxacin, or ceftriaxone is recommended for close contacts of individuals with severe disease, treatment of meningococcal carriers is not recommended. Unless selective media are employed, colonies of *N. meningitidis* may not be noticed among the other members of the resident flora. *N. meningitidis* recovered from CSF, blood, and other normally sterile sites should be identified and reported. Isolates from sputum cultures must be interpreted with regard to clinical presentation and in consultation with clinicians caring for the patient. Because meningococci may be isolated from anogenital sites infected by gonococci, it is necessary to identify neisserial isolates from these sites to the species level by using confirmatory tests.

OTHER *NEISSERIA* SPECIES

Neisseria lactamica

N. lactamica resembles *N. meningitidis* in colony morphology and was initially thought to be a lactose-positive variant of *N. meningitidis*. This species is found in the throat and is isolated more frequently from children than from adults (48). *N. lactamica* grows on all types of selective media and produces acid from glucose, maltose, and lactose (Table 3). ONPG is also hydrolyzed and can be used as a substitute for lactose. Some strains may cause false-positive reactions with commercial coagglutination tests.

N. cinerea

N. cinerea is part of the commensal flora of the upper respiratory tract and has been isolated from other sites, including the cervix, rectum, conjunctivae, blood, and CSF (37, 38, 67). *N. cinerea* grows on both blood and chocolate agar. On chocolate agar after 24 h of incubation, colonies of *N. cinerea* resemble the large-colony types of *N. gonorrhoeae*, are about 1 mm in diameter, and are smooth with entire edges. The organism does not produce acid from carbohydrates in either CTA-based media or the rapid acid production test (Table 3). Weak positive reactions with glucose after overnight incubation have been reported with some identification systems, and its positive prolyl-hydroxyprolyl aminopeptidase reaction may also result in misidentifications of *N. cinerea* as *N. gonorrhoeae* (45, 79). Most *N. cinerea* isolates, however, do not grow well on MTM or other selective media, which precludes testing using chromogenic substrate tests such as the BactiCard-*Neisseria* (Remel, Inc.). *N. cinerea* can be differentiated from the asaccharolytic species *N. flavescens* by its inability to produce polysaccharide from sucrose (see below) and the lack of a discernible yellow pigment. This species is differentiated from the asaccharolytic diplococcal species *M. catarrhalis* by its negative nitrate reduction, DNase, and tributyrin hydrolysis reactions.

The colistin susceptibility test is helpful for differentiating *N. cinerea* from *N. gonorrhoeae*. A suspension of the organism (0.5 McFarland turbidity standard) is prepared in broth and is swabbed onto a chocolate or blood agar plate as for a disk diffusion susceptibility test. A 10-μg colistin disk is placed on the inoculum, and the plate is incubated in CO_2 for 18 to 24 h. *N. cinerea* is colistin susceptible and has a zone that is larger than or equal to 10 mm around the disk. Generally, *N. gonorrhoeae* grows up to the edge of the disk.

Neisseria flavescens

N. flavescens is found in the upper respiratory tract and is rarely associated with infectious processes. This organism forms smooth, yellowish colonies on blood, chocolate, and nutrient agar at 35°C. Most strains also grow at room temperature on chocolate or blood agar. This organism synthesizes iodine-positive polysaccharide from sucrose (see discussion below) and can be differentiated from *M. catarrhalis* by its yellow pigmentation, inability to reduce nitrate, and negative DNase and tributyrin hydrolysis reactions.

Neisseria subflava Biovars, *Neisseria mucosa*, and *Neisseria sicca*

N. subflava, *N. mucosa*, and *N. sicca* make up part of the normal human upper respiratory tract flora and are occasional isolates from infectious processes, including endocarditis, bacteremia, meningitis, empyema, pericarditis, and pneumonia. Identification of the "nonpathogenic" *Neisseria* species is not generally necessary unless the organism is determined to be clinically significant or is isolated from a systemic site (e.g., blood or CSF) or in pure culture. Identification is based on colony morphology, growth on simple nutrient media, inability to grow on selective media, acid production from carbohydrates, reduction of nitrate and nitrite, and synthesis of a starch-like, iodine-staining polysaccharide from sucrose. Nitrate reduction and nitrite reduction are determined in tryptic-soy or heart infusion broth containing 0.1% (wt/vol) KNO_3 or 0.01% (wt/vol) KNO_2, respectively. Polysaccharide synthesis is determined by inoculating the organism onto brain heart infusion agar containing 1% sucrose. Medium lacking sucrose is inoculated as a negative control. After incubation at 35°C for 48 h, the plates are flooded with Gram's or Lugol's iodine (1:4 dilution). A positive test is indicated by the development of a deep blue color in and around the colonies synthesizing the polysaccharide.

N. subflava strains can be subdivided into three biovars (biovars subflava, flava, and perflava) based on acid production from fructose and sucrose, synthesis of iodine-positive polysaccharide from sucrose, and reduction of nitrate and nitrite (Table 3). All three biovars reduce nitrite but not nitrate. *N. mucosa* has a carbohydrate utilization pattern similar to that of *N. subflava* bv. perflava and also produces the iodine-positive polysaccharide, but *N. mucosa* is able to reduce both nitrate and nitrite to nitrogen gas. All of these organisms display varying degrees of yellow pigmentation. *N. sicca* strains are biochemically identical to *N. subflava* bv. perflava, but they grow as dry, adherent, leathery colonies on agar media.

Neisseria polysaccharea

N. polysaccharea is found in the human oropharynx. This organism is an oxidase-positive, catalase-positive, gram-negative

diplococcus that forms smooth yellow colonies (81). Growth on selective media for the pathogenic *Neisseria* is a variable characteristic of *N. polysaccharea* due to the colistin susceptibility of some strains (4). The organisms are resistant to vancomycin. After 24 h of growth, *N. polysaccharea* forms colonies of about 2 mm in diameter on chocolate or blood agar. Acid is produced from glucose and maltose but not from fructose or lactose. Acid production from sucrose is variable and appears to depend on the types of media used to determine this characteristic. *N. polysaccharea* produces an amylosucrase enzyme that synthesizes amylopectin, an extracellular polysaccharide from sucrose. Nitrate is not reduced, whereas nitrite frequently is reduced. *N. polysaccharea* can be differentiated from *N. meningitidis* by polysaccharide synthesis and the γ-glutamylaminopeptidase test. *N. polysaccharea* produces iodine-positive polysaccharide from sucrose and is γ-glutamyl aminopeptidase negative, whereas *N. meningitidis* does not produce iodine-positive polysaccharide from sucrose and is γ-glutamyl aminopeptidase positive. Like *N. gonorrhoeae*, *N. lactamica*, and some *N. meningitidis* strains, *N. polysaccharea* is L-hydroxyprolyl aminopeptidase positive (4). The organism requires cysteine for growth and does not grow on nutrient agar or on chocolate agar at 22°C.

Neisseria elongata Subspecies

N. elongata subspecies *elongata*, *glycolytica*, and *nitroreducens* are rod-shaped members of the genus *Neisseria*. All subspecies are members of the human upper respiratory tract flora, and all have been isolated from infectious processes, including endocarditis (51). These subspecies can be differentiated on the basis of catalase reactivity, acid production from glucose, and reduction of nitrate (Table 3).

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Actinobacillus, Capnocytophaga, Eikenella, Kingella, Pasteurella, and Other Fastidious or Rarely Encountered Gram-Negative Rods

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The bacterial genera covered in this chapter are taxonomically diverse, but common traits justify their discussion as a group. They are facultatively anaerobic gram-negative rods that belong neither to the families *Enterobacteriaceae, Vibrionaceae,* and *Aeromonadaceae* nor to the *Rickettsiales;* they are rather part of the families *Pasteurellaceae, Neisseriaceae, Cardiobacteriaceae, Flavobacteriaceae, Porphyromonadaceae,* and *Fusobacteriaceae* (51).

These bacteria grow aerobically but require, with the exception of *Chromobactrium* and some *Pasteurella* species, supplemented media for growth, grow slowly, often requiring 48 h at 37°C, and do not grow on enteric media such as MacConkey or desoxycholate agar. A 5 to 10% CO_2 atmosphere may be necessary for initial growth and improves growth on subcultures. Some isolates may be gram-variable; many species show limited viability. With the exception of *Chromobacterium*, they do not possess flagella but may show gliding or twitching motility, resulting in limited spreading of colonies and pitting of the agar (66).

Most species may be part of the flora of the nasopharynx and/or the oral cavity of animals and/or humans and, thus, are parasitic, in contrast to the environmental *Chromobacterium*. Only those isolated from humans are covered in this chapter. Transmission from animals to humans occurs by contact (e.g., bites or licking of wounds for *Pasteurella*), and from human to human by droplets (e.g., directly for *Kingella* or via paraphernalia or human bites for *Eikenella*); only *Chromobacterium* is transmitted by soil. Endogenous infections may occur as well (e.g., endocarditis).

The collection of specimens should follow the guidelines described in chapters 5 and 20. The low viability of many species makes use of transport media mandatory. Serological tests have not been tried on large numbers of cases.

The use of blood or chocolate agar and, wherever no normal flora is present, of enriched liquid media is mandatory. Selective media are mentioned below for each organism.

Phenotypic identification may present numerous difficulties. Identification to the species level often requires the use of multiple substrates not altogether available in many automated systems (17, 36, 60); databases of the latter also may not contain all relevant species. Similar biochemical characteristics, e.g., in *Actinobacillus* spp., may call for species confirmation by molecular methods (44, 52, 90). Triple-sugar iron or Kligler's agar may not support growth, e.g., of *Eikenella*. Gas formation, if present, is generally scant and may not be seen in these media. Media used to check acid formation from carbohydrates should be rich in peptones (e.g., cystine-trypticase agar), but serum should not be used, if at all possible, because it may split maltose. An alternative would be the use of Trypticase agar with 1% carbohydrate and a large inoculum (cell paste or agar block). Indole formation may take 2 days and may require extraction with xylene. The indole spot test is reliable only for *Pasteurella multocida* (103). Oxidase must be tested from colonies on blood-containing media with tetramethyl-*p*-phenylenediamine dihydrochloride (57). Catalase should preferably be checked in a liquid medium in order to avoid false-positive reactions on slides due to transfer of blood and false negatives due to weak reactions.

Except for a very recent report on *Pasteurella* spp. (30), there are no Clinical and Laboratory Standards Institute (CLSI) (formerly NCCLS) data for disk susceptibility testing of members of the group. MIC determinations require either dilution systems or E tests (55–56, 74, 80, 94). Most species are susceptible to many antimicrobials. There may even be small zones around 5-μg vancomycin disks (42).

Members of the group may cause infections anywhere in the human body. Those present in the oropharynx of animals often cause bite infections which may develop into systemic disease, particularly in immunosuppressed individuals. A disease particular to members of the group is HACEK endocarditis, named after the bacteria involved (*Haemophilus parainfluenzae*, *Haemophilus aphrophilus*, *Actinobacillus actinomycetemcomitans*, *Cardiobacterium*, *Eikenella*, and *Kingella* spp.) (19). It is characterized by a long interval between first symptoms and diagnosis (2 weeks to 6 months), frequent embolization, and large vegetations on left-side valves. Native and prosthetic valves may be affected. Prognosis is good with appropriate antibiotic treatment. In modern automated blood culture systems, HACEK organisms grow within 5 days (35, 62). In a few instances, blood cultures were negative but microscopy of the resected valve and/or broad-range PCR of valve or emboli led to the diagnosis (98, 101). *Cardiobacterium*, *Capnocytophaga*, and *Eikenella* may also participate in noma lesions (108), whereas the latter two genera and *Actinobacillus actinomycetemcomitans* (as the predominant agent) participate in periodontal lesions (102).

PASTEURELLA

The genus *Pasteurella* belongs, together with the genera *Haemophilus* and *Actinobacillus* and several nonhuman ones, to the family *Pasteurellaceae* (51). These genera have identical cellular fatty acid composition, with C16:1ω7c, C16:0, and C14:0 as the principal components. There is no single phenotypic feature that distinguishes the genera *Pasteurella* and *Actinobacillus*. The guanine-plus-cytosine (G+C) content of the DNA for *Pasteurella* is between 37.7 and 45.9 mol%. Members of one group of pasteurellae, however, are by DNA-DNA hybridization more closely related to the other two genera and have been marked *[P.]* (5), whereas the other group are called *Pasteurella* sensu stricto.

Pasteurellae are coccoid to small, nonmotile, facultatively anaerobic gram-negative rods that occur singly, in pairs, or in short chains. Bipolar staining is frequent. The "related" species generally have a more pronounced rod-like morphology and may even grow on MacConkey agar. All pasteurellae are hemin and CO_2 independent, but a few strains require factor V (nicotine adenine dinucleotide) (80). Colonies are 1 to 2 mm in diameter after 24 h of growth on blood agar at 37°C and opaque-grayish. Encapsulated strains tend to be mucoid. A slight greening underneath them may be observed. The indole-positive species exhibit a mouse-like odor.

Pasteurellae are widespread in healthy and diseased wild and domestic animals. Their habitats are the nasopharynx and the gingiva. Those occurring in animals only (particularly in birds and mammals) (25) are not covered here. *P. multocida*, the species most frequently isolated from humans, also occurs in many animals including dogs and cats. In spite of the genotypical homogeneity of *P. multocida*, phenotypically diverse lineages have been observed, e.g., infections of wounds from bites by large cats may be due to sucrose-negative variants (27). Virulence factors (capsule serotypes A to F, a cytotoxin, filamentous hemagglutinins, and iron acquisition proteins) have been identified in animal models (63), but their role in human pathology remains to be elucidated. Most human cases are due to serotypes A, D, and F (37).

Human infections are predominantly those of wounds or soft tissues following bites, scratches, or licking of skin lesions by carnivores. Of 159 human isolates of *Pasteurella* sensu stricto, 60% were *P. multocida* subsp. *multocida*, 18% were *P. canis,* 13% were *P. multocida* subsp. *septica*, 5% were *P. stomatis,* and 3% were *P. dagmatis* (all except *P. canis* are associated with both dogs and cats) (68). Infected cat bite wounds contain pasteurellae (mostly subsp. *multocida* and *septica*) significantly more often than infected dog bite wounds, reflecting a higher oropharyngeal colonization rate in cats (50 to 90% versus 55 to 60%) (24). Two species may even be encountered in one sample (142). The second most common site not only of infection but also of colonization is the respiratory tract, where pasteurellae sensu stricto may also cause sinusitis and bronchitis as well as pneumonia and empyema (with a poor prognosis), mostly in patients with underlying disease (24, 141). Finally, systemic diseases (meningitis, peritonitis, septicemia, arthritis, endocarditis, and osteomyelitis) have also been reported, with underlying medical conditions, particularly liver cirrhosis, present in many individuals affected (8, 143). An association exists between subspecies and clinical presentation: subsp. *septica* is more often associated with wounds than with respiratory and systemic infections, whereas subsp. *multocida* predominates in respiratory infections and bacteremias (24, 37). Cases of human infection with *P. gallinarum* (3, 6) (now *Avibacterium gallinarum*) (13) have to be regarded with circumspection (45); nevertheless, a case of neonatal meningitis (1) seems to be beyond reproach.

Of the "related" species, *[P.] caballi* has primarily been isolated from horses (120) and also from horse bite wounds in humans (41). *[P.] bettyae* (formerly HB-5), of uncertain habitat, has been isolated from infections of the male and female genital tracts and of newborns (15, 33). *[P.] aerogenes* has been detected primarily in pigs and hamsters and was isolated from bite wounds from those animals (39, 46). *[P.] pneumotropica* corresponds in habitat and transmission to *P. multocida,* but human infections (bites, systemic disease) are rare (44). The former *[P.] haemolytica*, also of animal origin, has now been subdivided into trehalose-positive strains, named *[P.] trehalosi,* and trehalose-negative strains, named *Mannheimia haemolytica* (5). Cases of human infection are somewhat doubtful (89, 111, 141); one case of endocarditis (132) seems to conform to the former description of the *[P.] haemolytica* complex.

Media containing vancomycin, clindamycin, and/or amikacin have been used to select pasteurellae (9). Biochemical reactions of species isolated from humans are listed in Table 1. In view of the phenotypic similarity of some species and the presence of some unclassified taxa (12, 25), species identification requires tests that may not be included in automated diagnostic systems (61). Beta-hemolysis is observed only in the former *[P.] haemolytica* complex. PCR (71), repetitive extragenic palindromic PCR (24), and 16S rRNA gene sequencing (44) have been used for diagnostic confirmation. Typing of pasteurellae has employed multilocus enzyme electrophoresis, PCR profiling, restriction endonuclease analysis, pulsed-field gel electrophoresis, and ribotyping (27, 71).

Pasteurellae are generally susceptible to β-lactam antibiotics including penicillin, cephalosporins (except oral narrow-spectrum agents), tetracyclines, quinolones, and trimethoprim-sulfamethoxazole and are generally resistant to macrolides and amikacin; other aminoglycosides are only moderately active (54, 55, 56, 57, 143). A few β-lactamase-positive strains of *P. multocida* (100) and *[P.] bettyae* (15) have been reported; they were susceptible to a combination of penicillin and clavulanic acid.

ACTINOBACILLUS

The genus *Actinobacillus,* in the family *Pasteurellaceae* (51), resembles *Pasteurella* in the composition of cellular fatty acids but has a narrower G+C content of the DNA (between 40 and 43%).

Actinobacilli are nonmotile, facultatively anaerobic coccoid to small gram-negative rods on solid media but are longer in liquid media or in those containing glucose or maltose and show a tendency to bipolar staining. Arrangement is single, in pairs, and (rarely) in short chains. Growth requires enriched media but not necessarily hemin and is improved by a 5 to 10% CO_2 atmosphere.

Many species have been isolated only from animals and are not discussed here. Species isolated from humans belong either to *Actinobacillus* sensu stricto (26) or to *A. actinomycetemcomitans*. The former include *A. lignieresii* (primary habitat in the oral cavity of sheep and cattle), *A. equuli* subsp. *equuli* (oral cavity of horses and pigs), *A. equuli* subsp. *haemolyticus* and *A. suis* (oral cavity of pigs), and the exclusively human species *A. ureae* (formerly *Pasteurella ureae*) and *A. hominis.* The species *A. actinomycetemcomitans* is of uncertain taxonomic status because 16S rRNA gene sequencing and DNA-DNA hybridization place it more

TABLE 1 Biochemical reactions of *Pasteurella* spp. and related species [a,b,c]

Reaction	*P. multocida*[d]	*P. canis*	*P. dagmatis*	*P. stomatis*	*Avibacterium gallinarum*	*[P.] aerogenes*	*[P.] bettyae*	*[P.] caballi*	*[P.] pneumotropica*	*[P.] trehalosi*[e]	*M. haemolytica*[e]
Catalase	+	+	+	+	+	+	−	−	+	v	+
Oxidase	+	+	+	+	+	+	v	+	+	+	+
Indole	+	+	+	+	−	−	+[w]	−	+	−	−
Urease	−	−	+	−	−	+	−	−	+	−	−
Ornithine decarboxylase	+	+	−	−	v	v	−	+	+	−	−
Growth on MacConkey agar	−	−	−	−	v	+	v	−	v	+	+
Gas from glucose	−	−	v	−	−	+	+[w]	+	−	−	−
Acid from:											
Lactose	−	−	−	−	−	v	−	+	−	−	ND
Sucrose	+	+	+	+	+	+	−	+	+	+	+
Xylose	v	−	−	−	v	v	−	+	+	−	+
Maltose	−	−	+	−	+	+	−	+	+	+	+
Mannitol	+	−	−	−	−	−	−	+	−	+	+

[a] Data from references 12, 25, 120, and 135.
[b] +, ≥90% of strains positive; −, ≤90% of strains negative; v, variable; w, weak; ND, no data available.
[c] All species reduce nitrate to nitrite and are negative for arginine dihydrolase and esculin hydrolysis.
[d] The three subspecies *multocida, septica,* and *gallicida* can be separated on the basis of sorbitol and dulcitol fermentation (+/− in subsp. *multocida*, −/− in subsp. *septica,* and −/+ in the mostly avian subsp. *gallicida*); weakly sorbitol-positive strains of subsp. *multocida* can be recognized by a negative α-glucosidase test and a specific PCR profile (52).
[e] Beta-hemolytic; *[P.] trehalosi* is trehalose positive; *Mannheimia haemolytica* is trehalose negative.

closely to the genus *Haemophilus* than to *Actinobacillus* (105). Its transfer to the genus *Haemophilus* (110), however, was not favored by the International Committee on Systematic Bacteriology Subcommittee on *Pasteurellaceae* and Related Organisms (72). Its habitat is the oral cavity of humans and of some primates (65).

Colonies of actinobacilli sensu stricto are approximately 2 mm in diameter after 24 h of growth at 37°C, are smooth or rough, are viscous, and often adhere to the agar surface. Smooth colonies are dome-shaped and have a bluish hue when viewed by transmitted light. *A. actinomycetemcomitans* colonies initially show a central dot and a slightly irregular edge, which, on further incubation, develops into a star-like configuration resembling "crossed cigars," and pit the agar (Fig. 1). After several subcultures, this rough morphology may give way to smooth and opaque, nonpitting colonies, reflecting loss of fimbriae. In liquid media, the bacterium forms granules which adhere to the sides and to the bottom of the tube.

A. lignieresii causes actinobacillosis, a granulomatous disease in cattle and sheep in which, in a fashion similar to actinomycosis, sulfur granules are formed in tissues (116). A few human soft tissue infections, originating from cattle or sheep bite wounds or contact, have been reported (109). The *A. equuli* subspecies and *A. suis* cause a variety of diseases in horses and pigs (25); human infections are mostly due to horse or pig bites or contact (7, 40). Both species have also been isolated from the human upper respiratory tract (117, 135). *A. ureae* has been isolated most often as a commensal from the human respiratory tract but also from meningitis patients following trauma or surgery (137) and other infections in immunocompromised patients (77). *A. hominis* was also encountered in such patients, as well as a commensal, albeit rarely (48).

A. actinomycetemcomitans is one of the major agents of adult and juvenile periodontitis (102). Furthermore, it has caused endocarditis (HACEK) (19) and soft tissue and other infections (75, 107). In sulfur granules, it may occur in combination with *Actinomyces* spp. (75).

Virulence factors of actinobacilli belong to the RTX (repeats-in-toxin) family, i.e., poreforming (hemolytic or cohemolytic) and cytolytic proteins (47). *A. actinomycetemcomitans* forms an RTX toxin termed leukotoxin, an epithelial distending toxin, some less well characterized immunomodulatory toxins, and fimbriae responsible for adherence (65).

Selective media have been devised for isolation of *A. actinomycetemcomitans* from dental samples. The clear Tryptic soy-serum-bacitracin-vancomycin medium allows observation of colonial morphology and catalase activity (127). It was recently modified (4). PCR has been used for detection, targeting the leukotoxin gene or specific 16S rRNA sequences (50).

Biochemical reactions are listed in Table 2. Species of *Actinobacillus* sensu stricto are easily separated from *A. actinomycetemcomitans* by urease production and sucrose fermentation. The latter and catalase and ONPG (*o*-nitrophenyl-β-D-galactopyranoside) reactions separate *A. actinomycetemcomitans* from *Haemophilus aphrophilus*, an important differentiation in the clinical laboratory which cannot always be accomplished if commercial systems are employed (17, 36). Separation of *A. equuli* subsp. *equuli* and *A. suis* may be impossible by phenotypic tests; the organisms share the same 16S rRNA gene sequence (26) but have different RTX toxin profiles (83).

On the basis of surface polysaccharides determined by tube agglutination or multiplex PCR (131), six serotypes of

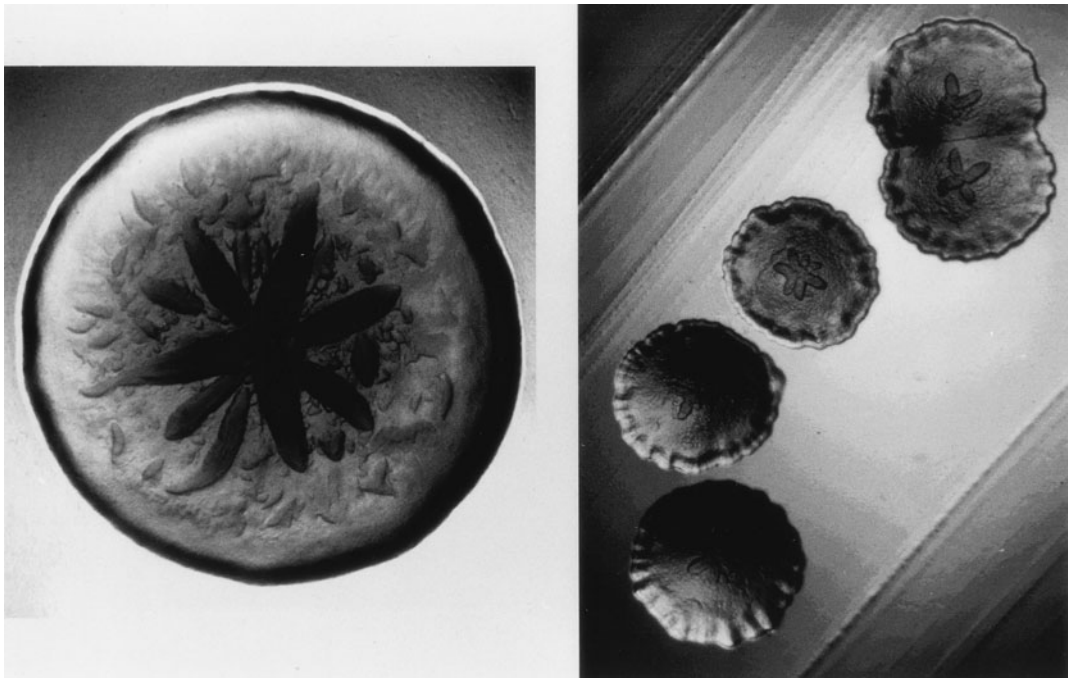


FIGURE 1 Star-shaped colonies of *Actinobacillus actinomycetemcomitans*.

A. actinomycetemcomitans can be distinguished, of which a, b, and c are most common. Serotype b is associated with periodontitis, penicillin resistance, and endocarditis, whereas serotype c is associated with other extraoral infections but also with periodontal health (106). Other typing systems (65, 107), also used on strains of *A. suis* and *A. equuli* (26), have been of less clinical relevance.

Susceptibility studies are extant for a few isolates of *A. ureae* and *A. hominis* that are susceptible to many antimicrobials including penicillin (48, 77). *A. actinomycetemcomitans* is usually susceptible to cephalosporins, ampicillin, doxycycline, and aminoglycosides; resistance to penicillin and macrolides is not uncommon (75, 80, 94, 107, 128). Penicillinase, however, has not been detected (94, 128).

CHROMOBACTERIUM

The genus *Chromobacterium,* in the *Neisseriaceae* family, contains one species, *C. violaceum* (51). It is a facultatively anaerobic, straight gram-negative rod which differs from all

TABLE 2 Biochemical reactions of *Actinobacillus* spp. and *Haemophilus aphrophilus*[a,b,c]

Reaction	*A. lignieresii*	*A. equuli* subsp. *equuli*	*A. equuli* subsp. *haemolyticus*	*A. suis*	*A. ureae*	*A. hominis*	*A. actinomycetemcomitans*	*H. aphrophilus*
Beta-hemolysis	−	−	+	+	−	−	−	−
Catalase	v	v	+[w]	+/+[w]	v	+	+	−
Oxidase	+	+	+	+	+	+	v	v
Esculin hydrolysis	−	−	−	+	−	−	−	−
Urease	+	+	+	+	+	+	−	−
ONPG	+	+	+	+	−	+	−	+
Growth on MacConkey agar	v	+	+	v	−	+	−	v
Gas from glucose	−	−	−	−	−	−	v	+
Acid from:								
Lactose	v	+	+	+	−	+	−	+[D]
Sucrose	+	+	+	+	+	+	−	+
Xylose	+	+	+	+	−	+	v	−
Maltose	+	+	+	+	+	+	v	+
Mannitol	+	+	v	−	+	+	v	−
Trehalose	−	+	+	+	−	+	−	+[D]
Melibiose	−	+	+	+	−	+	−	−

[a] Data from references 26, 48, 109, 117, and 135.
[b] +, ≥90% of strains positive; −, ≥90% of strains negative; D, delayed reaction; ONPG, *o*-nitrophenyl-β-D-galactosidase; v, variable; w, weak.
[c] All species are indole and ornithine decarboxylase negative and reduce nitrate to nitrite.

other species covered in this chapter by being motile by one polar and one to four lateral flagella, by growth on most enteric media, by an optimal growth temperature below 37°C, and an environmental origin. Colonies are 1 to 2 mm in diameter after 24 h of growth at 30 to 35°C, are round and smooth, have an almond-like smell, and may be beta-hemolytic. Most colonies produce a violet pigment called violacein, which is soluble in ethanol but not in water. Nonpigmented strains are known, however. The G+C value of the DNA is between 65 and 68 mol%.

C. violaceum inhabits soil and water in tropical and subtropical climates between latitudes of 35°N and 35°S (South Africa, Southeast Asia, Australia, southeastern United States). Human infections are rare. The portal of entry is usually the skin, but oral intake has also been reported. Wound infections, abscesses, and septicemia may develop. Infections are significantly associated with neutrophil dysfunction (glucose-6-phosphate dehydrogenase deficiency, chronic granulomatous disease [CGD]). Children without CGD and children with bacteremia show a high fatality rate (88, 123). A number of virulence factors other than endotoxin (adhesions, invasins, and cytolytic proteins) have been described (18).

Biochemical reactions are listed in Table 3. Identification of *C. violaceum* is easy if violacein is produced, although in those cases the oxidase reaction may be difficult to detect. Nonpigmented strains may be confused with *Aeromonas* spp. but are lysine, maltose, and mannitol negative. The principal cellular fatty acids do not differentiate between these genera.

C. violaceum is usually resistant to many antimicrobials but is often susceptible to imipenem, fluoroquinolones, gentamicin, tetracycline, and trimethoprim-sulfamethoxazole (2, 88).

EIKENELLA

The genus *Eikenella* belongs to the family *Neisseriaceae* (51). Thus far, only one species, *E. corrodens*, has been recognized, but DNA-DNA hybridization, composition of cellular carbohydrates, and the occurrence of biochemically aberrant (among them, catalase-positive) isolates suggest that there may be more than one genomospecies (76). The G+C content is between 56 and 58 mol%.

Eikenellae are slender, straight, small, nonmotile, facultatively anaerobic gram-negative rods (Fig. 2A). With a few exceptions, they require hemin for growth unless 5 to 10% CO_2 is present (53) and, therefore, grow poorly or not at all on triple-sugar iron or Kligler's agar. Colonies are 1 to 2 mm in diameter after 48 h of growth and show clear centers that are often surrounded by spreading growth, and they may pit the agar. They smell of hypochlorite and assume a slightly yellow hue after several days. In liquid media, granules are formed.

The habitat of *E. corrodens* is the oral cavity and perhaps the gastrointestinal tract of humans and some mammals from whom it can be transmitted via saliva (bites, syringes) to other individuals. The organism is associated with adult and juvenile periodontitis (102) and is also an agent of oral, pleuropulmonary, abdominal, joint, bone, and wound (e.g., human bite) infections (108, 122). These are often indolent and mixed with other members of the oropharyngeal flora. Risk factors are dental manipulations and intravenous drug abuse. Endocarditis is of the HACEK type if monomicrobial; polymicrobial cases, however, are known (19).

Detection is improved by use of a selective medium containing clindamycin (124) and by PCR (50). Biochemical reactions are recorded in Table 3. A typical isolate fails to form acid from carbohydrates and is ornithine decarboxylase and nitratase positive. Typing has been done by arbitrarily primed PCR (49) and restriction endonuclease analysis (23), demonstrating unstable polyclonality in the oral cavity.

E. corrodens is susceptible to many antimicrobials including penicillin, expanded- and broad-spectrum cephalosporins, carbapenems, doxycycline, and fluoroquinolones and is often resistant to macrolides (54, 55, 56). β-Lactamase-positive strains have become more frequent, but their resistance can be overcome by β-lactamase inhibitors (85).

KINGELLA

The genus *Kingella*, consisting of the species *K. kingae*, *K. denitrificans*, *K. oralis*, and *K. potus,* also belongs to the family *Neisseriaceae* (51). The G+C content of the DNA is between 47 and 58 mol%.

Its members are short, nonmotile, facultatively anaerobic gram-negative rods with square ends which lie together in pairs or clusters (Fig. 2B) and tend to decolorize unevenly on Gram stain. Colonies develop to 1- to 2-mm diameter in 48 h of growth, one type being smooth with a central papilla, the other showing spreading edges and pitting of the medium. *K. kingae*, as the only species, shows a small but distinct zone of hemolysis on blood agar. CO_2 (5 to 10%) enhances growth. Viability is limited.

K. kingae colonizes the throat but not the nasopharynx of many children aged 6 months to 4 years (139). The natural habitats of *K. denitrificans* and *K. potus* are unknown. *K. oralis* has been isolated from the healthy human mouth (22).

Infections with *K. kingae* show a predilection for bones and joints of previously healthy children under 4 years of age (139). Septic arthritis, diskitis, and osteomyelitis of the lower extremities but also occult bacteremia are conspicuous. Stomatitis and/or upper respiratory tract infections may precede systemic disease, which suggests possible interaction with viral disease(s) (139). Infections in adults occur more commonly in immunocompromised individuals (139) or may present as endocarditis (HACEK) (19). Ribotyping and pulsed-field gel electrophoresis have shown that person-to-person transmission via respiratory droplets may occur (125). *K. denitrificans* has been reported mostly as an agent of endocarditis (96). *K. oralis* has been isolated from patients with periodontitis, but its relationship to the disease is unclear (22). *K. potus* has caused a wound infection following a bite of a kinkajou (87).

Recovery of *K. kingae* from body fluids and pus can be difficult because these specimens seem to be inhibitory to the bacteria. The use of various blood culture media has significantly improved the detection rate (139). In a study using blood culture media for simulated synovial fluids, BacT/Alert (BioMérieux, Inc., Durham, N.C.) proved to be superior to BACTEC (Becton Dickinson, Cockeysville, Md.) blood cultures with regard to sensitivity and detection time (70), but comparative studies with clinical samples are not extant. Broad-range PCR has even detected *K. kingae*-specific sequences in synovial fluids that did not show growth in blood culture media (97). For isolation from areas with normal flora, solid media containing clindamycin (34) or vancomycin (140) as well as Thayer-Martin agar (140) have been recommended.

Biochemical test results are listed in Table 3. In addition to microscopic and colonial morphology, they serve to differentiate kingellae from rod-shaped members of the genus *Neisseria* (Table 4).

TABLE 3 Biochemical reactions of rod-shaped species of the *Neisseriaceae* and of the *Cardiobacteriaceae* [a,b,c]

Reaction	*Chromobacterium violaceum*	*Eikenella corrodens*	*Kingella kingae*	*Kingella denitrificans*	*Kingella oralis*	*Kingella potus*	*Simonsiella muelleri*	Group EF-4a	*Cardiobacterium hominis*	*Cardiobacterium valvarum*	*Suttonella indologenes*
Catalase	+	−	−	−	−	−	−	−	−	−	v
Oxidase	v	+	+	+	+	+	+	+	+	+	+
Indole	v	−	−	−	−	−	−	−	+[w]	v	+
Arginine dihydrolase	+	−	−	−	−	−	−	v	−	ND	−
Nitrate to nitrite	+	+	−	+/G	−	−	v	+/G	−	−[d]	−
Esculin hydrolysis	−	−	−	−	−	−	−	−	−	−	−
Ornithine decarboxylase	−	+	−	−	−	−	−	−	−	ND	−
Growth on MacConkey agar	+	−	−	−	−	−	−	v	−	ND	−
Alkaline phosphatase[e]	+	−	+	−	+	−	−	−	−	ND	+
Acid from:											
Glucose	+[f]	−[g]	+	+	+[w]	−	+	+	+	v	+
Lactose	−	−	−	−	−	−	−	−	−	−	−
Sucrose	v	−	−	−	−	−	−	−	+	v	+
Xylose	−	−	−	−	−	−	−	−	−	−	−
Maltose	−	−	+	−	−	−	+	−	+	v	+
Mannitol	−	−	−	−	−	−	−	−	+	−	−
Special features	Violacein v	LD v	Beta-hemolytic			DNase +, yellow pigment	Microscopic morphology	Yellowish or no pigment			
Main cellular fatty acids	18:1ω7c, 16:0, 14:0	16:0, 18:1ω7c, 16:1ω7c	14:0, 16:1ω7c, 16:0	16:0, 14:0, 18:2, 18:1ω9c	ND	16:0, 18:1ω7c	16:1ω7c, 12:0, 14:0	16:0, 16:1ω7c, 18:1ω7c	18:1ω7c, 16:0, 14:0	18:1ω7c, 16:0, 14:0	16:0, 18:1ω7c, 16:1ω7c, 14:0

[a] Data from references 34, 61, 76, 81, 87, and 135.
[b] +, ≥90% of strains positive; −, ≥90% of strains negative; G, gas; LD, lysine decarboxylase; ND, no data; v, variable; w, weak.
[c] All species are negative for urease.
[d] One possible isolate (69; see text) was nitratase positive.
[e] APIZYM system (76).
[f] Some strains form small amounts of gas.
[g] Weakly positive reactions may be observed in O/F media (76).

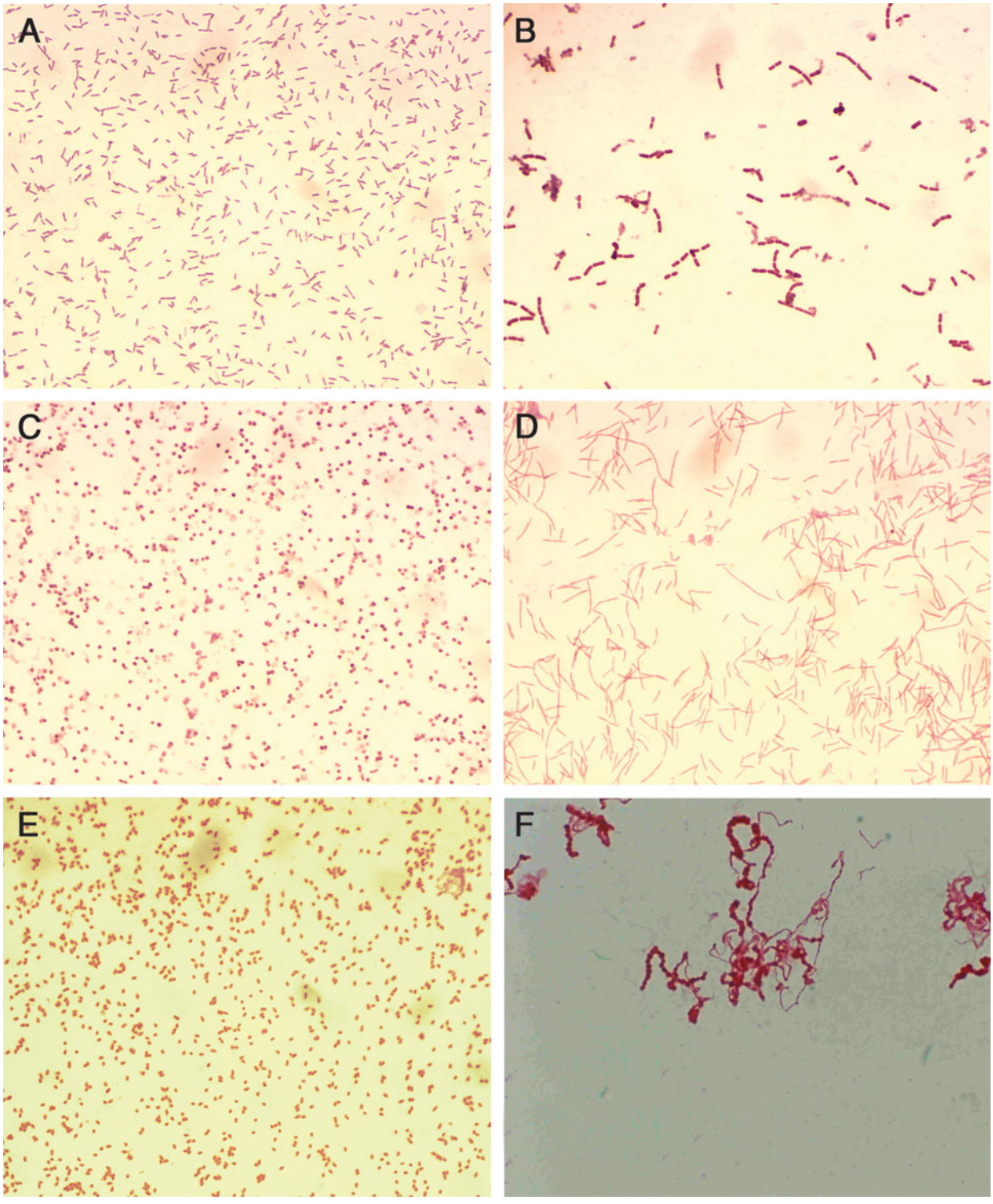


FIGURE 2 (A) *Eikenella corrodens* (Gram stain); (B) *Kingella kingae* (Gram stain); (C) Group EF-4a (Gram stain); (D) *Capnocytophaga ochracea* (Gram stain); (E) *Dysgonomonas capnocytophagoides* (Gram stain); (F) *Streptobacillus moniliformis* (Gram stain of a 48-h culture grown on sheep blood agar). (Courtesy of D. Fedorko, Bethesda, Md.)

TABLE 4 Differentiation between *Kingella* and rod-shaped *Neisseria* species[a,b]

Feature	*Kingella kingae*	*Kingella denitrificans*	*Kingella oralis*	*Kingella potus*	*Neisseria elongata*		*Neisseria weaveri*
					subsp. *glycolytica*	subsp. *nitroreducens*	
Catalase	−	−	−	−	+	−	+
Nitrate to nitrite	−	+	−	−	−	+	−
Nitrate to gas	−	+	−	−	−	−	−
Alkaline phosphatase[c]	+	−	+	ND	−	−	ND
Glucose acid	+	+	+[w]	−	+[w]	v	−
Maltose acid	+	−	−	−	−	−	−
Beta-hemolysis	+	−	−	−	−	−	−
Main cellular fatty acids	14:0, 16:1ω7c, 16:0	16:0, 14:0, 18:2, 18:1ω9c	ND	16:0, 18:1ω7c	16:0, 16:1ω7c, 18:1ω7c	16:0, 16:1ω7c, 18:1ω7c	16:0, 16:1ω7c, 18:1ω7c

[a] Data from references 22, 87, 114, and 135.
[b] +, ≥90% of strains positive; −, ≥90% of strains negative; ND, no data available; v, variable; w, weak.
[c] APIZYM system (76).

Kingellae are generally susceptible to β-lactam antibiotics, macrolides, doxycycline, trimethoprim-sulfamethoxazole, and quinolones (139). β-Lactamase-positive isolates exist; they are susceptible to the combination with β-lactamase inhibitors (96, 129).

SIMONSIELLA

The genus *Simonsiella* also belongs to the family *Neisseriaceae* and has a G+C content of the DNA of 40.3 to 50.1 mol% (51). Its members are strictly aerobic gram-negative, nonmotile, crescent-shaped rods. They are arranged in multicellular filaments, 10 to 50 μm by 2 to 8 μm, which are segmented into groups of mostly eight cells, resulting in a caterpillar-like appearance (Fig. 3). The long axis of each cell is perpendicular to the long axis of the filament, representing the width of the latter. Gliding motility and incomplete decolorization with Gram stain are usually observed. Simonsiellae grow well on blood agar but not on enteric media. Colonies are 1 to 2 mm in diameter after 24 h at 37°C and produce a pale yellow pigment.



FIGURE 3 Scanning electron micrograph of *Simonsiella* sp. with two multicellular filaments giving the appearance of caterpillars. (Courtesy of L. Corboz, Zürich, Switzerland.)

Three species which can be separated by biochemical tests (81) have been described. Their natural habitat is the oral cavity of sheep *(S. crassa)*, dogs *(S. steedae)*, and humans *(S. muelleri)*; simonsiellae from cats remain unnamed. *S. muelleri* has not been associated with human disease (20, 136).

An optimal medium for microscopic recognition is BSTSY agar, which contains bovine serum, glucose, tryptic soy broth, and yeast extract (82). *S. muelleri* (Table 3) is beta-hemolytic. The long axis of the cells measures 2.1 to 3.5 μm, and the short axis measures 0.5 to 0.9 μm.

One strain recovered from the gastric aspirate of a newborn was susceptible to β-lactam antibiotics, tetracycline, and gentamicin (136).

GROUP EF-4a

Groups EF-4a and EF-4b (EF, eugonic fermenter) also belong to the family *Neisseriaceae* (114), although they are not listed in the taxonomic outline of the bacteria (51). Group EF-4a consists of facultatively anaerobic, nonmotile, coccoid to short gram-negative rods (Fig. 2C) with a G+C content of 49.3 to 50.9 mol% (114). Colonies are slightly yellow or nonpigmented and often smell of popcorn.

The organisms are part of the normal flora of dogs, cats, and rodents, in whom they may cause pulmonary infection. Human infections are associated with cat or dog bite or contact (112, 133).

The two groups have identical habitats, microscopic and colony morphology, and fatty acid composition and cause the same infections in humans, but they differ biochemically. EF-4a (Table 3) is fermentative and arginine dihydrolase variable and reduces nitrate with or without gas formation (135). EF-4b is oxidative and arginine dihydrolase negative and always reduces nitrate without gas formation. Trimethoprim has been used in a selective medium (112). EF-4a organisms are susceptible to aminopenicillins, expanded- and broad-spectrum cephalosporins, fluoroquinolones, and trimethoprim-sulfamethoxazole and variably susceptible to penicillin, aminoglycosides, and macrolides (55, 92). β-Lactamases have not been found in any strain (92).

CARDIOBACTERIUM

The genus *Cardiobacterium*, which belongs to the family *Cardiobacteriaceae* (51), consists of *C. hominis* and a newly

described species, provisionally named *C. valvarum*, until now represented by five (61) and possibly another (69) isolate from which it differs in nitrate reduction but with whom it shares similar cellular fatty acids and a 99.4% 16S rRNA sequence similarity. The G+C content of the DNA is 59 to 60 mol%.

C. hominis occurs in short chains, pairs, or rosettes, showing bulbous ends and staining irregularly. Addition of yeast extract to the medium (e.g., in chocolate agar) seems to abolish this pleomorphism. Initial growth requires 5 to 10% CO_2. Colonies on blood agar attain a diameter of approximately 1 mm after 48 h at 37°C, are circular, smooth, and opaque, and may pit the agar. Colonies of *C. valvarum* are of the same or a smaller size (0.2-mm diameter), barely grow on chocolate agar, and show pleomorphism and sometimes pitting on that medium.

The normal habitat of *C. hominis* is the human upper respiratory tract and possibly also the gastrointestinal and genitourinary tracts (126). Human disease is mainly endocarditis (HACEK) (19, 31); on rare occasions, *C. hominis* has been isolated from other body sites (84, 108). In blood culture-negative cases, the diagnosis has been made by broad-range PCR and stains from heart valve or embolic tissue (98, 101).

Biochemical tests are recorded in Table 3. The indole test is weakly positive for *C. hominis* and was strongly positive for only one *C. valvarum* isolate (61). A positive indole reaction may initially suggest *Pasteurella* or *Suttonella;* identification must, therefore, include tests for catalase and mannitol fermentation.

C. hominis and *C. valvarum* are susceptible to many antimicrobials (30) including penicillin. β-Lactamase production is very rare and can be inhibited by clavulanic acid (93).

SUTTONELLA

The genus *Suttonella* is the second one in the family *Cardiobacteriaceae* (51). It contains one species, *S. indologenes* (formerly *Kingella indologenes*), a plump, irregularly staining, nonmotile, facultatively anaerobic gram-negative rod whose colonies may show spreading and/or pitting (135). The G+C value is 49 mol%. Characteristics differentiating *Suttonella* from morphologically and biochemically similar species are listed in Table 3. The organism, whose natural habitat is unknown, has only rarely been isolated from human sources, e.g., from diseased eyes (130, 135) and from blood cultures of patients with endocarditis (73, 135). Its susceptibility resembles that of *C. hominis.*

CAPNOCYTOPHAGA

The genus *Capnocytophaga,* in the family *Flavobacteriaceae* (51), consists of seven species. The G+C content of the DNA is 34 to 40 mol%.

Cells are mainly fusiform, medium to long, with tapered ends (Fig. 2D). Primary isolation requires 5 to 10% CO_2 and enriched media. Growth may differ depending on the composition of the blood agar base (38). Colonies are very small after 24 h at 37°C, reach 2 to 4 mm in diameter after 2 to 4 days, are convex or flat and often slightly yellow when scraped off the agar, show regular or spreading edges, and adhere to the agar surface.

The oxidase- and catalase-negative species *C. ochracea*, *C. gingivalis*, *C. sputigena*, *C. haemolytica*, and *C. granulosa* are normal inhabitants of the human mouth. The first three are also associated with juvenile and adult periodontitis (66, 102), whereas the latter two have been isolated from supragingival plaque of healthy adults as well as from subgingival plaque in adult periodontitis (28). All five species may cause septicemia and other infections (endocarditis, endometritis, osteomyelitis, abscesses, peritonitis, and keratitis) in immunocompetent and immunosuppressed (mainly neutropenic) patients (16, 108). They produce an immunosuppressive factor (104).

The oxidase- and catalase-positive species *C. canimorsus* and *C. cynodegmi* reside in the oral cavity of healthy dogs and cats (*C. canimorsus* is found in approximately 25% of dogs and 15% of cats) (14). Infections are associated mainly with dog and cat bites or contact. Patients infected with *C. canimorsus* most often present with septicemia and have been splenectomized or are alcoholics. In fulminant cases with a poor prognosis, disseminated intravascular coagulation, acute renal failure, respiratory distress syndrome, and shock may develop (91). Hemolytic-uremic syndrome and thrombotic thrombocytopenic purpura are other possible sequelae (78, 99). Meningitis, arthritis, eye infections (91), and endocarditis (118) have been reported as well. *C. cynodegmi* has rarely been isolated from localized and systemic infections (119). Both organisms are able to multiply intracellularly in mouse macrophages; *C. canimorsus* also produces a cytotoxin (43).

For detection of *Capnocytophaga* spp. in mixed cultures, various selective media containing bacitracin, polymyxin B, vancomycin, and trimethoprim (29) have been used, as has PCR (64). The organisms also grow on Thayer-Martin and Martin-Lewis agar. Inhibition by sodium polyanethol sulfonate has been reported (121).

Biochemical test results are listed in Table 5. Phenotypic species differentiation in the oxidase-negative group may be inconclusive (28), which has frequently resulted in the identification of the organism as "*Capnocytophaga* sp." 16S rRNA PCR-restriction fragment length polymorphism backed up by 16S rRNA gene sequencing has proven to be a reliable method for identification (28). Typing by restriction fragment length polymorphism has been undertaken (16).

Capnocytophaga spp. are usually susceptible to broad-spectrum β-lactams, macrolides, doxycycline, and fluoroquinolones but are resistant to aminoglycosides and to colistin (115). β-Lactamase-positive isolates have occasionally been found; they were susceptible to combinations with β-lactam inhibitors (74).

DYSGONOMONAS AND RELATED BACTERIA

In the genus *Dysgonomonas* of the family *Porphyromonadaceae*, three species have been recognized: *D. capnocytophagoides* (formerly CDC group DF-3), *D. gadei* (so far represented by one strain only) (68), and *D. mossii* (three strains) (86). The G+C content of the DNA has been approximately 38 mol%. The related DF-3-like bacteria have not been investigated further since their first description (32).

All resemble *Capnocytophaga* spp. in their growth characteristics; *D. mossii* has been reported to be dependent on heme (86). They are coccoid to small, nonmotile gram-negative rods (Fig. 2E). Colonies are entire, measure 1 to 2 mm in diameter after 24 h of growth, have a strawberry-like odor, and are neither adherent nor spreading.

Most *D. capnocytophagoides* strains have been isolated from stools of immunocompromised patients, and a few strains were from other sources (95). Diarrhea was reported to occur for one-half of 20 patients with fecal isolates, whereas routine stool cultures yielded the organism for 1.1 to 2.3% of individuals (95). One blood isolate was found by

TABLE 5 Biochemical reactions of *Capnocytophaga* spp., *Dysgonomonas* and related species, and *Streptobacillus*[a,b,c]

Reaction	C. *ochracea*	C. *sputigena*	C. *gingivalis*	C. *granulosa*	C. *haemolytica*	C. *canimorsus*	C. *cynodegmi*	*D. capnocytophagoides*[d]	D. *mossii*	DF-3-like	*S. moniliformis*
Catalase	−	−	−	−	−	+	+	−	−	−	−
Oxidase	−	−	−	−	−	+	+	−	−	−	−
Indole	−	−	−	−	−	−	−	−	+	+	−
Arginine dihydrolase	−	−	−	ND	ND	+	+	−	−	−	+
Nitrate to nitrite	−	v	−	−	+	−	v	−	−	−	−
Esculin hydrolysis	+	+	−	−	+	v	+	v	+	+	v
Gelatinase	−	v	−	ND	ND	−	−	−	−	v	−
Starch hydrolysis	+	−	−	ND	ND	ND	ND	ND	+	ND	ND
ONPG	+	+	−	+	+	+	+	+	+	+	−
Acid from:											
Lactose	v	v	−	+	+	+	+	+	+	+	−
Sucrose	+	+	+	+	+	−	+	+	+	−	−
Xylose	−	−	−	−	−	−	−	+	+	−	−
Main cellular fatty acids	i-15:0, 3OH-17:0	i-15:0, 3OH-17:0	i-15:0, 3OH-17:0	i-15:0, 3OH-17:0	i-15:0, 3OH-17:0	i-15:0, 3OH-17:0	i-15:0, 3OH-17:0	ai-15:0, i14:0, 15:0, i-3-OH-16:0	ND	ai-15:0, i-15:0, 16:0, 18:2, i-3-OH-17:0	16:0, 18:1, 18:2, 18:0

[a] Data from references 11, 32, 67, and 135.
[b] +, ≥90% of strains positive; −, ≥90% of strains negative; ND, no data available; v, variable.
[c] All species are negative for urease and ornithine decarboxylase and form acid from glucose.
[d] The isolate of *D. gadei* was catalase and indole positive (67).

ribotyping to be identical to one in the stool of the same patient (58). The natural habitat of the other species is unknown.

A selective medium containing cefoperazone, vancomycin, and amphotericin B has been used for stool cultures (58). The species show few biochemical differences (Table 5). Aerobically growing isolates of *Leptotrichia buccalis* may be confused with *Dysgonomonas;* however, the former have a different cellular fatty acid profile (mainly C16:0 and C11/t9/t6C18:1) and produce lactic acid from glucose whereas *Dysgonomonas* produces propionic and succinic acid (11).

Only *D. capnocytophagoides* strains have been checked for antimicrobial susceptibility, and they were found to be mostly susceptible to doxycycline, clindamycin, erythromycin, and trimethoprim-sulfamethoxazole, resistant to cephalosporins, aminoglycosides, and fluoroquinolones, and variably susceptible to other β-lactam antibiotics and imipenem (58, 95).

STREPTOBACILLUS

The genus *Streptobacillus,* in the family *Fusobacteriaceae,* consists of one species, *S. moniliformis,* with a G+C content of the DNA of 24 to 26 mol%.

The species is a facultatively anaerobic, nonmotile gram-negative rod with a pleomorphic appearance. For culture, media enriched with sheep or rabbit blood (15% seems to be optimal), serum, or ascitic fluid and a 5 to 10% CO_2 atmosphere are required. As a culture consisting of small- to medium-size rods ages, some organisms develop into 100- to 150-μm-long filaments which contain granules, bulbs (often in series), and bands (Fig. 2F). Coccal forms and Gram variability may also be observed. Eubacterial and L-phase colonies may be present in the same culture. The former are 1 to 3 mm in diameter after 48 h of growth on blood agar, are round and smooth, and develop optimally after approximately 72 h in 10% CO_2 at 37°C. L-phase colonies develop better on clear, serum-supplemented media and yield the characteristic "fried egg" appearance with irregular outlines and coarse granular lipid globules. In liquid media, growth occurs mainly in the form of "puff balls" at the bottom of the tube. The organism dies quickly unless subcultured.

S. moniliformis occurs naturally in the nasopharynx of wild and laboratory rats and other rodents (mice, squirrels, ferrets, weasels, and gerbils) and occasionally of dogs and cats that prey on rodents. Human infections result either from bites of those animals or from consumption of contaminated food or water. The former infection is called rat-bite fever, and the latter is called Haverhill fever. The characteristic picture of irregular fever, chills, myalgias, and arthralgias is followed in a few days by a maculopapular rash on the extremities and, sometimes, polyarthritis. Complications such as endocarditis (113), myocarditis, pericarditis, meningitis, pneumonia, amnionitis, and abscesses may develop (59).

The organism is best isolated from blood, joint fluid, or abscess material, which has to be collected and transported in the usual way (see chapters 5 and 20). It is inhibited by sodium polyanethol sulfonate present in blood culture media (138). Nalidixic acid has been used in selective media (138). In culture-negative cases, broad-range PCR of fluids has been employed (10, 134). Serological tests are unreliable (21).

S. moniliformis is biochemically inert (Table 5). Glucose is acidified weakly and in a delayed fashion (135). Identification may be confirmed by 16S rRNA gene sequencing (10) or by fatty acid analysis.

S. moniliformis is susceptible to many antimicrobials, particularly to penicillin and doxycycline, the mainstays of treatment (59). It is resistant to trimethoprim-sulfamethoxazole, nalidixic acid, and colistin. The MICs of aminoglycosides and ciprofloxacin are near breakpoint levels (138).

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Haemophilus

MOGENS KILIAN

41

TAXONOMY AND DESCRIPTION OF THE GENUS

Members of the genus *Haemophilus* are gram-negative, non-acid-fast, nonmotile, and non-spore-forming rods that may range from small coccobacilli to filamentous rods. They are obligate parasites that, with a few misclassified exceptions (e.g., *Haemophilus ducreyi*), are exclusively adapted to human mucosal membranes in the respiratory tract. Most species of animal origin originally included in the genus *Haemophilus* have been transferred to the genus *Pasteurella* or the genus *Actinobacillus,* which together with the genus *Haemophilus* and several newly described genera of bacteria from animals constitute the family *Pasteurellaceae* (62).

The currently recognized *Haemophilus* species associated with humans are *H. influenzae, H. aegyptius, H. haemolyticus, H. parainfluenzae, H. parahaemolyticus, H. ducreyi, H. aphrophilus, H. paraphrophilus, H. segnis*, *H. pittmaniae*, and the poorly defined species *H. paraphrohaemolyticus* (36, 60).

All *Haemophilus* species are facultatively anaerobic. The genus name refers to the fact that in vitro growth requires accessory growth factors contained in blood: X factor (hemin; "X" for unknown) and V factor (NAD; "V" for vitamin). *H. influenzae* requires both of these compounds, whereas most other species require only one of them (Table 1). Strains of a few species grow better in a humid atmosphere with added 5 to 10% CO_2. The optimal temperature is about 33 to 37°C (see below).

Haemophilus species ferment a characteristic range of carbohydrates; end products from the fermentation of glucose are succinic, lactic, and acetic acids. Strains of some species (e.g., *H. aphrophilus*) produce gas in fermentation media. All *Haemophilus* strains reduce nitrate and produce alkaline phosphatase (35). The G+C content of DNA of *Haemophilus* species ranges from 37 to 44 mol% (35). Predominant cell wall fatty acids are *n*-tetradecanoate (14:0), 3-hydroxy-tetradecanoate (3-OH-14:0), hexadecanoate (16:1), and *n*-hexadecanoate (16:0) (36).

Separate subpopulations of *H. influenzae* express polysaccharide capsules, of which six different serotypes, termed serotypes a through f, have been described.

By traditional taxonomic criteria, it is unjustified to maintain the recognition of *H. influenzae* and *H. aegyptius* as separate species (10). However, one formal obstacle to combining the two is that the name *H. aegyptius* has priority over *H. influenzae*. Moreover, population genetic analyses reveal that they do constitute distinct populations of bacteria, and clinical experience indicates that they have distinct pathogenic potentials. This is even truer for the so-called *H. influenzae* biogroup aegyptius, which is the closest relative of *H. aegyptius* (7, 37). Likewise, several studies have questioned the validity of *H. paraphrophilus* as a species separate from *H. aphrophilus* (37).

According to nucleic acid hybridization studies and 16S rRNA sequence homologies, the species *H. ducreyi* does not belong in the genus *Haemophilus*, although it is a valid member of the family *Pasteurellaceae* (37, 62). *Actinobacillus actinomycetemcomitans* is closely related to *H. aphrophilus, H. paraphrophilus,* and *H. segnis* (62, 71). However, as *A. actinomycetemcomitans* lacks significant homology to the type species *H. influenzae*, the proposal to transfer *A. actinomycetemcomitans* to the genus *Haemophilus* has gained limited support (71). Rather, *A. actinomycetemcomitans* and its close relatives among the *Haemophilus* species warrant recognition as a separate genus in the family *Pasteurellaceae.*

EPIDEMIOLOGY AND TRANSMISSION

Haemophilus bacteria constitute approximately 10% of the normal bacterial flora of the healthy upper respiratory tract. The predominant species is *H. parainfluenzae*, which accounts for three-fourths of the *Haemophilus* flora both in the oral cavity and in the pharynx but is absent in the nasal cavity. Noncapsulate *H. influenzae* strains, predominantly of biotypes II or III, are present in the pharynx of most healthy children but normally constitute less than 2% of the total bacterial flora (19, 37).

Nasopharyngeal colonization by *H. influenzae* is a dynamic phenomenon characterized by constant turnover of a mixture of clones, with a mean duration of carriage of 1.4 to 2 months (19, 74, 80). During local infection a single clone of *H. influenzae* usually dominates the bacterial flora in the pharynx and nasal cavity. With increasing age of the individual, carriage of *H. influenzae* in the upper respiratory tract becomes less frequent (37). In contrast to the situation in the upper respiratory tract, patients with chronic obstructive pulmonary disease are often persistently colonized by a single or multiple clones of noncapsulate *H. influenzae* (54). In most populations without vaccination, *H. influenzae* serotype b carriage by

TABLE 1 Principal differential characteristics of *Haemophilus* species

Species	Growth factor requirement		Hemolysis	Fermentation of:					Presence of catalase	β-Galactosidase (ONPG[b] test)	CO_2 enhances growth
	X[a]	V		Glucose	Sucrose	Lactose	Mannose	Xylose			
H. influenzae[c]	+	+	−	+	−	−	−	+[d]	+	−	−
H. aegyptius[c]	+	+	−	+	−	−	−	−	+	−	−
H. haemolyticus	+	+[e]	+/−	+	−	−	−	+[d]	+	−	−
H. ducreyi	+	−	−[f]	−	−	−	−	−	−	−	−
H. parainfluenzae	−	+	−	+	+	−	+	−	D[g]	D	−
H. parahaemolyticus	−	+[e]	+	+	+	−	−	−	+	−	−
H. segnis	−	+	−	w[h]	w	−	−	−	D	−	−
H. paraphrophilus[i]	−	+	−	+	+	+	+	D	−	+	+
H. aphrophilus[i]	−	−	−	+	+	+	+	−	−	+	+
H. pittmaniae	−	+[e]	+	+	+	−	+	−	−/w	+	−

[a] As determined by the porphyrin test.
[b] ONPG, *o*-nitrophenyl-β-D-galactopyranoside.
[c] For further characteristics, see Table 2.
[d] More than 90% of isolates are positive.
[e] Due to V factor being released from blood cells in blood agar (bovine, horse, and rabbit blood) by the bacterial hemolysin, symbiosis can be demonstrated only on blood-free media.
[f] Detection requires special media; see text.
[g] D, differences encountered.
[h] w, weak reaction.
[i] For further characteristics, see Table 3.

healthy individuals is below 1% during the first 6 months of life but averages 3 to 5% throughout the rest of childhood, although it may be considerably higher in selected populations (74, 86). Crowding seems to be a factor contributing to a higher carriage rate (33, 74). In most populations, vaccination of children resulted in a significantly reduced carriage of *H. influenzae* serotype b as a result of antibodies induced by the conjugate vaccine (81), but there are unexplained exceptions to this pattern (3, 33).

The association between pharyngeal colonization with *H. influenzae* type b and the occurrence and transmission of disease remains poorly understood. Factors that seem to contribute to the lack of direct association are differences in virulence of individual clones of *H. influenzae* type b (56, 57) and in the disease susceptibility of individuals (33, 66). Carriage in healthy individuals of *H. aegyptius* and *H. influenzae* biogroup aegyptius has not been demonstrated.

The species *H. parainfluenzae, H. pittmaniae, H. aphrophilus, H. paraphrophilus,* and *H. segnis,* but not *H. influenzae,* are part of the normal microflora of the oral cavity in front of the palatinal arches. The species *H. aphrophilus, H. paraphrophilus,* and *H. segnis* occur predominantly on tooth surfaces in the biofilm known as dental plaque. Carriage of *H. parahaemolyticus* and *H. haemolyticus* in healthy individuals appears to be rare. Human saliva contains a mean number of more than 10^7 haemophili per ml (36). Symptomless cervical carriage of the venereal pathogen *H. ducreyi* may occur (29, 85). Spread of the *Haemophilus* species that colonize the upper respiratory tract typically occurs by respiratory droplets, and spread of *H. ducreyi* is by sexual intercourse.

CLINICAL SIGNIFICANCE

H. influenzae

Until the implementation of vaccination in many countries, *H. influenzae* was one of the three leading causes of bacterial meningitis worldwide. Most of these cases were in young children between the ages of 3 months and 3 years, with a peak incidence of infection at 6 to 7 months of age. Virtually all were caused by serotype b, and the majority belonged to biotype I. Both characteristics make them distinct from commensal strains. Strains with the same characteristics were also a major etiological agent of acute epiglottitis (obstructive laryngitis) associated with septicemia. The annual number of invasive *H. influenzae* serotype b infections in the United States is now below 100, most of which occur in unvaccinated or incompletely vaccinated children (1, 11). However, disease still occurs in selected vaccinated populations (27, 28, 43) and *H. influenzae* type b remains a leading cause of meningitis among unvaccinated children in many Asian and developing countries. It is estimated that at least 3 million cases of serious disease and 400,000 to 700,000 deaths occur in young children per year worldwide (66). Occasional cases of invasive infection caused by noncapsulate strains or strains possessing a capsule, primarily of serotype f, occur, mostly in patients with significant underlying disease, such as malignancy, chronic obstructive pulmonary disease, alcoholism, and human immunodeficiency virus infection (22). In children, underlying diseases are less common (26% of cases), and pneumonia and meningitis are equally represented (88). Strains that carry a capsule of serotype a, c, d, or e are occasionally isolated from patients with infections as well as from the healthy respiratory tract (38, 51, 86, 92). Among Navajo and White Mountain Apache children a relatively high incidence (20.2 cases per 100,000 population aged <5 years) of invasive disease with serotype a

strains is observed, but with no absolute increase in numbers in the period after vaccination against *H. influenzae* type b (51).

Invasion of the bloodstream by encapsulated strains of *H. influenzae* may also result in septic arthritis, osteomyelitis, cellulitis, and pericarditis (66, 86). Primary *H. influenzae* pneumonia is being recognized with increasing frequency in both children and adults and is sometimes complicated by bacteremia (44, 53, 58). A recent literature review revealed that *H. influenzae* accounted for 7% of identified etiologic agents of childhood community-acquired pneumonia in North America and Europe and for 21% of such agents in Africa and South America (58). Most isolates from patients with pneumonia are non-type b strains. Pneumonia was the predominant clinical syndrome in patients with *H. influenzae* serotype f disease (88).

Noncapsulate *H. influenzae* strains (often collectively referred to by the misnomer "nontypeable"), indistinguishable from strains found in the healthy respiratory tract (predominantly biotypes II and III), are frequent causes of infections in children. Infections with noncapsulate *H. influenzae* usually occur at sites contiguous with the upper respiratory tract (21, 53) and typically do not include bacteremia, although occasional cases are reported (21, 63). Noncapsulate *H. influenzae* is, after *Streptococcus pneumoniae*, the second most frequent cause of otitis media, the most common cause of purulent bacterial conjunctivitis, and an important cause of sinusitis and chronic or acute exacerbations of lower respiratory tract infections in patients with and without cystic fibrosis (21, 24, 53, 86). *H. influenzae* otitis media is usually associated with significantly increased proportions (>50% of total bacterial flora) of the same clone in the nasopharynx (18, 42). *H. influenzae* occasionally causes obstetric and neonatal infections, and in some countries these are predominantly due to biotype IV strains that are genetically distinct from other *H. influenzae* (73, 93). Both *H. influenzae* and *H. parainfluenzae* have been implicated in urinary tract infections and peritonitis (2, 73, 86, 93). Occasional cases of meningitis caused by noncapsulate *H. influenzae* are often associated with underlying medical problems including ventriculoperitoneal shunts (21, 63).

H. aegyptius

H. aegyptius (Koch-Weeks bacillus) is associated with an acute purulent and contagious form of conjunctivitis ("pink eye") that occurs in seasonal endemics, especially in hot climates (69, 86). In the mid-1980s and early 1990s bacteria that share many of the properties of *H. aegyptius* caused small epidemics of a fulminant pediatric disease known as Brazilian purpuric fever (BPF), manifested by high fever, hemorrhagic skin lesions, septicemia, vascular collapse, hypotensive shock, and death, usually within 48 h of onset (7). The disease is characteristically preceded by purulent conjunctivitis that has resolved before the onset of fever. Although invasive, these bacteria are noncapsulate and have been designated *H. influenzae* biogroup aegyptius (7), a term sometimes erroneously also applied to strains of *H. aegyptius*. Cases clinically similar to BPF were observed in Australia and in the United States, but these were caused by strains genetically related to but distinct from Brazilian BPF isolates (7, 37).

H. ducreyi

H. ducreyi causes the venereal disease soft chancre or chancroid. The genital lesion begins as a tender papule that becomes pustular and then ulcerated over the course of 2 days. Lesions may merge to form larger ulcers that may be accompanied by unilateral inguinal lymphadenitis (bubo formation). *H. ducreyi* infection is an important cause of genital ulcers in Asia, Africa, and Latin America, but is a less important cause of such infections in North America and most parts of Europe. However, PCR detection of *H. ducreyi* suggests that the prevalence of chancroid is underreported (64, 88). The disease has received renewed attention because it facilitates the transmission of human immunodeficiency virus in populations in which it is endemic. Extragenital lesions may occur but are rare (39, 70, 88).

Other Species

In my experience *H. parahaemolyticus* is often isolated from patients with pharyngitis in whom no other bacterial pathogen is detected, from patients with lower respiratory tract infections, and from patients with local abscesses in the oral cavity. Some *Haemophilus* isolates from lower respiratory tract samples from patients with chronic obstructive pulmonary disease are *H. haemolyticus*, some of which, disturbingly, are nonhemolytic and impossible to distinguish from *H. influenzae* by phenotypic analysis (55). Further studies on the clinical significance of these species in causing respiratory tract infections are needed.

Some of the oral *Haemophilus* species *(H. parainfluenzae, H. pittmaniae, H. aphrophilus, H. paraphrophilus*, and *H. segnis)* are occasionally implicated in subacute endocarditis, brain abscesses, sinusitis, arthritis, and osteomyelitis (2, 15, 76), which may follow a temporary bacteremic condition in association with dental treatments that cause a break of the oral mucosal barrier. Reported cases of meningitis ascribed to *H. parainfluenzae* can probably be explained by misidentification of *H. influenzae* isolates as described below.

COLLECTION, TRANSPORT, AND STORAGE

Specimens of patient blood and cerebrospinal fluid (CSF) are obtained and processed as described in chapters 5 and 20. Prompt transport of these samples to the laboratory is mandatory to ensure the fastest possible diagnosis and survival of microorganisms in the sample. Additional specimens from which *Haemophilus* organisms may be isolated include aspirated synovial fluid, pericardial fluid, pleural fluid, pus, nasopharyngeal or throat swabs, sputum, purulent discharge from infected eyes, urine, and occasionally vaginal swabs. It is important that samples taken from the respiratory tract remain representative of the infecting flora by avoidance of contamination with commensals as far as possible. Thus, throat swabs must be collected from the pharynx and not surfaces in front of the palatinal arches. Because most children carry *H. influenzae* in the upper respiratory tract, its mere detection is of no value for establishment of the etiology of otitis media (18).

Samples must be transported to the laboratory in a suitable transport medium or should be spread directly onto appropriate agar media whenever possible (especially conjunctival specimens). The viability of most *Haemophilus* organisms is readily lost as a result of drying out, and they do not survive more than a few days in clinical samples. This is particularly true for the more fastidious species such as *H. aegyptius*.

Genital specimens for *H. ducreyi* culture should be collected from the base and the undermined margins of the chancroid lesion with a saline- or broth-moistened swab. Cultivation may be supplemented by aspiration of pus from infected bubonic lymph nodes, but isolation of *H. ducreyi* from pus is usually less successful than isolation from ulcer material (17, 85). Direct plating on selective media is preferable as it results in the highest recovery. The use of transport media is a viable alternative only if a reliable source of refrigeration is

available, as the viability of *H. ducreyi* in various transport media is completely lost within 1 day at room temperature. Best results were obtained by using thioglycolate-hemin-based transport media containing various combinations of selenium dioxide, albumin, and glutamine (14). After storage at 4°C for up to 4 days, 71% of samples from patients clinically diagnosed with chancroid yielded growth of *H. ducreyi*.

Haemophilus isolates may be stored for many years at room temperature after lyophilization in skim milk or at −135°C on dry cotton swabs heavily inoculated with bacteria from an agar culture transferred to a sterile empty vial. An alternative method of storage is freezing below −60°C of 24-h broth cultures or suspensions of freshly grown cells in broth medium containing 10% glycerol. Biweekly transfers on agar media can also maintain most strains. However, strains of *H. aegyptius* and *H. ducreyi* will rapidly die out.

DIRECT EXAMINATION

Microscopic Examination

CSF

A smear of CSF previously concentrated by centrifugation at 10,000 × *g* or by filtration is stained by Gram's method or with methylene blue. Cytocentrifugation of CSF significantly increases the sensitivity of the Gram stain (79). Care should be exercised in destaining because the coccobacillary form of *H. influenzae* may morphologically resemble pneumococci. However, in CSF the organism may be relatively pleomorphic, with coccoid, coccobacillary, short-rod, long-rod, and filamentous forms. The bacteria may be few in number but are usually detected along with granulocytes by careful examination.

Capsules present on bacteria in CSF can be demonstrated by several techniques, provided that a sufficient number of bacteria are present. The simplest method is by demonstration of the capsular swelling reaction (Quellung reaction). A drop of CSF is mixed on a slide with a drop of an antiserum against each of the capsular serotypes (Becton Dickinson Diagnostic Systems, Sparks, Md.). When mixed with the relevant antiserum, the capsule will appear swollen and sharply delineated by phase-contrast microscopy when it is compared with a control smear without added antiserum. Immunofluorescence staining achieved by addition of a secondary layer of fluorescein-conjugated anti-rabbit immunoglobulins (DAKO, Carpinteria, Calif.) provides another excellent means of identifying and serotyping encapsulated strains of *H. influenzae* in clinical samples. It is important to include controls that rule out direct binding of the fluorescent-labeled secondary antibodies to the bacteria.

Respiratory Tract Specimens

The presence of small, pleomorphic gram-negative rods in sputum samples in areas of the sample that contain polymorphonuclear leukocytes and no squamous epithelial cells strongly suggests that a *Haemophilus* species is etiologically involved, but the final diagnosis must be established by cultivation. Due to their small size and staining reaction, *Haemophilus* bacteria may easily be missed in Gram-stained smears of sputa. Direct microscopy is also an important tool in cases in which fluid is collected by bronchoalveolar lavage or transthoracic needle aspiration.

Other Specimens

Due to the fastidious nature of *H. aegyptius,* microscopic examination of Gram-stained smears of conjunctival scrapings, particularly from patients with seasonal conjunctivitis, is a useful supplement to cultivation. *H. aegyptius* organisms appear as long, slender gram-negative rods (Fig. 1).

There is some doubt about the value of direct examination of Gram-stained smears or dark-field microscopy as an aid in the diagnosis of chancroid. Most genital ulcers have a polymicrobial flora, and the arrangements of *H. ducreyi* cells as long chains or "schools of fish," previously considered typical, appear to be more characteristic of smears prepared from cells grown in vitro (85). The sensitivity of Gram staining does not exceed 50% (85). The development of assays for the direct detection of *H. ducreyi* in smears by immunofluorescence has been hindered, in part, by the poor specificities of polyclonal antisera. Monoclonal antibodies against various surface epitopes on *H. ducreyi* have been developed and used in field studies. Many show satisfactory specificities and sensitivities compared with the results of culture techniques (85), but none appear to be commercially available.

Antigen Detection

Various immunochemical techniques for detection of capsular antigen of serotype b in CSF and other body fluids have been developed, but with the rarity of this serotype in many countries and the limited sensitivity that these tests offer over microscopy of Gram-stained smears, they are now of limited clinical value (49).

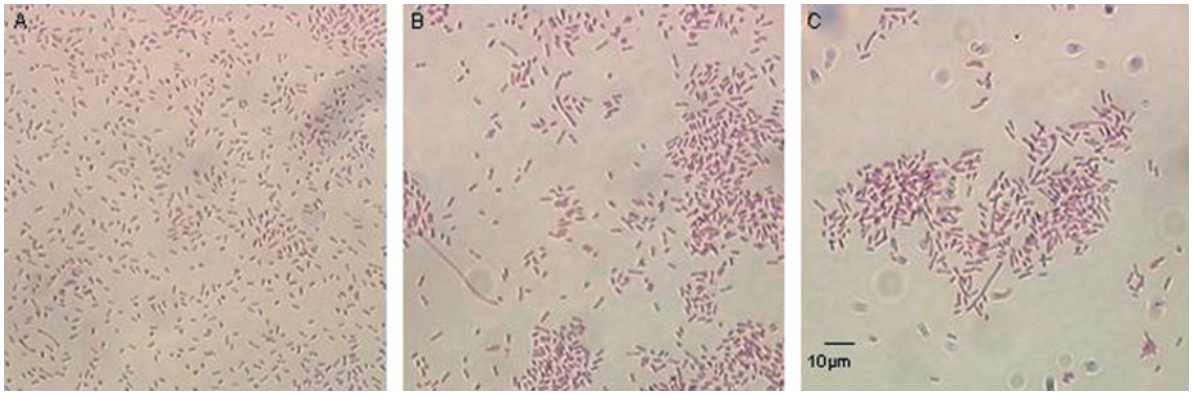


FIGURE 1 Gram-stained smears of *H. influenzae* (A), *H. aegyptius* (B), and *H. influenzae* biogroup aegyptius (C) showing the elongated slender rod morphology of the last two.

Nucleic Acid Detection Techniques

Real-time PCR-based technology for simultaneous detection of *H. influenzae* (serotypes b and c), *Neisseria meningitidis* (multiple serogroups), and *S. pneumoniae* (multiple serotypes) or for specific detection of *H. influenzae* type b in clinical samples of CSF, plasma, serum, and whole blood has been described (12, 47). Multiplex PCR may also be used for simultaneous detection of the four principal middle-ear pathogens *H. influenzae, S. pneumoniae, Moraxella catarrhalis*, and *Alloiococcus otitis* and of the major etiologic agents of genital ulcer disease, *H. ducreyi, Treponema pallidum*, and herpes simplex virus types 1 and 2. The sensitivity of these methods exceeds that of standard culture techniques, and their specificity is generally high (6, 12, 30, 47, 50, 64, 82). None of the methods are commercially available.

ISOLATION TECHNIQUES

Media

Attempts to isolate *Haemophilus* species must take into account the particular growth requirements of this group of bacteria (Table 1). The two growth factors hemin (X factor) and NAD (V factor) are contained in blood cells, but only X factor is directly available in conventional blood agar. To release V factor, the blood cells must be broken up by brief heating as in chocolate agar and in Levinthal's medium. In addition to liberating V factor, heat treatment inactivates V factor-destroying enzymes (NADase) present in blood. The heat treatment is obtained by adding the blood (5% sheep, bovine, or horse blood) to the medium base when its temperature is reduced to approximately 80°C immediately after autoclaving. A comparison of six medium bases showed that a medium consisting of GC agar base plus 5% chocolatized sheep blood and 1% yeast autolysate promoted the best growth of *H. influenzae, H. parainfluenzae,* and most other species that may be isolated from humans, excluding *H. ducreyi* (75).

For *H. aegyptius*, which is particularly fastidious, chocolate agar supplemented with 1% growth factor supplements (IsoVitaleX [Becton Dickinson Diagnostic Systems]; Vitox [Oxoid, Basingstoke, United Kingdom]; or cofactors-vitamin-amino acids [CVA; Gibco Diagnostics, Grand Island, N.Y.]) provides a good medium for isolation, although primary growth from clinical samples is never luxuriant (13, 91).

Growth on conventional blood agar may be achieved by adding a source of V factor, traditionally done by cross-streaking the inoculated plate with a staphylococcus or enterococcus strain. These bacteria excrete V factor and allow detection of *Haemophilus* species that grow as satellite colonies (Fig. 2). Alternatively, V factor may be supplied by applying a filter paper disk or a strip saturated with V factor (BD Diagnostic Systems, Franklin Lakes, N.J.; or Rosco A/S, Taastrup, Denmark) to the surface of the medium.

Due to the relatively slow growth and small size of *Haemophilus* colonies, their presence in cultures from sites with a mixed flora may easily be overlooked. When necessary, this problem may be solved by including selective agents or by a special incubation procedure. This is particularly important for attempts to detect *H. ducreyi.* GC-HgS agar, which consists of GC agar (GIBCO Laboratories) supplemented with 3 mg of vancomycin per liter, 1% hemoglobin, 5% fetal bovine serum, 1% IsoVitaleX (Becton Dickinson Diagnostics), or some other comparable enrichment, has a high sensitivity for isolation of *H. ducreyi* from clinical specimens, as does Mueller-Hinton agar (BBL) supplemented with 5% chocolatized horse blood, 1% IsoVitaleX, and 3 mg of vancomycin per liter (MH-HB agar) (14). Strains of *H. ducreyi* that are inhibited by 3 mg of vancomycin per liter have been reported (32). However, this observation needs to be confirmed in other laboratories. It is generally accepted that the combined use of two media, e.g., GC-HgS and MH-HB, is optimal for the detection and isolation of *H. ducreyi,* possibly because of differences in nutritional requirements between strains (85), but the sensitivity of culture techniques for *H. ducreyi* is never 100% (14, 17, 85). Some lots of fetal calf serum inhibit growth of *H. ducreyi*. Fetal calf serum can be replaced by either activated charcoal or bovine albumin, but not by newborn calf serum (84).

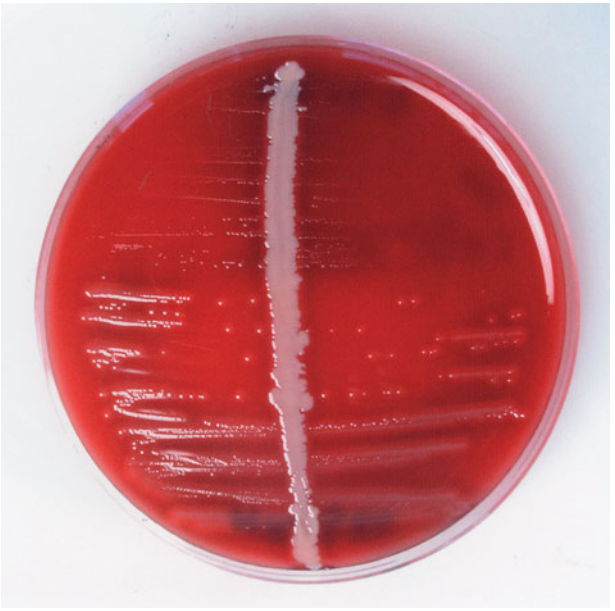


FIGURE 2 Growth of *Haemophilus* isolates on horse blood agar around a streak of *Staphylococcus aureus*. The upper half shows typical satellite growth characteristic of most *Haemophilus* species. The lower part shows characteristic lack of satellite growth of a strain of *H. parahaemolyticus* in spite of its requirement for V factor. Note that the colonies of *H. parahaemolyticus* resemble those of pyogenic streptococci on blood agar.

The problem of overgrowth of *H. influenzae* by *Pseudomonas aeruginosa* in cultures of sputa from patients with cystic fibrosis has been solved with some success by anaerobic incubation of inoculated chocolate agar plates containing 300 mg of bacitracin per liter. Detection of *Haemophilus* bacteria in cultures from the upper respiratory tract is also considerably improved by use of the latter medium (36). However, to be able to evaluate the relative proportion of haemophili in the microflora, a nonselective medium must be included.

Lower respiratory tract secretions and aspirates or swabs of pus from localized infections should be inoculated both on 5% blood agar cross-inoculated with a feeder strain and on chocolate agar.

Cultures of all *Haemophilus* organisms except *H. ducreyi* should be incubated at 35 to 37°C. *H. ducreyi* grows significantly better at 33°C. A moist atmosphere supplemented with 5 to 10% CO_2 is preferred by most strains and is mandatory for the isolation of *H. ducreyi* and many strains of *H. aphrophilus* and *H. paraphrophilus*. Given optimal conditions, most *Haemophilus* species grow to colonies of at least 1 to 2 mm in diameter after incubation for 18 to 24 h. Fresh isolates of *H. aegyptius* and *H. ducreyi* require incubation for 3 to 4 days.

Modern commercial blood culture systems show excellent recovery of *Haemophilus* species from blood samples. However, the same systems fail to recover *Haemophilus* species from normally sterile body fluids like pleural fluids, joint fluids, ascitic fluid, or dialysates unless blood (e.g., human blood, 1:8, vol/vol) or X- and V-factor enrichments are added (26, 67). Cultivation of spinal fluid or subcultivation from blood cultures should be performed on both chocolate agar and blood agar.

Appearance of Growth

With the exception of *H. aphrophilus* and *H. ducreyi,* all *Haemophilus* species that may be isolated from humans require V factor. Therefore, on blood agar these species will grow as satellite colonies around the staphylococcus streak. Colonies of *H. parahaemolyticus* and some strains of *H. haemolyticus* are surrounded by strong beta-hemolytic zones on horse, bovine, and rabbit blood agar. In contrast, *Haemophilus* species are nonhemolytic on sheep blood agar. Colonies of the three hemolytic species *H. parahaemolyticus*, *H. haemolyticus*, and *H. pittmaniae* usually do not show satellite growth on agar media with blood that they are capable of lysing and may be mistaken for pyogenic streptococci (Fig. 2).

H. influenzae colonies on chocolate agar are grayish, semiopaque, smooth, and flat convex and reach a diameter of 1 to 2 mm after incubation for 24 h. In dense areas of the plate, encapsulated strains tend to grow confluently, in contrast to colonies of noncapsulate strains, which remain separate. On clear agar media such as Levinthal's agar, colonies of encapsulated strains show a bright iridescence (red, blue, green, and yellow) when light is obliquely transmitted from behind. The phenomenon is most clearly detected in young (10- to 18-h) cultures and gradually disappears during prolonged incubation. In some strains with capsules of serotypes other than type b, iridescence is not always clear-cut. Noncapsulate strains examined in the same way show a more uniform bluish green color. Strains from cases of meningitis and epiglottitis virtually always produce indole, and so do many noncapsulate isolates (e.g., biovar II). Release of indole gives the growth on agar media a characteristic pungent smell similar to that of *Escherichia coli*. Otherwise, *Haemophilus* strains emit a "mouse-nest" smell.

Colonies of *H. parainfluenzae* may be up to 2 mm in diameter after incubation for 24 h and appear either smooth or rough and wrinkled. Most colonies are flat, grayish, and semiopaque. *H. aphrophilus* and *H. paraphrophilus* grow as rough, raised colonies that rarely attain a diameter exceeding 1 mm. When incubated in air without extra CO_2, these species grow, if at all, in colonies of varying sizes; the growth mimics that of a mixed culture. Fresh isolates of *H. aegyptius* and *H. segnis* grow as small (<1 mm), smooth colonies. Colonies of *H. ducreyi* are smooth and semitranslucently gray and attain a diameter of 0.1 to 0.5 mm on enriched chocolate agar after incubation for 3 days. They are characteristically cohesive and can be pushed intact across the surface of the agar.

Microscopy of Cultures

Gram-stained smears of *Haemophilus* isolates show small gram-negative rods with varying degrees of polymorphism (Fig. 1). The most extensive polymorphism, which may include long filamentous forms, is observed with the X-factor-independent species. *H. ducreyi* often appears as parallel rows of small rods in chains with a "school of fish," "railroad track," or "fingerprint" appearance.

IDENTIFICATION

X- and V-Factor Requirement

The satellite phenomenon, which may be detected in primary blood agar plate cultures, provides a convenient means for a tentative genus identification of all of the V-factor-requiring species that are regularly isolated from humans. However, other bacteria, such as occasional strains of *Pasteurella multocida,* some animal-pathogenic *Actinobacillus* and *Pasteurella* species (59, 62), and some streptococci may also show satellite growth, although it is not always due to V-factor requirement.

A small 5.25-kb plasmid that is normally present in *H. ducreyi* has been found to confer independence of V factor in occasional isolates of *H. parainfluenzae* (46, 94). How widespread such V-factor-independent strains of *H. parainfluenzae* are is not known.

A prerequisite for detection of the satellite phenomenon is an agar medium that lacks V factor. Since ordinary blood agar contains various amounts of free V factor, depending on the method of preparation and length of storage, it is sometimes difficult to achieve convincing satellite growth on this medium. The special problem associated with hemolytic strains has already been mentioned. In case of doubtful reactions, far better results are obtained on a blood agar medium to which the blood (5 to 10%) is added before autoclaving. Since NAD is heat labile, this medium is completely devoid of V factor but otherwise satisfies all growth requirements of *Haemophilus* species, including X factor.

Determination of a requirement for X factor is done in some laboratories by demonstrating growth around an X-factor-containing paper disk (Becton Dickinson Diagnostic Systems) on an agar medium or by comparing growth on medium with blood to growth on medium without blood. However, even when particular care is being exercised to avoid carrying over X factor with the inoculum, this method results in erroneous results in up to 20% of cases (M. Kilian, unpublished observations). If the identity is not confirmed by biochemical tests, *H. influenzae* strains are often misidentified as *H. parainfluenzae* and occasionally vice versa. This undoubtedly explains some of the reported cases of meningitis ascribed to *H. parainfluenzae.* The porphyrin test (34) provides a more accurate and rapid means of determining the X-factor requirement.

Porphyrin Test

The porphyrin test is based on the observation that hemin-independent *Haemophilus* strains excrete porphobilinogen and porphyrins, both of which are intermediates in the hemin biosynthetic pathway (Fig. 3), when supplied with δ-aminolevulinic acid. X-factor-requiring strains do not excrete these compounds because of a lack of enzymes involved in the biosynthesis of heme (36).

The substrate consists of 2 mM δ-aminolevulinic acid hydrochloride (Sigma Chemical Co., St. Louis, Mo.) and 0.8 mM $MgSO_4$ in 0.1 M phosphate buffer at pH 6.9. It is distributed in 0.5-ml quantities in small glass tubes and may be stored for several months in a refrigerator. Sterility is not required. The substrate is inoculated by suspending a heavy loopful of bacteria from an agar plate culture. After incubation for 4 h at 37°C, the mixture is exposed to UV light (wavelength, approximately 360 nm), preferably in a dark room. A red fluorescence from the bacterial cells or from the fluid indicates the presence of porphyrins; i.e., growth of the strain is not dependent on X factor. In cases of doubtful

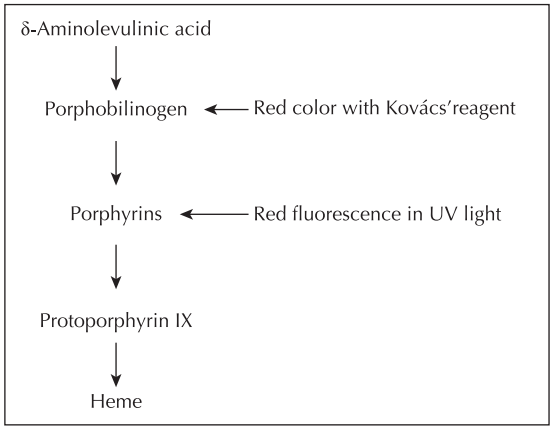


FIGURE 3 Principal steps of the heme biosynthetic pathway and methods for detection of intermediate compounds.

reactions, tubes may be reincubated for up to 24 h. An alternative way is to add 0.5 ml of Kovács' reagent (p-dimethylaminobenzaldehyde, 5 g; amyl alcohol, 75 ml; concentrated HCl, 25 ml), shake the mixture vigorously, and allow the phases to separate. A red color in the lower water phase is indicative of porphobilinogen. When this method of reading is used, it is advisable to include an inoculated tube without δ-aminolevulinic acid as a negative control. Kovács' reagent also gives a red color reaction with indole, which, however, will be present in the upper alcohol phase. Still, indole-positive strains of *H. influenzae* may erroneously be identified as X-factor independent in the absence of an appropriate control. Another disadvantage of this method of reading is that further incubation of samples with doubtful reactions is not possible.

Hemolysis

Hemolysis induced by isolates of *H. haemolyticus, H. parahaemolyticus,* and *H. pittmaniae* may be detected on agar plates with horse, bovine, or rabbit (quad plates) blood but not on sheep blood agar. Demonstration of the hemolytic activity of *H. ducreyi* requires specialized media. Reproducible results have been obtained with bilayer horse blood agar plates consisting of GC agar base, 1% X factor-V factor supplement, and 5% horse blood. All strains examined were less hemolytic on sheep blood agar (83).

Biochemical Tests, General

Table 1 shows key reactions for further differentiation of the *Haemophilus* species. Tests for fermentation of glucose, sucrose, and lactose are important for species identification. They are performed in 1% solutions of the respective carbohydrates in phenol red broth base (Becton Dickson Diagnostic Systems) supplemented with X and V factors (10 mg/liter each; Sigma Chemical Co.) after autoclaving (35). Reactions are usually clear-cut after 24 h of incubation, but some species such as *H. segnis* and *H. aegyptius* show weak reactions.

The ability to reduce nitrate, which is a characteristic of all *Haemophilus* species, can be demonstrated after 5 days of growth in Levinthal's broth with 0.1% (wt/vol) of potassium nitrate. Strains of *H. aphrophilus* and *H. paraphrophilus* may, however, reduce nitrate beyond nitrite, which may result in a negative reaction for nitrite (35).

Direct Enzyme Tests

Three biochemical reactions are important tools in the differentiation of some of the *Haemophilus* species and for differentiation from species of other *Pasteurellaceae* genera: indole production, urease, and ornithine decarboxylase activities (Table 2). The three biochemical reactions used in identification and in biotyping (see below) of *H. influenzae* and *H. parainfluenzae* may preferably be performed as rapid tests that bypass the problems of special growth requirements (35). All three test media (0.3- to 0.5-ml quantities) are inoculated with a heavy loopful of bacteria from an agar culture, and the results are read after incubation for 4 h. (The test for ornithine decarboxylase may in some cases require additional incubation for 18 to 20 h.)

Indole Test

The substrate for the indole test is 1% L-tryptophan in 0.05 M phosphate buffer at pH 6.8. After inoculation and incubation for 4 h, 1 volume of Kovács' reagent is added and the mixture is shaken. A red color in the upper alcohol phase indicates the presence of indole.

Urease Test

The substrate for the urease test is 0.1 g of KH_2PO_4, 0.1 g of K_2HPO_4, 0.5 g of NaCl, and 0.5 ml of 1:500 phenol red in 100 ml of distilled water. The pH is adjusted to 7.0 with NaOH, and 10.4 ml of a 20% (wt/vol) aqueous solution of urea is added (for 1:500 phenol red, dissolve 0.2 g of phenol red in NaOH and add distilled water to 100 ml). The development of a red color within 4 h after inoculation indicates urease activity.

TABLE 2 Differential tests for *H. influenzae, H. parainfluenzae, H. aegyptius*, *H. parahaemolyticus,* and *H. segnis* and for biotyping of *H. influenzae* and *H. parainfluenzae*

Species and biotype	Indole	Urease	Ornithine decarboxylase
H. influenzae			
Biotype I	+	+	+
Biotype II	+	+	−
Biotype III	−	+	−
Biotype IV	−	+	+
Biotype V	+	−	+
Biotype VI	−	−	+
Biotype VII	+	−	−
Biotype VIII	−	−	−
H. aegyptius[a]	−	+	−
H. influenzae biogroup aegyptius[a]	−	+	−
H. parainfluenzae			
Biotype I	−	−	+
Biotype II	−	+	+
Biotype III	−	+	−
Biotype IV	+	+	+
Biotype V	−	−	−
Biotype VI	+	−	+
Biotype VII	+	+	−
Biotype VIII	+	−	−
H. parahaemolyticus	−	+	−
H. segnis	−	−	−

[a] For differentiation of *H. aegyptius, H. influenzae* biotype III, and *H. influenzae* biogroup aegyptius, see text for further information.

Ornithine Decarboxylase Test

The substrate for ornithine decarboxylase is the medium used regularly for other bacteria (see chapter 21). For *Haemophilus* organisms it is to be inoculated with a heavy loopful of bacteria from an agar plate culture. A purple color developing within 4 to 24 h indicates ornithine decarboxylase activity.

Alternative Methods

Several commercial kits are available for identification of *Haemophilus* species once the V-factor requirement has been determined; e.g., API NH (bioMérieux Inc., Hazelwood, Mo.), Vitek NHI Card V1308 (bioMérieux), the Haemophilus ID Test kit (Remel, Lenexa, Kans.), the RIM-H system (Austin Biological Laboratories, Austin, Tex.), and RapidID NF (Innovative Diagnostics, Norcross, Ga.) (65, 72). However, most of these kits do not provide sufficient information to accurately identify an isolate to the species level without additional tests. Therefore, it is advisable that more unusual findings be confirmed with traditional tests.

DNA-Based Identification

If the technology is available, isolates of most *Haemophilus* species and related genera may easily be identified by determining a partial 16S rRNA gene sequence and comparing it with sequences of type strains available in databases accessible on the Internet (e.g., http://www.ncbi.nlm.nih.gov/BLAST/ or http://rdp.cme.msu.edu/index.jsp, which allows a BLAST search against only type strains). Because of their close similarities, the technique does not differentiate between *H. influenzae, H. aegyptius*, and *H. influenzae* biogroup aegyptius and between *H. aphrophilus* and *H. paraphrophilus*.

A review of various published methods for identification of virulence-associated properties and identification probes and a comprehensive compilation of primer and probe sequences have been prepared by Van Belkum and van Alphen (89).

Particular Identification Problems

The differentiation of *H. influenzae, H. aegyptius,* and *H. influenzae* biogroup aegyptius by standard laboratory techniques is impossible at present. The three taxa form distinct clusters by phylogenetic analysis based on multiple genetic and phenotypic traits including electrophoretic mobility of selected housekeeping enzymes (as determined by multilocus enzyme electrophoresis) (37). Both *H. aegyptius* and *H. influenzae* biogroup aegyptius have the same key biochemical characteristics as *H. influenzae* biotype III (Table 2). Since the latter does not possess the invasive potential of BPF strains or the ability to cause serious endemic conjunctivitis, as *H. aegyptius* strains do, it is important to develop means for exact identification. *H. aegyptius* and *H. influenzae* biogroup aegyptius lack the ability to ferment xylose, in contrast to the vast majority of *H. influenzae* strains (7, 35). Additional characteristics that may be of use in the separation of *H. aegyptius* from *H. influenzae* are its poorer in vitro growth, its more slender and rod-like shape (Fig. 1), and its ability to agglutinate human erythrocytes as a result of more stable pilus expression (36). It has been reported that the distinct outer membrane protein profiles of *H. aegyptius* and *H. influenzae* biotype III may be used as an adjunct to differentiate the two species (9).

Recently it has become clear that identification of *H. haemolyticus* poses special problems. Some strains of *H. haemolyticus* are clearly nonhemolytic and cannot be distinguished from *H. influenzae* by phenotypic analysis (55). The prevalence and clinical significance of *H. haemolyticus* are therefore not clear.

Isolates of *H. ducreyi* may be presumptively identified by their adherent colony characteristics, by their appearance in Gram-stained smears, and by a positive oxidase and negative catalase reaction. Confirmation can be achieved by demonstrating a negative porphyrin test result, the inability to ferment carbohydrates, and a positive reaction for nitrate reduction. However, commercial identification systems fail to reveal the ability to reduce nitrate, and a positive oxidase reaction is obtained only with tetramethyl-*p*-phenylenediamine (36, 52).

H. aphrophilus and *A. actinomycetemcomitans* are not easily distinguished from certain *Pasteurella* species, which potentially may explain two reports of the isolation of *Pasteurella gallinarum* from human cases of endocarditis and bacteremia (25). Criteria for the identification of such bacteria may be found elsewhere (62; see also chapter 40).

Biochemical reactions that are valuable in separating *H. aphrophilus* and *H. paraphrophilus* from the closely related *A. actinomycetemcomitans* and other resembling species are provided in Table 3.

TYPING SYSTEMS

Capsular Serotyping

Due to significant differences in pathogenic potentials of noncapsulate and individual serotypes of encapsulated strains of *H. influenzae,* capsule detection and serotyping have been important in the analysis of clinical isolates. The separation of the six serotypes of *H. influenzae* is based on structurally distinct capsular polysaccharides. Until vaccination against *H. influenzae* type b was introduced, identification of serotype b strains was of particular practical significance, as invasive pathogenicity was almost exclusively associated with that serotype. There is no doubt that the exclusive focus on serotype b in many laboratories has resulted in an underestimation of infections caused by other serotypes.

The evaluation of cases of apparent vaccine failure necessitates definitive serotyping. Furthermore, the decline of *H. influenzae* serotype b carriage raised concerns about an increase of *H. influenzae* carriage and disease caused by other serotypes and by noncapsulate strains. A study of Alaskan residents aged 10 years and older suggests such an increase (from 0.5 to 1.1 per 100.000 per year) (68).

Capsular serotyping may be carried out by slide agglutination, by coagglutination with staphylococci or latex particles coated with type-specific antibodies, by a capsular swelling test, and by immunofluorescence microscopy. A recent multicenter comparison of the outcome of slide agglutination serotyping with PCR-based detection of capsule biosynthesis genes in 141 invasive *H.* influenzae isolates showed discrepancies in 40% of the isolates characterized. Discrepancies were due mainly to false-positive reactions obtained with isolates that were noncapsulate. An overall 94% agreement was achieved when the slide agglutination method was performed correctly (38). It is therefore advisable that laboratories that are unable to perform appropriate serotyping of isolates refer these to reference laboratories.

Bacterial suspensions used for serological typing of isolates must be prepared from a young (12- to 18-h) agar culture, since the capsular structure tends to deteriorate in older cultures. A smooth suspension of bacteria is made in normal saline containing formalin (0.5%, vol/vol) and must be of sufficient density to permit the antigen-antibody reaction to proceed to completion within 1 min. In a strong positive slide

TABLE 3 Differential tests for *H. aphrophilus*, *H. paraphrophilus*, and some related species

Species	X factor required	V factor required	Indole	Urease	Ornithine decarboxylase	Lysine decarboxylase	Fermentation of:				Nitrate reduction	Presence of catalase
							Glucose	Sucrose	Lactose	Mannitol		
H. aphrophilus	−	−	−	−	−	−	+	+	+	−	+	−
H. paraphrophilus	−	+	−	−	−	−	+	+	+	−	+	−
A. actinomycetemcomitans	−	−	−	−	−	−	+	−	−	D[a]	+	+
Eikenella corrodens	−	−	−	−	+	+	−	−	−	−	+	−
Cardiobacterium hominis	−	−	+	−	−	−	+	+	−	+	−	−
Suttonella indologenes	−	−	+	−	−	−	+	+	−	−	−	−
H. haemoglobinophilus	+	−	+	−	−	−	+	+	−	+	+	+

[a] D, differences encountered.

agglutination reaction, all bacteria are agglutinated, and the fluid between the clusters is clear.

As polyclonal antisera contain various proportions of antibodies to somatic antigens, agglutination may occur as a result of reaction with somatic antigens. This problem may result in simultaneous agglutination in antisera against several serotypes. Only strong reactions occurring within 1 min should be taken as a positive reaction. Antisera for serotyping of encapsulated *H. influenzae* isolates are available from Becton Dickinson Diagnostic Systems and from some state laboratories. A coagglutination test for detection of serotype b is available under the product name Phadebact (Boule Diagnostics AB, Huddinge, Sweden).

PCR-Based Capsule Typing

While traditional serotyping in inexperienced hands is liable to frequent misinterpretations, the capsule serotype of an isolate may be unequivocally determined by detection of serotype-specific gene sequences. The most attractive method is PCR capsular genotyping, which is easier to perform than probe analysis as there is no need for lengthy DNA extractions, Southern blotting, and hybridization. This PCR methodology may also be used to detect deletion mutants and the amplification pattern of the *capB* locus (21, 90), which differentiates two distinct evolutionary divisions (divisions I and II) of *H. influenzae* type b with different pathogenic potentials (56). Primers specific for the *bex*A gene, which is required for capsule transport and present in strains of all six serotypes, can be used to differentiate between noncapsulate and capsulate isolates (90). Primers for the *bex*A gene (Primers HI-1 and HI-2) and three-primer sets for amplification of each of the six serotype-specific gene sequences are shown in Table 4.

TABLE 4 Capsule transport gene *(bexA)* and serotype-specific three-primer sets for demonstration of capsule production and capsular serotype-specific genes in *H. influenzae*[a]

Primer name	Primer sequences[b]							
HI-1 (*bex*A)	.....CGT	TTG	TAT	GAT	GTT	GAT	CCA	GAC
HI-2 (*bex*A)	.....TGT	CCA	TGT	CTT	CAA	AAT	GAT	G
a_1	.....CTA	CTC	ATT	GCA	GCA	TTT	GC	
a_2	.....GAA	TAT	GAC	CTG	ATC	TTC	TG	
a_3	.....AGT	GGA	CTA	TTC	CTG	TTA	CAC	
b_1	.....GCG	AAA	GTG	AAC	TCT	TAT	CTC	TC
b_2	.....GCT	TAC	GCT	TCT	ATC	TCG	GTG	AA
b_3	.....ACC	ATG	AGA	AAG	TGT	TAG	CG	
c_1	.....TCT	GTG	TAG	ATG	ATG	GTT	CA	
c_2	.....CAG	AGG	CAA	GCT	ATT	AGT	GA	
c_3	.....TGG	CAG	CGT	AAA	TAT	CCT	AA	
d_1	.....TGA	TGA	CCG	ATA	CAA	CCT	GT	
d_2	.....TCC	ACT	CTT	CAA	ACC	ATT	CT	
d_3	.....CTC	TTC	TTA	GTG	CTG	AAT	TA	
e_1	.....GGT	AAC	GAA	TGT	AGT	GGT	AG	
e_2	.....GCT	TTA	CTG	TAT	AAG	TCT	AG	
e_3	.....CAG	CTA	TGA	ACA	AGA	TAA	CG	
f_1	.....GCT	ACT	ATC	AAG	TCC	AAA	TC	
f_2	.....CGC	AAT	TAT	GGA	AGA	AAG	CT	
f_3	.....AAT	GCT	GGA	GTA	TCT	GGT	TC	

[a] The data are from references 20 and 31.

[b] Primers labeled "3" represent sequences between primers labeled "1" and "2" and may, in combination with one of the primary primers, be used for confirmation in second rounds of PCR using the first-round PCR product as a template.

Occasional strains that have lost the ability to produce a serotype b capsule (b⁻ mutants) are identified by a positive result in the type-b-specific PCR and a negative result with the *bexA* gene primers (21).

Other Typing Methods

H. influenzae and *H. parainfluenzae* may be subdivided into eight biotypes or biovars each (35, 36) on the basis of indole production, urease, and ornithine decarboxylase activities (Table 2). Biotypes of *H. influenzae* show a relationship to source of isolation (35, 61) and to capsular serotype (35) in agreement with the fact that the serotypes constitute separate evolutionary lineages, in contrast to serotypes of *S. pneumoniae* (48, 56, 57). The vast majority of serotype a, b, and f strains belong to biotype I, serotype c strains are usually biotype II, and strains with serotype d or e capsules are biotype IV (35). Subtyping on the basis of outer membrane proteins, lipopolysaccharides, or isoenzymes has also been done. However, for epidemiological purposes, typing by DNA-based techniques (DNA fingerprinting, ribotyping, pulsed-field gel electrophoresis, and multilocus sequence typing [MLST]) is preferred because of their superior discriminatory power (41, 48, 77). The optimal typing method is MLST, which is based on sequencing of parts of seven selected housekeeping genes (*adk, atpG, frdB, fucK, mdh, pgi,* and *recA*) (48). The method has the advantage that results are electronically portable and easily comparable between laboratories. In addition, by taking advantage of the MLST database (http://haemophilus.mlst.net/) it is possible to see one's own observations in a global context.

ANTIBIOTIC SUSCEPTIBILITY

Wild-type strains of the *Haemophilus* species are susceptible to ampicillin, cephalosporins, chloramphenicol, sulfonamides, tetracycline, and the macrolide-azalide-ketolide group of antimicrobials. However, mainly because of the spread of conjugative plasmids, a large proportion of clinical isolates are resistant to ampicillin and most other β-lactam antibiotics, to chloramphenicol, and to the tetracyclines (5, 8, 23, 45). The likelihood that an *H. influenzae* isolate will be resistant to ampicillin varies from 5 to 60% in different countries. This development has effectively removed the aminopenicillins (without the addition of a β-lactamase inhibitor) and certain cephalosporins as options for empirical treatment of infections in which *H. influenzae* is a suspected pathogen. Resistance to β-lactams is in most cases a result of a TEM-1 and, to a lesser extent, ROB-1-type β-lactamase production. There are also β-lactamase-negative strains for which ampicillin MICs are increased due to mutations in the penicillin-binding proteins (4, 5, 78, 87). Those strains are referred to as β-lactamase-negative, ampicillin resistant (BLNAR). The prevalence of BLNAR *H. influenzae* in the United States is still below 1%, but recent surveys from several European countries show prevalences from 2 to 20% among *H. influenzae* isolates (33). In contrast to other cephalosporins tested, cefixime is still fully active against *H. influenzae* with the BLNAR phenotype (31).

The rate of macrolide nonsusceptibility is about 1% for azithromycin and 10% for clarithromycin and is in most cases categorized as intermediate resistance. Resistance to fluoroquinolones is still rare (the reported rate in Spain is 0.1%) (16, 45).

Considerable geographical and temporal differences in the antimicrobial susceptibilities of *H. ducreyi* isolates have been recorded. There is very little information concerning chromosomally mediated resistance in *H. ducreyi*. However, most clinical isolates contain plasmids, which may encode resistance, either separately or in combination, to sulfonamides, aminoglycosides, tetracyclines, chloramphenicol, and β-lactam antibiotics. It is not unusual for a single isolate to contain multiple resistance plasmids. Both TEM-1 and ROB-1 β-lactamases have been identified in isolates from Thailand (85). Recommended drugs are azithromycin, ceftriaxone, ciprofloxacin, or erythromycin (95). Strains for which the erythromycin MIC is 4 mg/liter have been isolated in Thailand and Singapore (85). In general, the prevalence and spectrum of antimicrobial resistance make it important that clinical isolates of *H. ducreyi* be routinely monitored for resistance.

Comprehensive and up-to-date in vitro susceptibility data for other *Haemophilus* species are not available. However, there are indications that the prevalence of resistance in these species is higher than in *H. influenzae*. Both β-lactamase-mediated resistance and non-β-lactamase-mediated resistance to ampicillin have been observed in *H. parainfluenzae*. A PCR-based screening of nasopharyngeal haemophili of a group of individuals in the United Kingdom revealed that 59% carried plasmids encoding β-lactamase. Of these, 83% were in *H. parainfluenzae* and 17% were in *H. influenzae* (40). Transmissible resistance to chloramphenicol and aminoglycosides in *H. parainfluenzae, H. parahaemolyticus,* and *H. paraphrophilus* has been described (36).

EVALUATION, INTERPRETATION, AND REPORTING

Detection of *Haemophilus* species by culture is uncomplicated and reliable provided that correct media and incubation procedures are being applied. Only for *H. ducreyi* do isolation procedures show suboptimal sensitivity. Once they become commercially available, PCR methods will become important in the diagnosis of *H. ducreyi* infections.

Interpretation of laboratory findings is also, in most cases, uncomplicated. Isolation of *Haemophilus* species from sites other than the upper respiratory tract and from normally sterile sites is almost always clinically significant. The mere detection of *H. influenzae* in a sample from the nasopharynx is of no clinical significance. However, strong predominance in the flora suggests an impaired balance that may be associated with sinusitis, otitis media, or other local infection in the area. It is therefore important that findings be interpreted on this basis and that they be reported in semiquantitative terms. It is also unavoidable that sputum samples are contaminated with haemophili during passage through the pharynx and oral cavity. The sample must be evaluated by microscopy prior to culture to make sure that small gram-negative rods in the sample are associated with inflammatory cells and not with squamous epithelial cells from the upper respiratory tract. Even in samples that bypass the upper respiratory tract, a mixture of bacteria often renders the interpretation difficult.

Detection of *H. ducreyi* in samples from genital ulcers or in inguinal lymph node aspirates is always clinically relevant and should be followed by proper antibacterial therapy.

With the virtual elimination of *H. influenzae* serotype b disease in many countries, the demands on the clinical microbiology laboratory in this area have changed dramatically. Methods specifically designed to detect antigens of *H. influenzae* type b are of less importance, and more emphasis should be placed on other serotypes. In this context, it is important to realize that observations made for one human population do not necessarily apply to other populations. Population genetic analyses of *H. influenzae* suggest that individual clones of *H. influenzae* may be strictly adapted to

humans with a particular genetic background and do not spread freely among human populations (57). As there is significant coupling of gene loci (linkage disequilibrium) in the *H. influenzae* population, such differences may include antibiotic resistance markers as well as virulence traits.

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Enterobacteriaceae: Introduction and Identification

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In the fifth edition of this Manual in 1991, Farmer and Kelly commented that it was becoming more difficult to cover the family *Enterobacteriaceae* in a single chapter. The family includes many important organisms (see Tables 1 to 7) such as the plague bacillus *Yersinia pestis,* the typhoid bacillus *Salmonella enterica* serotype Typhi *(Salmonella typhi),* four genera with species that often cause diarrhea and other intestinal infections, seven species that frequently cause nosocomial infections, many other organisms that occasionally cause human or animal infections, dozens of species that occasionally occur in human clinical specimens, and many other species that do not occur in human clinical specimens but can be confused with those that do. In the sixth edition, the material on *Enterobacteriaceae* was divided among three chapters: an introduction to the family that described the overall plan for isolation and identification; a chapter that covered *Salmonella, Shigella, Escherichia coli,* and *Yersinia,* the enteric pathogens; and a chapter that covered the remaining genera and species in the family. In the seventh edition, a fourth chapter was added that covered *Klebsiella, Enterobacter, Citrobacter,* and *Serratia.* In the eighth edition, there were also four chapters. However, *Yersinia* was assigned to its own chapter, and *Klebsiella, Enterobacter, Citrobacter, Serratia,* and *Plesiomonas* (see this chapter) and the remaining *Enterobacteriaceae* were grouped together. This organization has been maintained for the ninth edition.

Because of space limitations, many topics in the present chapter are discussed briefly and only a few primary literature citations are given. Several books, reviews, and chapters are recommended for more detailed information (5, 12–14, 16, 24, 32, 37, 38, 43, 45, 49, 55, 63, 68, 69, 82, 90, 92).

NOMENCLATURE AND CLASSIFICATION

The nomenclature and classification of the genera, species, subspecies, biogroups, and serotypes of *Enterobacteriaceae* have always been topics for heated debate and differing opinions (12–14, 24, 31–34, 37, 38, 55, 63). Until recently, genera and species were defined by biochemical and antigenic analysis. Today, newer techniques such as nucleic acid hybridization and nucleic acid sequencing, which measure evolutionary distance (see chapters 16 and 19 in this Manual), have made it possible to determine the evolutionary relationships among organisms in the family (12–16, 21, 37, 38, 55). The use of these molecular techniques has led to the discovery of many new species and has resulted in the proposed reclassification of others (12–14, 37).

This chapter includes the different names and classifications that clinical microbiologists are likely to encounter in the scientific literature and in material accompanying commercial products. The nomenclature and classification given in Tables 1 to 5 are a compromise based on all available evidence. They include most of the genera, species, subspecies, biogroups, and unnamed Enteric Groups included in the family. If two names are widely used for the same organism, both are mentioned in this chapter with one in parentheses. Many of the "nonclinical" organisms in the family are also included, because there is a possibility that they will be isolated from a human clinical specimen in the future (12–14, 16, 37, 55).

Most of the newly described organisms are very rarely found in clinical specimens (26, 32, 37). This is illustrated by the published listings of organisms that most often cause bacteremia, nosocomial infections, and infections of the gastrointestinal tract (see Tables 6 and 7). The National Library of Medicine's Internet taxonomy database has a useful list of organisms in the family *Enterobacteriaceae* and its relatives (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=543). This list may also be accessed through http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Taxonomy by selecting "Taxonomy" in the "Search" field and typing "Enterobacteriaceae" in the adjacent "for" field. The Internet site of J. P. Euzéby (http://www.bacterio.cict.fr) gives nomenclature, classifications, original literature citations, and other information for all of the genera and species in the family *Enterobacteriaceae* and its relatives. Unfortunately, the Euzéby site has no alphabetical list of the genera included in the family.

New Species That Occur in Human Clinical Specimens

Names of several new organisms and several "proposed alternative classifications" have been published since the previous edition of this Manual (Table 2), and several of the new organisms occur in human clinical specimens. It is becoming more and more difficult to update the biochemical reaction table (Table 3) of clinically important *Enterobacteriaceae.* For example, *Klebsiella granulomatis (Calymmatobacterium granulomatis)* does not grow on most bacteriological media and lacks a type strain that can be grown and described based on

TABLE 1 Genera and species of *Enterobacteriaceae* that cause,[a] or are associated with, specific or unusual human diseases, syndromes, or conditions[b]

Disease, syndrome, or condition	Organism(s)	Comment
Brain abscesses	*Citrobacter diversus, Enterobacter sakazakii*	Severe, often fatal disease in neonates, survivors have severe mental impairment; cause outbreaks in hospital nurseries.
Dysentery[c]	*Shigella*	One the most important human diarrheal diseases ("invasive" strains of *Escherichia coli* cause a similar but often milder disease)
Granuloma inguinale	*Klebsiella granulomatis (Calymmatobacterium granulomatis)*	Chronic genital ulcerative disease; organism is difficult to demonstrate microbiologically because it does not grow on laboratory media (see text).
Hemorrhagic colitis[c]	*Escherichia coli* O157:H7	Other Shiga toxin-producing strains can also causes a similar, but often milder disease.[b]
Histamine poisoning (scombroid poisoning)	*Proteus morganii, Klebsiella=Raoultella,* others	Caused by bacteria that produce large amounts of histamine and histamine-like substances (scombrotoxin) when they multiply and spoil fish tissues (via bacterial histidine decarboxylase)
Intestinal infection preceded by *Entamoeba histolytica* infection	*Edwardsiella tarda*	Several interesting studies suggest that the protozoan infection must precede *Edwardsiella tarda* in order for it to cause infection.
Meningitis and sepsis in neonates caused by ingesting contaminated infant formula	*Enterobacter sakazakii*	Nursery outbreaks in which infants acquire the strain from dried infant formula that is contaminated with the bacterium; other coliform organisms have also been isolated from formula samples.
Neutropenic patients—initial fever and fever after empirical antibiotic treatment	*Escherichia coli, Klebsiella, Enterobacter*	
Ozena	*Klebsiella ozaenae*	Chronic atrophic rhinitis (ozena), foul smelling discharge from the nose: causative role is uncertain; it may be just colonization.
Paratyphoid fever	*Salmonella* serotypes Paratyphi A, B, C, and others	Causation: paratyphoid fever is an enteric fever that is similar to typhoid fever[b].
Plague (pneumonic and bubonic plague)	*Yersinia pestis*	One of the most important human diseases—the "Black Death" of the Middle Ages
Pneumonia associated with alcoholism—"Friedländer's pneumonia"	*Klebsiella pneumoniae*	Capsular types K1–K6 are most frequently isolated.
Pseudoappendicitis	*Yersinia enterocolitica*	The appendix is normal after surgical removal.
Rhinoscleroma	*Klebsiella rhinoscleromatis*	Chronic granulomatous infection of the nasal passages and respiratory tract; usually seen in the tropics
Reiters syndrome (reactive arthritis)	*Salmonella, Shigella,* enteropathogenic strains of *Yersinia enterocolitica*	Sometimes occurs after gastrointestinal infection; more common in patients with the HLA-B27 histocompatibility antigen
Salmonellosis[c]	*Salmonella*—any of the named or numbered serotypes	One of the most important human diarrheal diseases
Shigellosis[c]	*Shigella*—any of the named and provisional serotypes	One of the most important human diarrheal diseases
Tropical sprue/enteropathy	*Klebsiella, Enterobacter, Hafnia,* others	"Syndrome of enigmatic origin" characterized by prolonged diarrhea and malabsorption by certain residents of the tropics (68); strong presumption for causation (68)
Typhoid fever[c]	*Salmonella* serotype Typhi	One of the most important human diseases

[a] See reference 68 for more details and the evidence for causation versus association in each.

[b] "Adherent-invasive *Escherichia coli*–strains" have been isolated from some patients with Crohn's disease (10), and this organism is being investigated for a causal role (along with many other unrelated organisms).

[c] See reference 68 and chapter 43 for discussions.

TABLE 2 Newly proposed genera, species, and subspecies of *Enterobacteriaceae,*[a] including several "proposed alternative classifications" for previously described organisms

Organism	Occurrence in human clinical specimens	Proposed as: comments	Reference
Averyella dalhousiensis (formerly classified as Enteric Group 58)	Yes	New genus and new species that colonize or infect traumatic injuries; septicemia in a patient receiving total parenteral nutrition (TPN) through a subcutaneous port	56
Citrobacter Group 139	Yes	Proposed alternative classification[b] for Enteric Group 139, which caused a small hospital outbreak	94[b]
Enterobacter cloacae subsp. *dissolvens*	Yes	Proposed alternative classification for *Enterobacter dissolvens;* isolated from plants, blood, skin abscess, abdomen	51
Enterobacter ludwigii	Yes	New species, 16 clinical isolates from blood, urine, etc.; formerly included in *Enterobacter cloacae;* now part of this species complex	52
Enterobacter radicincitans	No	New species isolated from phyllosphere of winter wheat; fixes nitrogen, promotes plant growth	59
Escherichia albertii	Yes	New species; originally misidentified as "Shiga toxin-producing *Hafnia alvei*"	53
Klebsiella singaporensis	No	New species represented by a single isolate from soil	67
Klebsiella variicola	Yes	New species that is phenotypically almost identical to *Klebsiella pneumoniae;* isolated from plants but also from human blood	81
Kluyvera intermedia	No	Proposed alternative classification for *Enterobacter intermedius*	74
Photorhabdus asymbiotica subsp. *australis*	Yes	New subspecies isolated from blood and wounds of patients in Australia	2
Photorhabdus luminescens subsp. *kayaii* and subsp. *thracensis*	No	Two new subspecies isolated from nematodes in Turkey	48
Salmonella enterica	Yes	An old species, but newly made legitimate (see text)	58
Samsonia erythrinae	No	New species isolated from diseased erythrina trees (*Erythrina* sp.)	89
Serratia marcescens subsp. *sakuensis*	No	New subspecies; reported to produce endospores; isolated from wastewater	1
Serratia quinivorans	No	Proposed alternative classification of *Serratia proteamaculans* subsp. *quinivora;* isolated from plants and insects	3
Yersinia aleksiciae	Yes (feces only)	New species represented by only five strains, formerly included in *Yersinia kristensenii* serogroup O:16; isolated from human feces, pork products, and rats/moles	87
Yersinia enterocolitica subspecies *palearctica*	Yes	New subspecies that was proposed to include one of the three evolutionary groups in the species	72
Xenorhabdus budapestensis, X. ehlersii, X. inexii, X. szentirmaii	No	Four new species that are symbiotic in nematodes of the genus *Steinernema* (family *Steinernematidae*) and that are insect pathogens	65

[a] This table includes organisms that were not included in this chapter in the 8th edition of the Manual.
[b] This chapter should be cited as the reference for *Citrobacter* Group 139 as a proposed alternative classification for Enteric Group 139.

simple phenotypic methods. Another problem has been the unavailability of certain strains (62). Other new organisms are almost identical to older organisms in their phenotypic properties. For example, *Klebsiella variicola* will be very difficult to differentiate from *K. pneumoniae* and other *Klebsiella* species. All the newly described species of *Enterobacteriaceae* need to be characterized and added to Table 3.

Organisms That Do Not Occur in Human Clinical Specimens

New or unusual *Enterobacteriaceae* that do not occur in human clinical specimens are listed in Tables 1 to 5, and more information and literature citations can be found at the Internet sites previously cited and in the new edition of *Bergey's Manual of Systematic Bacteriology* (16). These new organisms should be characterized and added to Table 3.

The Expanding Number of *Enterobacteriaceae* Species

How many species of *Enterobacteriaceae* are there? There are probably many hundreds, if not thousands. This is becoming more apparent as methods such as DNA-DNA hybridization and 16S rRNA sequencing (14, 16) are being used to study strains isolated from human clinical specimens, plants, animals,

TABLE 3 Biochemical reactions[a] of the named species, subspecies, biogroups, and Enteric Groups of the family *Enterobacteriaceae*

Organism	Indole production	Methyl red	Voges-Proskauer	Citrate (Simmons)	Hydrogen sulfide (TSI)	Urea hydrolysis	Phenylalanine deaminase	Lysine decarboxylase	Arginine dihydrolase	Ornithine decarboxylase	Motility	Gelatin hydrolysis (22°C)	Growth in KCN	Malonate utilization	D-Glucose, acid	D-Glucose, gas	Lactose fermentation	Sucrose fermentation	D-Mannitol fermentation	Dulcitol fermentation	Salicin fermentation	Adonitol fermentation	*myo*-Inositol fermentation	D-Sorbitol fermentation	L-Arabinose fermentation	Raffinose fermentation	L-Rhamnose fermentation	Maltose fermentation	D-Xylose fermentation	Trehalose fermentation	Cellobiose fermentation	α-Methyl-D-glucoside fermentation	Erythritol fermentation	Esculin hydrolysis	Melibiose fermentation	D-Arabitol fermentation	Glycerol fermentation	Mucate fermentation	Tartrate, Jordan's	Acetate utilization	Lipase (corn oil)	DNase (25°C)	Nitrate → nitrite	Oxidase, Kovacs	ONPG test	Yellow pigment (25°C)	D-Mannose fermentation	Tyrosine hydrolysis
Genus *Averyella*																																																		
*A. dalhousiensis**[b]	0	100	0	85	0	70	0	100	0	85	100	0	100	85	100	85	30	0	100	85	100	0	0	100	100	0	100	100	100	100	100	55	0	0	0	0	30	0	60	45	0	0	100	0	100	0	100	0
Genus *Budvicia*																																																			
*B. aquatica**	0	93	0	0	80	33	0	0	0	0	27	0	0	0	100	53	87	0	60	0	0	0	0	0	80	0	100	0	93	0	0	0	0	0	0	27	0	20	27	0	0	0	100	0	93	0	0	0
Genus *Buttiauxella*																																																		
B. agrestis	0	100	0	100	0	0	0	0	0	100	100	0	80	60	100	100	100	0	100	0	100	0	0	0	100	100	100	100	100	100	100	0	0	100	100	0	60	100	60	0	0	0	100	0	100	0	100	0
B. brennerae	0	100	0	0	0	0	0	0	0	33	100	0	100	100	100	100	0	0	100	0	100	67	0	0	100	100	33	100	100	100	100	0	0	100	100	67	67	67	0	0	0	0	100	0	100	0	100	0
B. ferragutiae	0	100	0	0	0	0	0	100	0	80	60	0	40	0	100	100	0	0	100	0	100	0	0	100	100	0	100	100	100	100	100	40	0	100	0	0	0	60	0	0	0	0	100	0	100	0	100	0
B. gaviniae	0	100	0	20	0	0	0	0	20	0	80	0	60	100	100	40	60	0	100	0	100	100	0	0	100	0	100	60	100	100	100	0	0	100	0	80	0	80	40	0	0	0	100	0	100	0	100	0
B. izardii	0	100	0	0	0	0	0	0	0	100	100	0	67	100	100	100	100	0	100	0	100	0	0	0	100	33	100	100	100	100	100	0	0	100	67	0	33	100	67	0	0	0	100	0	100	0	100	0
*B. noackiae**	33	100	0	33	0	0	100	0	67	0	100	0	100	100	100	100	0	0	100	0	100	0	0	0	100	0	100	100	100	100	100	33	0	100	0	0	0	100	100	0	0	0	100	0	100	0	100	0
B. warmboldiae	0	100	0	33	0	0	100	0	0	0	100	0	33	100	100	100	0	0	100	0	100	0	67	0	100	0	100	100	100	100	100	0	0	100	0	0	0	0	0	0	0	0	100	0	100	0	100	0
Genus *Cedecea*																																																		
*C. davisae**	0	100	50	95	0	0	0	0	50	95	95	0	86	91	100	70	19	100	100	0	99	0	0	0	0	10	0	100	100	100	100	5	0	45	0	100	0	0	0	0	91	0	100	0	90	0	100	0
*C. lapagei**	0	40	80	99	0	0	0	0	80	0	80	0	100	99	100	100	60	0	100	0	100	0	0	0	0	0	0	100	0	100	100	0	0	100	0	100	0	0	0	60	100	0	100	0	99	0	100	0
*C. neteri**	0	100	50	100	0	0	0	0	100	0	100	0	65	100	100	100	35	100	100	0	100	0	0	100	0	0	0	100	100	100	100	0	0	100	0	100	0	0	0	0	100	0	100	0	100	0	100	0
C. species 3*	0	100	50	100	0	0	0	0	100	0	100	0	100	0	100	100	0	50	100	0	100	0	0	0	0	100	0	100	100	100	100	50	0	100	100	100	0	0	0	50	100	0	100	0	100	0	100	0
C. species 5*	0	100	50	100	0	0	0	0	50	50	100	0	100	0	100	100	0	100	100	0	100	0	0	100	0	100	0	100	100	100	100	0	0	100	100	100	0	0	0	50	50	0	100	0	100	0	100	0
Genus *Citrobacter*																																																		
*C. freundii** (A)[c]	33	100	0	78	78	44	0	0	67	0	89	0	89	11	100	89	78	89	100	11	0	0	0	100	100	44	100	100	89	100	44	11	0	0	100	0	100	100	100	44	0	0	100	0	89	0	100	0
*C. youngae** (A)	15	100	0	75	65	80	0	0	50	5	95	0	95	5	100	75	25	20	100	85	10	0	5	100	100	10	100	95	100	100	45	0	0	5	10	5	90	100	100	65	0	0	85	0	90	0	100	0
*C. braakii** (A)	33	100	0	87	60	47	0	0	67	93	87	0	100	0	100	93	80	7	100	33	0	0	0	100	100	7	100	100	100	100	73	33	0	0	80	0	87	100	93	53	0	0	100	0	80	0	100	0
*C. murliniae** (A)	100	100	0	100	67	67	0	0	67	0	100	0	100	0	100	100	67	33	100	100	33	0	0	100	100	33	100	100	100	100	100	0	0	0	33	0	100	100	100	33	0	0	100	0	100	0	100	0
*C. werkmanii** (A)	0	100	0	100	100	100	0	0	100	0	100	0	100	100	100	100	17	0	100	0	0	0	0	100	100	0	100	100	100	100	0	0	0	0	0	0	100	100	100	100	0	0	100	0	100	0	100	0
*C. gillenii** (A)	0	100	0	33	67	0	0	0	33	0	67	0	100	100	100	100	67	33	100	0	0	0	0	100	100	0	100	100	100	100	67	0	0	0	67	0	67	67	100	0	0	0	100	0	67	0	100	0
*C. amalonaticus** (B)	100	100	0	95	5	85	0	0	85	95	95	0	99	1	100	97	35	9	100	1	30	0	0	99	99	5	100	99	99	100	100	2	0	5	0	0	60	96	96	86	0	0	99	0	97	0	100	0
*C. farmeri** (B)	100	100	0	10	0	59	0	0	85	100	97	0	93	0	100	96	15	100	100	2	9	0	0	98	100	100	100	100	100	100	100	75	0	0	100	0	65	100	93	80	0	0	100	0	100	0	100	0
C. Group 137* (B)	100	100	0	0	0	70	0	0	20	100	100	0	100	0	100	0	100	100	100	0	100	0	0	100	100	100	100	100	100	100	100	80	0	100	100	0	100	100	50	100	0	0	100	0	100	0	100	0
*C. rodentium** (B)	0	100	0	0	0	100	0	0	0	100	0	0	0	100	100	100	100	0	100	0	0	0	0	100	100	0	100	100	100	100	100	0	0	0	0	0	0	100	100	0	0	0	100	0	100	0	100	0
*C. sedlakii** (B)	100	100	0	95	5	85	0	0	85	95	95	0	99	1	100	97	35	9	100	1	30	0	0	99	99	5	100	99	99	100	100	2	0	5	0	0	60	96	96	86	0	0	99	0	97	0	100	0
*C. diversus (C. koseri)** (C)	100	100	0	10	0	59	0	0	85	100	97	0	93	0	100	96	15	100	100	2	9	0	0	98	100	100	100	100	100	100	100	75	0	0	100	0	65	100	93	80	0	0	100	0	100	0	100	0
Genus *Edwardsiella*																																																		
*E. tarda**	99	100	0	1	100	0	0	100	0	100	98	0	0	0	100	100	0	0	0	0	0	0	0	0	0	9	0	0	100	0	0	0	0	0	0	0	0	30	0	25	0	0	100	0	0	0	100	0
E. tarda biogroup 1*	100	100	0	0	0	0	0	100	0	100	100	0	0	0	100	50	0	100	100	0	0	0	0	0	0	100	0	0	100	0	0	0	0	0	0	0	0	0	0	0	0	0	100	0	0	0	100	0
*E. hoshinae**	50	100	0	0	0	0	0	100	0	95	100	0	0	100	100	35	0	100	100	0	50	0	0	0	13	0	0	100	0	100	0	0	0	0	0	0	65	0	0	0	0	0	100	0	0	0	100	0
E. ictaluri	0	0	0	0	0	0	0	100	0	65	0	0	0	0	100	50	0	0	0	0	0	0	0	0	0	0	0	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	0	0	0	100	0
Genus *Enterobacter*																																																		
*E. aerogenes**	0	5	98	95	0	2	0	98	0	98	97	0	98	95	100	100	95	100	100	5	100	98	95	100	100	96	99	99	100	100	100	95	0	98	99	100	98	90	95	50	0	0	100	0	100	0	95	0
*E. cloacae**	0	5	100	100	0	65	0	0	97	96	95	0	98	75	100	100	93	97	100	15	75	25	15	95	100	97	92	100	99	100	99	85	0	30	90	15	40	75	30	75	0	0	99	0	99	0	100	0
"*E. agglomerans* complex"*	20	50	70	50	0	20	20	0	0	0	85	2	35	65	100	20	40	75	100	15	65	7	15	30	95	30	85	89	93	97	55	7	0	60	50	50	30	40	25	30	0	0	85	0	90	75	98	0
*E. gergoviae**	0	5	100	99	0	93	0	90	0	100	90	0	0	96	100	98	55	98	99	0	99	0	0	0	99	97	99	100	99	100	99	2	0	97	97	97	100	2	97	93	0	0	99	0	97	0	100	0
*E. sakazakii**	11	5	100	99	0	1	50	0	99	91	96	0	99	18	100	98	99	100	100	5	99	0	75	0	100	99	100	100	100	100	100	96	0	100	100	0	15	1	1	96	0	0	99	0	100	98	100	0
*E. taylorae (E. cancerogenus)**	0	5	100	100	0	1	0	0	94	99	99	0	98	100	100	100	10	0	100	0	92	0	0	1	100	0	100	99	100	100	100	1	0	90	0	0	1	75	0	35	0	0	100	0	100	0	100	
E. amnigenus biogroup 1*	0	7	100	70	0	0	0	0	9	55	92	0	100	91	100	100	70	100	100	0	91	0	0	9	100	100	100	100	100	100	100	55	0	91	100	0	0	35	9	0	0	0	100	0	91	0	100	0
E. amnigenus biogroup 2*	0	65	100	100	0	0	0	0	35	100	100	0	100	100	100	100	35	0	100	0	100	0	0	100	100	0	100	100	100	100	100	100	0	100	100	0	0	100	0	0	0	0	100	0	100	0	100	0
*E. asburiae**	0	100	2	100	0	60	0	0	21	95	0	0	97	3	100	95	75	100	100	0	100	0	0	100	100	70	5	100	97	100	100	95	0	95	0	0	11	21	30	87	0	0	100	0	100	0	100	0

TABLE 3 *(Continued)*

Organism	Indole production	Methyl red	Voges-Proskauer	Citrate (Simmons)	Hydrogen sulfide (TSI)	Urea hydrolysis	Phenylalanine deaminase	Lysine decarboxylase	Arginine dihydrolase	Ornithine decarboxylase	Motility	Gelatin hydrolysis (22°C)	Growth in KCN	Malonate utilization	D-Glucose, acid	D-Glucose, gas	Lactose fermentation	Sucrose fermentation	D-Mannitol fermentation	Dulcitol fermentation	Salicin fermentation	Adonitol fermentation	*myo*-Inositol fermentation	D-Sorbitol fermentation	L-Arabinose fermentation	Raffinose fermentation	L-Rhamnose fermentation	Maltose fermentation	D-Xylose fermentation	Trehalose fermentation	Cellobiose fermentation	α-Methyl-D-glucoside fermentation	Erythritol fermentation	Esculin hydrolysis	Melibiose fermentation	D-Arabitol fermentation	Glycerol fermentation	Mucate fermentation	Tartrate, Jordan's	Acetate utilization	Lipase (corn oil)	DNase (25°C)	Nitrate → nitrite	Oxidase, Kovacs	ONPG test	Yellow pigment (25°C)	D-Mannose fermentation	Tyrosine hydrolysis
*E. hormaechei**	0	57	100	96	0	87	4	0	78	91	52	0	100	100	100	83	9	100	100	87	44	0	0	0	100	0	100	100	96	100	100	83	0	0	0	0	4	96	13	74	0	0	100	0	95	0	100	0
E. cancerogenus	0	0	100	100	0	0	0	0	100	100	100	0	100	100	100	100	0	0	100	0	100	0	0	0	100	0	100	100	100	100	100	0	0	100	0	0	0	100	0	33	0	0	100	0	100	0	100	0
E. dissolvens	0	0	100	100	0	100	0	0	100	100	0	0	100	100	100	100	0	100	100	0	100	0	0	100	100	100	100	100	100	100	100	100	0	100	100	0	0	100	0	100	0	0	100	0	100	0	100	0
E. nimipressuralis	0	100	100	0	0	0	0	0	0	100	0	0	100	100	100	100	0	0	100	0	100	0	0	100	100	0	100	100	100	100	100	100	0	100	100	0	0	100	0	0	0	0	100	0	100	0	100	0
E. pyrinus	0	29	86	0	0	86	0	100	0	100	43	0	0	86	100	100	14	100	100	0	100	0	100	0	100	0	100	100	0	100	100	0	0	100	0	0	0	0	0	0	0	0	100	0	100	0	100	0
Genera *Escherichia* and *Shigella*																																																	
*E. coli**	98	99	0	1	1	1	0	90	17	65	95	0	3	0	100	95	95	50	98	60	40	5	1	94	99	50	80	95	95	98	2	0	0	35	75	5	75	95	95	90	0	0	100	0	95	0	98	0
E. coli, inactive*	80	95	0	1	1	1	0	40	3	20	5	0	1	0	100	5	25	15	93	40	10	3	1	75	85	15	65	80	70	90	2	0	0	5	40	5	65	30	85	40	0	0	98	0	45	0	97	0
*S. dysenteriae** (Serogroup A)	45	99	0	0	0	0	0	0	2	0	0	0	0	0	100	0	0	0	0	5	0	0	0	30	45	0	30	15	4	90	0	0	0	0	0	0	10	0	75	0	0	0	99	0	30	0	100	0
*S. flexneri** (Serogroup B)	50	100	0	0	0	0	0	0	5	0	0	0	0	0	100	3	1	1	95	1	0	0	0	29	60	40	5	30	2	65	0	0	0	0	55	1	10	0	30	8	0	0	99	0	1	0	100	0
*S. boydii** (Serogroup C)	25	100	0	0	0	0	0	0	18	2	0	0	0	0	100	0	1	0	97	5	0	0	0	43	94	0	1	20	11	85	0	0	0	0	15	0	50	0	50	0	0	0	100	0	10	0	100	0
*S. sonnei** (Serogroup D)	0	100	0	0	0	0	0	0	2	98	0	0	0	0	100	0	2	1	99	0	0	0	0	2	95	3	75	90	2	100	5	0	0	0	25	0	15	10	90	0	0	0	100	0	90	0	100	0
*E. fergusonii**	98	100	0	17	0	0	0	95	5	100	93	0	0	35	100	95	0	0	98	60	65	98	0	0	98	0	92	96	96	96	96	0	0	46	0	100	20	0	96	96	0	0	100	0	83	0	100	0
*E. hermannii**	99	100	0	1	0	0	0	6	0	100	99	0	94	0	100	97	45	45	100	19	40	0	0	0	100	40	97	100	100	100	97	0	0	40	0	8	3	97	35	78	0	0	100	0	98	98	100	0
*E. vulneris**	0	100	0	0	0	0	0	85	30	0	100	0	15	85	100	97	15	8	100	0	30	0	0	1	100	99	93	100	100	100	100	25	0	20	100	0	25	78	2	30	0	0	100	0	100	50	100	0
E. blattae	0	100	0	50	0	0	0	100	0	100	0	0	0	100	100	100	0	0	0	0	0	0	0	0	100	0	100	100	100	75	0	0	0	0	0	0	100	50	50	0	0	0	100	0	0	0	100	0
Genus *Ewingella*																																																	
*E. americana**	0	84	95	95	0	0	0	0	0	0	60	0	5	0	100	0	70	0	100	0	80	0	0	0	0	0	23	16	13	99	10	0	0	50	0	99	24	0	35	10	0	0	97	0	85	0	99	0
Genus *Hafnia*																																																	
*H. alvei**	0	40	85	10	0	4	0	100	6	98	85	0	95	50	100	98	5	10	99	0	13	0	0	0	95	2	97	100	98	95	15	0	0	7	0	0	95	0	70	15	0	0	100	0	90	0	100	0
H. alvei biogroup 1	0	85	70	0	0	0	0	100	0	45	0	0	0	45	100	0	0	0	55	0	55	0	0	0	0	0	0	0	0	70	0	0	0	0	0	0	0	0	30	0	0	0	100	0	30	0	100	0
Genus *Klebsiella*																																																
*K. pneumoniae** (A)	0	10	98	98	0	95	0	98	0	0	0	0	98	93	100	97	98	99	99	30	99	90	95	99	99	99	99	98	99	99	98	90	0	99	99	98	97	90	95	75	0	0	99	0	99	0	99	0
*K. ozaenae** (A)	0	98	0	30	0	10	0	40	6	3	0	0	88	3	100	50	30	20	100	2	97	97	55	65	98	90	55	95	95	98	92	70	0	80	97	95	65	25	50	2	0	0	80	0	80	0	100	0
*K. rhinoscleromatis** (A)	0	100	0	0	0	0	0	0	0	0	0	0	80	95	100	0	0	75	100	0	98	100	95	100	100	90	96	100	100	100	100	0	0	30	100	100	50	0	50	0	0	0	100	0	0	0	100	0
*K. oxytoca** (B)	99	20	95	95	0	90	1	99	0	0	0	0	97	98	100	97	100	100	99	55	100	99	98	99	98	100	100	100	100	100	100	98	2	100	99	98	99	93	98	90	0	0	100	0	100	1	100	0
*K. ornithinolytica** (C)	100	96	70	100	0	100	0	100	0	100	0	0	100	100	100	100	100	100	100	10	100	100	95	100	100	100	100	100	100	100	100	100	0	100	100	100	100	96	100	95	0	0	100	0	100	1	100	0
*K. planticola** (C)	20	100	98	100	0	98	0	100	0	0	0	0	100	100	100	100	100	100	100	15	100	100	100	92	100	100	100	100	100	100	100	100	0	100	100	100	100	100	100	62	0	0	100	0	100	1	100	0
K. terrigena (C)	0	60	100	40	0	0	0	100	0	20	0	0	100	100	100	80	100	100	100	20	100	100	80	100	100	100	100	100	100	100	100	100	0	100	100	100	100	100	100	20	0	0	100	0	100	0	100	0
Genus *Kluyvera*																																																
*K. ascorbata**	92	100	0	96	0	0	0	97	0	100	98	0	92	96	100	93	98	98	100	25	100	0	0	40	100	98	100	100	99	100	100	98	0	99	99	0	40	90	35	50	0	0	100	0	100	0	100	0
*K. cryocrescens**	90	100	0	80	0	0	0	23	0	100	90	0	86	86	100	95	95	81	95	0	100	0	0	45	100	100	100	100	91	100	100	95	0	100	100	0	5	81	19	86	0	0	100	0	100	0	100	0
*K. georgiana**	100	100	0	100	0	0	0	100	0	100	100	0	83	50	100	17	83	100	100	33	100	0	0	0	100	100	83	100	100	100	100	100	0	100	100	0	33	83	50	83	0	0	100	0	100	0	100	0
*K. intermedia**	0	100	100	65	0	0	0	0	0	89	89	0	65	100	100	100	100	65	100	100	100	0	0	100	100	100	100	100	100	100	100	100	0	100	100	0	100	100	100	0	0	0	100	0	100	0	100	0
Genus *Leclercia*																																																
*L. adecarboxylata**	100	100	0	0	0	48	0	0	0	0	79	0	97	93	100	97	93	66	100	86	100	93	0	0	100	66	100	100	100	100	100	0	0	100	100	96	3	93	83	28	0	0	100	0	100	37	100	0
Genus *Leminorella*																																																
*L. grimontii**	0	100	0	100	100	0	0	0	0	0	0	0	0	0	100	33	0	0	0	83	0	0	0	0	100	0	0	0	83	0	0	0	0	0	0	0	17	100	100	0	0	0	100	0	0	0	0	0
*L. richardii**	0	0	0	0	100	0	0	0	0	0	0	0	0	0	100	0	0	0	0	0	0	0	0	0	100	0	0	0	100	0	0	0	0	0	0	0	0	50	100	0	0	0	100	0	0	0	0	0
Genus *Moellerella*																																																
*M. wisconsensis**	0	100	0	80	0	0	0	0	0	0	0	0	70	0	100	0	100	100	60	0	0	100	0	0	0	100	0	30	0	0	0	0	0	0	100	75	10	0	30	10	0	0	90	0	90	0	100	0

(Continued)

TABLE 3 Biochemical reactions[a] of the named species, subspecies, biogroups, and Enteric Groups of the family *Enterobacteriaceae (Continued)*

Organism	Indole production	Methyl red	Voges-Proskauer	Citrate (Simmons)	Hydrogen sulfide (TSI)	Urea hydrolysis	Phenylalanine deaminase	Lysine decarboxylase	Arginine dihydrolase	Ornithine decarboxylase	Motility	Gelatin hydrolysis (22°C)	Growth in KCN	Malonate utilization	D-Glucose, acid	D-Glucose, gas	Lactose fermentation	Sucrose fermentation	D-Mannitol fermentation	Dulcitol fermentation	Salicin fermentation	Adonitol fermentation	*myo*-Inositol fermentation	D-Sorbitol fermentation	L-Arabinose fermentation	Raffinose fermentation	L-Rhamnose fermentation	Maltose fermentation	D-Xylose fermentation	Trehalose fermentation	Cellobiose fermentation	α-Methyl-D-glucoside fermentation	Erythritol fermentation	Esculin hydrolysis	Melibiose fermentation	D-Arabitol fermentation	Glycerol fermentation	Mucate fermentation	Tartrate, Jordan's	Acetate utilization	Lipase (corn oil)	DNase (25°C)	Nitrate → nitrite	Oxidase, Kovacs	ONPG test	Yellow pigment (25°C)	D-Mannose fermentation	Tyrosine hydrolysis
Genus *Morganella*																																																	
M.*morganii* subsp. *morganii**	95	95	0	0	20	95	95	1	0	95	95	0	98	1	99	90	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	95	0	0	0	90	0	10	0	98	99+
M. *morganii* biogroup 1*	100	95	0	0	15	100	100	100	0	80	0	0	90	5	100	93	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	0	100	0	0	0	90	0	20	0	100	99+
M. *morganii* subsp. *sibonii**	50	86	0	0	7	100	93	29	0	64	79	0	79	0	100	86	0	7	0	0	0	0	0	0	0	0	0	0	0	100	0	0	0	0	0	0	7	7	100	0	0	0	100	0	0	0	100	99+
Genus *Obesumbacterium*																																																	
O. proteus biogroup 2	0	15	0	0	0	0	0	100	0	100	0	0	0	0	100	0	0	0	0	0	0	0	0	0	0	0	15	50	15	85	0	0	0	0	0	0	0	0	15	0	0	0	100	0	0	0	85	0
Genus *Pantoea*																																																	
P. dispersa	0	82	64	100	0	0	9	0	0	0	100	0	82	9	100	0	0	1	100	0	0	0	0	0	100	0	91	82	100	100	55	0	0	0	0	100	27	0	9	100	0	0	91	0	91	27	100	0
Genus *Photorhabdus*																																																	
P. luminescens (25°C)	50	0	0	50	0	25	0	0	0	0	100	50	0	0	75	0	0	0	0	0	0	0	0	0	0	0	0	25	0	0	0	0	0	0	0	0	0	0	50	0	0	0	0	0	0	50	100	0
*P. asymbiotica**	0	0	0	20	0	60	0	0	0	0	100	80	20	0	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	60	20	0	0	0	0	0	60	100	0
Genus *Plesiomonas*																																																	
*P. shigelloides**	100	90	0	0	0	0	3	100	98	99	95	0	1	0	100	0	80	0	0	0	0	0	95	0	0	0	0	95	0	100	0	0	0	0	70	0	35	0	50	8	0	0	100	99	90	0	10	0
Genus *Pragia*																																																	
P. fontium	0	100	0	89	89	0	22	0	0	0	100	0	0	0	100	0	0	0	0	0	78	0	0	0	0	0	0	0	0	0	0	0	0	78	0	0	0	0	0	0	0	0	100	0	0	0	0	0
Genus *Proteus*																																																	
*P. mirabilis**	2	97	50	65	98	98	98	0	0	99	95	90	98	2	100	96	2	15	0	0	0	0	0	0	0	1	1	0	98	98	1	0	0	0	0	0	70	0	87	20	92	50	95	0	0	0	0	99+
*P. vulgaris**	98	95	0	15	95	95	99	0	0	0	95	91	99	0	100	85	2	97	0	0	50	0	0	0	0	1	5	97	95	30	0	60	1	50	0	0	60	0	80	25	80	80	98	0	1	0	0	99+
*P. penneri**	0	100	0	0	30	100	99	0	0	0	85	50	99	0	100	45	1	100	0	0	0	0	0	0	0	1	0	100	100	55	0	80	0	0	0	0	55	0	85	5	45	40	90	0	1	0	0	99+
P. myxofaciens	0	100	100	50	0	100	100	0	0	0	100	100	100	0	100	100	0	100	0	0	0	0	0	0	0	0	0	100	0	100	0	100	0	0	0	0	100	0	100	0	100	50	100	0	0	0	0	99+
Genus *Providencia*																																																	
*P. rettgeri**	99	93	0	95	0	98	98	0	0	0	94	0	97	0	100	10	5	15	100	0	50	100	90	1	0	5	70	2	10	0	3	2	75	35	5	100	60	0	95	60	0	0	100	0	5	0	100	99+
*P. stuartii**	98	100	0	93	0	30	95	0	0	0	85	0	100	0	100	0	2	50	10	0	2	5	95	1	1	7	0	1	7	98	5	0	0	0	0	0	50	0	90	75	0	10	100	0	10	0	100	99+
*P. alcalifaciens**	99	99	0	98	0	0	98	0	0	1	96	0	100	0	100	85	0	15	2	0	1	98	1	1	1	1	0	1	1	2	0	0	0	0	0	0	15	0	90	40	0	0	100	0	1	0	100	99+
*P. rustigianii**	98	65	0	15	0	0	100	0	0	0	30	0	100	0	100	35	0	35	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	50	25	0	0	100	0	0	0	100	99+
P. heimbachae	0	85	0	0	0	0	100	0	0	0	46	0	8	0	100	0	0	0	0	0	0	92	46	0	0	0	100	54	8	0	0	0	0	0	0	92	0	0	69	0	0	0	100	0	0	0	100	99+
Genus *Rahnella*																																																	
*R. aquatilis**	0	88	100	94	0	0	95	0	0	0	6	0	0	100	100	98	100	100	100	88	100	0	0	94	100	94	94	94	94	100	100	0	0	100	100	0	13	30	6	6	0	0	100	0	100	0	100	0
Genus *Salmonella*																																																	
*S. enterica** (Group I[d])	1	100	0	95	95	1	0	98	70	97	95	0	0	0	100	96	1	1	100	96	0	0	35	95	99	2	95	97	97	99	5	2	0	5	95	0	5	90	90	90	0	2	100	0	2	0	100	0
Serotype Typhi*	0	100	0	0	97	0	0	98	3	0	97	0	0	0	100	0	1	0	100	0	0	0	0	99	2	0	0	97	82	100	0	0	0	0	100	0	20	0	100	0	0	0	100	0	0	0	100	0
Serotype Choleraesuis*	0	100	0	25	50	0	0	95	55	100	95	0	0	0	100	95	0	0	98	5	0	0	0	90	0	1	100	95	98	0	0	0	1	0	45	1	0	0	85	1	0	0	98	0	0	0	95	0
Serotype Paratyphi A*	0	100	0	0	10	0	0	0	15	95	95	0	0	0	100	99	0	0	100	90	0	0	0	95	100	0	100	95	0	100	5	0	0	0	95	0	10	0	0	0	0	0	100	0	0	0	100	0
Serotype Gallinarum*	0	100	0	0	100	0	0	90	10	1	0	0	0	0	100	0	0	0	100	90	0	0	0	1	80	10	10	90	70	50	10	0	1	0	0	0	0	50	100	0	0	10	100	0	0	0	100	0
Serotype Pullorum*	0	90	0	0	90	0	0	100	10	95	0	0	0	0	100	90	0	0	100	0	0	0	0	10	100	1	100	5	90	90	5	0	0	0	0	0	0	0	0	0	0	0	100	0	0	0	100	0
Group II strains*	2	100	0	100	100	0	0	100	90	100	98	2	0	95	100	100	1	1	100	90	5	0	5	100	100	0	100	100	100	100	0	8	0	15	8	0	25	96	50	95	0	0	100	0	15	0	95	0
Group IIIa strains*	1	100	0	99	99	0	0	99	70	99	99	0	1	95	100	99	15	1	100	0	0	0	0	99	99	1	99	98	100	99	1	1	0	1	95	1	10	90	5	90	0	2	100	0	100	0	100	0
Group IIIb strains*	2	100	0	98	99	0	0	99	70	99	99	0	1	95	100	99	85	5	100	1	0	0	0	99	99	1	99	98	100	99	1	1	0	1	95	1	10	30	20	75	0	2	100	0	92	0	100	0
Group IV strains*	0	100	0	98	100	2	0	100	70	100	98	0	95	0	100	100	0	0	98	0	60	5	0	100	100	0	98	100	100	100	50	0	0	0	100	5	0	0	65	70	0	0	100	0	0	0	100	0
S. bongori (Group V)*	0	100	0	94	100	0	0	100	94	100	100	0	100	0	100	94	0	0	100	94	0	0	0	100	94	0	88	100	100	100	0	0	0	0	94	0	0	88	0	100	0	0	100	0	94	0	100	0
Group VI strains*	0	100	0	89	100	0	0	100	67	100	100	0	0	0	100	100	22	0	100	67	0	0	0	0	100	0	100	100	100	100	0	0	0	0	89	0	33	89	100	89	0	0	100	0	44	0	100	0
Genus *Serratia*																																																	
*S. marcescens**	1	20	98	98	0	15	0	99	0	99	97	90	95	3	100	55	2	99	99	0	95	40	75	99	0	2	0	96	7	99	5	0	1	95	0	0	95	0	75	50	98	98	98	0	95	0	99	0
S. marcescens biogroup 1*	0	100	60	30	0	0	0	55	4	65	17	30	70	0	100	0	4	100	96	0	92	30	30	92	0	0	0	70	0	100	4	0	0	96	0	0	92	0	50	4	75	82	83	0	75	0	100	0

TABLE 3 *(Continued)*

Organism	Indole production	Methyl red	Voges-Proskauer	Citrate (Simmons)	Hydrogen sulfide (TSI)	Urea hydrolysis	Phenylalanine deaminase	Lysine decarboxylase	Arginine dihydrolase	Ornithine decarboxylase	Motility	Gelatin hydrolysis (22°C)	Growth in KCN	Malonate utilization	D-Glucose, acid	D-Glucose, gas	Lactose fermentation	Sucrose fermentation	D-Mannitol fermentation	Dulcitol fermentation	Salicin fermentation	Adonitol fermentation	*myo*-Inositol fermentation	D-Sorbitol fermentation	L-Arabinose fermentation	Raffinose fermentation	L-Rhamnose fermentation	Maltose fermentation	D-Xylose fermentation	Trehalose fermentation	Cellobiose fermentation	α-Methyl-D-glucoside fermentation	Erythritol fermentation	Esculin hydrolysis	Melibiose fermentation	D-Arabitol fermentation	Glycerol fermentation	Mucate fermentation	Tartrate, Jordan's	Acetate utilization	Lipase (corn oil)	DNase (25°C)	Nitrate → nitrite	Oxidase, Kovacs	ONPG test	Yellow pigment (25°C)	D-Mannose fermentation	Tyrosine hydrolysis
S. liquefaciens complex*	1	93	93	90	0	3	0	95	0	95	95	90	90	2	100	75	10	98	100	0	97	5	60	95	98	85	15	98	100	100	5	5	0	97	75	0	95	0	75	40	85	85	100	0	93	0	100	0
*S. rubidaea**	0	20	100	95	0	2	0	55	0	0	85	90	25	94	100	30	100	99	100	0	99	99	20	1	100	99	1	99	99	100	94	1	0	94	99	85	20	0	70	80	99	99	100	0	100	0	100	0
S. odorifera biogroup 1*	60	100	50	100	0	5	0	100	0	100	100	95	60	0	100	0	70	100	100	0	98	50	100	100	100	100	95	100	100	100	100	0	0	95	100	0	40	5	100	60	35	100	100	0	100	0	100	0
S. odorifera biogroup 2*	50	60	100	97	0	0	0	94	0	0	100	94	19	0	100	13	97	0	97	0	45	55	100	100	100	7	94	100	100	100	100	0	7	40	96	0	50	0	100	65	65	100	100	0	100	0	100	0
*S. plymuthica**	0	94	80	75	0	0	0	0	0	0	50	60	30	0	100	40	80	100	100	0	94	0	50	65	100	94	0	94	94	100	88	70	0	81	93	0	50	0	100	55	70	100	100	0	70	0	100	0
*S. ficaria**	0	75	75	100	0	0	0	0	0	0	100	100	55	0	100	0	15	100	100	0	100	0	55	100	100	70	35	100	100	100	100	8	0	100	40	100	0	0	17	40	77	100	92	8	100	0	100	0
S. entomophila	0	20	100	100	0	0	0	0	0	0	100	100	100	0	100	0	0	100	100	0	100	0	0	0	0	0	0	100	40	100	0	0	0	100	0	60	0	0	100	80	20	100	100	0	100	0	100	0
*"Serratia" fonticola**	0	100	9	91	0	13	0	100	0	97	91	0	70	88	100	79	97	21	100	91	100	100	30	100	100	100	76	97	85	100	6	91	0	100	98	100	88	0	58	15	0	0	100	0	100	0	100	0
Genus *Tatumella*																																																	
*T. ptyseos**	0	0	5	2	0	0	90	0	0	0	0	0	0	0	100	0	0	98	0	0	55	0	0	0	0	11	0	0	9	93	0	0	0	0	25	0	7	0	0	0	0	0	98	0	0	0	100	0
Genus *Trabulsiella*																																																	
*T. guamensis**	40	100	0	88	100	0	0	100	50	100	100	0	100	0	100	100	0	0	100	0	13	0	0	100	100	0	100	100	100	100	100	0	0	40	0	0	0	100	50	88	0	0	100	0	100	0	100	0
Genus *Xenorhabdus*																																																	
X. nematophilus	40	0	0	0	0	0	0	0	0	0	100	80	0	0	80	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	60	0	0	20	20	0	0	60	80	0
Genus *Yersinia*																																																	
*Y. pestis** *(A)*	0	80	0	0	0	5	0	0	0	0	0	0	0	0	100	0	0	0	97	0	70	0	0	50	100	0	1	80	90	100	0	0	0	50	20	0	50	0	0	0	0	0	85	0	50	0	100	0
*Y. pseudotuberculosis** *(A)*	0	100	0	0	0	95	0	0	0	0	0	0	0	0	100	0	0	0	100	0	25	0	0	0	50	15	70	95	100	100	0	0	0	95	70	0	50	0	50	0	0	0	95	0	70	0	100	0
*Y. enterocolitica** *(B)*	50	97	2	0	0	75	0	0	0	95	2	0	2	0	100	5	5	95	98	0	20	0	30	99	98	5	1	75	70	98	75	0	0	25	1	40	90	0	85	15	55	5	98	0	95	0	100	0
*Y. frederiksenii** *(B)*	100	100	0	15	0	70	0	0	0	95	5	0	0	0	100	40	40	100	100	0	92	0	20	100	100	30	99	100	100	100	100	0	0	85	0	100	85	5	55	15	55	0	100	0	100	0	100	0
*Y. intermedia** *(B)*	100	100	5	5	0	80	0	0	0	100	5	0	10	5	100	18	35	100	100	0	100	0	15	100	100	45	100	100	100	100	96	77	0	100	80	45	60	6	88	18	12	0	94	0	90	0	100	0
*Y. kristensenii** *(B)*	30	92	0	0	0	77	0	0	0	92	5	0	0	0	100	23	8	0	100	0	15	0	15	100	77	0	0	100	85	100	100	0	0	0	0	45	70	0	40	8	0	0	100	0	70	0	100	0
*Y. rohdei** *(B)*	0	62	0	0	0	62	0	0	0	25	0	0	0	0	100	0	0	100	100	0	0	0	0	100	100	62	0	0	38	100	25	0	0	0	50	0	38	0	100	0	0	0	88	0	50	0	100	0
Y. aldovae (B)	0	80	0	0	0	60	0	0	0	40	0	0	0	0	100	0	0	20	80	0	0	0	0	60	60	0	0	0	40	80	0	0	0	0	0	0	0	0	100	0	0	0	100	0	0	0	100	0
*Y. bercovieri** (B)	0	100	0	0	0	60	0	0	0	80	0	0	0	0	100	0	20	100	100	0	20	0	0	100	100	0	0	100	100	100	100	0	0	20	0	0	0	0	100	0	0	0	100	0	80	0	100	0
*Y. mollaretii** (B)	0	100	0	0	0	20	0	0	0	80	0	0	0	0	100	0	40	100	100	0	20	0	0	100	100	0	0	60	60	100	100	0	0	0	0	0	20	0	100	0	0	0	100	0	20	0	100	0
*"Yersinia" ruckeri** (C)	0	97	10	0	0	0	0	50	5	100	0	30	15	0	100	5	0	0	100	0	0	0	0	50	5	5	0	95	0	95	5	0	0	0	0	0	30	0	30	0	30	0	75	0	50	0	100	0
Genus Yokenella																																																
*Y. regensburgei** (*K. trabulsii*)	0	100	0	92	0	0	0	100	8	100	100	0	92	0	100	100	0	0	100	0	8	0	0	0	100	25	100	100	100	100	100	0	0	67	92	0	0	0	0	25	0	0	100	0	100	0	100	0
CDC Enteric Groups																																																
Enteric Group 59*	10	100	0	100	0	0	30	0	60	0	100	0	80	90	100	100	80	0	100	0	100	0	0	0	100	0	100	100	100	100	100	10	0	100	0	10	10	60	50	50	0	0	100	0	100	25	100	0
Enteric Group 60*	0	100	0	0	0	50	0	0	0	100	75	0	0	100	100	100	0	0	50	0	0	0	0	0	25	0	75	0	0	100	0	0	0	0	0	0	0	75	0	75	0	0	100	0	100	0	100	0
Enteric Group 63	0	100	0	0	0	0	0	100	0	100	65	0	0	0	100	100	0	0	100	0	100	0	0	100	100	0	100	100	100	100	100	65	0	100	0	0	0	65	0	0	0	0	100	0	100	0	100	0
Enteric Group 64	0	100	0	50	0	0	0	0	50	0	100	0	100	100	100	50	100	0	100	0	100	100	0	0	100	0	100	100	100	100	100	0	0	100	0	100	0	100	50	0	0	0	100	0	100	0	100	0
Enteric Group 68*	0	100	50	0	0	0	0	0	0	0	0	0	100	0	100	0	0	100	100	0	50	0	0	0	0	0	0	50	0	100	0	0	0	0	0	0	50	0	0	0	0	0	100	0	100	0	100	0
Enteric Group 69	0	0	100	100	0	0	0	0	100	100	100	0	100	100	100	100	100	25	100	100	100	0	0	100	100	100	100	100	100	100	100	100	0	100	100	0	0	100	0	25	0	0	100	0	100	0	100	0

Matrix 1, Version 11, February 2006

[a] Each number is the percentage of positive reactions after 2 days of incubation at 36°C (unless a different temperature is indicated). The vast majority of these positive reactions occur within 24 h. Reactions that become positive after 2 days are not considered. Abbreviations: TSI, triple sugar iron agar; ONPG, *o*-nitrophenyl-β-D-galactopyranoside. Several "species" in the table are composed of two or more DNA-DNA hybridization groups, so the term "complex" should be understood to follow the species name (see text).

[b] An asterisk by the name of an organism indicates that it occurs in human clinical specimens.

[c] Species in the same genus are grouped with their closest phenotypic and evolutionary relatives (14, 16, 38) rather than being listed alphabetically. For example, the three "subgroups" of the genus *Citrobacter* are defined as "A," "B," and "C" and are listed together.

[d] The Roman numerals refer to the seven *Salmonella* groups that are also biochemically and genetically distinct (see text).

TABLE 4 New, unusual, fastidious, or unculturable genera and species that have been classified[a] (14) in the family *Enterobacteriaceae*

Human pathogen
Klebsiella granulomatis (Calymmatobacterium granulomatis)—causes donovanosis (granuloma inguinale) (see text)
Associated with plants, but some species may occasionally cause or be associated with human clinical infections (see text)
Pantoea, 7 species, 2 subspecies
Pathogenic for or associated with plants; not isolated from human clinical specimens
Brenneria, 6 species; causes a variety of diseases of deciduous trees and walnut trees
Dickeya, 6 species (4 new species plus 2 additional species from other genera) (83)
Erwinia, 9 to 28 species, several named subspecies; major plant pathogen, also exists as a saprophyte and epiphyte
Pectobacterium, 4 to 6 species, several named subspecies; causes soft rot and a variety of other plant diseases, including blights, cankers, die back, leaf spot, and wilts
Phlomobacter fragariae—unculturable species that occurs in the sieve tubes of phloem tissue of plants.
Pathogenic for or associated with insects; not isolated from human clinical specimens
Arsenophonus—2 species, *A. nasoniae,* which is culturable and was originally described as causing the "son-killer" trait in a parasitic wasp, and *A. tiatominarum,* which has not been cultured
Buchnera aphidicola—Cannot be cultivated outside of the aphid host; it is essential for the survival of the aphid.
Photorhabdus—3 species, 3 subspecies. Two species are found only in nematodes and insects that are infected by the nematode. One species, *P. asymbiotica,* causes bacteremia and wound infections in humans (see text).
Wigglesworthia glossinidia—Obligate intracellular endosymbiont of tsetse flies
Xenorhabdus—several species; found only in nematodes and the insects that the nematodes infect; insect pathogens
Associated with extreme environments, not isolated from human clinical specimens
Alterococcus agarolyticus—habitat is coastal hot springs

[a] The unculturable and extremely fastidious organisms have been classified in the family *Enterobacteriaceae* based mainly on 16S rRNA sequencing data. This table includes some organisms whose eventual classification may be as "relatives" of *Enterobacteriaceae*. For more information about each organism, see the Internet site of J. P. Euzéby at http://www.bacterio.cict.fr.

and the environment. One example is the study by Müller et al. (71), who found six new species of *Buttiauxella* and two new species of *Kluyvera* in a large collection of strains isolated from snails. Similarly, additional DNA-DNA hybridization subgroups, which are probably new species (sometimes called genomospecies), have been found in systematic studies of *Enterobacter cloacae* (12), *Proteus vulgaris* (73), *Rahnella aquatilis* (17), *Klebsiella* (67, 81), *Enterobacter* (48, 52, 54, 59), *Yersinia* (7, 8), and *Citrobacter* (15, 18, 94). Most of the *Enterobacteriaceae* that clinical microbiologists encounter every day belong to just a few of the many species described (32). However, the expanding number of *Enterobacteriaceae* species is becoming a serious problem for reference laboratories and for commercial identification systems, whose identification methods are becoming inadequate for complete and accurate identification. When a commercial identification system gives an unusual organism for a final identification, there are several possibilities to consider (56): the identification is correct, just unusual; the identification is incorrect because another aerobic or anaerobic organism is present (42) and the biochemical profile is the result of the metabolic activities of the mixture; or a handling or coding error was made somewhere along the way. Before a final report of an unusual organism is issued, it is advisable to do as much checking as possible. This checking could include repeating the biochemical tests with the same commercial system after confirming the absence of a contaminating aerobic or anaerobic organism (42), testing the isolate with another commercial identification system (56) or with tube tests, and comparing the strain's antibiogram with known patterns reported for this organism. If these steps do not resolve the problem, the state health department or a reference laboratory can be contacted for advice, and the culture will often be accepted for further study. Different commercial systems often give different identifications for the same strain. The "gold standard" for identification is DNA-DNA hybridization; however, it is unavailable except in a few research laboratories. A different standard is evolving that is based on 16S rRNA sequencing. Although less accurate, it is a readily available alternative, and unusual strains can be submitted to a commercial laboratory (Accugenix, Newark, Del. [http://www.accugenix.com] or Midi, Newark, Del. [http://www.midi-inc.com]) for a "fee-for-service" identification. Clinical isolates reported with results obtained with these commercial tests should be reported with a disclaimer to indicate their research ("non-Clinical Laboratory Improvement Amendment [CLIA]") status. We suspect that a reference laboratory's identification based on phenotypic characteristics will be the final result for most difficult strains and will be done at state or national health departments or commercial reference laboratories.

Changes in Classification: "Proposed Alternative Classifications"

Contrary to popular opinion, there is no designated international body that considers every proposed change in classification and then issues an official classification. For almost 75 years, the Subcommittee on *Enterobacteriaceae* (http://www.the-icsp.org/subcoms/Enterobacteriaceae.htm) of the International Committee on Prokaryotes (http://www.the-icsp.org/default.htm) has studied and discussed the nomenclature and classification of the family. When the *Enterobacteriaceae* Subcommittee studies a specific "proposed reclassification," it can only make a recommendation, which can then be accepted or rejected by individuals in the scientific community. It should be emphasized that changes in classification are decided by usage, not by a judicial decision or action (see chapter 19 for further discussion). Sometimes two classifications are widely used, and both can be "correct." Classifications are correct if they conform to all the

TABLE 5 *Enterobacteriaceae* that are difficult to differentiate and identify completely; use of the term "complex"[a] as a solution for reporting cultures[b]

Vernacular name	Organisms included, definition, and comment
Citrobacter freundii complex	In addition to *C. freundii,* this term includes *C. braakii, C. gillenii, C. murliniae, C. rodentium, C. sedlakii, C. werkmanii,* and *C. youngae,* which are difficult to differentiate (15, 18).
Enterobacter agglomerans complex	This term includes over 60 named organisms: over a dozen "*Enterobacter agglomerans* DNA-DNA hybridization groups,"[c] the species of *Brenneria, Dickeya, Erwinia, Pectobacterium, Pantoea,* and perhaps also *Enterobacter cowanii,* all of which are difficult or impossible to differentiate.
Enterobacter cloacae complex	*E. cloacae* is made up of at least five DNA-DNA hybridization groups (12). The definition of the complex would include *Enterobacter ludwigii* plus these unnamed groups. For practical identification schemes, the term includes *Enterobacter amnigenus* and *Enterobacter kobei,* which are difficult to differentiate.
Klebsiella pneumoniae complex	In addition to *K. pneumoniae,* the term includes the closely related species (subspecies) *K. ozaenae* and *K. rhinoscleromatis* and the new species *K. ludwigii.* For practical identification schemes, the term includes *Klebsiella (Raoultella) planticola* and *K. terrigena,* which are very difficult to differentiate. *Klebsiella (Raoultella) ornithinolytica* is ornithine$^+$ and thus phenotypically distinct.
Kluyvera-Buttiauxella complex	This complex includes two genera with almost a dozen species (Table 3) and now includes *Kluyvera intermedia,* formerly classified as *Enterobacter intermedium.*
Proteus vulgaris complex	*P. vulgaris* is made up of at least four DNA-DNA hybridization groups. The definition of the complex could be expanded to include the closely related species *P. penneri* and *P. hauseri,* which can often be differentiated.
Rahnella aquatilis complex	*R. aquatilis* is made up of at least three DNA-DNA hybridization groups.
Serratia liquefaciens complex	The term includes *S. liquefaciens* and three closely related species *S. grimesii, S. proteamaculans,* and S. *quinovorans,* which are difficult to differentiate.
Yersinia enterocolitica complex	In addition to *Y. enterocolitica,* the term includes the closely related species *Y. aldovae, Y. bercovieri, Y. frederiksenii, Y. intermedia, Y. kristensenii,* and *Y. mollaretii,* which are difficult to differentiate.

[a] The word "group" is an alternative term for complex; e.g., the *Enterobacter agglomerans* group, which is also a vernacular name.
[b] An alternative approach would be to report only the genus name (i.e., *Citrobacter* species, *Kluyvera* species, or *Kluyvera-Buttiauxella* species). However, the terms in this table have a narrower definition.
[c] Some of the *Enterobacter agglomerans* DNA-DNA hybridization groups can rarely occur in human clinical specimens.

rules in the *Bacteriological Code (International Code of Nomenclature of Bacteria).* However, classifications can be useful or not useful and can be frequently used in the literature or rarely used (14, 16, 38).

Proposed Changes in Classification and Other Changes in Table 3

Several "alternative classifications" have been proposed in the literature. Some of these appear to be totally justified and have been incorporated into Table 3. However, others have not been fully discussed or widely accepted by the scientific community (28). Table 3 gives the nomenclature and classification that one of us (J.J.F.) has incorporated into tables, data matrices, and computer programs used to identify clinical and nonclinical isolates of *Enterobacteriaceae.* It will differ from other nomenclatures and classifications.

In the seventh edition, the genus *Plesiomonas* was classified in the family *Vibrionaceae* along with *Aeromonas.* Because *Plesiomonas* is closer to *Enterobacteriaceae* than to *Vibrionaceae* based on 16S rRNA sequencing and because it contains the enterobacterial common antigen, it was included in the family *Enterobacteriaceae* in the eighth edition, and this classification has been maintained in the ninth edition. However, *Plesiomonas* is oxidase positive, a characteristic not shared with other species of *Enterobacteriaceae,* and is a distant relative of *E. coli,* the type species of the type genus of *Enterobacteriaceae* (14). Thus, the classification of *Plesiomonas* in the family *Enterobacteriaceae* might best be viewed as tentative.

In Table 3, the organism originally classified (9, 40) as *Xenorhabdus luminescens* DNA hybridization group 5 is now classified as *Photorhabdus asymbiotica* (44). It has caused rare cases of bacteremia and wound infection in the United States (40) and Australia (75). These Australian strains are distinct in some ways and have been proposed (Table 2) as *Photorhabdus asymbiotica* subspecies *australis* (2).

The names *Citrobacter diversus* and *Citrobacter koseri* have both been used in the literature for some time, but the name *Citrobacter diversus* has been used much more frequently. Many workers recognized the phenotypic similarity of these two organisms and thought that they might be the same. The species have different type strains, and so considering them to be the same will always be a subjective matter. They can be considered subjective synonyms but not objective synonyms (which must have the same type strain). The name *Citrobacter diversus* became the correct name for this organism on 1 January 1980, when the *Approved Lists of Bacterial Names* was issued, because under the laws of priority it was the older name. However, in 1993 the Judicial Commission of the International Committee on Systematic Bacteriology issued an Opinion (57) that the name *Citrobacter koseri* should be conserved over the name *Citrobacter diversus,* even though the name *Citrobacter diversus* was the older name, was on the

TABLE 6 *Salmonella* isolates in the United States for 1993 to 2003[a]

Rank for 2003	Serotype name (or formula[b])	No. of isolates: 1993–2002	No. of isolates: 2003
1	Typhimurium	83,873	6,631
2	Enteritidis	74,241	4,863
3	Newport	24,637	3,847
4	Heidelberg	19,837	1,810
5	Javiana	9,186	1,659
6	Montevideo	7,918	849
7	Saintpaul	4,838	823
8	Munchen	6,904	781
9	Oranienburg	6,093	554
10	Infantis	5,477	539
11	Branderup	4,824	530
12	Agona	6,068	510
13	1, 4, [5], 12:i:−[b]	628	498
14	Thompson	5,766	494
15	Mississippi	2,389	438
16	Typhi	3,979	359
17	Paratyphi B (L-tartrate$^+$)	3,014	331
18	Hadar	6,197	280
19	Bareilly	1,419	234
20	Stanley	2,157	224
	Paratyphi A	822	108
	Paratyphi C	9	0
	Choleraesuis	675	19
	Choleraesuis variety Kunzendorf		13
	Other serotypes		5,239
	Incomplete or no typing		2,105
	Total	354,093	33,589

[a] Surveillance data are from the Centers for Disease Control and Prevention. A free copy of the annual *Salmonella* surveillance reports can be obtained from the Centers for Disease Control and Prevention, Foodborne and Diarrheal Diseases Branch, Mail Stop A38, 1600 Clifton Rd., Atlanta, GA 30333. Recent surveillance reports can also be viewed on the Internet at http://www.cdc.gov/ncidod/dbmd/phlisdata/salmonella.htm. Note: the entire document for a given year can be very large, so it may be necessary to download individual tables to avoid time and computer data storage problems. A paper copy (619 pages in color) of CDC's very useful *Salmonella* atlas (22) is also available without charge (contact Richard Bishop at rqb7@cdc.gov).

[b] This serotype is referred to by its antigenic formula because it does not have a formal serotype name, unlike the other 19 serotypes. Its O antigens are 1, 4, [5], 12 ([5] indicates that serofactor 5 may or may not be present); its H antigen for phase one is "i," and it does not have a phase two H antigen.

Approved Lists of Bacterial Names, was the correct name under the rules of the *Bacteriological Code,* and was the name used most frequently in the literature. This opinion needs much more discussion by the scientific community, which is beyond the scope of this chapter; therefore, both names are included in Table 3.

Phenotypic and 16S rRNA sequencing data indicate that *Kluyvera cochleae* is almost identical to *Enterobacter intermedium,* and the proposed reclassification of this organism as *Kluyvera intermedia* (74) appears to solve several problems (Tables 2 and 3).

Another change in Table 3 is that species in the same genus are now grouped with their closest phenotypic and evolutionary relatives (14, 16, 38) rather than listed alphabetically. For example, three subgroups of the genus *Citrobacter* are defined as A, B, and C and listed together. In addition, we propose that Enteric Group 139 be reclassified as *Citrobacter* Group 139. A similar notation is used in Table 3 for other genera; Tables 2 and 4 and the text give explanations.

Other Proposed Changes in Nomenclature and Classification

Proposed Classification of Three *Klebsiella* Species in *Raoultella*

In 2001, Drancourt et al. (28) proposed that *Klebsiella planticola, K. ornithinolytica,* and *K. terrigena* be classified in a new genus, *Raoultella,* as *R. planticola, R. ornithinolytica,* and *R. terrigena.* These three species are extremely similar to *Klebsiella pneumoniae* in their phenotypic properties (37), making differentiation very difficult (Table 3). This proposed alternative classification needs further evaluation; however, we agree that these three species should be grouped together and have done this in Table 3.

Enterobacter agglomerans Group–*Pantoea*

The *Enterobacter agglomerans–Pantoea* complex is a confusing subject, and writers continue to make errors in the definition

TABLE 7 *Shigella* isolates in the United States for 2003[a]

Rank	Serotype	Isolates
1	*Shigella sonnei* (serogroup D)	9,263
2	*Shigella flexneri* (serogroup B)	1,660
3	*Shigella boydii* (serogroup C)	125
4	*Shigella dysenteriae* (serogroup A)	41
	Not (completely) serotyped	463
	Total *Shigella* isolates	11,552

[a] Data are from the Centers for Disease Control and Prevention at http://www.cdc.gov/ncidod/dbmd/phlisdata/shigella.htm. Surveillance reports for previous years are also at this Internet address. Note: the entire document for a given year is typically very large so it may be prudent to download individual tables to avoid time and computer data storage problems.

and circumscription (boundaries) of *Pantoea agglomerans.* In 1972, Ewing and Fife redefined the name *Enterobacter agglomerans* to include a wide variety of organisms known under many different names (32). These investigators also defined 11 different biogroups to recognize the phenotypic diversity of the many strains included in *Enterobacter agglomerans.* This name has become useful for clinical microbiologists, and it has been used extensively in the literature. Systematic analysis by Brenner and coworkers using DNA-DNA hybridization indicated that *Enterobacter agglomerans* is very heterogeneous, with at least 14 DNA hybridization groups (12). For this reason, the names "*Enterobacter agglomerans* complex" and "*Enterobacter agglomerans* group" (37) have been used to better indicate the heterogeneity of this "species" (Tables 3 and 5). However, it has been very difficult to find simple tests to differentiate and identify all of the DNA hybridization groups (37). For this reason, workers have been reluctant to subdivide the *Enterobacter agglomerans* group until a definitive classification could be proposed (37). Gavini et al. (46) took the first step toward more logical classification for this complex group by proposing that the group of six strains defined by Brenner et al. as "DNA hybridization group 13 of *Enterobacter agglomerans*" be classified in a new genus, *Pantoea,* as *P. agglomerans.* They also defined a new species in the genus, *Pantoea dispersa* (46), previously classified as *Enterobacter agglomerans* DNA hybridization group 3 by Brenner (12).

However, this new classification has caused communication problems. Some authors have broadened the original definition of Gavini et al. for *Pantoea agglomerans* to include organisms that are not phylogenetically related. Since DNA-DNA hybridization is not routinely done and since simple tests are not available to definitively identify strains to the level of DNA hybridization group, it seems prudent to retain the vernacular name "*Enterobacter agglomerans* complex" as a convenient name for clinical microbiologists to use in reporting clinical isolates (Tables 3 and 5). This term is defined biochemically in Table 3, and it should be emphasized that it is used merely for convenience because the name *Enterobacter agglomerans* is well understood and widely used in the literature. Eventually, this term will be replaced with a better classification. When definitive testing in a reference laboratory (usually including DNA hybridization) is done, more precise names can be used in reporting. Examples could include *Pantoea agglomerans* (limited to strains that fall into DNA hybridization group 13), *Pantoea dispersa* (limited to strains that fall into DNA hybridization group 3), and *Enterobacter agglomerans* DNA hybridization group 1, etc. Tables 3 and 5 use and define the vernacular name *Enterobacter agglomerans* complex, a term that may prove useful for reporting isolates in most microbiology laboratories because almost none can do DNA-DNA hybridization. A less desirable vernacular name for this group of organisms is the "*Pantoea agglomerans* complex."

Enterobacter taylorae–Enterobacter cancerogenus

Enterobacter taylorae and *Enterobacter cancerogenus* may be two names for the same organism (47). However, they have different type strains; therefore, they are not objective synonyms under the rules of the *Bacteriological Code.* Until the identity of these two organisms is universally accepted, both names will be used (Table 3).

Nomenclature, Classification, and Reporting of the Genus *Salmonella*

After much study and a lengthy judicial process (58), there is now good agreement on many issues in the nomenclature and classification of the genus *Salmonella* (32, 41, 58, 70, 76, 78, 79). The recent decision of the Judicial Commission on the International Committee on Systematics of Prokaryotes (58) to replace *Salmonella choleraesuis* with *Salmonella enterica* stabilizes this issue which has caused confusion and the use of illegitimate names in the literature for many years. In 2005 (58), *Salmonella enterica* finally became the legitimate species name to include most of the most important serotype names. Three 2005 references (58, 78, 91) and the Internet site of J. P. Euzéby (http://www.bacterio.cict.fr) provide additional historical insights and alternative perspectives on the issues.

Until the 1970s, the species concept in the genus *Salmonella* was based on epidemiology, host range, biochemical reactions, and antigenic structure (the O antigen, phases 1 and 2 of the H antigen, and the Vi antigen, if present), and strains that differed in one or all of these properties were given distinct names. Names such as *Salmonella typhi, Salmonella cholerae-suis* (originally some names such as this one were written with a hyphen, which was eventually dropped because of changes in the *Bacteriological Code*), *Salmonella paratyphi* A, *Salmonella paratyphi* A var. *durazzo, Salmonella typhimurium, Salmonella typhimurium* var. *copenhagen, Salmonella enteritidis,* and *Salmonella newport* began to appear, and the list rapidly expanded to include hundreds of names. Some workers believed that these names really represented biological species, but others thought that they were antigenic and biochemical varieties with an uncertain evolutionary relationship. However, there was universal agreement that the names were an extremely useful way to communicate about the particular serotypes and the diseases they caused. Most authors wrote the serotype names in italics as a species in the genus *Salmonella,* for example, *Salmonella typhimurium* (32, 41). Several proposals to the Judicial Commission of the International Committee on Systematic Bacteriology have requested that important serotype names be preserved (31, 33, 34) to preserve stability in nomenclature, but it is not clear whether this is a matter that will be decided by judicial action or by usage.

In 1973, Crosa et al. (25) used DNA-DNA hybridization to show that *Salmonella* strains could be grouped into five main evolutionary groups. Two (possibly three) additional groups are now known (11, 78, 79). The vast majority of strains that cause human infections occur in DNA hybridization group 1 (*Salmonella* group I). Strains isolated from animals and the environment clustered into the four other groups, designated DNA groups 2 (II), 3a (IIIa), 3b (IIIb), and 4 (IV). Over the

years, different authors have used different terms to refer to these evolutionary groups: DNA-DNA hybridization groups (25, 41), multilocus enzyme electrophoresis clusters (11, 79), subgenera, species (see the *Approved Lists of Bacterial Names* and http://www.bacterio.cict.fr), and subspecies (70, 76, 77). Crosa et al. (25) showed that all five groups of *Salmonella* were very highly related genetically. With the operational species definition usually used in DNA hybridization, these five groups were considered to belong to the same species. Under the rules of the *Bacteriological Code,* the name of this species had to be *Salmonella choleraesuis.* However, this species name is a cause of confusion, since *Salmonella choleraesuis* would have two totally different meanings, a broad one as a species and a narrow one as a serotype. There was support for making an exception to the rules of the *Bacteriological Code* and using a name that has never been used as a serotype name to avoid confusion. There was a formal proposal in 1999 to coin a new name, *Salmonella enterica* (31), which would replace the name *Salmonella choleraesuis* as the species name to represent most of the serotypes of *Salmonella.* However, the proposal to replace the name *Salmonella choleraesuis* with *Salmonella enterica* was denied by the Judicial Commission of the International Committee on Systematic Bacteriology. Thus, *Salmonella choleraesuis* remained the correct name until a variation of the original proposal to the Judicial Commission was approved in 2005 (58). Even though it was an illegitimate name, the name *Salmonella enterica* had already been used by the World Health Organization's International Center for *Salmonella* (76) and by many of the World Health Organization's national centers for *Salmonella.* The name had also been used widely in the literature. Fortunately, the 2005 decision of the Judicial Commission has made *Salmonella enterica* the correct name, which is gaining universal acceptance.

Another point of confusion concerns the method of writing serotype names. For almost 100 years, serotype names have been written as species (the serotype-as-species nomenclature), for example, *Salmonella enteritidis.* The World Health Organization's International Center for *Salmonella* at the Institut Pasteur, Paris, France, introduced a different nomenclature in which the serotype name is capitalized and not written in italics. In this nomenclature, the name *Salmonella enteritidis* would be written in one of the following ways: "*Salmonella enterica* serovar Enteritidis," "*Salmonella* serovar Enteritidis," "*Salmonella* ser. Enteritidis," or "*Salmonella* Enteritidis." The nomenclature described by McWhorter-Murlin and Hickman-Brenner (70) is similar, but these authors use the term "serotype" instead of "serovar." The main advantage of these nomenclatures is that they do not artificially treat the serotypes as species. The main disadvantage is that they create a new nomenclature that differs from one that has been widely accepted and used for more than 70 years. There have been literally hundreds of thousands of uses of the serotype-as-species nomenclature in the literature. The International Center for *Salmonella*'s nomenclature appears in the second edition of *Bergey's Manual* (78) and is being used (sometimes with modifications) by the national centers for *Salmonella* (19, 70). However, many published articles and books continue to use the nomenclature. Since *Salmonella* names are being written differently by different authors and different national centers for *Salmonella,* it is not surprising that the literature is beginning to reflect this confusion. Recent examples of the way "serotype Typhimurium" is being written include *Salmonella* serotype Typhimurium, *Salmonella* ser. Typhimurium, *Salmonella typhimurium, Salmonella* Typhimurium, *Salmonella* typhimurium, *Salmonella* serovar Typhimurium, and *Salmonella* serovar *Typhimurium* or simply Typhimurium (omitting the genus name *Salmonella* entirely) (88). When the variations are combined with the four species and subspecies possibilities, i.e., *Salmonella choleraesuis, Salmonella choleraesuis* subspecies *choleraesuis, Salmonella enterica,* and *Salmonella enterica* subspecies *enterica,* the number of possible variations is multiplied considerably. One example of the almost endless possibilities is *Salmonella enterica* subspecies *enterica* serovar Typhimurium.

The current disagreements in *Salmonella* nomenclature and classification include the use of the term "serotype" (19, 70) versus "serovar" (76, 77) (both terms are often abbreviated as "ser."); the best way to write the names of the serotypes; the use of names versus antigenic formulas for some of the serotypes; the argument over whether some well-known serotype names should be eliminated and combined with other serotypes (19, 70, 76, 77); and the question of how to name the distinct DNA hybridization groups.

Most clinical microbiology laboratories identify *Salmonella* isolates with a commercial identification system and then with commercial *Salmonella* "polyvalent grouping antisera," which will agglutinate only those strains with the O antigen groups contained in the polyvalent serum (often only groups A through E). These two methods usually give definitive results, and a simple report can be issued such as "*Salmonella* serogroup B," avoiding the problems described above. Abbreviating "serotype" and "serovar" as "ser." would be a further simplification and would avoid the disagreement over these two terms. Reference laboratories that do complete serotyping and biochemical testing can issue a definitive report such as "*Salmonella* serotype Typhimurium" or "*Salmonella enterica* serotype Typhimurium."

Nomenclature for Shiga Toxins/Verotoxins Produced by *E. coli* and *Shigella*

Several different names are being used in the literature for the cytotoxins produced by *E. coli* and *Shigella.* This topic is critical because of the importance of *E. coli* O157 and other strains that produce these toxins (see chapter 43 of this Manual). Several different commercial assays for these toxins are being marketed; therefore, it is essential to read the package insert carefully to determine exactly which toxin(s) the kit is detecting and to word laboratory reports accordingly.

For almost 100 years, it has been known that *Shigella dysenteriae* serogroup O1 produces a potent cytotoxin known as Shiga toxin. More recently, it has been shown that certain strains of *E. coli* that cause intestinal infections produce a similar toxin, which was first detected because it was cytotoxic for Vero cells in tissue culture. A number of recent studies have defined these proteins from *S. dysenteriae* O1 and *E. coli,* and there is agreement that they constitute a family of toxins. They are being referred to in the literature as Shiga toxin (ST), Shiga-like toxins (SLT), verocytotoxin(s), and verotoxin(s) (VT), and at least five different toxins are involved (20, 86). This complex subject was recently reviewed by Scheutz and Strockbine (86). The *E. coli* strains that produce these toxins are often referred to as STEC and VTEC. Calderwood et al. (20) summarized the data available and proposed that strains of *E. coli* that produce these toxins be called "Shiga toxin-producing" *E. coli,* which would replace the previous term, "Shiga-like toxin producing." They also recommended that the new toxin name be cross-referenced with the corresponding verotoxin name. With this nomenclature, a laboratory report for a stool culture might be worded, "Positive for *E. coli* O157:H7, which produces Shiga

toxins Stx1 (VT1) and Stx2 (VT2)." Hopefully, the differences between those using the two different nomenclatures will be resolved, resulting in a single nomenclature.

Proposed Reclassification of *Calymmatobacterium granulomatis* as *Klebsiella granulomatis*

Calymmatobacterium granulomatis has received little attention in industrialized countries. In the seventh edition of this Manual, *Calymmatobacterium* was mentioned only twice (pages 25 and 50). It was listed as an aerobic bacterium that can be found in the genital area, and under the topic "Specimen Management" it was mentioned under the disease granuloma inguinale, or ulcerative donovanosis, with the notes "mostly a tropical disease" and "culture is nonproductive." *Calymmatobacterium granulomatis* has been described as a highly pleomorphic gram-negative rod that does not grow on laboratory media. Diagnosis of granuloma inguinale has been based on showing the presence of "Donovan bodies" in Giemsa-stained smears of mononuclear cells or histiocytes from the patient's genital ulcers.

It had been assumed for almost a century that *Calymmatobacterium granulomatis* has no relationship to the "easy-to-culture" organisms of the family *Enterobacteriaceae.* However, Carter et al. (21) proposed that *Calymmatobacterium granulomatis* be reclassified in the genus *Klebsiella* as *Klebsiella granulomatis.* This proposal was based both on nucleotide sequence relatedness and on disease similarity. Granuloma inguinale is a disease similar to rhinoscleroma, also a tropical disease (nasal infection) caused by (or associated with) *Klebsiella rhinoscleromatis.* While this alternative classification is being evaluated and tested, it would be helpful to write both scientific names, with the writer's preference listed first: *"Klebsiella granulomatis (Calymmatobacterium granulomatis)"* (which we prefer) or *"Calymmatobacterium granulomatis (Klebsiella granulomatis)."* Other diseases of unknown etiology may be caused by unculturable *Enterobacteriaceae.*

DESCRIPTION OF THE FAMILY *ENTEROBACTERIACEAE*

Most genera and species in the family *Enterobacteriaceae* share the following properties: they are gram negative and rod shaped; do not form spores; are motile with peritrichous flagella or are nonmotile; grow on peptone or meat extract media without the addition of other supplements or sodium chloride; grow well on MacConkey agar; grow both aerobically and anaerobically; are often active biochemically; ferment (rather than oxidize) D-glucose and other sugars, often with gas production; are catalase positive and oxidase negative; reduce nitrate into nitrite; contain the enterobacterial common antigen; and have 39 to 59% guanine-plus-cytosine (G+C) contents in DNA (5, 12–14, 38, 55). Host-adapted species that are unculturable, difficult to culture, or slow growing appear to have evolved in some genera (14) (Table 4).

When techniques that measure evolutionary distance are used, genera and species in the family should also be more closely related to *E. coli,* the type species of the type genus of the family, than they are to organisms in other families (14, 38). Tables 1 to 5 expand on this definition and give most of the exceptions.

NATURAL HABITATS

Enterobacteriaceae are widely distributed on plants and in soil, water, and the intestines of humans and animals (5, 14, 43, 55). Some species occupy very limited ecological niches. *Salmonella* serotype Typhi causes typhoid fever and is found only in humans (50, 68). In contrast, strains of *Klebsiella pneumoniae* are distributed widely in the environment and contribute to biochemical and geochemical processes (63). However, strains of *K. pneumoniae* also cause human infections, ranging from asymptomatic colonization of the intestinal, urinary, and respiratory tracts to fatal pneumonia, septicemia, and meningitis.

CLINICAL SIGNIFICANCE

Some *Enterobacteriaceae* are associated with or cause specific humans diseases (Table 1) (14, 55, 68, 69, 82). Many cause, or are isolated from, abscesses, pneumonia, meningitis, septicemia, and infections of wounds, the urinary tract, and the intestine (68, 69). They are a major component of the normal intestinal flora of humans but are relatively uncommon as normal flora of other body sites. Several species of *Enterobacteriaceae* are very important causes of nosocomial infections (69). *Enterobacteriaceae* may account for 80% of clinically significant isolates of gram-negative bacilli and 50% of clinically significant bacteria in clinical microbiology laboratories (30). They account for nearly 50% of septicemia cases, more than 70% of urinary tract infections, and a significant percentage of intestinal infections (68, 69).

Human Extraintestinal Infections

Except for the species of *Shigella,* which rarely cause infections outside the gastrointestinal tract, many species of *Enterobacteriaceae* commonly cause extraintestinal infections. However, a small number of species, i.e., *E. coli, Klebsiella pneumoniae, Klebsiella oxytoca, Proteus mirabilis, Enterobacter aerogenes,* the *Enterobacter cloacae* complex, and *Serratia marcescens,* account for most of these infections. Urinary tract infections, primarily cystitis, are the most common (85), followed by respiratory, wound, bloodstream (27), and central nervous system infections. Many of these infections, especially sepsis and meningitis, are life threatening and are often hospital acquired. Because of the severity of these infections, prompt isolation, identification, and susceptibility testing of *Enterobacteriaceae* isolates are essential.

Human Intestinal Infections

Several organisms in the family *Enterobacteriaceae* are also important causes (Tables 6 and 7) of intestinal infections of humans and animals worldwide. Although other species in the family have been associated with diarrhea (93) or even implicated as causes of diarrhea, only organisms in four genera, *Escherichia* (29, 36, 55, 61), *Salmonella* (25, 41, 50, 78), *Shigella* (32, 68), and *Yersinia* (7, 60, 68, 80), have been clearly documented as enteric pathogens. These four genera are discussed in chapters 43 and 44 of this Manual. Other *Enterobacteriaceae* such as *Citrobacter, Edwardsiella, Hafnia, Morganella, Proteus, Klebsiella, Enterobacter,* and *Serratia* may have an association with diarrhea in certain studies (39, 93), and some authors have gone as far as to implicate them as actually causing diarrhea (5, 93). Strains of these *Enterobacteriaceae* that produce "biologically active" compounds (often vastly overstated as being "enterotoxin-producing strains") have been isolated from people with diarrhea (93), but the causal role of these strains in diarrhea is uncertain. One possible way to emphasize the drastic change in the stool flora would be to issue a report such as "*Klebsiella pneumoniae* isolated in essentially pure culture

(10 of 10 colonies tested); please consult the laboratory to discuss possible significance." The patient's antibody response, or lack of one, would be a helpful way to assess the particular organism's causative role. There is no evidence that strains of these other genera are important causes of diarrhea.

In contrast to the arguable role of the organisms listed above, the evidence for the causal role of *Plesiomonas shigelloides* (see chapter 45 in this Manual) in diarrhea is somewhat stronger. A safe generalization would be that "certain strains of *P. shigelloides* may cause diarrhea in certain people under certain conditions, but it is probably not an intrinsic pathogen." For an intrinsic pathogen, most strains would cause diarrhea in most people, under most conditions (39).

Surveillance at the National and International Levels

Many countries provide surveillance data on the Internet for plague, typhoid fever, salmonellosis, shigellosis, diarrheagenic *E. coli*, institutional infections, bacteremia, meningitis, antibiotic resistance, and other enteric and nonenteric infections. Often the word "infection" or a similar word is used when the term "clinical microbiology isolate" would be more appropriate. Care must be used in interpreting these data because "association" and "clinical microbiology isolate" do not equate with "causation" and "infection" in each instance (39). For example, few would argue with the use of the term "infection" for a clinical isolate of *Salmonella* serotype Typhi from the stool of a patient with typhoid fever. In contrast, the word "infection" would be an overstatement if used to describe a stool isolate of a nonenteropathogenic *Yersinia enterocolitica* serotype (such as O10) or one of the other six species of the *Y. enterocolitica* group (Table 3). Check to see if surveillance data make these important distinctions.

SPECIMEN COLLECTION, TRANSPORT, AND PROCESSING

Extraintestinal Specimens

Enterobacteriaceae are recovered from infections at many different body sites, and normal practices (see chapters 5 and 20 of this Manual) for collecting blood, respiratory, wound, urine, and other specimens should be followed.

Intestinal Specimens

Stool cultures are usually submitted to the laboratory with a request to isolate and identify the cause of a possible intestinal infection, usually manifested as diarrhea (see chapter 20). The groups of *Enterobacteriaceae* usually associated with diarrhea in the United States are *Salmonella* (22), *Shigella* (23), and certain pathogenic strains of *E. coli* and *Yersinia enterocolitica.*

Stool specimens require special attention to both collection and transportation and should be obtained early in the course of illness, when the causative agent is likely to be present in the largest numbers in feces. At this stage, the use of enrichment broths should be unnecessary. If rapid processing (within 2 h of collection) is not possible, a small portion of feces or a swab coated with feces should be placed in transport medium, such as Stuart, Amies, Cary-Blair, or buffered glycerol saline. Cary-Blair is probably the best overall transport medium for diarrheal stools. In cases of diarrhea that do not yield a causative agent, a tube of frozen stool can be invaluable for looking for new causative agents or for testing against the patient's convalescent serum. More information about the isolation, identification, typing, and virulence testing of isolates of *Salmonella, Shigella, E. coli,* and *Y. enterocolitica* is given in chapters 43 and 44.

Macroscopic and Microscopic Examination

Stool specimens should be examined visually for the presence of blood or mucus, but microscopic examination is less helpful because of its lack of specificity (84). Although identification by fluorescent-antibody staining is theoretically possible for all enteric pathogens, it has been of limited success because the method is difficult and there are many serological cross-reactions among the species of *Enterobacteriaceae* (32). This technique was most often used to detect *Salmonella* strains (primarily in the food industry) and certain serogroups of *E. coli* and to aid in outbreak investigations.

ISOLATION

Extraintestinal Specimens

Most strains of *Enterobacteriaceae* grow readily on the plating media commonly used in clinical microbiology laboratories (see chapter 20). MacConkey agar, generally interchangeable with eosin methylene blue agar, is usually used, because it allows a preliminary grouping of enteric and other gram-negative bacteria. The most common isolates of *Enterobacteriaceae* have a characteristic appearance on blood agar and MacConkey agar that is useful for preliminary identification (Table 8). Broth enrichment can increase the isolation rate if small numbers of *Enterobacteriaceae* are present, but this step is not normally required.

Intestinal Specimens

Media that can be used routinely for intestinal specimens include a nonselective medium such as blood agar, a differential medium of low to moderate selectivity such as MacConkey agar, and a more selective differential medium such as xylose-lysine-deoxycholate (XLD) agar or Hektoen enteric agar (HE). A broth enrichment substance such as selenite (or GN [gram-negative broth] or tetrathionate) can be included, particularly if the specimen is not optimal. A highly selective medium such as brilliant green agar, bismuth sulfite, Rambach, or CHROM agar *Salmonella* (BD Diagnostics, Sparks, Md.) can also be included for isolating strains of *Salmonella.* A special plate, such as sorbitol-MacConkey agar (or one of its modifications), can be added to enhance the isolation of Shiga toxin-producing strains of *E. coli* O157:H7. This medium should be used if the stool is frankly bloody or if the patient has a diagnosis of hemolytic-uremic syndrome, and it can be used for all fecal specimens if resources permit (see chapter 42). When the presence of *Yersinia enterocolitica* is suspected, a selective-differential medium, such as CIN (cefsulodin-Irgasan-novobiocin) agar (also called *Yersinia* selective agar), can be added (see chapter 44). A complete stool culture procedure should also include media for isolation of *Campylobacter* and possibly *Vibrio* strains in areas where cholera and other *Vibrio* infections are common. Several new plating media appear to be more sensitive or specific and are gaining in popularity (see chapters 43 and 44).

IDENTIFICATION

There are many different approaches to identifying strains of *Enterobacteriaceae* (14, 37, 38).

TABLE 8 Colonial appearance of the most common *Enterobacteriaceae* on MacConkey agar and sheep blood agar[a]

Genus or species	Appearance and typical colony diameter on:	
	MacConkey agar	Sheep blood agar[b]
Salmonella and *Shigella*	Colorless, flat, 2–3 mm	Smooth, 2–3 mm
Escherichia coli (lactose positive)	Red, usually surrounded by precipitated bile, 2–3 mm	Smooth, 2–3 mm
Escherichia coli (lactose negative)	Colorless, 2–3 mm	Smooth, 2–3 mm
Yersinia enterocolitica	Colorless, less than 1 mm	Smooth, less than 1 mm
Klebsiella pneumoniae	Pink, mucoid, 3–4 mm	Mucoid, 3–4 mm
Enterobacter	Pink, not as mucoid as *Klebsiella*, 2–4 mm	Smooth, 3–4 mm
Proteus vulgaris and *Proteus mirabilis*	Colorless, flat, often swarm slightly, 2–3 mm	Swarm in waves to cover plate
Other *Proteus*, *Providencia*, and *Morganella* species	Colorless, flat, no swarming, 2–3 mm	Flat, 2–3 mm, no swarming

[a] Most strains appear this way, but there are exceptions.
[b] Most strains of *Enterobacteriaceae* are nonhemolytic, which is in contrast to many strains of *Aeromonas* and *Vibrio,* which are hemolytic. A few strains of *E. coli* are strongly hemolytic, as are occasional strains of other *Enterobacteriaceae*.

Conventional Biochemical Tests in Tubes

Tube testing was once used by all clinical microbiology laboratories, and it is still widely used in reference and public health laboratories (32, 37). Although some laboratories prepare their own media from commercial dehydrated powders, most of the common media are also available commercially in glass tubes that are ready to use. Growth from a single colony is inoculated into each tube, and the tests are read at 24 h and usually also at 48 h. In many reference laboratories, most tests are often kept for 7 days to detect delayed reactions. Unfortunately, the media and tests are not completely standardized, and few laboratories use exactly the same formulations or procedures. Even with these variables, this approach usually results in correct identifications of the common species of *Enterobacteriaceae.* Table 3 gives the results for *Enterobacteriaceae* in 48 tests (for the media and methods used to generate the data in this table, see references 32, 35, and 37).

Computer Analysis To Assist in Identification

Two microcomputer programs were developed in the 1980s at the Centers for Disease Control and Prevention (CDC)'s Enteric Reference Laboratories to assist with the identification of *Enterobacteriaceae* cultures. "George" and "Strain Matcher" were described in the 1985 review of the family (37). One of us (J.J.F.) plans to revise and update these programs to run on current operating systems and make them more available. These plans include modifying the *Enterobacteriaceae* data matrix in Table 3 and other data matrices to be compatible with the probabilistic identification program *PIBWin* that is free and can be downloaded from the Internet (http://www.som.soton.ac.uk/staff/tnb/pib.htm).

Screening Tests, Using All Information Available

Over the years, the Enteric Reference Laboratories at CDC have found that many genera, species, and serotypes can be tentatively identified with a number of screening tests (Table 9). More precise identification can be made by using a complete set of tests or commercial identification systems. Because of the limited availability of certain reagents (bacteriophage O1 and *Yersinia* typing sera, etc.), these screening tests may be more useful in a reference or research laboratory.

Example 1. A urine isolate has the following properties: colonies on MacConkey agar are 2 to 3 mm in diameter, are bright red and nonmucoid, and have precipitated bile around them; it is indole positive and 4-methylumbelliferyl-β-D-glucuronidase (MUG) positive; it grows at 44.5°C; and it is antibiotic resistant. These results are completely compatible with *E. coli.*

Example 2. An isolate from the feces of a diarrhea patient has the following properties: colonies on MacConkey agar are 2 to 3 mm in diameter and colorless; colonies on XLD agar are 2 to 3 mm and black; the isolate agglutinates in *Salmonella* polyvalent O serum and in O-group B serum; the MUCAP test (hydrolysis of 4-methylumbelliferyl caprylate; Biolife, Milan, Italy) and lysis by bacteriophage O1 are positive; and it is antibiotic resistant. All these results are compatible with *Salmonella* serogroup B.

Commercial "Kits" for Identification

A commercial kit is defined as a panel of miniaturized or standardized tests that are available commercially. The tests incorporated in the kits are often a subset of those given in Table 3. The approach for using kits is similar to the conventional tube method, with the main differences being in the miniaturization, the number of tests available, the suspending medium, and the method of reading and interpreting results (sometimes by machine). Kits are now used by most American laboratories and are discussed in chapter 15. Kits often give the correct identification for the most common species of *Enterobacteriaceae,* but they may not be as accurate for some of the new species. It is important to check the instruction manual to determine which organisms have been included in the database and the number of strains that were used to define each organism. The main problem with kit-based identification is that the tests used (usually about 20 tests) are becoming inadequate to differentiate all of the current species of *Enterobacteriaceae* given in Tables 1 to 5. This is also becoming a problem with conventional tube tests, even when the 48 tests listed in Table 3 are used. Unusual identifications or "no identification" obtained with a kit could be verified by other methods or approaches (56), but referral to a reference laboratory may be the best alternative. Other methods might

TABLE 9 Screening test for the enteric pathogens *Salmonella, Shigella, Escherichia coli, Yersinia,* and for the other important *Enterobacteriaceae* and those most frequently isolated from human clinical specimens[a]

Organism (genus, species, or serotype)	Test or property[b]
Salmonella	Lactose$^-$, sucrose$^-$, H_2S^+, O1 phage$^+$[c], MUCAP$^+$[d], agglutinates in polyvalent serum,[b] typical colonies on media selective/differential for *Salmonella* (brilliant green agar, SS agar, Rambach agar, CHROM agar, etc.), lysed by the *Salmonella*-specific "bacteriophage O1"[c], often antibiotic resistant
Salmonella typhi	Ornithine$^-$, H_2S^+ (trace amount only), L-rhamnose$^-$, no gas produced during fermentation, agglutinates in group D serum, Vi serum, and flagella "d" serum
Shigella	Nonmotile, lysine$^-$, gas$^-$, agglutinates in polyvalent serum, biochemically inactive, often antibiotic resistant, PhoE$^+$ (molecular test)[d]
Shigella dysenteriae	Agglutinates in group A serum, D-mannitol$^-$
Shigella dysenteriae O1	Catalase$^-$, agglutinates in O1 serum, Shiga toxin$^+$
Shigella flexneri	Agglutinates in group B serum, D-mannitol$^+$
Shigella boydii	Agglutinates in group C serum, D-mannitol$^+$
Shigella sonnei	Agglutinates in group D serum, D-mannitol$^+$, ornithine decarboxylase$^+$, lactose$^+$ (delayed), characteristic colony variation from smooth to rough
Escherichia coli	Extremely variable biochemically, indole$^+$, MUG$^+$, grows at 44.5°C, sometimes antibiotic resistant, PhoE$^+$ (molecular test)[d]
Escherichia coli O157:H7	Colorless colonies on sorbitol-MacConkey agar (SMAC), red colonies on MacConkey agar, MUG$^-$, D-sorbitol$^-$ (or delayed), agglutinates in O157 serum and H7 serum; many commercial media and tests are available (95)
Escherichia coli—invasive strains	Many strains resemble *Shigella* because they are "inactive" biochemically: lactose$^-$, nonmotile, lysine$^-$; O antigen groups O28, O29, O112, O124, O136, O143, O144, O152, O164, O167, and others; no commercial assay or simple way to isolate and identify
Yersinia	Grows on CIN agar, often more active biochemically at 25 than 36°C ; motile at 25°C, nonmotile at 36°C, urea$^+$
Yersinia enterocolitica, pathogenic serotypes	Smaller colonies (often less than 1 mm) than other *Enterobacteriaceae* species on enteric plating media, CR-MOX$^+$, pyrazinamidase$^-$, salicin$^-$, esculin$^-$, agglutinate in O-typing sera for "enteric pathogenic" serotypes: 3; 4,32; 5,27; 8; 9; 13a,13b; 18; 20; or 21
Yersinia enterocolitica O3 (a pathogenic serotype)	D-Xylose$^-$, agglutinates in O3 serum, tiny colonies at 24 h on plating media; in most countries it is the most frequently isolated pathogenic serotype
Yersinia enterocolitica, nonpathogenic serotypes	CR-MOX$^-$, pyrazinamidase$^+$, salicin$^+$, esculin$^+$, do not agglutinate in O-typing sera for "enteric pathogenic" serotypes: 3; 4,32; 5,27; 8; 9; 13a,13b; 18; 20; or 21
Citrobacter	Citrate$^+$, lysine decarboxylase$^-$, often grows on CIN agar, strong characteristic odor
Enterobacter	Variable biochemically, citrate$^+$, VP$^+$, resistant to cephalothin
Enterobacter sakazakii	Yellow colonies (more pigmented at 25 than 36°C), often "tough as leather"; grows on several selective media designed for its isolation; D-sorbitol negative, delayed positive DNase at 36°C
Hafnia	Lysed by *Hafnia*-specific bacteriophage 1672,[c] often more active biochemically at 25 than 36°C
Klebsiella	Mucoid colonies, encapsulated cells, nonmotile, lysine$^+$, very active biochemically, ferment most sugars, VP$^+$, malonate$^+$, resistant to carbenicillin and ampicillin
Proteus-Providencia-Morganella	Phenylalanine$^+$, tyrosine hydrolysis$^+$, often urea$^+$, resistant to colistin
Proteus	Swarms on blood agar, pungent odor, H_2S^+, gelatin$^+$, lipase$^+$
Proteus mirabilis	Urea$^+$, indole$^-$, ornithine$^+$, maltose$^-$
Proteus vulgaris	Urea$^+$, indole$^+$, ornithine$^-$, maltose$^+$
Providencia	No swarming, H_2S^-, ornithine$^-$, gelatin$^-$, lipase$^-$
Morganella	Very inactive biochemically, no swarming, citrate$^-$, H_2S^-, ornithine$^+$, gelatin$^-$, lipase$^-$, urea$^+$
Plesiomonas shigelloides	Oxidase$^+$, lysine$^+$, arginine$^+$, ornithine$^+$, *myo*-inositol$^+$
Serratia	DNase$^+$, gelatinase$^+$, lipase$^+$, resistant to colistin and cephalothin
Serratia marcescens	L-Arabinose$^-$
Serratia, other species	L-Arabinose$^+$

[a] This table gives only the general properties of the genera, species, and serogroups, so there will be exceptions. See Table 3 in this chapter and following chapters for more details and more precise data. The properties listed for a genus or group of genera generally apply to each of its species, and the properties listed for a species generally apply to each of its serotypes.

[b] See Table 3 for biochemical test results given as percentages. The serological tests refer to slide agglutination in group or individual antisera (O1, O3, etc.) for *Salmonella, Shigella, Escherichia coli,* or *Yersinia,* respectively.

[c] These are two bacteriophage tests useful for identification. The *Hafnia*-specific bacteriophage 1672 (HER 272) is available from the American Type Culture Collection (ATCC 51873-B1).

[d] Abbreviations: CIN, cefsulodin-Irgasan-novobiocin agar (a plating medium selective for *Yersinia*); CR-MOX, Congo red, magnesium oxalate agar (a differential medium useful for distinguishing pathogenic from nonpathogenic strains of *Yersinia*); MUCAP, 4-methylumbelliferyl caprylate (a genus-specific test for *Salmonella*); MUG, 4-methylumbelliferyl-β-D-glucuronidase; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; PhoE, a research test done by PCR that is sensitive and specific for *E. coli*/*Shigella* (57); VP, Voges Proskauer.

include a different kit (which will have similar limitations), a kit that contains more tests (such as those in 96-well plastic plates), or more expensive research techniques such as molecular tests or 16S rRNA sequencing (56).

Molecular Methods of Identification

Molecular methods have proved extremely useful for identification to the level of family, genus, species, serotype, clone, and strain and for differentiating pathogenic from nonpathogenic strains (see chapter 16 of this Manual). For example, a PCR test for the *phoE* gene appears to be a sensitive and specific test for determining if a strain belongs to the *Escherichia-Shigella* group (88). However, few if any of these molecular methods are commercially available. In the United States, commercial diagnostic tests must often be approved by the Food and Drug Administration if they are used on human clinical specimens. Regulatory and cost limitations have greatly restricted the use of molecular methods in clinical microbiology laboratories. However, they have proved extremely useful in a research setting. In the United States, to conform to the CLIA regulations of 1988, also called CLIA '88, it is necessary to report these research results with a disclaimer unless all the CLIA requirements have been met.

Problem Strains

Most strains of *Enterobacteriaceae* grow rapidly on plating media and on media used for biochemical identification, but occasionally a slow-growing or fastidious strain is encountered. Some strains grow poorly on blood agar but much better on chocolate agar incubated in a candle jar. This characteristic suggests a possible nutritional requirement or a mutation involving respiration. There are slow-growing strains of *E. coli, Klebsiella pneumoniae,* and *Serratia marcescens,* and typical biochemical reactions of these strains usually require extended incubation. Another type of problem organism is sometimes isolated from patients being treated with antimicrobial agents. Li et al. described such "pleiotropic" (having multiple phenotypic expression) mutants of *S. marcescens* (66) and *Salmonella* after exposure to gentamicin. These strains react atypically in many of the standard biochemical tests and are difficult to identify. A different type of pleiotropic mutant induced by chemical exposure was reported by Lannigan and Hussian (64). A *Salmonella* strain lost the ability to produce hydrogen sulfide, reduce nitrate to nitrite, and produce gas from glucose because of chlorate resistance acquired after exposure to Dakin's solution (a solution that contains chlorate and is found in hospitals). Similarly, "dwarf" colony forms of *Salmonella* serotype Typhi have been known for many years. They are only 0.2 to 0.3 mm in diameter after 24 h of incubation but are normal size if the medium is supplemented with sulfite or thiosulfate. Some atypical and slowly growing strains become more typical and grow better after they have been transferred several times. Laboratories occasionally isolate strains that grow rapidly but have a biochemical reaction profile that does not fit (Table 3) any of the described species, biogroups, or Enteric Groups of *Enterobacteriaceae* (56). At present, this type of culture can be reported only as "unidentified." It may be an atypical strain of one of the organisms listed in Tables 1 to 5, or it may belong to a new species that has not been described (37, 56, 94). Additional testing at a state, national, or international reference laboratory can often answer the question about the culture's identity and has led to the discovery of new causes of human infections (12–18, 37, 55, 56, 71, 94).

Commercial Products and Services

A wide variety of commercial products and diagnostic services are available for *Enterobacteriaceae,* but availability is constantly changing. The best approach is to go to a suppliers' Internet site to check availability, technical information, and price. Products include routine and reference identification products and kits (with or without antimicrobial susceptibility tests), combination isolation-identification products, dehydrated media, ready-to-use media in tubes and plates, antisera, reagents, antibiotic products, cultures, and bacteriophages. Services include serodiagnosis, isolation, identification, antimicrobial susceptibility testing, molecular testing, serotyping, and subtyping. For more information, see chapters 15 and 16 of this Manual, the U. S. Food and Drug Administration's *BAM Manual Online* (http://www.cfsan.fda.gov/~ebam/bam-toc.html), and references 55 and 92.

ANTIBIOTIC SUSCEPTIBILITY

Several methods are available for testing the antibiotic susceptibility of *Enterobacteriaceae,* but the most popular are disk diffusion (6) and broth dilution (see chapters 17 and 70 to 78). Several textbook and infectious disease reviews describe antibiotic usage in clinical practice (4, 68, 69, 82).

When antibiotics were first introduced, there was only slight resistance among the species of *Enterobacteriaceae.* Today, antibiotic resistance is much more common among strains isolated from humans and animals. Resistance patterns vary depending on the organism and its origin (4, 68, 69, 82).

Intrinsic Resistance

Intrinsic resistance is a genetic property of most strains of a species and evolved long before the clinical use of antibiotics. For example, essentially all strains of *Serratia marcescens* have intrinsic resistance to penicillin G, colistin, and cephalothin. This evolution of resistance can best be shown by studying strains isolated and stored before the antibiotic era or by studying a large collection of strains from a wide variety of sources including strains that have had little or no exposure to antibiotics. Table 10 lists some common *Enterobacteriaceae* and their intrinsic resistance patterns.

The Antibiogram as a Marker in Epidemiological Studies

Antibiotic susceptibility testing is usually done on isolates that are clinically significant and provides an antibiogram that is useful for comparing isolates in epidemiologic studies. When the selective ecological pressure of antibiotics is changed, the resistance patterns of epidemic (or endemic) strains may also change (4, 68, 69, 82). These changes have been documented in outbreaks that have lasted for several months or longer. Even with these limitations in stability, the antibiogram is probably the most useful and practical laboratory marker for comparing strains and can be extremely helpful in recognizing and analyzing infection problems.

Use of Antibiograms for Identification

The antibiogram of a culture can be compared with those of known isolates (Table 10) to provide a different approach to identification. When the antibiogram and identification are incompatible (for example, a strain of *Klebsiella* that is susceptible to ampicillin and carbenicillin or a culture of *Enterobacter* that is susceptible to cephalothin), the culture should be streaked and checked for purity. In addition, both the identification and the antibiogram may have to be repeated.

TABLE 10 Intrinsic antimicrobial resistance in some common *Enterobacteriaceae* species

Genus/species	Most strains are resistant to:
Buttiauxella species	Cephalothin
Cedecea species	Polymyxins, ampicillin, cephalothin
Citrobacter amalonaticus	Ampicillin
Citrobacter freundii	Cephalothin
Citrobacter diversus (*C. koseri*)	Cephalothin, carbenicillin
Edwardsiella tarda	Colistin
Enterobacter cloacae	Cephalothin
Enterobacter aerogenes	Cephalothin
Many other *Enterobacter* species	Cephalothin
Escherichia hermannii	Ampicillin, carbenicillin
Ewingella americana	Cephalothin
Hafnia alvei	Cephalothin
Klebsiella pneumoniae	Ampicillin, carbenicillin
Kluyvera ascorbata	Ampicillin
Kluyvera cryocrescens	Ampicillin
Proteus mirabilis	Polymyxins, tetracycline, nitrofurantoin
Proteus vulgaris	Polymyxins, ampicillin, nitrofurantoin, tetracycline
Morganella morganii	Polymyxins, ampicillin, cephalothin
Providencia rettgeri	Polymyxins, cephalothin, nitrofurantoin, tetracycline
Other *Providencia*[a] species	Polymyxins, nitrofurantoin
Serratia marcescens[b]	Polymyxins, cephalothin, nitrofurantoin
Serratia fonticola	Ampicillin, carbenicillin, cephalothin
Other *Serratia* species	Polymyxins,[c] cephalothin

[a] Most strains of *Providencia stuartii* are also resistant to cephalothin and tetracycline.
[b] *Serratia marcescens* can also be resistant to ampicillin, carbenicillin, streptomycin, and tetracycline.
[c] Most *Serratia* species are resistant to polymyxins, but some strains have unusual zones of inhibition from 10 to 12 mm or larger, even though they are resistant when tested by other methods.

Some of the material in this chapter was taken from or adapted from chapters and reviews of the family Enterobacteriaceae *that J.J.F. wrote while an employee or "guest researcher" of the U.S. Government. Under U.S. copyright law, these publications are defined to be "works of the U.S. Government" and thus are not subject to copyright under the U.S. Code.*

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Escherichia, Shigella, and *Salmonella**

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TAXONOMY

Escherichia, Shigella, and *Salmonella* are classified in the family *Enterobacteriaceae,* which is addressed in chapter 42 of this Manual (35). Species in these three genera are gram-negative rods that grow well on MacConkey agar (MAC). When these organisms are motile, it is by peritrichous flagella; however, all strains of *Shigella* spp. and some strains of *Escherichia* and *Salmonella* are nonmotile. All ferment D-glucose; *Escherichia* and *Salmonella* strains usually produce gas. *Shigella* is phenotypically similar to *Escherichia coli* and, with the exception of *Shigella boydii* serotype 13, would be considered the same species by DNA-DNA hybridization analysis (16). Findings from recent phylogenetic studies with nucleotide sequences of internal fragments from 14 housekeeping genes show that *S. boydii* 13 strains cluster in a neighbor-joining tree with *Escherichia albertii*, a newly described species of *Escherichia* associated with diarrheal disease in Bangladeshi children (48, 49).

NATURAL HABITATS

Escherichia, Shigella, and *Salmonella* are most frequently isolated from the intestines of humans and animals. Because *E. coli* is ubiquitous in human and animal feces, the presence of this species in water is considered to be an indicator of fecal contamination. Some species or serotypes are isolated primarily from humans (e.g., all species of *Shigella* and *Salmonella* serotype Typhi), while others (e.g., *Salmonella* serotype Gallinarum and *Salmonella* serotype Marina) are strongly associated with certain animal hosts. These genera can be isolated from fecally contaminated foods or water but probably do not occur as free-living organisms in the environment. *Salmonella* strains can, however, survive for long periods of time, perhaps years, in the environment (58).

COLLECTION, TRANSPORT, AND STORAGE OF FECAL SPECIMENS

Information on the collection, transport, and storage of specimens from extraintestinal sites is provided in chapters 5 and 20 of this Manual. Fecal specimens can include whole stools, swabs prepared from whole stools, or rectal swabs with visible fecal staining. Transport of fecal specimens to the laboratory in a timely fashion is critical, particularly for more delicate organisms such as *Shigella* (119). Ideally, fecal specimens should be examined as soon as they are received in the laboratory, but if not processed immediately, they should be either refrigerated or frozen at −70°C. Fecal specimens that will not be examined within 1 to 2 h after collection and all rectal swabs should be immediately placed in chilled transport medium and stored refrigerated. If specimens in transport medium are not to be examined within 3 days, they should be frozen immediately at −70°C.

Many of the commercially available transport media (e.g., Cary-Blair, Stuart's, and Amies transport media) are satisfactory for these organisms. Although acceptable for the transport of *E. coli, Salmonella,* and *Shigella,* buffered glycerol saline should not be used for specimens that must also be tested for *Campylobacter* and *Vibrio*.

ESCHERICHIA

Description of the Genus

The genus *Escherichia* is composed of motile or nonmotile bacteria that conform to the definitions of the family *Enterobacteriaceae* (35). There are six species in this genus: *Escherichia albertii, E. blattae, E. coli, E. fergusonii, E. hermannii,* and *E. vulneris*. The type species is *E. coli*. Typical biochemical reactions are listed in Table 1.

Clinical Significance

Of the six *Escherichia* species, *E. coli* is the species usually isolated from human specimens. It is a nearly ubiquitous constituent of the bowel flora of healthy individuals; however, certain strains may cause extraintestinal and intestinal infections in healthy as well as immunocompromised individuals. Urinary tract infections, bacteremia, meningitis, and diarrheal disease are the most frequent clinical syndromes, caused primarily by a limited number of pathogenic clones of *E. coli* (53). In particular, *E. coli* organisms bearing the K1 capsule are isolated with high frequency from cases of neonatal sepsis and meningitis. Uropathogenic *E. coli* strains are not easily identified by conventional microbiologic methods. *E. hermannii* and *E. vulneris* are most often obtained from

* This chapter contains information presented in chapter 42 by Cheryl A. Bopp, Frances W. Brenner, Patricia I. Fields, Joy G. Wells, and Nancy A. Strockbine in the eighth edition of this Manual.

TABLE 1 Biochemical reactions of the six species of *Escherichia* and selected members of the family *Enterobacteriaceae*[a]

Species/biogroup	Indole production	Voges-Proskauer	Motility (35°C)	Yellow pigment	Lysine decarboxylase	Ornithine decarboxylase	Growth in KCN	Acetate utilization	Mucate utilization	D-Glucose, gas	Adonitol, acid	L-Arabinose, acid	D-Arabitol, acid	Cellobiose, acid	Dulcitol, acid	Lactose, acid	Sucrose, acid	D-Mannitol, acid	Raffinose, acid	L-Rhamnose, acid	D-Sorbitol, acid	D-Xylose
Escherichia albertii/ biogroup 1 [*n* = 5] (e.g., Albert 19982)	0	0	0	0	100	100	0	40	0	100	0	100	0	0	0	0	0	100	0	0	100	0
Escherichia albertii/ biogroup 2 [*n* = 10] (e.g., former *S. boydii* 13)	100	0	0	0	0	100	0	0	0	40	0	100	0	0	0	0	0	100	0	0	0	0
Escherichia blattae	0	0	0	0	100	100	0	0	50	100	0	100	0	0	0	0	0	0	0	100	0	100
Escherichia coli	98	0	95	0	90	65	0	90	95	95	5	99	5	2	60	95	50	98	50	80	94	95
Escherichia coli (inactive biotypes)	80	0	5	0	40	20	1	40	30	5	3	85	5	2	40	25	15	93	15	65	75	70
Escherichia fergusonii	98	0	93	0	95	100	0	96	0	95	98	98	100	96	60	0	0	98	0	92	0	96
Escherichia hermannii	99	0	99	98	6	100	94	78	97	97	0	100	8	97	19	45	45	100	40	97	0	100
Escherichia vulneris	0	0	100	50	85	0	15	30	78	97	0	100	0	100	0	15	8	100	99	93	1	100
Shigella boydii[b]	23	0	0	0	0	0	0	0	0	0	0	94	0	0	12	1	0	7	0	1	34	16
Shigella dysenteriae	40	0	0	0	0	0	0	0	0	0	0	45	0	0	4	0	0	0	0	30	29	3
Shigella flexneri	42	0	0	0	0	0	0	8	0	3	0	60	1	0	2	0	1	91	33	5	30	3
Shigella sonnei	0	0	0	0	0	98	0	0	10	0	0	95	15	5	0	2	1	99	3	77	1	1
Hafnia alvei	0	85	85	0	100	98	95	15	0	98	0	95	0	15	0	5	10	99	2	97	0	98
Hafnia alvei/biogroup 1	0	70	0	0	100	45	0	0	0	0	0	0	0	0	0	0	0	55	0	0	0	0
Salmonella serotype Paratyphi A	0	0	95	0	0	95	0	0	0	99	0	100	0	5	90	0	0	100	0	100	95	0
Salmonella serotype Choleraesuis	0	0	95	0	95	100	0	1	0	95	0	0	1	0	5	0	0	98	1	100	90	98
Yersinia ruckeri	0	10	0	0	50	100	15	0	0	5	0	5	0	5	0	0	0	100	5	0	50	0

[a] Values are percentages of isolates tested with positive test results within 1 or 2 days of incubation at 35 to 37°C. Reactions for isolates that become positive after 2 days are not considered. Data were compiled from findings published by Ewing (34), Wathen-Grady et al. (117, 118), Ansaruzzaman et al. (5), Pryamukhina and Khomenko (88), and Farmer (37) and from unpublished findings from the reference laboratory at the CDC, 1972 to 2005.

[b] Excludes strains previously identified as *S. boydii* 13.

wound infections but have also been isolated from infections at other body sites, while *E. fergusonii* is most frequently identified from human feces (9). *E. albertii* has recently been implicated as a possible diarrheal pathogen in humans (see below) (104). *E. blattae,* which is a commensal organism of cockroaches, is not recovered from human specimens.

Diarrheagenic *E. coli*

There are at least five categories of recognized diarrheagenic *E. coli:* Shiga toxin (ST)-producing *E. coli* (STEC) (also referred to as enterohemorrhagic *E. coli* [EHEC]), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), and enteroinvasive *E. coli* (EIEC) (53, 72). The clinical significance of several other groups of putative diarrheagenic *E. coli*, particularly diffusely adherent *E. coli* (DAEC), is unclear.

STEC: O157 and Other STEC Serogroups

We refer to the STEC category of diarrheagenic *E. coli* according to the toxins that these organisms produce, e.g., STEC rather than EHEC, because the essential genetic features that define organisms capable of causing hemorrhagic colitis and hemolytic-uremic syndrome (HUS) are not clear. *E. coli* serotypes O157:H7 and O157:nonmotile (NM) (O157 STEC) produce one or more Shiga toxins, also called verocytotoxins, and are the most frequently identified diarrheagenic *E. coli* serotypes in North America and Europe. Each year an estimated 73,000 cases of illness and 60 deaths are caused by O157 STEC in the United States (69).

E. coli O157:H7 and other STEC serotypes cause illness that can present as mild nonbloody diarrhea, severe bloody diarrhea (hemorrhagic colitis), and HUS (reviewed in reference 42). Additional symptoms of *E. coli* O157:H7 infection include abdominal cramps and lack of a high fever. Of patients with O157 STEC diarrhea, 4% or more develop HUS (90), a condition characterized by microangiopathic hemolytic anemia, thrombocytopenia, and acute renal failure. The fatality rate of HUS has declined in recent years due to improvements in case management.

O157 STEC is thought to cause at least 80% of cases of HUS in North America and is recognized as a common cause of bloody diarrhea in developed countries (90). In the United States, the rate of isolation of O157 STEC from fecal specimens is highest in the Northern tier states, where it may approach the rates of common diarrheal pathogens. Many U.S. clinical laboratories do not routinely culture stools for O157 STEC; as a result, many illnesses are not detected (24).

O157 STEC colonizes dairy and beef cattle and, therefore, ground beef has caused more O157 STEC outbreaks than any other vehicle of transmission (101). Other known vehicles of transmission include raw milk, sausage, roast beef, unchlorinated municipal water, apple cider, raw vegetables, and sprouts; these vehicles are typically exposed to water contaminated by bovine manure. O157 STEC spreads easily from person to person because the infectious dose is low (<200 CFU); outbreaks associated with person-to-person spread have occurred in schools, long-term-care institutions, families, and day-care facilities.

More than 150 non-O157 STEC serotypes have been isolated from persons with diarrhea or HUS (http://www.microbionet.com.au/frames/feature/vtec/brief01.html). In some countries, non-O157 STEC strains, particularly *E. coli* serotypes O111:NM and O26:H11, are more commonly isolated than O157 STEC, although most outbreaks and cases of HUS are attributed to the latter (serotypes characteristic of diarrheagenic *E. coli* pathotypes are presented in Table 2). In the United States, *E. coli* O157:H7 is the most frequently isolated STEC but increasingly non-O157 STEC are identified as causes of outbreaks and sporadic illness (23). At the CDC's *E. coli* Reference Laboratory, 72% of all non-O157 STEC isolates received between 1983 and 2000 belonged to eight serogroups (O26, O111, O103, O121, O45, O145, O165, and O113) (N. A. Strockbine, unpublished data). Because most laboratory methods for the detection of O157 STEC do not detect non-O157 STEC, the numbers of infections with serotypes other than O157:H7 or O157:NM are probably underestimated.

ETEC

ETEC, which produces heat-labile *E. coli* enterotoxin (LT) and/or heat-stable *E. coli* enterotoxin (ST), is an important cause of diarrhea in developing countries, particularly among young children (72). ETEC also is a frequent cause of traveler's diarrhea. Ten U.S. outbreaks were reported to the CDC from 1995 to 2001, whereas only 15 outbreaks occurred during the preceding 25 years (C. A. Bopp, unpublished data) (30). ETEC is infrequently identified in the United States, but this may be attributable in part to the fact that few laboratories are capable of identifying this pathogen. ETEC strains, particularly those associated with outbreaks, tend to cluster in a few serotypes (Table 2).

The most prominent symptoms of ETEC illness are diarrhea and abdominal cramps, sometimes accompanied by nausea and headache, but usually with little vomiting or fever (30). Although ETEC is usually associated with relatively mild watery diarrhea, illness in some recent ETEC outbreaks has been notable for its prolonged duration.

EPEC

In the past, EPEC strains were defined as certain *E. coli* serotypes that were epidemiologically associated with infantile diarrhea but did not produce enterotoxins or Shiga toxins and were not invasive. The traditional EPEC serotypes are listed in Table 2; typically these serotypes show a distinct pattern of localized adherence to HeLa and HEp-2 cells (115). These serotypes usually also demonstrate actin aggregation in the fluorescent actin stain test, which correlates with the attaching-and-effacing lesion in vivo (72). Because of the lack of simple diagnostic methods for EPEC, few laboratories attempt to identify these organisms. Full EPEC pathogenicity requires two genetic elements: the EPEC adherence factor (EAF) plasmid, which encodes most importantly the bundle-forming pilus, and the chromosomal locus of enterocyte effacement (LEE), which mediates the attaching-and-effacing phenotype. The term "typical EPEC" has been suggested for those organisms harboring both the EAF plasmid and the LEE pathogenicity island (see below). Typical

TABLE 2 Frequently encountered serotypes of diarrheagenic *E. coli*[a]

ETEC	EPEC	EIEC	STEC		EAEC
O6:NM	**O55:NM**	O28:NM	O22:H5	O118:H16	O3:H2
O6:H16	**O55:H6**	O29:NM	O22:H8	O119:NM	O15:H18
O8:H9	O55:H7	O112:NM	**O26:NM**	O119:H4	**O44:H18**
O15:H11	O86:NM	O124:NM	**O26:H11**	O119:H25	O51:H11
O20:NM	O86:H34	O124:H7	O28:H25	**O121:H19**	O77:H18
O25:NM	**O111:NM**	**O124:H30**	O45:H2	O128:NM	O86:H2
O25:H42	**O111:H2**	O136:NM	O55:H7	O128:H2	O111ab:H21
O27:NM	O111:H12	**O143:NM**	O84:NM	O128:H45	O126:H27
O27:H7	O111:H21	O144:NM	O88:H25	**O145:NM**	O141:H49
O27:H20	**O114:NM**	O152:NM	O91:NM	O146:H21	ONT:H21
O49:NM	**O114:H2**	**O164:NM**	O91:H14	O153:H2	ONT:H33
O63:H12	**O119:H6**	O167:NM	O91:H21	O153:H25	
O78:H11	**O125:H21**	**ONT:NM**	**O103:H2**	**O157:NM**	
O78:H12	O126:NM		**O104:H21**	**O157:H7**	
O128:H7	O126:H27		**O111:NM**	O165:NM	
O148:H28	**O127:NM**		**O111:H2**	O165:H25	
O153:H45	**O127:H6**		**O111:H8**	O172:NM	
O159:NM	O127:H9		**O113:H21**	O174:H21	
O159:H4	O127:H21		**O118:H2**	O174:H28	
O159:H20	**O128:H2**		O118:H12		
O167:H5	O128:H7				
O169:NM	O128:H12				
O169:H41	**O142:H6**				
	O157:H45				

[a] Outbreak-related serotypes are shown in bold type. NM, nonmotile; NT, not typeable.

EPEC correspond to EPEC of the classical serotypes and are important causes of diarrhea in developing countries (32, 72); these organisms were implicated in highly lethal nursery outbreaks in the United States and the United Kingdom before 1970. The infection is currently rare in the industrialized world. More recently, atypical EPEC have been implicated as enteric pathogens in the United States, including in several outbreaks of diarrheal disease (113). These strains possess a functional LEE apparatus but do not carry the EAF plasmid. The full role of these pathogens has yet to be elucidated, but they may be considered as potential causes of diarrheal outbreaks when no other pathogens are identified.

The symptoms of often severe, prolonged, and nonbloody diarrhea, vomiting, and fever in infants or young toddlers are characteristic of EPEC illness (72). Infection with EPEC has been associated with chronic diarrhea; sequelae may include malabsorption, malnutrition, weight loss, and growth retardation.

EIEC

EIEC strains invade cells of the colon and produce a generally watery, but occasionally bloody, diarrhea by a pathogenic mechanism similar to that of *Shigella*. EIEC is rare in the United States and is less common than ETEC or EPEC in the developing world (72). EIEC strains, like ETEC and EPEC, are associated with a few characteristic serotypes (Table 2). Three large outbreaks of diarrhea caused by EIEC have been reported in the United States (72).

EAEC

EAEC, as originally defined by its specific pattern of aggregative adherence to HEp-2 cells in culture, has been associated with diarrhea in a variety of clinical settings, including endemic diarrhea in children of both impoverished and industrialized countries, epidemic diarrhea, diarrhea of travelers to developing countries, and persistent diarrhea among patients with human immunodeficiency virus/AIDS infection (47). The pathogenicity of EAEC has been confirmed in volunteer studies (73) and by implication of EAEC in diarrhea outbreaks (28). Early studies frequently failed to find an association of EAEC with pediatric diarrhea, but this association has been strengthened by the use of molecular techniques, which discriminate the true pathogens exhibiting the aggregative pattern (29, 95). The term "typical EAEC" describes organisms harboring virulence genes under the control of the global EAEC regulator AggR (95). Typical EAEC may be a common cause of pediatric diarrhea in U.S. infants (29) and should be considered as a potential cause of foodborne outbreaks and diarrhea in human immunodeficiency virus/AIDS patients (47). EAEC diarrhea is accompanied by signs and symptoms of mild inflammation (abdominal pain and fever), but stools usually do not contain blood or fecal leukocytes (47).

Putative Diarrheagenic *E. coli*

Several putative pathotypes have been described. For none of these types has virulence clearly been demonstrated in volunteer strudies or outbreak investigations. DAEC strains, which exhibit a characteristic diffuse pattern of adherence to HEp-2 cells, have been implicated as causes of diarrhea in some epidemiologic studies but not others (72), and a prototype DAEC strain did not elicit diarrhea in adult volunteers (109). In several studies, DAEC infections have been significantly associated with watery diarrhea among children 1 to 5 years of age but were not associated with illness among infants (59). DAEC may occur in industrialized countries (72). A complex signal transduction cascade has been suggested as the mechanism of DAEC pathogenesis (99).

Cytotoxic necrotizing factor (CNF)-producing *E. coli* strains produce a toxin that induces morphological alterations (multinucleation) and death in tissue cultures (20). Two forms have been described: CNF1 and CNF2. CNF1-producing strains were originally detected in infants with enteritis and later from humans with extraintestinal infections (13, 20). Most CNF1-producing strains are also hemolytic, although the toxin is distinct from hemolysin. CNF2-producing strains have been isolated from animals with diarrhea (31, 80, 102). The role of these strains in human diarrheal disease has not been definitively determined (72).

Cytolethal distending toxin-producing *E. coli* strains produce a heat-labile factor that induces cytotonic and cytotoxic changes in Chinese hamster ovary cells similar to those caused by LT (51). This factor does not affect Y-1 cells. The results of one study in Bangladesh suggested that cytolethal distending toxin-producing *E. coli* strains are associated with diarrhea (3), but other studies are needed to establish their status as etiologic agents.

Several diarrheal outbreaks have been linked to *E. coli* strains that do not belong to any of the established pathotypes. Some of these strains carry the gene encoding the enteroaggregative ST-like toxin (EAST1), which is related to the ETEC ST enterotoxin. Further studies are needed to prove the pathogenicity of these strains, but the EAST1 gene can be identified using molecular techniques (68).

Isolation Procedures

Isolation procedures for extraintestinal infections are covered in chapter 20.

Isolation Procedures for STEC

All fecal specimens submitted for culture of bacterial enteric pathogens in areas of high endemicity or from patients with bloody diarrhea should be examined for O157 STEC (106). Culture for non-O157 STEC is indicated for patients with HUS and/or bloody diarrhea and should be considered for other patients with diarrhea based on severity of illness, age, and epidemiologic or exposure information.

Because there is no selective isolation medium for non-O157 STEC, testing for the presence of Shiga toxin in fecal specimens is the best approach for detecting these organisms. Commercial enzyme-linked immunoassays are a sensitive means of detecting Shiga toxin (33, 63). Isolation and serotyping of STEC from fecal specimens that are positive by nonculture assays should always be attempted because serotype information is important for public health purposes and may also help in clinical decisions.

Enrichment

Although broth enrichment is widely used for the recovery of O157 STEC from foods, there is little evidence that it enhances isolation from human fecal specimens. However, immunomagnetic separation (IMS), a technique shown to increase the rate of isolation of O157 STEC from food specimens, has been adapted to culture of fecal specimens (54). IMS enhances the detection of O157 STEC from patients with HUS, patients presenting an extended period of time after the onset of illness, asymptomatic carriers, or specimens that have been stored or transported improperly. IMS beads for O157, O111, and O26 are available commercially (Table 3), or laboratories may produce beads with other O-specific antibodies (81).

TABLE 3 Partial listing of commercial suppliers of reagents for detection of STEC[a]

Antisera for tube agglutination
Difco Laboratories (Division of Becton Dickinson and Co., Sparks, Md.)
O157 and H7 antisera
SA Scientific, San Antonio, Tex.
O157 and H7 antisera
Denka Seiken Co., Ltd., Tokyo, Japan
O157, H7, O145, O128, O111, O103, O91, O26, and other *E. coli* O antisera
Latex slide agglutination reagents
Denka Seiken Co., Ltd., Tokyo, Japan
O157, O111, and O26 reagents
Oxoid Inc., Ogdensburg, N.Y.
O157, O145, O128, O111, O103, O91, and O26 reagents
ProLab Diagnostics, Inc., Ontario, Canada
O157 and H7 reagents
Remel, Inc., Lenexa, Kans.
O157 and H7 reagents
Immunomagnetic beads
Dynal Biotech Inc., Lake Success, N.Y.
Anti-O157 labeled beads
Denka Seiken Co., Ltd., Tokyo, Japan
Anti-O157, anti-O111, and anti-O26 labeled beads
O157 immunoassays
Meridian Diagnostics Inc., Cincinnati, Ohio
For testing stool specimens or enrichment broths for O157 antigen
Denka Seiken Co., Ltd., Tokyo, Japan
For testing colony sweeps or individual colonies for O157, O111, or O26 antigens
Shiga toxin immunoassays
Meridian Diagnostics Inc., Cincinnati, Ohio
For testing stool specimens, enrichment broths, colony sweeps, or individual colonies for Shiga toxin
Remel, Inc., Lenexa, Kans.
For testing stool specimens or enrichment broths for Shiga toxin
Denka Seiken Co., Ltd., Tokyo, Japan
For testing colony sweeps or individual colonies for Shiga toxin
Oxoid Inc., Ogdensburg, N.Y.
For testing individual colonies for Shiga toxin
Chromogenic agars (for visual detection of O157:H7 colonies upon direct inoculation of agar plates)
Merck KGaA, Darmstadt, Germany
Biomerieux Inc., Hazelwood, Mo.
Biosynth International, Inc., Naperville, Il.
Biolog, Inc., Hayward, Calif.
CHROMagar; available under license to Becton Dickinson and Co., Franklin Lakes, N.J.

[a] Not intended to be a comprehensive listing. The U.S. Food and Drug Administration has not approved all of these reagents for use with clinical specimens. This table does not include reagents or tests specifically intended for examination of food, water, or environmental specimens. The online version of the *Bacteriological Analytical Manual* lists many tests for food specimens (http://www.fda.gov). Inclusion does not constitute endorsement by the CDC or ASM.

Plating Media

Because O157 STEC strains ferment lactose, they are impossible to differentiate from other lactose-fermenting organisms on lactose-containing media. Most O157 STEC strains do not ferment the carbohydrate D-sorbitol overnight, in contrast to the approximately 80% of other *E. coli* strains that ferment sorbitol rapidly. Thus, sorbitol-containing MacConkey agar (SMAC) is used for isolation of O157 STEC. Sorbitol-nonfermenting colonies are suspected (but not definitively) to be O157:H7 (67). In some areas of central Europe, sorbitol-fermenting O157 STEC strains are commonly isolated from patients with HUS (12); these organisms are very rare in North America (Strockbine, unpublished).

Specific culture media have been developed to exploit biochemical and antibiotic resistance traits that are characteristic of STEC strains. Several chromogenic agar media are available commercially to assist in rapid identification (Table 3); these media generally perform well for O157:H7 and for some non-O157 STEC (10, 65, 77) Cefixime-tellurite SMAC (CT-SMAC) has also proved to be a useful selective medium for O157:H7. It has been used mainly for culture of animal and food specimens because of its selectivity, but it has also been applied to culture of human fecal specimens (25, 124). It has been reported that some O157:NM strains fail to grow on CT-SMAC (54).

As noted above, *E. coli* strains with particular virulence in the urinary tract cannot be easily distinguished from organisms of lower virulence, and any *E. coli* isolated in high numbers (particularly $>10^5$ CFU/ml of urine) should be considered a potential pathogen. *E. coli* with increased virulence in the urinary tract is commonly hemolytic on sheep blood agar and expresses one or more of several urinary tract adhesins. Several chromogenic media have been proposed for use in detecting uropathogenic *E. coli;* these have been compared in published studies (6, 26).

Commercial Rapid Diagnostic Methods

A number of commercial immunoassays (Table 3) are now available for detecting Shiga toxin or O157 antigen in fecal specimens, enrichment broth cultures, colony sweeps, or individual colonies. Isolation of STEC from fecal specimens that are positive by one of these rapid diagnostic methods is important for public health purposes. Determination of the subtype of O157 STEC and the serotype of a non-O157 STEC isolate is valuable for outbreak investigations and surveillance purposes (see "Subtyping" and "Identification" below).

Screening Procedures for STEC Strains

For the isolation of O157 STEC from SMAC, colorless (nonfermenting) colonies are tested with O157 antiserum or latex reagent (103) (Table 3). If the O157 latex reagent is used, it is important to test positive colonies with the latex control reagent to rule out nonspecific reactions. The manufacturers of these kits recommend that strains reacting with both the antigen-specific and control latex reagents be heated and retested. However, in a study that followed this procedure, none of the nonspecifically reacting strains were subsequently identified as O157 STEC (14).

Unlike most other *E. coli* strains, O157 STEC do not express beta-glucuronidase; therefore, the MUG reaction (4-methylumbelliferryl-beta-D-glucuronide for detection of beta-glucuronidase activity) is helpful for screening for

O157 STEC (98). MUG-positive, urease-positive O157 STEC strains have been isolated in the United States but are still rare (45) (Strockbine, unpublished).

For the recovery of STEC from stool specimens which test positive for Shiga toxin, either SMAC or MacConkey agar should be inoculated. It is advantageous to use SMAC because O157 STEC can be quickly and easily identified. If sorbitol-nonfermenting colonies are negative with O157 latex, then sorbitol-fermenting colonies (because most non-O157 STEC ferment sorbitol) and a representative sample of sorbitol-nonfermenting colonies may be selected for Shiga toxin testing. Latex reagents and antisera (Table 3) for detecting certain non-O157 STEC serotypes are now available and could also be used to test colonies from Shiga toxin-positive specimens or to serogroup Shiga toxin-positive isolates.

Virtually all O157 STEC and 60 to 80% of non-O157 STEC produce a characteristic *E. coli* hemolysin, referred to as enterohemolysin (Ehly), which is distinct from the α-hemolysin, produced by other *E. coli* strains (11). A special medium, washed sheep blood agar supplemented with calcium (WSBA-Ca), is used as a differential medium for the detection of enterohemolytic activity (11). Ehly-producing colonies can be differentiated from α-hemolysin-producing colonies on WSBA-Ca because the latter are visible after 3 to 4 h of incubation. After 3 to 4 h, colonies are marked for the appearance of α-hemolysin, and the plates are examined again after 18 to 24 h. Incorporation of mitomycin C into the WSBA-Ca enhances the appearance of the Ehly hemolysis and increases the proportion of non-O157 STEC that exhibit this activity (107). Because many non-O157 STEC strains do not demonstrate the enterohemolytic phenotype and because enterohemolytic nontoxigenic strains have been reported, additional screening methods should be used in conjunction with WSBA-Ca medium (96).

Presumptive STEC isolates should be sent to a reference laboratory or a public health laboratory for further characterization.

Isolation Methods for Diarrheagenic *E. coli*

Methods for the identification of ETEC, EPEC, EIEC, EAEC, and the putative diarrheagenic *E. coli* are generally available only in reference or research settings. Public health and reference laboratories usually examine specimens for these pathogens only when an outbreak has occurred and specimens are negative for routine bacterial pathogens. EAEC should be considered as a possible etiologic agent of watery diarrhea for which no other pathogen has been identified (29), and ETEC should be considered for travelers. EPEC should be considered as a possible pathogen in outbreaks of severe nonbloody diarrhea occurring in infants or young toddlers, particularly in nursery or day care settings. EIEC and EAEC should be considered as possible etiologic agents in outbreaks of diarrhea, bloody or nonbloody.

To capture *E. coli* for further testing, fecal specimens should be plated on a differential medium of low selectivity (e.g., MAC). Five to 20 colonies, mostly lactose fermenting but with a representative sample of nonfermenting colonies, should be selected and inoculated to nonselective agar slants (such as L agar or nutrient agar). These colonies are then sent to a reference laboratory for testing or are screened for virulence-associated characteristics if assays are available. Strains can be kept frozen for long periods in L broth with 15 to 50% glycerol at −80°C. Arrangements for sending *E. coli* isolates from well-characterized outbreaks to the CDC for testing can be made through local and state health departments.

Screening Procedures for ETEC, EPEC, EAEC, and EIEC Strains

E. coli pathotypes other than STEC cannot be distinguished from other *E. coli* strains by biochemical screening techniques. Many EIEC strains are nonmotile and fail to decarboxylate lysine; however, some EIEC strains are motile or lysine positive. Use of commercial antisera to the classical EPEC somatic (O) and capsular (K) antigens is no longer recommended.

Identification

Biochemical Identification

Biochemical identification of presumptive O157 STEC isolates is necessary because other species may cross-react with O157 antiserum or latex reagents, including *Salmonella* O group N, *Yersinia enterocolitica* serotype O9, *Citrobacter freundii*, and *E. hermannii*. Special biochemical tests (cellobiose fermentation and growth in the presence of KCN) may be necessary to differentiate *E. hermannii* from *E. coli*, but because *E. hermannii* is rarely detected in stool specimens, use of these tests is not cost-effective for most laboratories.

Identification of *E. albertii* with commercial identification systems is problematic at present because representative strains of this species are not yet included in commercial databases (1). Abbott and colleagues, who extensively characterized five strains of *E. albertii* by conventional biochemical methods and by commercial identification panels, reported that *E. albertii* is an indole-negative species that ferments D-mannitol but not D-xylose (1). In their study, *E. albertii* strains were identified by commercial systems as *Hafnia alvei; Salmonella* or *S. enterica* serotype Cholerasuis; *E. coli*, inactive or serotype O157:H7; or *Yersinia ruckeri*. Although some strains would have been clearly misidentified, the majority of the strains generated probability scores for the final identification that were unacceptable or the identification was inconsistent with the source of the specimen (e.g., the fish pathogen *Y. ruckeri* from a human specimen), which should have triggered additional biochemical tests to establish a more reliable identification. The authors found that the most reliable clue to the possible presence of *E. albertii* was an unacceptable first-choice identification of *H. alvei* for an isolate that is both L-rhamnose and D-xylose negative.

Biochemical tests that can help discriminate *E. albertii* strains from selected members of the *Enterobacteriaceae* family with similar biochemical phenotypes are shown in Table 1. Two biogroups of *E. albertii* are listed in Table 1. These correlate with two of the distinct clusters of strains identified in the *E. albertii* lineage by phylogenetic studies (49). Biogroup 1 is comprised of the five strains isolated from Bangladeshi children with diarrhea, while biogroup 2 is comprised of strains formerly identified as *S. boydii* 13. The strains in the two biogroups differ from each other in their abilities to produce indole from tryptophane, decarboxylate lysine, and ferment D-sorbitol. Antigenic relationships between members of the *E. albertii* lineage and other members of the *Enterobacteriaceae* family have been observed (e.g., *S. boydii* 7 and *E. coli* O28). A diagnostic PCR assay using three housekeeping genes was described by Hyma et al. (49) for *E. albertii;* this assay is independent of biochemical or antigenic phenotypes and should facilitate studies to learn about the diversity within the lineage, their natural habitat, and their role in enteric disease.

Serotyping

The serologic classification of *E. coli* is generally based on the O antigen (somatic) and the H antigen (flagellar) (9).

The O and H antigens of *E. coli* are stable and reliable strain characteristics, and although 181 O antigens and 56 H antigens have been described (a few of which are no longer recognized), the actual number of serotype combinations associated with diarrheal disease is limited (Table 2). Determination of the O and H serotypes of *E. coli* strains implicated in diarrheal disease is particularly useful in epidemiologic investigations (Table 2). Even though antisera for the tube agglutination test are available from several manufacturers, most laboratories do not attempt to complete *E.* coli serotyping because it is costly. For well-characterized outbreaks with no identified etiologic agent, arrangements may be made through state health departments to send *E. coli* isolates to the CDC for virulence testing and serotyping.

Serologic Confirmation of O157 STEC

Confirmation of *E. coli* O157:H7 requires identification of the H7 flagellar antigen. H7-specific antisera and latex reagents are commercially available (Table 3), but detection of the H7 flagellar antigen often requires multiple passages (103). Isolates that are nonmotile or negative for the H7 antigen should be tested for the production of Shiga toxins or the presence of Shiga toxin gene sequences.

Approximately 85% of O157 isolates from humans received by the CDC are serotype O157:H7, 12% are nonmotile, and 3% are H types other than H7 (Strockbine, unpublished). *E. coli* O157:NM strains frequently produce Shiga toxin and are otherwise very similar to O157:H7, but no O157 strain from human illness with an H type other than H7 has been found to produce Shiga toxin (Strockbine, unpublished) (38).

Virulence Testing

Detection of diarrheagenic pathotypes is typically performed on *E. coli* colonies selected from selective or nonselective media. If PCR techniques are used, a sweep of confluent growth from a MAC plate may be screened; if the PCR assay is positive, isolated colonies may then be picked and screened individually. Multiplex PCR assays are capable of simultaneously detecting multiple *E. coli* pathotypes (75).

STEC

Two distinct Shiga toxins, Stx1 and Stx2, also referred to as verocytotoxins, have been described. In addition, there are several variant forms of Stx2, including Stx2c, Stx2d, Stx2e, and Stx2f, which in one study were more frequently identified from asymptomatic carriers than HUS (40). All of these toxins are similar to the Shiga toxin expressed by *Shigella dysenteriae* serotype 1, and the Stx1 toxins produced by O157 STEC and other STEC serotypes are virtually identical. STEC may produce either Stx1 or Stx2 or both toxins. The production of Stx or the genes encoding Stx can be detected by a variety of biologic, immunologic, or nucleic acid-based assays (72). Protocols for several of these tests (e.g., cell culture, DNA probing, and PCR) are available (79). Stx has also been directly detected in the blood of HUS patients by using flow cytometry even in the absence of serologic or microbiologic evidence of STEC infection (111).

STEC strains represent a spectrum of virulence potential ranging from the highly virulent O157:H7 serotype that has been responsible for the majority of outbreak cases to avirulent serotypes that have been isolated only from nonhuman sources. The presence of additional virulence factors other than Stx correlates with disease potential. The most important of these virulence factors are the intimin adhesin and the type III secretion system encoded on the LEE pathogenicity island (53). The *eae* gene probe for intimin and the *hly*A (*ehx*A) gene probe for a plasmid-encoded hemolysin have been the most frequently employed methods to determine virulence potential, but probes for at least 25 different virulence-associated genes have been employed to characterize STEC strains (86). STEC have been classified into five "seropathotypes" (A through E) based on the occurrence of serotypes in human disease, in outbreaks, and in severe disease (HUS or hemorrhagic colitis) and on possession of specific virulence genes (55).

ETEC

The ST and LT enterotoxins produced by ETEC may be detected by a variety of biologic, immunologic, and nucleic acid-based assays (72). Two distinct ST variants (STh and STp) have been identified in human strains. Strains that produce ST only or ST in combination with LT have caused most ETEC outbreaks in the United States (30).

Immunoassays for the identification of ST or LT from culture supernatants of ETEC strains are available from at least two commercial sources (Table 3). The ST EIA assay (Denka Seiken Co., Ltd., and Oxoid Ltd.) is a competitive enzyme immunoassay for the detection of ST only (97). A reversed passive latex agglutination assay (VET-RPLA; Oxoid; a similar kit is available from Denka Seiken) detects both cholera toxin and LT, which are highly related antigenically. The effectiveness of the VET-RPLA may be optimized by use of a culture medium designed for LT production such as Biken's medium rather than the medium recommended by the manufacturer (122).

EPEC

EPEC, EAEC, and DAEC can be detected by the characteristic patterns of adherence to HEp-2 or HeLa cells in culture (115). These patterns are also observed on formalin or glutaraldehyde-fixed cells, obviating the need to prepare cells expressly for the assay (70).

EPEC are defined on the basis of the attaching/effacing (A/E) histopathology produced on epithelial cells and the lack of Stx (reviewed in references 32 and 53). The A/E phenotype can be detected by tissue culture cell assays or by DNA probe or PCR tests for the *eae* gene encoding intimin or the LEE pathogenicity island. The EAF plasmid of typical EPEC (see above) is detected by fragment or oligonucleotide probes or PCR primers (72). Atypical EPEC possess only the A/E phenotype/LEE pathogenicity island but do not possess the EAF plasmid.

EAEC

Several simple assays have been described as surrogates to the cell adherence test for identification of EAEC. These include a simple biofilm formation assay on polystyrene (123) and screening for the presence of a pellicle at the surface of broth media (4). EAEC can be more definitively identified by a specific DNA probe (the AA or CVD432 probe) (7), which is superior to tissue culture adherence assays in identifying pathogenic strains of EAEC (29). More recent data suggest that the AA probe corresponds to a putative virulence gene called *aat*A (76), which is under the control of a regulator termed AggR. AggR in turn controls several other virulence factors (95). Thus, the *aggR* gene (which defines typical EAEC) may represent a superior diagnostic target.

EIEC

EIEC can be identified by various in vivo assays, immunoassays, and nucleic acid-based assays for invasiveness, but

no commercial kits or reagents are available. Cell culture invasion assays or DNA-based assays for the *ipa*C or *ipa*H invasion-related factors are, for the most part, practical only in research settings (72). Plasmid DNA electrophoresis may be used to detect the large 120- to 140-MDa plasmid associated with invasiveness, but this plasmid is easily lost when the isolate is subcultured. Because of shared invasiveness-related characteristics, these assays also detect *Shigella* strains.

DAEC

DAEC were initially defined on the basis of a diffuse adherence pattern to cultured epithelial cells, but this phenotype is not specific for enteric strains (99). Various DNA probes and PCR assays have been proposed for DAEC identification as reviewed (72).

Extraintestinal *E. coli*

The presence of any *E. coli* in urine specimens at 10^5 CFU/ml (or lower in some situations) or in cerebrospinal fluid or blood specimens in any amount is indicative of infection and the need for treatment. Numerous virulence factors have been identified for extraintestinal *E. coli* (53), particularly the K1 antigen, but these are usually identified only in epidemiological studies.

Subtyping

Several methods of subtyping have been used for *E. coli* O157:H7 isolates. In particular, pulsed-field gel electrophoresis (PFGE) methods are useful (72). A national molecular subtyping network, PulseNet, was established in 1996 by the CDC to facilitate subtyping of bacterial foodborne pathogens, including *E. coli* O157:H7, *Shigella,* nontyphoidal *Salmonella* serotypes, and *Listeria monocytogenes* (108). Successful detection of outbreaks by this network of state and local public health laboratories is dependent upon submission of isolates by clinical laboratories for confirmation and subtyping.

Determination of the serotype and the antimicrobial susceptibility pattern is usually adequate for defining outbreak strains of ETEC, EPEC, and EIEC. Plasmid typing or PFGE methods may also be helpful for distinguishing between sporadic isolates and outbreak strains, but neither method has been widely used for these groups of *E. coli*.

Serodiagnostic Tests

At the present time, serodiagnostic tests for diarrheagenic *E. coli* are valuable only for seroepidemiology surveys and are not useful for the diagnosis of sporadic infections. Assays that measure serum antibody response to lipopolysaccharide (LPS) have been used to detect STEC infection in culture-negative HUS patients (72). Enzyme-linked immunosorbent assays have been described to detect saliva antibodies to LPS (62) and serum antibodies to the secreted EspB protein in HUS patients (100).

Antimicrobial Susceptibilities

STEC

Antimicrobial therapy for O157 STEC diarrhea or HUS is controversial: some publications have suggested that antibiotics increase the risk of HUS (42, 121), while a meta-analysis of published reports found no significantly increased risk (94). Until recently, *E. coli* O157:H7 isolates were almost uniformly sensitive to antimicrobial agents. However, since the early 1990s, O157 and other STEC strains have demonstrated slowly increasing levels of resistance to certain antibiotics, particularly streptomycin, sulfonamides, and tetracycline (see http://www.cdc.gov/narms/).

ETEC, EPEC, EIEC, EAEC, and Other Diarrheagenic *E. coli* Strains

Treatment with an appropriate antibiotic can reduce the severity and duration of symptoms of ETEC infection (72). Antimicrobial resistance, particularly to tetracycline, is common among ETEC strains isolated from outbreaks in the United States (30). Antibiotic treatment may be helpful for diarrhea caused by EPEC (72). Most EPEC strains associated with outbreaks are resistant to multiple antimicrobial agents (32). EAEC are commonly resistant to most antibiotics, though these strains are typically sensitive to fluoroquinolones. Clinical studies have demonstrated the effectiveness of ciprofloxacin for travelers with diarrhea caused by EAEC (41). Little information about the efficacy of antimicrobial treatment or the prevalence of resistance is available for EIEC or other putative diarrheagenic *E. coli* strains, but determination of the antimicrobial susceptibility pattern may be helpful in establishing whether the isolates are associated with an outbreak.

Interpretation and Reporting of Results

STEC

A presumptive diagnosis of an O157 STEC (isolate positive for O157 antigen) or a non-O157 STEC (isolate positive for Shiga toxin) infection should be reported to the clinician as soon as the laboratory obtains this result. It is advisable to indicate on negative reports that non-O157 STEC strains can cause diarrhea and HUS. Clusters and outbreaks of STEC should be reported to public health authorities. Presumptive STEC isolates should be confirmed by demonstration of the O157 and H7 antigens or assay for Shiga toxin and should be identified biochemically as *E. coli*. STEC isolates should be forwarded to a local or state public health laboratory for serotyping and/or molecular subtyping.

ETEC, EPEC, EAEC, and EIEC

Generally, the ETEC, EPEC, EAEC, and EIEC classes of diarrheagenic *E. coli* are identified only during outbreak investigations. A laboratorian reporting these results, which usually will be a retrospective diagnosis obtained by a reference laboratory, should provide an explanation of the clinical significance of these organisms and may refer the clinician to the reference laboratory for further information. All suspected outbreaks should be reported to public health authorities.

SHIGELLA

Description of the Genus and Taxonomy

The genus *Shigella* is composed of nonmotile bacteria that conform to the definition of the family *Enterobacteriaceae* (35). There are four subgroups of *Shigella* that historically have been treated as species: subgroup A as *S. dysenteriae,* subgroup B as *Shigella flexneri,* subgroup C as *Shigella boydii,* and subgroup D as *Shigella sonnei*. From a genetic standpoint, the four species of *Shigella* and *E. coli* represent a single genomospecies (56). Using a genetic definition for species, the four species of *Shigella* would be regarded as serologically defined anaerogenic biotypes of *E. coli.* The current nomenclature of *Shigella* is maintained largely for medical purposes because of the useful association of the genus epithet with

TABLE 4 Differentiation of *E. coli* and *Shigella*

Test	Result[a] of test with:		
	Shigella	Inactive *E. coli*[b]	*E. coli*
Lysine decarboxylase	−	d	+
Motility	−	−	+
Gas from glucose	−	−	+
Acetate utilization	−	d	+
Mucate	−	d	+
Lactose	−	d	+

[a] Abbreviations: +, 90% or more positive within 1 or 2 days; −, no reaction (90% or more) in 7 days; d, different reactions [+, (+), −]. Adapted from Ewing (35).

[b] Nonmotile, anaerogenic biotypes sometimes referred to as Alkalescens-Dispar bioserotypes.

the distinctive disease (shigellosis) caused by these organisms. The type species is *S. dysenteriae*. *Shigella* does not form gas from fermentable carbohydrates, with the exception of certain strains of *S. flexneri* serotype 6 and *S. boydii* serotype 14. Compared with *Escherichia, Shigella* strains are less active in their use of carbohydrates (Table 4). *S. sonnei* strains ferment lactose on extended incubation, but other species generally do not use this substrate in conventional medium.

Recent findings from phylogenetic studies show that *S. boydii* 13 strains, some of which can produce gas from glucose, are more appropriately regarded as *E. albertii* (49). *S. boydii* 13 strains were first described in 1952 and then added to the *Shigella* scheme in 1958 (36).

Epidemiology and Transmission

Humans and other large primates are the only natural reservoirs of *Shigella* bacteria. Most transmission is by person-to-person spread, but infection is also caused by ingestion of contaminated food or water. Shigellosis is most common in situations in which hygiene is compromised (e.g., child care centers and other institutional settings). In developing populations without running water and indoor plumbing, shigellosis can become endemic. Sexual transmission of *Shigella* among men who have sex with men also occurs.

In the United States, an estimated 450,000 cases of shigellosis occur each year, with 70 deaths (69). Up to 20% of all U.S. cases of shigellosis are related to international travel. Most infections in the United States and other developed countries are caused by *S. sonnei; S. flexneri* is the second most common serogroup (44). In the developing world, the most prevalent *Shigella* species are *S. flexneri* and *S. dysenteriae* 1, with the latter being the most frequent cause of epidemic dysentery. Infection with *S. dysenteriae* 1 is associated with high rates of morbidity and mortality in developing countries, particularly when antimicrobial resistance or its misdiagnosis delays appropriate treatment.

Clinical Significance

Members of the genus *Shigella* have been recognized since the late 19th century as causative agents of bacillary dysentery. *Shigella* causes bloody diarrhea (dysentery) and nonbloody diarrhea. Shigellosis often begins with watery diarrhea accompanied by fever and abdominal cramps but may progress to classic dysentery with scant stools containing blood, mucus, and pus. Ulcerations, which are restricted to the large intestine and rectum, typically do not penetrate beyond the lamina propria. Bloodstream infections can occur but are rare. All four subgroups of *Shigella* are capable of causing dysentery, but *S. dysenteriae* serotype 1 has been associated with a particularly severe form of illness thought to be related in part to its production of Shiga toxin. Infection can occasionally be asymptomatic, particularly infection with *S. sonnei* strains. Complications of shigellosis include HUS, which is associated with *S. dysenteriae* 1 infection, and Reiter chronic arthritis syndrome, which is associated with *S. flexneri* infection (2). The identification of *Shigella* species is important for both clinical and epidemiologic purposes.

Isolation Procedures

Enrichment and Plating Media

There is no reliable enrichment medium for all *Shigella* isolates, but gram-negative broth and Selenite broth are frequently used. For the optimal isolation of *Shigella,* two different selective media should be used: a general-purpose plating medium of low selectivity (e.g., MAC) and a more selective agar medium (e.g., xylose lysine desoxycholate agar [XLD]). Desoxycholate citrate agar (DCA) and Hektoen Enteric agar (HE) are suitable alternatives to XLD as media with moderate to high selectivities. Salmonella-Shigella agar (SS) should be used with caution because it inhibits the growth of some strains of *S. dysenteriae* 1.

Screening Procedures

Shigella strains appear as lactose- or xylose-nonfermenting colonies on the isolation media described above. *S. dysenteriae* 1 colonies may be smaller on all of these media, and these strains generally grow best on media with low selectivities (e.g., MAC). *S. dysenteriae* 1 colonies on XLD agar are frequently very tiny, unlike other *Shigella* species. *S. sonnei* colonies often appear flattened and spread out on blood agar plates.

Suspect colonies may be screened biochemically or serologically on Kligler iron agar [KIA] or triple sugar iron agar [TSI]. *Shigella* species characteristically produce an alkaline slant and an acid butt (K/A) but do not produce gas or H_2S. A few strains of *S. flexneri* 6 and a very few strains of *S. boydii* produce gas in KIA or TSI. The motility and the lysine decarboxylase tests are characteristically negative for *Shigella* and can be used to further screen isolates before serologic testing (Table 4). Isolates that react appropriately with the screening biochemicals should then be identified with a complete set of biochemical tests, with automated systems or self-contained commercial kits being satisfactory, and should be tested with grouping antisera. Confirmation requires both biochemical and serologic identification, and laboratories that do not perform both types of tests should send *Shigella* isolates to a reference laboratory for confirmation.

Identification

Biochemical

Because the somatic antigens of most serotypes of *Shigella* are either identical or related to those of *E. coli*, suspicious cultures that are serologically negative should be tested further biochemically (35). *Shigella* and inactive *E. coli* (anaerogenic or lactose nonfermenting) are frequently difficult to distinguish by routine biochemical tests. See Table 4 for the biochemical reactions characteristic of *Shigella* spp. Although *S. dysenteriae* and *S. sonnei* are biochemically distinct, *S. flexneri* and *S. boydii* are often biochemically indistinguishable, so that serologic grouping is essential.

Serotyping

Serologic testing is essential for the identification of *Shigella*. Three of the four subgroups, A (*S. dysenteriae*), B (*S. flexneri*), and C (*S. boydii*), are made up of a number of serotypes. Subgroup A has 15 serotypes; subgroup B has 8 serotypes (with serotypes 1 to 5 subdivided into 11 subserotypes); and subgroup C has 19 serotypes numbered 1 through 20, with *S. boydii* 13 reclassified as *E. albertii*. Subgroup D (*S. sonnei*) is made up of a single serotype. Subgroups A and C are rare. Several provisional *Shigella* serotypes have also been described, which are held sub judice until findings from the characterization of representative isolates show them to be unique. Antisera for the identification of provisional serotypes are typically available only at reference laboratories.

Serologic identification is typically performed by slide agglutination with polyvalent somatic (O) antigen grouping sera, followed, in some cases, by testing with monovalent antisera for specific serotype identification. Monovalent antiserum to *S. dysenteriae* 1 is required to identify this serotype and is not widely available. Because of the potentially serious nature of illness associated with this serotype, isolates that agglutinate in subgroup A reagent should be sent to a reference laboratory immediately for further serotyping.

Biochemically typical *Shigella* isolates that agglutinate poorly or that do not agglutinate at all should be suspended in saline and heated in a water bath at 100°C for 15 to 30 min. After cooling, the antigen suspension is tested in normal saline to determine if it is rough (agglutinates spontaneously). If the heated and cooled suspension is not rough, it may then be retested for agglutination in antisera.

Subtyping

A variety of methods have been used to subtype *Shigella,* including colicin typing (particularly for *S. sonnei*), plasmid profiling, restriction fragment length polymorphism analysis, PFGE, and ribotyping (105). For an overview of the epidemiologic use of typing methods, refer to chapter 12 in this Manual.

Serodiagnostic Tests

Several serodiagnostic assays based on different antigens possessed by *Shigella* have been described (61, 114). These assays are practical only in research settings for seroepidemiology surveys and are not currently used for the diagnosis of infection in individual patients.

Antimicrobial Susceptibilities

Shigella infections are often treated with antimicrobial agents. Because of the widespread antimicrobial resistance among *Shigella* strains, all isolates should undergo susceptibility testing (http://www.cdc.gov/narms/). Reporting of susceptibility results to the clinician is particularly important for *S. dysenteriae* 1 isolates. Infections caused by these strains are often acquired during international travel to areas where most strains are multidrug resistant (110). In many areas of Africa and Asia, *S. dysenteriae* 1 strains are resistant to all locally available antimicrobial agents, including nalidixic acid, but are still susceptible to the fluoroquinolones (93); however, fluoroquinolone-resistant strains have been reported in south Asia.

Interpretation and Reporting of Results

A preliminary report of suspected *Shigella* infection may be issued if biochemical or serologic screening tests are positive. If serotyping results are available, these should also be reported, particularly if the isolate is *S. dysenteriae* 1. All *Shigella* isolates should be tested for antimicrobial susceptibility. Before issuing a final report, isolates should be confirmed by both serologic and biochemical methods. Isolates, particularly those from individuals with dysentery-like illness, that are biochemically identified as *Shigella* but that are serologically negative may be new serotypes of *Shigella* and should be sent to a reference laboratory for further characterization. Isolates from sites other than the gastrointestinal tract and which resemble *Shigella* should be carefully scrutinized for gas production and other differentiating characteristics. These isolates should be sent to a reference laboratory for confirmation because they are more likely to be anaerogenic *E. coli,* certain strains of which may cross-react with *Shigella* antiserum.

SALMONELLA

Description of the Genus

The genus *Salmonella* is composed of motile bacteria that conform to the definition of the family *Enterobacteriaceae* (35). The nomenclature employed to describe the genus *Salmonella* had been problematic for many years, due to the use of multiple schemes in the literature and the historical practice of considering different serotypes of *Salmonella* to be different species. The publication of Judicial Opinion 80 in 2005 (112) will hopefully serve to clarify nomenclatural issues regarding the genus *Salmonella,* and the conventions set forth in that opinion are used here. *Salmonella* history and nomenclature are reviewed at the following website: http://www.bacterio.cict.fr/s/salmonella.html.

The genus *Salmonella* is composed of two species, *Salmonella enterica* and *Salmonella bongori* (formerly subspecies V) (91). *Salmonella enterica* has been subdivided into six subspecies: *S. enterica* subsp. *enterica*, designated subspecies I; *S. enterica* subsp. *salamae,* subspecies II; *S. enterica* subsp. *arizonae,* subspecies IIIa; *S. enterica* subsp. *diarizonae*, subspecies IIIb; *S. enterica* subsp. *houtenae,* subspecies IV; and *S. enterica* subsp. *indica,* subspecies VI. The type species is *S. enterica* subsp. *enterica*. Subspecies IIIa and IIIb represent organisms originally described in the genus "*Arizona*"; subspecies IIIa contains the monophasic strains and subspecies IIIb contains the diphasic strains of "*Arizona*" (92). Despite their common history, subspecies IIIa and IIIb are more closely related to some of the other subspecies of *Salmonella enterica* than they are to each other and thus should be considered separate entities (120).

Subspecies I strains are commonly isolated from humans and warm-blooded animals. Subspecies II, IIIa, IIIb, IV, and VI strains and *S. bongori* are usually isolated from cold-blooded animals and the environment. Non-subspecies I strains are typically considered rare human pathogens; they make up about 1 to 2% of *Salmonella* isolates reported to the U.S. National *Salmonella* surveillance system (22). The biochemical tests useful for identification of *Salmonella* and for subspecies differentiation are given in Table 5.

Salmonella Serotypes

Salmonella serotyping is a subtyping method based on the immunologic characterization of three surface structures: O antigen, which is the outermost portion of the LPS layer that covers the bacterial cell; H antigen, which is the filament portion of the bacterial flagella; and Vi antigen, which is a capsular polysaccharide present in specific serotypes. Serotyping of *Salmonella* is commonly performed to facilitate public health

TABLE 5 Biochemical reactions useful for differentiating *Salmonella* species and subspecies[a]

Test	Species or subspecies (no. of strains tested)						
	S. enterica						*S. bongori* (16)
	I (650)	II (146)	IIIa (120)	IIIb (155)	IV (120)	VI (9)	
Dulcitol	+	+	−	−	−	d[b]	+
Lactose	−	−	−[c]	+[d]	−	d[e]	−
ONPG (*o*-nitrophenyl-β-D-galactopyranoside)	−	−[f]	+	+	−	d[g]	+
Salicin	−	−	−	−	+[h]	−	−
Sorbitol	+	+	+	+	+	−	+
Galacturonate	−	+	−	+	+	+	+
Malonate	−	+	+	+	−	−	−
Mucate	+	+	+	−[i]	−	+	+
Growth in KCN	−	−	−	−	+	−	+
Gelatin (strip)	−	+	+	+	+	+	−
L(+)-Tartrate (*d*-tartrate[j])	+	−	−	−	−	−	−

[a] Reactions after incubation at 37°C. +, 90% or more positive within 1 or 2 days; (+), positive reaction after 3 or more days; −, no reaction (90% or more) in 7 days; d, different reactions [+, (+), −]; KCN, potassium cyanide. Adapted from Ewing (35).
[b] A total of 67% were positive.
[c] A total of 15% were positive.
[d] A total of 85% were positive.
[e] A total of 22% were positive.
[f] A total of 15% were positive.
[g] A total of 44% were positive.
[h] A total of 60% were positive.
[i] A total of 30% were positive.
[j] Sodium potassium tartrate (35).

surveillance for *Salmonella* infection and to aid in the recognition of outbreaks. The serotype of an isolate often correlates with a particular disease syndrome or food vehicle, making serotype data particularly useful in identifying cases and defining outbreaks. For example, *Salmonella* serotype Typhi causes typhoid fever, a more severe disease syndrome than those caused by most other *Salmonella* serotypes. *Salmonella* serotype Enteritidis is associated with infections acquired from chicken or egg products (82). Further, *Salmonella* serotyping is performed worldwide and has aided in the recognition of international outbreaks (64). *Salmonella* serotypes Enteritidis and Typhimurium are the two most common serotypes in the United States, making up approximately 35 to 40% of all culture-confirmed infections (22).

Clinical Significance

Strains of *Salmonella* are categorized as typhoidal and nontyphoidal, corresponding to the disease syndrome with which they are associated. Strains of nontyphoidal *Salmonella* usually cause an intestinal infection (accompanied by diarrhea, fever, and abdominal cramps) that often lasts 1 week or longer (46). Less commonly, nontyphoidal *Salmonella* can cause extraintestinal infections (e.g., bacteremia, urinary tract infection, or osteomyelitis), especially in immunocompromised persons. Persons of all ages are affected; the incidence is highest in infants and young children. *Salmonella* is ubiquitous in animal populations, and human illness is usually linked to foods of animal origin. Salmonellosis also is transmitted by direct contact with animals, by nonanimal foods, by water, and occasionally, by human contact. Each year, an estimated 1.4 million cases of illness and 600 deaths are caused by nontyphoidal salmonellosis in the United States (69).

Typhoid fever, caused by *Salmonella* serotype Typhi, is a serious bloodstream infection common in the developing world. However, it is rare in the United States, where an estimated 800 cases, with fewer than 5 deaths, occur each year; >70% of U.S. cases are related to foreign travel (69). Typhoid fever typically presents with a sustained debilitating high fever and headache. Adults characteristically present without diarrhea. Illness is milder in young children, where it may manifest as nonspecific fever. Humans are the only reservoir for *Salmonella* serotype Typhi, indicating that this serotype is adapted to the human host; healthy carriers have been noted. Typhoid fever typically has a low infectious dose ($<10^3$) and a long, highly variable incubation period (1 to 6 weeks). It is transmitted through person-to-person contact or fecally contaminated food and water. Fatal complications of typhoid most commonly occur in the second or third week of illness.

A syndrome similar to typhoid fever is caused by "paratyphoidal" strains of *Salmonella, Salmonella* serotypes Paratyphi A, Paratyphi B, and Paratyphi C. Serotypes Paratyphi A and Paratyphi C are rare in the United States (22). Serotype Paratyphi B is a diverse serotype that is associated with both paratyphoid fever and gastroenteritis (87). The two pathovars are typically differentiated on the basis of the ability to ferment tartrate; isolates causing paratyphoid fever, the systemic pathovar, are tartrate negative. Isolates associated with gastroenteritis, the enteric pathovar, are typically tartrate positive and are referred to as *Salmonella* Paratyphi B var. L(+)-tartrate + or *Salmonella* Paratyphi B var. Java. The systemic pathovar of *Salmonella* Paratyphi B is considered to be rare in the United States; however, the tartrate reaction is often not reported, making it impossible to distinguish between the two pathovars (22).

Salmonella serotypes Choleraesuis and Dublin are host adapted to cattle and pigs, respectively, causing serious disease in these two animal species. They rarely cause human infection, but such infections are typically severe, with spread to extraintestinal sites (66, 116). *Salmonella* serotype

Dublin has been shown to share virulence traits with *Salmonella* serotype Typhi, which may contribute to its invasiveness in humans (71, 83).

Isolation Procedures

Enrichment

Maximal recovery of *Salmonella* from fecal specimens is obtained by using an enrichment broth, although isolation from acutely ill persons is usually possible by direct plating of specimens. Enrichment broths for *Salmonella* are usually highly selective and inhibit certain serotypes of *Salmonella,* particularly *Salmonella* serotype Typhi. The three selective enrichment media most widely used to isolate *Salmonella* from fecal specimens are tetrathionate broth, tetrathionate broth with brilliant green, and Selenite broth (SEL). SEL may also be used for the recovery of *Salmonella* serotype Typhi and *Shigella,* although its value as enrichment for the latter has not been clearly established. Specimens that might contain organisms inhibited by selective enrichment broths should be plated directly or cultured in a nonselective enrichment broth (e.g., gram-negative broth).

A number of commercial rapid diagnostic tests are available for the testing of foods, but to our knowledge, none has been evaluated in the literature for use with fecal specimens.

Plating Media

Many differential plating media, varying from slightly selective to highly selective, are available for isolation of *Salmonella* from fecal specimens. Media of low selectivity include MAC and eosin methylene blue. Media of intermediate selectivity include XLD, desoxycholate citrate agar, Salmonella-Shigella agar, and HE. Highly selective media include bismuth sulfite agar, the preferred medium for the isolation of *Salmonella* serotype Typhi, and brilliant green agar. Bismuth sulfite agar, XLD, and HE all have H_2S indicator systems, which are helpful for the detection of lactose-positive *Salmonella*. Most laboratories today use HE or XLD because these media may also be used for the isolation of *Shigella*.

In the developing world, typhoid fever is frequently diagnosed solely on clinical grounds, but isolation of the causative organism is necessary for a definitive diagnosis. *Salmonella* serotype Typhi is more frequently isolated from blood cultures than from fecal specimens. Blood cultures are positive for 80% of typhoid patients during the first week of fever but show decreasing positive results thereafter.

Screening Procedures

A latex agglutination kit has been described for screening for *Salmonella* from SEL enrichment broth (Wellcolex Color *Salmonella;* Remel Inc., Lenexa, Kans.) (15). This kit can also be used to screen individual colonies from primary plates. In using this kit, it should be kept in mind that it identifies only those *Salmonella* isolates belonging to the more common O serogroups and it does not differentiate between O groups C_1 and C_2.

Suspect colonies may be inoculated onto a screening medium such as KIA or TSI. On KIA or TSI, most *Salmonella* strains produce a K/AG + reaction, indicating that glucose is fermented with gas, and H_2S is produced. On these media, *Salmonella* serotype Typhi isolates are characteristically K/A but do not produce gas and only a small amount of H_2S is visible at the site of the stab and in the stab line. Lysine iron agar is also a useful screening medium because most *Salmonella* isolates, even those that ferment lactose, decarboxylate lysine and produce H_2S. Alternately, isolates may be identified by a battery of biochemical tests or by slide agglutination with antisera for *Salmonella* O groups. Isolates suspected of being *Salmonella* serotype Typhi should be tested serologically with *Salmonella* Vi and O group D antisera (see below).

If the biochemical reactions for a particular isolate are not characteristic but *Salmonella* antigens are found, the cultures should be plated on MAC or eosin methylene blue to obtain a pure culture, tested with a complete set of biochemical tests, or forwarded to a reference laboratory.

Identification

Clinical laboratories may issue a preliminary report of *Salmonella* when an isolate is positive either with *Salmonella* O group antisera or by biochemical identification methods. An isolate is confirmed as *Salmonella* when the specific O serogroup has been determined and biochemical identification has been completed.

Biochemical Identification

Suspect colonies from one of the differential plating media mentioned above can be identified biochemically as *Salmonella* spp. with traditional media in tubes or commercial biochemical systems. Methods of biochemical identification and specific commercial manual and automated identification systems are covered in chapter 15. The species and subspecies of *Salmonella* can be identified biochemically, as indicated in Table 5.

Serotyping

O serogroup determination is adequate for confirmation of isolates as *Salmonella.* Full serotype determination is useful for public health surveillance but is beyond the scope of most routine clinical laboratories. The methods described below for serotyping are intended primarily for reference laboratories. *Salmonella* isolates are serotyped based on the antigenic properties of their O (somatic) antigens, H (flagellar) antigens, and Vi (capsular) antigen (17). O antigen is a carbohydrate antigen and is the outermost component of LPS. It is a polymer of O subunits; each O subunit is typically composed of four to six sugars depending on the O antigen. O antigens are designated by numbers and are divided into O serogroups based on antigenic factors associated with the O subunit. Many of the common O groups were originally designated by letter and are still commonly referred to by letter (e.g., serotype Typhimurium belongs to group O:4 or group B, serotype Enteritidis belongs to group O:9 or group D1). Additional O antigenic factors have been identified for specific O groups. They are typically associated with a side sugar that is added to the basic O subunit structure, and they are often variably present or variably expressed within O groups or within serotypes.

H antigen is a protein antigen called flagellin; multiple flagellin subunits make up the flagellar filament. The ends of flagellin are conserved and give the flagellum its characteristic filament structure; the antigenically variable portion of flagellin is the middle region, which is surface exposed. Salmonellae are unique among the enteric bacteria in that they commonly express two different flagellin antigens, although specific serotypes such as Typhi and Enteritidis possess only one flagellar antigen. The two flagellar antigens are referred to as phase 1 and phase 2; monophasic and diphasic strains express one or two flagellar antigens, respectively. Individual flagellar antigens can be composed of multiple antigenic factors. For example, the phase 2 flagellar antigen of serotype Typhimurium is antigen 1,2, which is composed of two antigenic factors, 1 and 2.

Many of the O and H antigenic types are found in multiple subspecies, and isolates from different subspecies can have the same antigenic profile. Thus, subspecies determination is an integral component of serotype determination for *Salmonella*. The serotype for all *Salmonella* strains can be designated by an antigenic formula; additionally, serotypes belonging to subspecies I are given a name which is typically related to the geographical place where the serotype was first isolated (84). The antigenic formulae of *Salmonella* serotypes are listed in the Kauffmann-White scheme and are expressed as follows: O antigen(s), Vi (when present): phase 1 H antigen(s): phase 2 H antigen(s) (when present). For example, the antigenic formula for *Salmonella* serotype Typhimurium is 4,5,12:i:1,2. Serotype names for subspecies I serotypes are written in roman (not italicized) letters, and the first letter is a capital letter (for example, *Salmonella* serotype [ser.] Typhimurium or *Salmonella* Typhimurium [19]). Serotypes belonging to other subspecies are designated by their antigenic formulae following the subspecies name (for example, *S. enterica* subsp. *salamae* ser. 50:z:e,n,x or *Salmonella* serotype II 50:z:e,n,x).

The WHO Collaborating Centre for Reference and Research on *Salmonella,* which is located at the Pasteur Institute in Paris, France, maintains the Kauffmann-White Scheme for the designation of *Salmonella* serotypes (84). The Kauffmann-White scheme is updated annually with a listing of new serotypes (85). Currently, there are 2,541 recognized *Salmonella* serotypes; the majority belong to subspecies I (1,504 serotypes [85]). Most common serotypes belong to O groups A, B, C_1, C_2, D_1, and E_1 (also known as groups O:2, O:4, O:7; O:8; O:9, and O:3,10, respectively). Serotypes belonging to subspecies II (502 serotypes), IIIa (95 serotypes), IIIb (333 serotypes), IV (72 serotypes), VI (13 serotypes), and *S. bongori* (22 serotypes) are primarily found in O groups O:11 (F) through O:67 (commonly referred to as the higher O groups).

Determination of O Antigens

O (heat-stable, somatic) antigens are identified by first testing the isolate in antisera that detects one or multiple antigenic factors corresponding to the O groups (O grouping antisera). Once the O group is determined, antisera that recognize single antigenic factors are used to confirm the O group and identify any additional antigenic factors that are associated with that O group (O single-factor antisera) (18). The approach most commonly used for determining O antigens is to initially test the isolates by slide agglutination in antisera against O groups A to E_1 because approximately 95% of *Salmonella* isolates belong to one of these O groups. If no agglutination occurs in antisera for these O groups, the isolate is tested in pools containing the remaining *Salmonella* O antisera, O:11 through O:67.

Determination of H Antigens

H (flagellar) antigens are typically determined by tube agglutination tests using broth cultures. Isolates are initially tested with H typing antisera, which recognize individual or multiple antigenic factors, and then with H single-factor antisera, which recognize individual antigenic factors. Typically, the flagellar antigens in a diphasic strain are coordinately regulated so that only one is expressed at time in a single bacterial cell; however, both phases may be detected in the whole culture, particular with a fresh clinical isolate. When only one phase is detected (either phase 1 or phase 2), the strain should be inoculated into a semisolid medium to which sterile antiserum to the detected flagellar antigen has aseptically been added. Growth of the strain in this semisolid agar immobilizes cells expressing the detected antigen and allows the growth of bacteria expressing the antigen in the other phase. After phase reversal, the strain is tested in appropriate H typing and single-factor antisera to complete the serotyping. A strain must be actively motile to ensure the good expression of H antigens; sometimes it must be passed through one or more tall tubes of semisolid agar to enhance motility before H antigens can be detected.

Detection of the Vi Antigen and Identification of *Salmonella* Serotype Typhi (9,12,[Vi]:d:-)

The Vi antigen, a heat-labile capsular polysaccharide, is useful for the identification of *Salmonella* serotype Typhi. It is also occasionally detected in *Salmonella* serotype Dublin, *Salmonella* serotype Paratyphi C, and some *Citrobacter* strains, so its detection does not constitute definitive evidence of *Salmonella* serotype Typhi. Vi antigen is identified by slide agglutination with specific antiserum.

If *Salmonella* serotype Typhi is suspected, the culture is first tested live (unheated) in O group D antiserum (which contains antibodies to O antigens 9 and 12) and Vi antiserum on a slide. The Vi capsular polysaccharide can mask the O antigens, blocking their reactivity with the O grouping antiserum. If only the Vi antiserum is positive, the bacterial suspension is heated in boiling water for 15 min to remove the capsule, cooled, and tested again in the same antisera. After heating, *Salmonella* serotype Typhi isolates will be negative in the Vi antiserum but positive in the O group D antiserum. Expression of the Vi antigen by *Salmonella* serotype Typhi is variable but tends to occur more frequently in freshly isolated cultures than in cultures that have been subcultured. If the strain is typical for *Salmonella* serotype Typhi on TSI or KIA (see "Screening Procedures" above), is urease negative, and reacts in O group D or Vi antiserum, a presumptive report is made. The identity of the isolate is confirmed by biochemical testing (Table 5) and determination of the H (flagellar) antigen (see below) before a final report is issued. *Salmonella* serotype Typhi strains typically express only one flagellar antigen, Hd.

Identification Problems

Several potential problems may prevent accurate serotype determination. The strain may express the Vi capsular antigen, which can block the binding of antibodies against the O antigens. The strain may be rough, i.e., fails to make complete O antigens. Rough strains have a tendency to weakly agglutinate in multiple O grouping antisera. The strain may be mucoid and not agglutinate in any O antisera, or isolates can be nonmotile and not express any flagellar antigens. Among isolates submitted to the National *Salmonella* Reference Laboratory at the CDC, isolates from urine are frequently rough, mucoid, and/or nonmotile. When O antigen and/or H antigens are not detected, a strain is confirmed as a *Salmonella* species by characterization of any antigens that are expressed and by biochemical testing (Table 5).

Many laboratories are likely to overlook *Salmonella* serotype Paratyphi A because they do not screen with O group A antiserum or because it is H_2S negative, lysine negative, and citrate negative. *Salmonella* serotypes Paratyphi B and Paratyphi B var. L(+)-tartrate + (var. Java) can be confused because they have an identical antigenic formula (4,5,12:b:1,2), but they are distinguished biochemically by their tartrate reaction. Similarly, *Salmonella* serotype Choleraesuis and *Salmonella* serotype Paratyphi C have the same antigenic formula (6,7:c:1,5) but are differentiated biochemically. *Salmonella* serotype Paratyphi C may express the

Vi antigen. *Citrobacter* and *E. coli* strains may possess O, H, or Vi antigens that are related to those of *Salmonella;* biochemical identification may be necessary to confirm that an isolate is *Salmonella* (see Table 5 in this chapter and Table 1 in chapter 42).

Subtyping

For rarer serotypes, serotype identification may be all that is necessary to identify clusters of temporally related isolates. However, additional subtyping methods are typically required for more common serotypes (e.g., Typhimurium, Enteritidis, and Newport). A variety of phenotypic (e.g., phage typing, antimicrobial susceptibility pattern determination, and biotyping) and genotyping methods (e.g., plasmid fingerprinting, PFGE, IS*200* profiling, and random amplified polymorphic DNA analysis) have been developed for subtyping within serotypes of *Salmonella* (74, 108). PFGE is the current method of choice for the subtyping of most *Salmonella* serotypes since it is universally applicable and provides good strain discrimination for most serotypes. PulseNet, an international subtyping network that tracks *Salmonella,* is based on PFGE (108). *Salmonella* serotype Enteritidis has limited diversity in PFGE analysis; as a result, phage typing is still commonly used to characterize strains, particularly in an outbreak setting (39, 82).

Serodiagnostic Tests

The Widal test, which measures agglutinating antibodies to the O and H antigens of *Salmonella* serotype Typhi, produces false-negative and false-positive reactions and does not provide a definitive diagnosis of individual cases of infection. Two other rapid serodiagnostic tests have proved more useful than the Widal test for the serodiagnosis of typhoid fever (78) (Tubex; IDL Biotech, Sollentuna, Sweden; and TyphiDot; Malaysian Biodiagnostic Research SDN BHD, Kuala Lumpur, Malaysia).

Antimicrobial Susceptibilities

Antimicrobial therapy is not recommended for uncomplicated *Salmonella* gastroenteritis, and routine susceptibility testing of fecal isolates is not warranted for treatment purposes. However, determination of antimicrobial resistance patterns is often valuable for surveillance purposes and may be performed periodically to monitor the development and spread of antimicrobial resistance among *Salmonella* isolates.

In contrast to uncomplicated salmonellosis, treatment with the appropriate antimicrobial agent can be crucial for patients with invasive *Salmonella* and typhoidal infections, and the susceptibilities of these isolates should be reported as soon as possible (57). Testing methods are detailed in chapter 73. The untreated case mortality rate for typhoid fever is >10%; when patients with typhoid fever are treated with appropriate antibiotics, the rate should be <1%. However, increasing levels of resistance to one or more antimicrobial agents in *Salmonella* isolates, particularly in *Salmonella* serotype Typhi, make selection of an appropriate antibiotic problematic. In particular, reduced susceptibility to ciprofloxacin among *Salmonella* serotype Typhi isolates and increasing numbers of treatment failures are of concern (52, 89).

Antimicrobial resistance, particularly multiple drug resistance, has been noted in several nontyphoidal serotypes of *Salmonella*. A strain of *Salmonella* serotype Typhimurium phage type DT104 resistant to five antimicrobials (ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline, or ACSSuT) emerged in the late 1990s and is now recognized worldwide. In 2002, 21% of *Salmonella* serotype Typhimurium isolates in the United States had the ACSSuT resistance profile (21). The ACSSuT resistance determinant has been found in *Salmonella* serotype Agona strains (27). The genomic element that carries this ACSSuT determinant has been found to harbor these and other resistance determinants in a variety of serotypes, indicating that the element may spread horizontally to other serotypes and acquire additional resistance determinants (60).

The emergence of a clone of *Salmonella* serotype Newport resistant to at least nine antimicrobials, including expanded-spectrum cephalosporins, was first noted in 2000 in the northeastern United States (43) and has now been found in many regions of the United States (8). In 2002, this strain made up 22% of all serotype Newport strains in the United States. Similarly resistant strains of *Salmonella* serotype Newport were recently reported in Japan, documenting the potential for worldwide spread of multiply resistant strains (50). Additional information regarding these and other antimicrobial resistant strains can be found at the CDC's NARMS website (http://www.cdc.gov/narms/).

Interpretation and Reporting of Results

A preliminary report can be issued as soon as a presumptive identification of *Salmonella* is obtained. In most situations, a presumptive identification would be based on biochemical findings obtained either by traditional or commercial systems or by a serologic reaction in *Salmonella* O grouping antisera. A confirmed identification requires both biochemical and serologic identification methods. Because the National *Salmonella* Surveillance System depends on the receipt of serotype information for *Salmonella* strains isolated in the United States for the tracking of outbreaks of infection, laboratories should follow the procedures recommended by their state health departments for submitting isolates for further characterization, including complete serotyping. The antimicrobial susceptibility of typhoidal *Salmonella* strains and strains from normally sterile sites should be determined, and the strains should be forwarded to a reference or public health laboratory for complete biochemical and serologic characterization.

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*Yersinia**

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44

TAXONOMY AND HISTORY OF THE GENUS

Yersinia pseudotuberculosis and *Y. pestis* were included in the genus *Pasteurella* until 1944, when van Loghem suggested that a new genus be formed due to the significant phenotypic and genotypic differences among the organisms. It was not until 1964 that *Y. enterocolitica* was renamed by Frederiksen from the previous name of *Bacterium enterocolitica* assigned in 1939 following a report of several cases of gastrointestinal disease. This diverse group of organisms was further divided into a subgroup initially designated *Y. enterocolitica*-like organisms and later into an additional four species *(Y. intermedia*, *Y. frederiksenii*, *Y. kristensenii,* and *Y. aldovae)* based on sugar fermentation and DNA relatedness. Subsequent species designations included *Y. ruckeri*, *Y. rohdei*, *Y. mollaretii*, and *Y. bercovieri.* The newest member of the genus is *Y. aleksiciae,* named after the German scientist Stojanca Aleksic (54) and previously included in the species *Y. kristensenii*. Although phenotypically identical, the two species were differentiated by multilocus enzyme electrophoresis (MLEE) typing and 16S rRNA analysis. A further breakdown of *Y. enterocolitica* into subspecies *(enterocolitica* and *palearctica)* differentiable based only on 16S rRNA gene sequencing has been proposed (37).

Although most species of the genus have been isolated from humans, with the exception of *Y. aldovae*, the only species that are considered human pathogens are *Y. pestis*, *Y. pseudotuberculosis,* and *Y. enterocolitica.* These species, in addition to *Y. ruckeri* serogroup 01, which is the cause of enteric red mouth disease in rainbow trout, are the only members of the genus that are pathogenic for animals. All of the other species are considered to be nonpathogenic, environmental isolates.

As a group, members of the genus *Yersinia* have a G+C content of 46 to 50% and are related to the rest of the members of the family *Enterobacteriaceae* by 10 to 32%. Intraspecies relatedness is very variable, ranging from 55 to 74% with the exception of that of *Y. pestis* and *Y. pseudotuberculosis,* which demonstrate more than 90% relatedness. Based on multilocus sequence typing (MLST) of the housekeeping genes, the two species are very closely related. *Y. pestis* is believed to have evolved from *Y. pseudotuberculosis* prior to the first plague pandemic (1, 12, 64). Detailed analysis of genetic differences between the species was later described by Hinchliffe et al. using DNA microarrays (22). The entire genome of *Y. pestis* has recently been sequenced, and work is in progress to sequence the genome of *Y. pseudotuberculosis*. Data collected from these studies will likely provide definitive information regarding the ancestry of these organisms (42). However, despite the high degree of relatedness between the two species, they have not been combined due to the significant epidemiologic and clinical differences that exist between them.

DESCRIPTION OF THE AGENTS

As are all members of the family *Enterobacteriaceae*, *Yersinia* is a gram-negative, non-spore-forming bacillus that exhibits bipolar staining particularly when seen in primary specimens stained with Giemsa or Wayson's dye, although Wayson's dye is not readily available in most routine clinical laboratories. The bacilli are smaller than other members of their family (0.5 to 0.8 μm in diameter and 1 to 3 μm in length) and tend to grow more slowly as well. With the exception of *Y. pestis*, which is nonmotile, all of the members of the genus are motile at room temperature and nonmotile at 37°C due to the presence of peritrichous or paripolar flagella.

Yersinia species are facultative anaerobes that grow at a wide range of temperatures (4 to 43°C), but optimal growth conditions are 25 to 28°C. *Yersinia* ferments glucose with the production of acid and no gas. Most strains will grow on MacConkey agar as well as various selective media; however, most exhibit poor growth in liquid media and do not form a turbid suspension (62). *Yersinia* is catalase positive and oxidase negative.

The cell walls and antigenic structures of *Yersinia* species are also very similar to those of other members of the *Enterobacteriaceae* family, with an O-specific side chain and only minor variations in the lipopolysaccharides of various serogroups. *Y. enterocolitica* has more than 70 serotypes, *Y. pseudotuberculosis* has 15 (7), and *Y. pestis* lacks the O antigen.

Although all three pathogenic *Yersinia* species, *Y. pestis*, *Y. enterocolitica,* and *Y. pseudotuberculosis*, are associated with different clinical entities, they all have in common the possession of a 70- to 75-kb virulence plasmid that contains the major virulence factors, including the *Yersinia* outer membrane proteins (Yops) and the processing and regulatory proteins for the Yops: Ysc (*Yersinia* secretion) and Lcr (low-calcium response) (6).

The gene for enterotoxin production in *Y. enterocolitica* and *Y. pseudotuberculosis* as well as those for other invasion factors such as invasin is located on the chromosome. *Y. pestis* has two

*This chapter contains information presented in chapter 43 by Jochen Bockemühl and Jane D. Wong in the eigth edition of this Manual.

plasmids that carry the genes for plasminogen activator (Pla), the bacteriocin pesticin (Pst), murine toxin (Ymr), and fraction 1 protein capsule (F1). *Y. pestis*, *Y. pseudotuberculosis,* and some strains of *Y. enterocolitica* also have a chromosomally located high-pathogenicity island that carries the genes for yersiniabactin, a siderophore that provides the organisms with iron. Additionally, *Y. pestis* contains the gene *hsm*, responsible for a hemin storage system. A comprehensive review of the virulence mechanisms and pathogenesis of *Yersinia* species has been recently published (61).

EPIDEMIOLOGY AND TRANSMISSION

The natural reservoir for *Y. pestis* is rodents, which have an inapparent infection and are not a common source of infection for humans. Although many different species of fleas infect more than 100 rodent species that can be specific to geographic locations, transmission of plague is classically associated with the rat flea *(Xenopsylla cheopis)* (18). Approximately 3,000 human cases of plague are identified every year in the world, and about 12 to 15 are identified in the United States (35), where 90% of cases occur in New Mexico, Arizona, Colorado, and California. Transmission to humans is usually through the bite of an infected rodent flea and regurgitation by the flea during a blood meal. *Yersinia* can survive in the stomach and proventriculus of the flea and actually multiply there and block the proventriculus, causing the flea to bite its host mammal repeatedly, thereby increasing chances for disease transmission (43). Interestingly, when the environmental temperature is above 80°F, *Y. pestis* does not produce coagulase, so blockage of the proventriculus is unlikely to occur in the flea, which makes transmission to humans less likely to occur (51). The most common reservoir in the United States for plague transmission is the squirrel. A secondary mode of transmission of disease to humans is through contact with infected cats, either by scratches, bites, or inhalation of aerosolized organisms. Cats become infected by ingestion of contaminated rodents. Human-to-human transmission is very rare and occurs only with the pneumonic form by inhalation of aerosolized particles, which might occur in a bioterrorism attack (see chapter 9). Death can occur within 2 to 4 days. Pneumonic plague is a very rare sequela in patients appropriately treated with antibiotics (<5%) (29). Rare cases of person-to-person transmission from patients with pneumonic plague have been documented. Several of the virulence factors of *Y. pestis* aid in dissemination, including plasminogen activator (Pla), the Yops, and pH 6 antigen. These virulence factors are involved in cell lysis, suppression of the immune system, and survival of the organisms within macrophages. Once *Y. pestis* enters the human host, other virulence factors such as F1 capsular antigen and the yersiniabactin iron scavenging system aid the organism in survival within phagocytes. Endotoxin produced by the organism is associated with the subsequent septic shock and systemic response. *Y. pestis* has been known to survive for days to weeks outside of the vector or mammalian host (in flea feces, dead rodents, or soil), which allows for further transmission.

Plague Pandemics

There have been three plague pandemics, each in different parts of the world and all believed to have been due to one of three biovars of *Y. pestis* differentiated by their abilities to ferment glycerol and to reduce nitrate (see "Identification"). The earliest pandemic, called the Justinian plague, in the fifth through seventh centuries in Africa was associated with biovar Antiqua. Biovar Medievalis, which was identified in Central Asia, was associated with the pandemic known as the Black Death during the 13th to 15th centuries. Modern plague, which originally was identified in southern China and is now worldwide, is associated with biovar Orientalis (29). Using microarray analysis, Zhou et al. (63) proposed a new, fourth biovar, Microtus, which includes strains previously part of the biovar Medievalis but separated based on biochemical, molecular, and pathogenicity differences. Biovar Microtus is pathogenic to the rodent genus *Microtus* (voles), mice, and other small rodents but not to large mammals, including humans (65).

There are more than 70 serotypes of *Y. enterocolitica,* and only a few are associated with disease in animals or humans, five of which are associated with human disease. Serotypes seem to be location specific. *Y. enterocolitica* organisms are widely distributed in nature and are found in the gastrointestinal tracts of many animal species, most commonly swine, rodents, and dogs. Due to their enhanced growth in cold temperatures, geographic distribution is mostly in the northern portions of the United States and in colder portions of Europe. Food products, particularly raw and poorly cooked meats, are frequently found to contain these organisms, although the majority of them are of nonpathogenic serotypes.

Y. pseudotuberculosis is also found in the environment (soil and water) and in a diverse group of wild and domesticated animal species. The main reservoirs for the organism are rodents, rabbits, and wild birds. The mode of transmission of *Y. pseudotuberculosis* is unknown, although due to its similarity to *Y. enterocolitica* (both are found in the environment), it has been speculated that transmission is via contaminated food or water.

CLINICAL SIGNIFICANCE

There are three forms of plague: pneumonic, septicemic, and bubonic. Bubonic plague is the most common clinical presentation. Following the bite of an infected flea, *Y. pestis* migrates through the blood and proliferates in the regional lymph nodes. Following a 2- to 8-day incubation period, the patient develops a fever and a painful swelling (bubo) in the area of the affected node.

Pneumonic plague can be a rare complication of bubonic plague or can be the primary infection following inhalation of aerosolized organisms either from contact with infected animals or from a bioterrorism event. After an incubation period of 1 to 6 days, symptoms include high fever and cough with hemoptysis and chest pain (24). An even higher mortality rate is associated with this form of plague than with bubonic plague.

Septicemic plague can occur when the organisms inoculated by the infected flea spread to the bloodstream without localizing in regional lymph nodes. This form of the disease is more common in children and is rapidly fatal. Septicemic plague can also occur following bubonic plague that is not adequately treated.

The most common form of disease due to *Y. enterocolitica* is gastroenteritis associated with consumption of contaminated food or water. It is not unusual to isolate *Y. enterocolitica* from raw meats including beef, lamb, pork, and chicken. The organism has also been found as a contaminant of cooked, prepackaged deli meat. The majority of strains isolated from human food sources are of the nonpathogenic serotypes. Carriage of the pathogenic serotypes of *Y. enterocolitica* is more common in swine; therefore, consumption of raw or poorly cooked pork, such as chitterlings, is the main risk factor for gastroenteritis (11, 26). Severity of disease is related to the serotype and can range from self-limited gastroenteritis to terminal ileitis and

mesenteric lymphadenitis, often misdiagnosed as appendicitis. Young children most commonly develop gastroenteritis and present with fever, diarrhea, and abdominal pain following consumption of food contaminated by *Yersinia.* Although symptoms typically resolve within approximately 7 days, patients can carry the organism in their gastrointestinal tracts for as long as several months. Organisms can migrate out of the gut via the lymphatics into local lymph nodes. An uncommon complication of gastroenteritis is septicemia, which is associated with the patient's HLA type. Persons at high risk for septicemia include the elderly and immunocompromised patients, particularly those with underlying metabolic diseases that are associated with iron overload (hemochromatosis), cancer, liver disease, and steroid therapy.

The production of urease allows the organism to survive in the stomach and colonize the small intestine of the human host. Pathogenic strains contain YoPs which allow them to resist the normal phagocytic and complement killing process that takes place in the Peyer's patches (58).

Y. enterocolitica is the most common cause of transfusion-related infections due to contaminated red blood cells. Since the organism is able to survive and multiply at refrigeration temperatures, donated blood contaminated with small numbers of organisms from an asymptomatic person can transmit infection to the transfused patient (31).

Reactive arthritis is an uncommon sequela of diarrhea due to *Y. enterocolitica*. Patients at increased risk include those who are carriers of the HLA-B27 allele and those with other immunologic disorders. Reactive arthritis is differentiated from rheumatoid arthritis by the asymmetrical involvement of multiple joints, most commonly the sacroiliac and the spine. Symptoms appear several days to months after the onset of diarrhea and may persist for months. Other, less common diseases associated with *Y. enterocolitica* infection include inflammatory bowel disease, most commonly associated with serotype O:3 (48), and autoimmune thyroid disorders such as Graves' disease and Hashimoto's thyroiditis (14). Both *Y. enterocolitica* and *Y. pseudotuberculosis* have been isolated from patients with Crohn's disease, although a causal relationship has not been proven (23).

Y. pseudotuberculosis usually produces a self-limiting disease, particularly in children and young adults. Rarely, *Y. pseudotuberculosis* can cause mesenteric lymphadenitis that clinically mimics appendicitis and septicemia and that occurs usually in immunocompromised patients (diabetics and those with liver cirrhosis or iron overload) (15). Long-term sequlae of *Y. pseudotuberculosis* infection include erythema nodosum, Reiter's syndrome, and nephritis. *Y. pseudotuberculosis* has also recently been implicated as a cause of foodborne illness following an outbreak of gastroenteritis-pseudoappendicitis associated with consumption of contaminated lettuce (40).

The other *Yersinia* species are not known to be human pathogens. Some of the other *Yersinia* species have also been shown to produce an enterotoxin and therefore may be associated with enteric disease in some patients (58). Elderly patients, more commonly women and those being treated with acid blockers, are more likely to develop gastroenteritis due to *Yersinia* species other than *Y. enterocolitica* and to have a prolonged course of disease related to these organisms (33).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Y. pestis is on the list of agents of bioterrorism (see chapter 9), and therefore all routine procedures performed with this organism should be done in a facility with a biosafety level of at least 2. Processes which are high risk for creating an aerosol should be performed under biosafety level 3 conditions. Routine clinical laboratories or sentinel laboratories should notify their local public health departments in the case of a presumptive diagnosis of plague. Clinical laboratories should be aware of the protocols as outlined by the American Society for Microbiology (http://www.asm.org/Policy/index.asp?bid=520).

Diagnosis of plague can be made by detection of *Y. pestis* in a bubo aspirate or by growth of *Y. pestis* from the blood. Patients with bubonic plague may shed organisms into the blood intermittently, so obtaining multiple sets of blood cultures over a 24-h period increases the sensitivity of detection. Blood cultures should be incubated at both 28 and 35°C to increase chances of recovery of the organism (3, 24). Other sources appropriate for culture include respiratory samples such as sputum, throat swabs, and throat washing specimens; however, due to contamination of these specimens with normal flora, a bronchial alveolar washing or lavage specimen would be preferable (3). Tissue samples should be collected in a sterile container with a small amount of sterile nonbactericidal saline to avoid drying of tiny pieces. Swab specimens are not recommended due to the poor recovery of the organisms. Blood or tissue specimens can also be collected from animals suspected to have died from *Y. pestis* infection for culture or direct fluorescent-antibody testing for the presence of the F1 antigen. Sera can also be collected from dead infected animals to test for antibody to *Y. pestis*. Material from fleas can be inoculated into mice for culture or direct fluorescent-antibody testing. If clinical suspicions are high and cultures of animal material fail to yield *Y. pestis,* PCR can be attempted. Specimens should be sent to the laboratory immediately, and if a delay in transit of more than 2 h is expected, the sample should be transported at 2 to 8°C, with the exception of blood, which should be transported under ambient conditions.

The appropriate specimen for culture of *Y. enterocolitica* and *Y. pseudotuberculosis* as well as other *Yersinia* species is stool, blood, or lymph nodes, depending on the disease form suspected. If food is suspected as the source of an outbreak, the local health department should be involved in processing of such specimens (see chapter 12). Maintain food at 4°C, and transport it as soon as possible. Swabs should be transported to the laboratory at 4°C in Cary-Blair, Amies, or Stuart's medium. Stool specimens can also be placed in transport media and should be maintained at 4°C if transport is expected to take longer than 2 to 4 h.

MICROSCOPY

Yersinia species are small (1 to 2 μm by 0.5 μm) gram-negative bacilli that appear either as single cells or as pairs or short chains, particularly when stains are prepared from liquid medium. Direct microscopy of a tissue specimen by using either Wright, Giemsa, Wayson's, or methylene blue stain may be helpful in presumptive identification of *Y. pestis* since the organisms appear to be safety pin shaped due to bipolar staining (Fig. 1). This morphology is not always evident following Gram staining or staining of colonies from culture media. Other, more specific direct staining methods include the use of fluorescently labeled antibody to the capsular F1 antigen, which is confirmatory for *Y. pestis*. However, rare strains of *Y. pestis* may lack the F1 antigen, and therefore the LcrV antigen, a key virulence factor that is secreted by the organism, has been used to develop a more sensitive diagnostic tool.

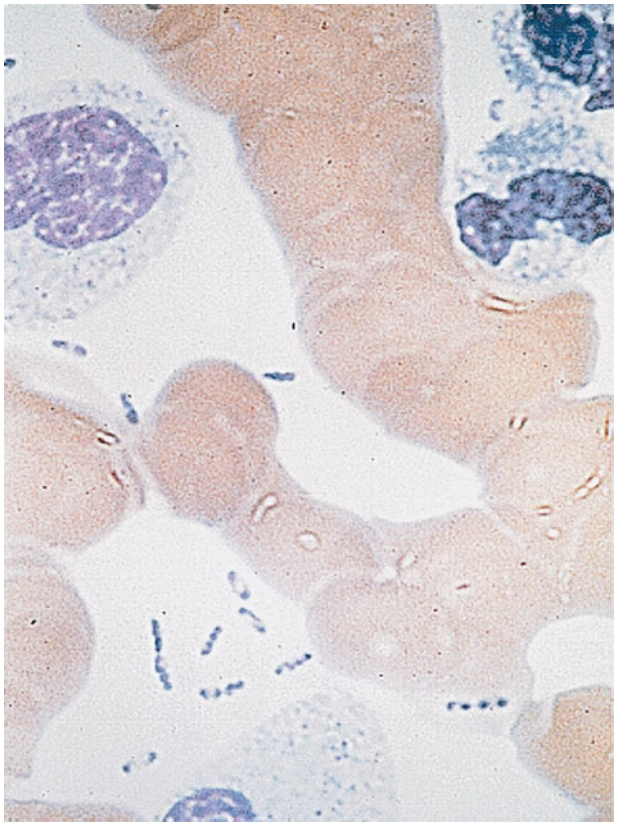


FIGURE 1 Giemsa stain of a blood smear from a patient with *Y. pestis* infection. Note the bipolar-staining "closed safety pin"-shaped cells. (Courtesy of the Centers for Disease Control and Prevention.)

DIRECT DETECTION

An antigen capture enzyme-linked immunosorbent assay (ELISA) using monoclonal antibody to the LcrV antigen detects as little as 0.5 ng of purified *Y. pestis* LcrV in blood or sputum (20). The only disadvantage is that the assay cannot distinguish *Y. pestis* from the other *Yersinia* species, although it would be less likely to find other species in respiratory specimens or blood cultures. A capture ELISA for detection of *Y. pestis* in bubo aspirates can also be applied to serum. This assay was tested on patients in Madagascar and was found to have a high sensitivity (100% with bubos and 90% with sera; limit of detection, 4 ng/ml) and specificity (approximately 99%) (52). Chanteau et al. modified the ELISA to use two different monoclonal antibodies, both directed at the F1 capsular antigen and designed in a dipstick format, to be used in the field for rapid diagnosis of plague in humans or animals (13). The only disadvantage of this assay is the inability to detect strains that lack the F1 capsule; however, this is a rare occurrence (53). Unfortunately, commercial sources for this antigen are not currently available.

Tissue biopsy specimens fixed in formalin can also be stained with an immunohistochemical stain that is based on a monoclonal antibody to the F1 capsular antigen of *Y. pestis*. This is a rapid method for diagnosis of plague that does not rely on having fresh tissue or live organisms (21).

Assays for the direct detection of nucleic acid in patient specimens have been developed to increase the sensitivity of detection of *Y. pestis*, especially in cases of suspected bioterrorism. Initial assays designed to detect the 16S rRNA gene were not found to be useful due to cross-reactivity with *Y. pseudotuberculosis* (45). The most sensitive assay at this time uses primers directed at the plasminogen activator gene (*pla*) that is located in a high copy number on a *Y. pestis*-specific plasmid. Development of a real-time PCR assay to detect the *pla* gene in sputum samples spiked with *Y. pestis* decreased the time to detection to 2 h and increased the sensitivity to 1.5 bacteria per ml (34). This assay also has the advantage of being useful for real-time diagnosis in the field in regions of the world where plague is endemic and culture methods are impractical (45). Following the sequencing of the entire genome of *Y. pestis* (42), so-called signature genes could be identified on the chromosome of *Y. pestis* that allow for its distinction from *Y. pseudotuberculosis* (63).

PCR has also been used as a sensitive method to detect small numbers of *Y. enterocolitica* in foodstuff (59), as well as in stored red blood cells to prevent transfusion reactions (49). Use of a multiplex PCR assay containing primer sets directed at four different virulence genes to test food samples allows for the distinction between pathogenic and nonpathogenic serotypes and specifically identifies the presence of *Y. enterocolitica* serotype O:3 (59).

ISOLATION PROCEDURES

Yersinia species grow on most routine media including blood, chocolate, and MacConkey agars incubated at 35°C in ambient air. Eosin-methylene blue, xylose-lysine-deoxycholate agar, and Hektoen enteric agars do not provide any advantage in the isolation of *Y. enterocolitica* and the differentiation of *Yersinia* species from other normal stool flora. Due to their ability to ferment sucrose and the fact that *Yersinia* species grow more slowly than most *Enterobacteriaceae*, a selective medium is recommended for specifically culturing for *Yersinia* from nonsterile sites. There are various selective media for the recovery of *Y. enterocolitica*, including cefsulodin-Irgasan-novobiocin (CIN) agar, which inhibits the growth of many other organisms from the family *Enterobacteriaceae*. Another selective medium is salmonella-shigella deoxycholate calcium chloride agar (17). CIN agar has been found to provide better recovery rates for *Yersinia* than either MacConkey or salmonella-shigella agar incubated at room temperature. Growth of many strains of *Y. pseudotuberculosis* can be inhibited on CIN agar, and therefore MacConkey agar is preferred for isolation.

Recovery of *Y. enterocolitica* from food is more difficult than recovery from human clinical specimens, and samples are usually referred to a public health laboratory (see chapter 12). Food must be enriched with saline (or a selective broth such as modified Rappaport broth containing magnesium chloride, malachite green, and carbenicillin [MRB]) at cold temperatures for approximately 21 days (2 to 4 days in MRB) (17).

Selective media are not commonly used for the isolation of *Y. pestis*, since when isolated from sterile sites organisms will grow on MacConkey agar and seem to be inhibited to some extent on CIN agar selective for *Y. enterocolitica*. Ber et al. developed a new medium, BIN (brain heart infusion supplemented with Irgasan, cholate salt, crystal violet, and nystatin), for the isolation of *Y. pestis* from specimens likely to be contaminated with normal flora, such as respiratory specimens (4), bubo aspirates, and stool specimens. Plates should be incubated at room temperature in 5% CO_2 for fastest growth. Cultures from suspected plague patients should be incubated for 5 days and up to 7 days if the patient has been treated for more than a few days with an appropriate antimicrobial.

Acute and convalescent serum samples should be collected for serologic testing from pretreated patients for whom cultures do not grow after this period of time.

Y. pestis colonies are slow growing and are only 1 to 2 mm in diameter after 48 h of incubation, with an irregular, "hammered copper" appearance. No hemolysis is seen on blood agar media. Viewed with a dissecting microscope, the colonies are raised with irregular edges, appearing as a "fried egg" (Fig. 2). Organisms growing in broth appear in clumps along the side of the tube in flocculant or stalactite-like formations if the tube is not shaken. After 24 h of incubation, the clumps settle to the bottom of the tube. Colonies of *Y. pestis* growing on BIN agar have a bluish color in the center and a transparent precipitate around the colony.

Colonies of *Y. enterocolitica* have a bull's eye appearance with a red center on CIN agar. Other members of the *Enterobacteriaceae* family, which grow on CIN agar, such as *Serratia*, *Morganella,* and *Citrobacter* spp., produce colonies similar in appearance to those of *Yersinia*, but larger. The use of pectin agar has also been described for isolation of *Y. enterocolitica* from stool and differentiation from other *Enterobacteriaceae*. Although the medium was more sensitive than other currently used selective media and the only other member of the family able to grow and demonstrate a similar colony morphology was *Klebsiella oxytoca,* the medium is not currently commercially available (9).

IDENTIFICATION

Yersinia are catalase positive and oxidase negative and ferment glucose, as do all other members of the family *Enterobacteriaceae*. *Y. enterocolitica* and *Y. pseudotuberculosis* can be presumptively identified by reactions on triple sugar iron (TSI) and lysine iron agar slants. *Y. enterocolitica* produces a yellow color in the entire TSI tube without gas production, and *Y. pseudotuberculosis* produces an alkaline slant and an acid butt, similar to *Shigella*. Both species are lysine decarboxylase negative and therefore produce a yellow butt in lysine iron agar slants. *Yersinia* species are included in the databases of some automated systems; however, most species have not been thoroughly evaluated due to the small number of *Yersinia* isolates tested. Although *Y. pestis* is included in the databases of many of the manual and automated systems (41), commercial systems may not adequately identify *Yersinia* species (particularly *Y. pestis*) due in part to their slow growth and biochemical inactivity. In addition, *Y. pestis* has been misidentified as *Y. pseudotuberculosis* and as *Shigella, Salmonella,* and *Acinetobacter* species. API 20E was shown to have the highest sensitivity and specificity for the identification of *Y. enterocolitica* and *Y. pseudotuberculosis* (39). A miniaturized, automated system (MICRONAUT; Merlin, Bornheim-Hersel, Germany) available only outside of the United States shows promise in the identification of *Yersinia* species based on enzyme reactivity (38).

Presumptive identification of *Y. pestis* is based on the detection of bipolar-staining, small gram-negative bacilli forming pinpoint colonies after 24 h on blood, with better growth at 28 than at 35°C, and non-lactose-fermenting colonies on MacConkey agar that are catalase positive and indole, oxidase, and urease negative. See "Basic Protocols for Sentinel Laboratories" on the American Society for Microbiology website for specific information, pictures, and flowcharts (http://www.asm.org/Policy/index.asp?bid=520). Following presumptive identification, routine clinical laboratories should notify the local public health laboratory and refer the isolate for confirmatory testing. *Y. pseudotuberculosis* can be differentiated from *Y. pestis* by negative urease activity.

Y. pestis can further be separated into biovars based on phenotypic methods. Biovars Antiqua and Medievalis are glycerol and arabinose positive, and biovar Antiqua is nitrate positive and biovar Medievalis is negative. Biovar Orientalis is glycerol negative and arabinose and nitrate positive, and the newest biovar, Microtus, is glycerol positive and arabinose and nitrate negative (65).

Presumptive identification of *Y. enterocolitica* can be made based on typical morphology on CIN agar, reactivity on TSI agar, and urease positivity. Identification of the other *Yersinia* species can be performed by biochemical analysis (Table 1).

Y. enterocolitica has six biogroups which can be differentiated based on reactivity to esculin, indole, D-xylose, trehalose, pyrazinamidase, β-D-glucosidase, and lipase. Although the issue is controversial, biogroup 1A is thought to be nonpathogenic and biotypes 1B and 2 through 5 are pathogenic. Strains belonging to biotype 1A can be differentiated from the

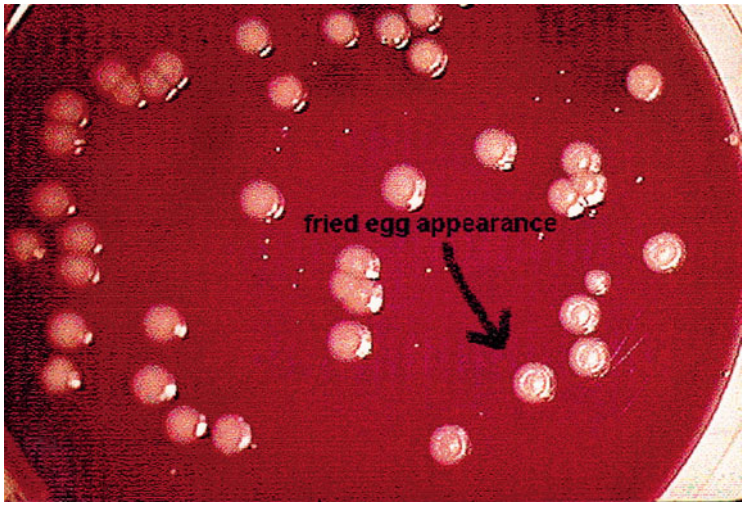


FIGURE 2 Typical fried egg-shaped colonies of *Y. pestis* on sheep blood agar. (Courtesy of the Centers for Disease Control and Prevention.)

TABLE 1 Biochemical reactivity of *Yersinia* species[a]

Yersinia species	Reaction or characteristic[b]												
	Motility (25°C)	Ornithine decarboxylase	Urease	VP (25°C)	Citrate (25°C)	Indole	Rhamnose	Sucrose	Cellobiose	Sorbose	Sorbitol	Melibiose	Raffinose	Fucose
Y. pestis	−	−	−	−	−	−	−	−	−	−	−	V	−	ND
Y. pseudotuberculosis	+	−	+	−	−	−	+	−	−	−	−	+	V	−
Y. enterocolitica	+	+	+	+	−	V	−	+	+	+	+	−	−	V
Y. frederiksenii	+	+	+	+	V	+	+	+	+	+	+	−	−	+
Y. kristensenii	+	+	+	−	−	V	−	−	+	+	+	−	−	V
Y. ruckeri	V	+	−	V	+	−	−	−	−	−	−	−	−	ND
Y. mollaretii	+	+	+	−	−	−	−	+	+	+	+	−	−	−
Y. bercovieri	+	+	+	−	−	−	−	+	+	−	+	−	−	+
Y. rohdei	+	V	V	−	−	−	−	+	+	ND	+	V	V	ND
Y. aldovae	+	+	+	+	+	−	+	−	−	ND	+	−	−	V
Y. intermedia	+	+	+	+	+	+	+	+	+	ND	+	+	+	V

[a]From references 56 and 62.
[b]Incubation is at 35°C except where indicated. VP, Voges-Proskauer; V, variable; ND, not done; −, negative; +, positive.

others by salicin and pyrazinamidase positivity (Table 2) (25). Serotyping could also help determine the pathogenicity of the isolate since only 11 of the 60 known serotypes are pathogenic; however, antisera are not readily available. Other methods that have been evaluated to determine the pathogenicity of *Y. enterocolitica* are based on the presence of the virulence plasmid and include autoagglutination, calcium dependency testing, and Congo red binding. Selective media containing Congo red as well as PCR assays have been evaluated for differentiation of virulent from avirulent strains but are currently being used only in research laboratories (5, 60).

TYPING SYSTEMS

Methods used for the evaluation of the relatedness of *Yersinia* species include serotyping, biotyping, antibiogram analysis, and bacteriophage typing as well as genetic methods. *Y. enterocolitica* can be divided into six biogroups, 1A, 1B, 2, 3, 4, and 5, by using biochemical analysis. These biogroups vary in geographic locations and pathogenic potentials (50). *Y. enterocolitica* also contains more than 70 serotypes, although serotyping is not often performed in routine clinical laboratories since the antisera are not readily available. Pulsed-field gel electrophoresis has long been considered the "gold standard" for typing of *Yersinia* species. Pulsed-field gel electrophoresis was found to be a more useful tool than ribotyping for typing of pathogenic isolates of *Y. enterocolitica* (59). Patterns generated by the restriction enzyme NotI alone or combined with ApaI and XhoI were highly discriminatory (36).

Recently, MLEE has been evaluated for its discriminatory power among *Yersinia* species. Sequencing of a single gene unique to an organism, such as 16S rRNA, has also been used for strain analysis of *Yersinia* species (30). An approach that combines the ease of MLEE testing with the specificity of genome sequencing is MLST. Comparing the sequences of multiple unique genes allows for better discrimination than comparing only 16S rRNA. This technique has recently been applied to *Yersinia* (2). MLST analysis of a large collection of *Yersinia* species was further studied by Kotetishvili et al. (30). With the use of six genes, all of the *Y. pestis* isolates appeared to be homogeneous and also were found to be identical to *Y. pseudotuberculosis*. Most of the other species of *Yersinia* tested also appeared to be homogeneous, with the exception of *Y. enterocolitica*, *Y. frederiksenii*, *Y. kristensenii,* and *Y. mollaretii,* which were more heterogeneous.

SEROLOGIC TESTS

The gold standard for the diagnosis of plague is isolation of the organism from blood or aspirate; however, serology can play a role, particularly for retrospective diagnostic and epidemiologic studies in areas where plague is endemic (46). Most patients with plague seroconvert 1 to 2 weeks following the onset of symptoms (47). Diagnosis can be made based only on a fourfold rise in antibody titers between acute and convalescent serum samples. Assay methods include passive hemagglutination, which is the method recommended by the World Health Organization due to its low cost and ease of performance. However, the assay lacks both sensitivity and specificity due to lack of standardization and lack of commercialization of reagents. The most commonly used antigen in serologic assays for *Y. pestis* is the capsular F1 antigen, which is a highly immunogenic, stable antigen and present in high concentrations in sera and bubo fluids of plague patients even after several days of appropriate antimicrobial therapy.

TABLE 2 Biotypes of *Y. enterocolitica*[a] after incubation at 25°C for 48 h

Test	Reaction[b] for biotype:					
	1A	1B	2	3	4	5
Lipase (Tween esterase)	+	+	−	−	−	−
Esculin	+	−	−	−	−	−
Salicin	+	−	−	−	−	−
Indole	+	+	(+)	−	−	−
Xylose	+	+	+	+	−	d
Trehalose	+	+	+	+	+	−
$NO_3 \rightarrow NO_2$	+	+	+	+	+	−
DNase	−	−	−	−	+	+
Pyrazinamidase[c]	+	−	−	−	−	−

[a] Modified from reference 62a with permission of the publisher, S. Karger AG, Basel, Switzerland.

[b] +, ≥90% of strains positive; d, 11 to 89% of strains positive; −, ≥90% of strains negative; (+), weakly positive reaction.

[c] According to Kandolo and Wauters (27a).

Other assays include ELISA, which is not standardized either and must be confirmed by using a Western blot assay.

Serology can be used as an adjunct in the diagnosis of disease due to *Y. enterocolitica* or *Y. pseudotuberculosis*. Antibody is detectable within the first week of illness and returns to normal levels 3 to 6 months later. The specificity of serologic assays ranges from 82 to 95% due to cross-reactivity between the two species and also with *Brucella*, *Franciscella,* and *Vibrio* species, *Borrelia burgdorferi, Chlamydia pneumoniae,* and some *Escherichia coli* serogroups. Another disadvantage of using serology for diagnosis is that antibodies to *Y. enterocolitica* O antigens can be found in many normally healthy patients due to the frequency of exposure to nonpathogenic serotypes. Most human infections with *Y. enterocolitica* involve serotypes O:3, O:5, 27, O:8, and O:9. Serotype O:3 is the most common cause of gastroenteritis. However, as mentioned above, antisera are available only to public health and research laboratories.

Antibody to outer membrane proteins (Yops) that are present only in virulent strains of *Y. enterocolitica* may be more helpful. In a small study of normally healthy blood donors, immunoglobulin M (IgM) antibody to Yops was 97% specific for acute infection (57). Testing of blood donors for anti-Yop IgA in New Zealand, which has a high incidence of *Y. enterocolitica* gastroenteritis, showed promise in preventing transfusion-related infections (28). The presence of IgG and IgA antibodies to *Y. enterocolitica* Yops is also used as an aid in the diagnosis of autoimmune disorders that occur post-infection, such as reactive arthritis, erythema nodosum, Graves' disease, and Hashimoto's thyroiditis (14). Increased sensitivity and specificity are seen with the Western blot assay compared to complement fixation and ELISA; however, cross-reactivity is still seen, particularly with *B. burgdorferi*, which can be associated with arthritis that is clinically indistinguishable from that due to *Y. enterocolitica* (47).

ANTIMICROBIAL SUSCEPTIBILITIES

Pneumonic plague is nearly 100% fatal if not treated within the first 24 h of development of symptoms. The drug of choice for the treatment of plague, pneumonic, septicemic, or bubonic, is streptomycin. However, due to the lack of availability of streptomycin and the threats of *Y. pestis*'s being used as an agent of bioterrorism, other agents have been evaluated in vitro and in animal models. The only other antibiotic currently approved for treatment of plague is doxycycline; however, other alternatives would be gentamicin and a fluoroquinolone (8). Antibiotic resistance among isolates of *Y. pestis* has been described, but only in rare case reports of resistance to tetracycline or streptomycin. Fluoroquinolone resistance has been induced in vitro, but naturally occurring resistance has not been documented (16). Steward et al. documented the efficacy of fluoroquinolones in the mouse model of systemic and pneumonic plague (55). Rapid detection of ciprofloxacin-resistant strains of *Y. pestis* can be accomplished by using real-time PCR and assessing point mutations in the DNA gyrase gene (32). An isolate of *Y. pestis* from a single infected patient in Madagascar was found to be multidrug resistant, with resistance to streptomycin, sulfonamides, tetracycline, and chloramphenicol (19). The treatment of choice for plague meningitis is chloramphenicol.

Most cases of *Y. enterocolitica* gastroenteritis do not require treatment; however, treatment is necessary in cases of systemic disease, especially in immunosuppressed patients. Treatment options include trimethoprim-sulfamethoxazole and a fluoroquinolone. *Y. enterocolitica* produces two different β-lactamases, one of which is a class A constitutive enzyme and the other of which is an inducible class C enzyme that is not inhibited by β-lactamase inhibitors. The presence of one or both of these enzymes varies depending on the biogroup (10). Although the β-lactamase confers resistance to penicillin on *Y. enterocolitica,* the organism remains uniformly susceptible to the extended-spectrum cephalosporins (44). Resistance to fluoroquinolones is due to either a mutation in the *gyrA* gene or efflux mechanisms. In a study conducted in Spain, 23% of *Y. enterocolitica* strains isolated from patients with gastroenteritis were nalidixic acid resistant. All resistant isolates had a mutation in *gyrA,* and some were resistant based on an efflux mechanism as well (10). *Y. enterocolitica* strains are susceptible in vitro to aminoglycosides, chloramphenicol, tetracycline, trimethoprim-sulfamethoxazole, and extended-spectrum cephalosporins.

Y. pseudotuberculosis is susceptible to ampicillin, tetracycline, chloramphenicol, cephalosporins, and aminoglycosides. Although infections due to *Y. pseudotuberculosis* are not usually treated, patients with septicemia should be treated with ampicillin, streptomycin, or tetracycline.

Y. aldovae and *Y. ruckeri* are also susceptible to penicillin. *Y. frederiksenii*, *Y. intermedia,* and *Y. rhodei* produce a β-lactamase similar to that of *Y. enterocolitica*, which is expressed at different levels in different strains (56).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Y. pestis, *Y. enterocolitica,* and *Y. pseudotuberculosis* are the primary pathogens in the genus *Yersinia.* Isolation of *Y. pestis* from any body site warrants further investigation. Isolation of *Y. enterocolitica* or *Y. pseudotuberculosis* from stool culture is not sufficient for causal evidence of disease since nonpathogenic serotypes may be normal stool flora. However, no readily available methods except those using routine biochemicals, which are not usually maintained in routine clinical laboratories, are available for differentiation of pathogenic serotypes. Isolation of either species in pure culture from a symptomatic patient with no other diagnosis should be considered suspect. Isolation of either species from blood or other normally sterile sites should also be considered significant.

It has not been shown to be cost-effective to screen all stools for *Y. enterocolitica* by using CIN agar. Isolation rates vary based on geographic locations, with the highest incidence in the colder portions of the country (27), so the decision to routinely rule out these organisms in stool cultures should be evaluated in individual laboratories after consultation with the infectious disease physicians.

Although the other *Yersinia* species besides *Y. pestis*, *Y. enterocolitica,* and *Y. pseudotuberculosis* are not considered human pathogens, they have been isolated from the gastrointestinal tracts of symptomatic patients with no other diagnosis. It has been recommended that the presence of these *Yersinia* species in pure culture be reported and that antibiotic susceptibility testing be performed to assess these organisms' pathogenic potentials. These organisms may be underrecognized pathogens (33).

Due to the lack of accuracy of commercial systems for the identification of *Y. pestis* and the implications related to bioterrorism with its identification, isolates should be sent to a local public health laboratory for confirmation.

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Klebsiella, Enterobacter, Citrobacter, Serratia, Plesiomonas, and Other *Enterobacteriaceae*

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45

TAXONOMY

Taxonomic changes within the *Enterobacteriaceae* have become commonplace and undoubtedly will continue at a rapid pace for the foreseeable future. Three major taxonomic changes in the *Enterobacteriaceae* included in the eighth edition of this Manual merit mention again. The oxidase-positive organism, *Plesiomonas shigelloides,* which clusters with *Proteus* in phylogenetic studies using 16S and 5S rRNA sequencing is now a member of the family *Enterobacteriaceae* (98, 99, 125). The *Enterobacteriaceae* will be redefined in the next edition of *Bergey's Manual* to accommodate an oxidase-positive organism and other organisms that defy the traditional definition of this family. Inclusion of *Calymmatobacterium granulomatis,* a nonculturable organism causing a sexually transmitted disease, in the genus *Klebsiella* as *K. granulomatis* was another major change (28, 85). This move was based on 16S rRNA and *phoE* gene sequencing. Lastly, it should be remembered that *Enterobacter agglomerans* now resides in the genus *Pantoea; P. agglomerans* remains heterogeneous at the DNA level (46). Members of the *Enterobacteriaceae* covered in this chapter, including new taxa or taxonomic changes, are listed in Tables 1 and 2. When laboratories adopt taxonomic changes, which are dictated by common usage in the literature, the older epithet should be included in parentheses following the new name.

Because of the genetic heterogeneity within the genus, taxonomic changes occur frequently in *Enterobacter*. To start, two new species, *E. radicincitans* and *E. ludwigii,* have been added to the genus (64, 81). *E. radicincitans* is a plant growth-promoting bacterium isolated from the phyllosphere of wheat, and *E. ludwigii,* which is isolated from human clinical specimens, was formerly a genomovar within the *E. cloacae* complex. Two other clusters of organisms previously included in the *E. cloacae* complex are now subspecies of *E. hormaechei*. Strains previously known as *E. hormaechei* are now subsp. *hormaechei,* and there are two newly created subspecies, subsp. *oharae* and subsp. *steigerwaltii;* all three subspecies are isolated from human clinical specimens from multiple sites and from plants (63). *Enterobacter intermedius,* which clustered with the type strain of *Kluyvera cochleae* by 16S rRNA sequencing, has been moved to the genus *Kluyvera* (118). The older legitimate epithet, *intermedius,* has replaced *cochleae* as the species name, resulting in the new designation *Kluyvera intermedia*. *E. dissolvens,* which was previously suspected to belong in *E. cloacae,* is now *E. cloacae* subsp. *dissolvens;* strains formerly known as *E. cloacae* are now *E. cloacae* subspecies *cloacae* (62). *E. cloacae* remains a heterogeneous species with several DNA groups residing within it, and as with *P. agglomerans* groups, these groups remain unnamed because they are not separable by phenotypic tests (61). *Hafnia alvei* is composed of two distinct DNA hybridization groups, and studies are in progress to name DNA hybrization group 2 (74).

Several additions have been proposed for the genus *Serratia*. *S. marcescens* subsp. *sakuensis* is a strain isolated from activated sludge from a wastewater treatment plant in Japan and reportedly is a spore former; strains known as *S. marcescens* assume subspecies status as subsp. *marcescens* (2). Of the two new species added to *Serratia,* one is a urea-utilizing organism isolated from water in India, named *S. ureilytica,* of which only a single strain is known, and the other is a nonculturable symbiont found in aphids and provisionally designated "*Candidatus* Serratia symbiotica" (17, 104).

In the genus *Klebsiella,* a group of nitrogen-fixing strains isolated from plants (banana, rice, sugar cane, and maize) and human clinical samples, primarily blood, have been named *K. variicola* (124). A second new species, *K. singaporensis,* has been proposed for a single strain isolated from soil collected from sugar cane roots in Singapore (93).

Two new species have been created within the genus *Photorhabdus, P. temperata* and *P. asymbiotica,* and three subspecies (*luminescens, laumondii,* and *akhurstii*) were described for the existing species, *P. luminescens* (43). *P. temperata,* like *P. luminescens,* is a symbiont of nematodes that causes disease in insects. However, *P. asymbiotica* contains only isolates from human infections and has two subspecies, *asymbiotica* and *australis,* isolated in the United States and Australia, respectively (3). Four new species, all symbionts in entomopathogenic nematodes, have been proposed for the genus *Xenorhabdus* including *X. budapestensis, X. ehlersii, X. innexi,* and *X. szentirmaii* (92).

Two new genera, *Averyella* and *Dickeya*, have been proposed for the *Enterobacteriaceae* (78, 127). *Averyella dalhousiensis* strains, previously known as enteric group 58, are isolated primarily from human wound specimens, although the organism was recently isolated from blood (78). The genus *Dickeya* includes two species transferred from *Pectobacterium (chrysanthemi)* and *Brenneria (paradisiaca)* as well as four novel species *(dadantii, dianthicola, dieffenbachiae,*

TABLE 1 Nomenclature, isolation source, and significance of selected genera of the family *Enterobacteriaceae*[a]

Current (previous) designation	Clinical data			Environmental data
	Frequency	Source	Significance	
Averyella dalhousiensis (enteric group 58)	Unk	Wound, blood	2	Unk
Citrobacter				
C. amalonaticus	++	**Feces,** blood, wound, UT, RT	2	Unk, one isolate from an animal
C. braakii	++	**Feces,** UT, wound	2	Similar to *C. freundii*
C. farmeri (*C. amalonaticus* biogroup 1)	++	**Feces,** UT, blood, wound, RT	2	Unk
C. freundii	++++	All sites, feces most common	1	Water, soil, fish, animals, food
C. koseri (*C. diversus*)	++	All sites, CSF most common	1	Unk
C. rodentium	−			Pathogenic for mice
C. sedlakii	+	Feces, UT, blood, wound	3	Same as for *C. braakii*
C. werkmanii	+	**Feces,** blood, wound	3	Same as for *C. braakii*
C. youngae	+++	**Feces,** UT, blood, wound	3	Same as for *C. braakii*
C. gillenii (*Citrobacter* DNA group 10)	+	**Feces,** UT, blood	3	Same as for *C. braakii*
C. murlinae (*Citrobacter* DNA group 11)	+	Feces, blood, UT, wound	3	Same as for *C. braakii*
Enterobacter				
E. aerogenes	++++	All sites	1	Water, soil, sewage, animals, dairy products
E. amnigenus biogroup 1	−			Plants
E. amnigenus biogroup 2	−			Water
E. asburiae	++	**UT,** RT, feces, wound, blood	2	Water
E. cancerogenus (*E. taylorae*)	++	**Wound,** RT, feces	2	Animals, water
E. cloacae subsp. *cloacae*	++++	All sites	1	Water, soil, sewage, meat
E. cloacae subsp. *dissolvens* (*Enterobacter dissolvens*)	−			Diseased corn stalks
E. cowanii (*P. agglomerans*/ Japanese NIH group 42)	Unk	UT, RT, blood, wound	3	Unk
E. gergoviae	++	RT, UT, blood	2	Water, cosmetics
E. hormaechei subsp. *hormaechei*	++	RT, wound, blood	1	Unk, one isolate from a frog
E. hormaechei subsp. *oharae*	++	All sites	1	Plants
E. hormaechei subsp. *steigerwaltii*	++	All sites	1	Plants
E. kobei	Unk	Unk	Unk	Food
E. ludwigii (*E. cloacae*)	Unk	UT, RT, blood, stool	2	Food
E. nimipressuralis (*Erwinia*)	−			Diseased elms
E. pyrinus (*Erwinia*)	−			Diseased pear trees
E. radicincitans	−			Phyllosphere of winter wheat
E. sakazakii	++	RT, wound, CSF	1	Unk
Hafnia alvei DNA Group 1 and DNA Group 2	++	**Feces,** blood, RT	2	Ubiquitous
Klebsiella				
K. pneumoniae	++++	All sites, RT and UT most common	1	Ubiquitous, including foods and water
K. granulomatis (*Calymmatobacterium*)	++	Genital tract	1	None
K. pneumoniae subsp. *ozaenae*	++	**Nasal discharge,** RT, UT, blood	1	Unk
K. pneumoniae subsp. *rhinoscleromatis*	++	Nasal discharge	2	Unk
K. oxytoca	++++	All sites	2	Ubiquitous, including foods and water
K. variicola	++	Blood	1	Plants
K. singaporensis	−			Soil

(Continued on next page)

TABLE 1 Nomenclature, isolation source, and significance of selected genera of the family *Enterobacteriaceae*[a] *(Continued)*

Current (previous) designation	Clinical data			Environmental data
	Frequency	Source	Significance	
Morganella morganii subsp. *morganii* and subsp. *sibonii*	++	All sites	1	Unk, isolations from mammalian and reptile gastrointestinal tracts
Pantoea agglomerans (*Enterobacter*)	+++	All sites	2	Plants
Photorhabdus	++	Wound	2	Unknown, possibly insects
P. asymbiotica subsp. *asymbiotica* and subsp. *australis*				
P. luminescens	−			Nematodes infecting insects
P. temperata	−			Nematodes infecting insects
Plesiomonas shigelloides	+++	**Feces,** blood	1	Aquatic habitats and animals
Proteus				
P. hauseri (*P. vulgaris* genomospecies 3)	+	Unk	Unk	Unk, probably like *P. mirabilis*
P. mirabilis	++++	**UT,** blood, CSF	1	Animals, birds, fish, foods
P. myxofaciens	−			Gypsy moth
P. penneri	++	**UT,** blood, wound, feces, eye	1	Probably similar to *P. mirabilis*
P. vulgaris	+++	**UT,** wound, stool, RT	1	Probably similar to *P. mirabilis*
Providencia				
P. alcalifaciens	+++	All sites, UT and feces common	1	Mammals, water
P. heimbachae	+	Feces	3	Penguins
P. rettgeri	+++	All sites, UT most common	1	Same as for *P. alcalifaciens*, insects
P. rustigianii	+	Feces	3	Unk
P. stuartii	+++	All sites, UT most common	1	Mammals
Raoultella				
R. ornithinolytica (*Klebsiella*)	+	Wound, UT, blood	2	Food
R. planticola (*Klebsiella*)	Unk	Similar to *K. pneumoniae*?	3	Plants, water
R. terrigena (*Klebsiella*)	Unk	Similar to *K. pneumoniae*?	3	Soil, water
Serratia				
S. entomophila	−			New Zealand grass grub, water
S. ficaria	+	RT, wound	3	Fig wasps, figs, plants
S. fonticola	+	**Wound,** RT	3	Water, birds
S. liquefaciens complex (*S. liquefaciens* sensu stricto, *S. proteamaculans*, *S. grimesii*)	+++	RT, wound	2	Plants, insects, mammals, birds, dairy products
S. marcescens subsp. *marcescens*	++++	All sites, RT most common	1	Water, soil, plants, vegetables, animals, insects
S. marcescens subsp. *marcescens* biogroup 1	+	**UT,** RT	2	Unk
S. marcescens subsp. *sakuensis*	−			Wastewater
S. odorifera biogroup 1 and biogroup 2	+	RT, wound, feces, blood, UT	2	Plants
S. plymuthica	+	RT	3	Water, plants, small mammals
S. rubidaea	+	RT, wound, blood, UT, feces	2	Water, plants
S. ureilytica	−			Water
"*Candidatus* Serratia symbiotica"	−			Aphid symbiont

[a] Abbreviations and symbols: ++++, frequent; +++, occasional; ++, rare; +, very rare; −, not yet isolated from humans; CSF, cerebral spinal fluid; RT, respiratory tract; Unk, unknown; UT, urinary tract; 1, major pathogenic species of humans; 2, proven cause of disease in rare instances; 3, isolated from humans, significance unknown. Bold denotes most common source. Data are from references 2, 3, 17, 21, 23, 37, 42, 46, 50, 51, 52, 68, 71, 74, 78, 89, 97, 104, 106, 111, 112, 113, 118, 130, and 133.

TABLE 2 Other members of the family *Enterobacteriaceae*[a]

Human pathogens or opportunists	Primarily environmental strains[b]	Nonhuman isolates
Edwardsiella tarda	*Budvicia aquatica*[c] (9, 20)	*Alterococcus agarolytica*[c] (131)
Ewingella americana	*Buttiauxella noackiae*[d] (105)	*Arsenophonus nasoniae*[e] (47, 140)
Cedecea davisae	*Edwardsiella hoshinae*[c,d] (55)	*Brenneria* species[f] (57)
Cedecea lapagei	*Trabulsiella guamensis*[c] (101)	*Buchnera aphidicola*[e] (107)
Cedecea neteri	*Pragia fontium*[c] (8)	*Buttiauxella* species[b,c,e] (34, 105)
Cedecea genomospecies 3		"*Candidatus* Hamiltonella defensa"[e] (104)
Cedecea genomospecies 5		"*Candidatus* Regiella insecticola"[e] (104)
Kluyvera ascorbata		"*Candidatus* Phlomobacter fragariae"[f] (147)
Kluyvera cryocrescens		*Dickeya* species[f] (127)
Kluyvera georgiana		*Erwinia* species[b,f] (79, 114, 129)
Leclercia adecarboxylata		*Edwardsiella ictaluri*[d] (58)
Leminorella grimontii		*Kluyvera intermedia*[f] (*Enterobacter intermedius* and *Kluyvera cochleae*) (105, 118)
Leminorella richardii		*Obesumbacterium proteus*[c] (71)
Leminorella genomospecies 3		*Pantoea* species[b,c,f] (33, 57 ,80)
Moellerella wisconsensis		*Pectobacterium* species[f] (57)
Rahnella aquatilis		*Samsonia erythrinae*[f] (134)
Rahnella genomospecies 2 (22)		*Sodalis glossinidius*[e] (32, 100)
Rahnella genomospecies 3		*Wigglesworthia glossinidia*[e] (4)
Tatumella ptyseos		*Xenorhabdus* species[e] (18, 92)
Yokenella regenburgei		

[a] References are given in parentheses. Genomospecies listed cannot be biochemically separated from other species within their genus and/or only a single strain exists.
[b] Rare human isolates of no, or questionable, significance.
[c] Environmental isolates.
[d] Fish, marine, animal, or bird isolates.
[e] Insect isolate or pathogen.
[f] Plant isolate or phytopathogens.

and *zeae); Dickeya* species are not isolated from human specimens (127).

Miscellaneous genera within the *Enterobacteriaceae* are given in Table 2. Numbered genomospecies designations within a genus are given when they are comprised of a single strain, if too few organisms exist to be named, or if they cannot be differentiated from other members of their genus with available phenotypic tests. The genera listed in the last two columns of Table 2 are not isolated from human clinical specimens or are isolated but may not be significant, and most of these taxa will be unfamiliar to clinical microbiologists. However, it should be noted that a *Pantoea dispersa*-like organism and a strain of *Pantoea ananatis* have been reported recently from bacteremic patients (33, 129). *Erwinia persicinus* and *Buttiauxella gaviniae* have been isolated from a urinary tract infection (UTI) and a spinal cord patient with urinary bladder pathology, respectively (34, 114). A number of the organisms in the third column cannot be grown on laboratory media, so they are unlikely to be confused with, or necessitate separation from, those genera seen in humans. References have been included for those organisms that are isolated only from nonhuman sources, and they will not be discussed further.

DESCRIPTION OF GENERA

Members belonging to the family *Enterobacteriaceae* are gram-negative, facultative anaerobic rods or coccobacilli ranging in width from 0.3 to 1.0 μm and in length from 0.6 to 6.0 μm. A proposed subspecies of *Serratia, S. marcescens* subsp. *sakuensis,* is the only reportedly spore-forming organism in this family (2). Prototrophic strains grow readily on ordinary media. Among these genera, auxotrophic strains from clinical specimens are rare. However, cysteine-requiring urinary isolates of *K. pneumoniae,* which grow as pinpoint colonies on routine media, do occur. If encountered, these strains require supplementation of biochemical media or commercial identification systems with 0.63 mM cysteine for accurate identification. A number of newer genera are not culturable in vitro but have been assigned to the *Enterobacteriaceae* based upon analysis of the genetic sequences of their genome. A proposal to create a "*Candidatus*" status for these types of organisms has been made (108). Other genera, including *K. (C.) granulomatis* and many insect symbionts, are culturable only by cell culture techniques.

Of the organisms in Tables 1 and 2 isolated from human specimens, all *Klebsiella, Leminorella, Moellerella, Tatumella,* and *Enterobacter asburiae* strains are nonmotile, although any strain of any genus may be nonmotile and recent data for *E. asburiae* indicate that some strains may be motile (62). Some strains of *S. plymuthica* may not grow at 37°C, but most other members of the genera discussed in this chapter grow well between 25 and 37°C. Essentially only *Klebsiella* and *Raoultella* (previously *Klebsiella*) spp. are encapsulated, but strains from all genera may grow as mucoid or rough colonies. Five genera produce pigment. Some strains of *S. marcescens* and most *S. rubidaea* and *S. plymuthica* strains produce a red pigment, prodigiosin, which may appear throughout the entire colony or only as a red center or margin. Most strains of *E. sakazakii* and some strains of *P. agglomerans, Leclercia adecarboxylata,* and *Photorhabdus asymbiotica* form yellow-pigmented colonies that range from bright to pale yellow. Weak pigment producers may be detected only by observing growth placed on a swab or filter paper. Yellow pigment may be enhanced by incubation at 25°C. *Photorhabdus luminescens* and *P. asymbiotica* cultures are luminescent, giving a visible glow in a darkroom after 5 min. *S. odorifera,* as indicated by its name, and some *Cedecea* spp. strains, produce a pungent (potato-like) odor due to the

production of alkyl-methoxypyrazines (50). Species of *Proteus* and *Providencia* oxidatively deaminate α-amino acids, producing pyruvic acids. L-phenylalanine deamination yields a green color when ferric chloride is added; however, deamination of *dl*-tryptophan produces the deep reddish brown pigment often seen in media inoculated with these organisms without the addition of ferric chloride (120). *Proteus* species also produce swarmer cells, which are elongated forms that are created when cells fail to septate or divide. These cells, which are profusely covered with flagella, act in concert to produce swarming motility on solid media (14).

Plesiomonas shigelloides organisms are also gram-negative, facultative anaerobes growing as straight rods similar in size to other *Enterobacteriaceae*. However, unlike other *Enterobacteriaceae, P. shigelloides* strains are oxidase positive, do not produce gas from glucose (*Enterobacteriaceae* are variable), and are susceptible to vibriostatic agent O/129 (2,4-diamino-6,7-diisopropylpteridine). Both *P. shigelloides* and enterobacteria grow at similar salt concentrations (0 to 5%) and pH ranges (4.0 to 8.0).

NATURAL HABITAT AND CLINICAL SIGNIFICANCE

The *Enterobacteriaceae* are widespread throughout the environment (Tables 1 and 2). Many species of the genera in Table 1 are commonly recognized nosocomial pathogens. The *Enterobacteriaceae* comprise 50% of all isolates from hospital-acquired infections and 80% of all gram-negative isolates (83). *Enterobacter* and *Klebsiella* spp. rank between fifth and ninth among common agents of bloodstream infections (35, 82, 143). Although less prevalent than a number of other etiologic agents, *Klebsiella* and *Enterobacter* cause significant infections. In one study, when found intraoperatively, *Klebsiella* and *Enterobacter* species had a 68 and 100% probability, respectively, of causing a wound infection; probability rates for *Escherichia coli* or *Staphylococcus aureus,* which were isolated three times more often during surgery, were only 31 and 55%, respectively (137). While *K. pneumoniae* and *E. cloacae* cause sepsis four to five times less often than gram-positive organisms, they are twice as likely to cause patient mortality (138). Species of both genera were among the most common organisms involved in relapse or reinfection in a study on recurrent bacteremia (139). *S. marcescens* and *Citrobacter* spp. constitute 1 to 2% and <1% of bloodstream infections, respectively (143). *Klebsiella* spp. rank as the second to fourth most common species in hospital-acquired UTIs and are the third to fourth most common agent in community-acquired UTIs (42, 49). In 2000, *Klebsiella* spp. accounted for 20, 23, and 58% of UTIs in Latin America, Europe, and North America, respectively (49). In this survey, *P. mirabilis, Enterobacter* spp., and *Citrobacter* spp. ranked as the fifth, sixth, and seventh most common agents of UTIs in Latin America, Europe, and North America, respectively (49). *Proteus mirabilis* continues to be an important cause of UTIs in Europe and North America, causing 4 to 6% of community-acquired UTIs and 3 to 6% of nosocomial infections including pyelonephritis (42, 49).

Klebsiella is carried in the nasopharynx and the bowel; however, feces are probably the most significant source of patient infections (103). Approximately one-third of patients carry *Klebsiella* in their stool, but rates may increase as much as threefold with hospitalization and antimicrobial usage in adults. Fecal carriage rates for children may be as high as 90 to 100% without antimicrobial therapy. *K. pneumoniae* has emerged as an important cause of community-acquired pyogenic liver abscess (PLA) worldwide (91, 121, 146). Diabetes and underlying biliary disease are major risk factors. The majority of patients with PLA due to *Klebsiella* are Asian males, 50 to 60 years of age, who present primarily with a right-lobe, solitary, monomicrobial abscess. *Klebsiella*-associated PLA mortality rates are lower than rates seen with other bacterial agents. *K. rhinoscleromatis* and *K. ozaenae* cause granulomatous infections termed rhinoscleroma and atrophic rhinitis, respectively. Both are chronic diseases of the upper respiratory tract; although atrophic rhinitis is restricted to the nose, rhinoscleroma may spread to the trachea and larynx (71). Both diseases occur most frequently in tropical areas of the world; transmission is thought to be from person to person, although prolonged contact with persons producing airborne nasal secretions is required. *K. (C.) granulomatis* causes chronic genital ulcers and also occurs predominantly in tropical countries (56). It is believed that *K. (C.) granulomatis* is sexually transmitted and that the only known reservoir is humans. *Raoultella (Klebsiella) planticola* and *Raoultella (Klebsiella) terrigena* are difficult to distinguish from *Klebsiella pneumoniae* without special tests. Studies in Europe have found that 3.5 to 19% of clinical strains initially identified as *Klebsiella* are actually *R. planticola* and that these strains have pathogenicity characteristics similar to those found in *K. pneumoniae* (119). In a survey of 436 *Klebsiella* strains isolated from newborns in the United States, however, only one isolate could be identified as *R. planticola,* indicating that its prevalence may vary geographically (141). Notably, all of the human strains of the new species *K. variicola* were reportedly isolated from blood (124). No further information on the human isolates was presented, but it would appear that this species is, at the very least, an opportunistic pathogen. As with other klebsiellae, plants appear to be the natural habitat of this organism.

Nosocomial *Enterobacter* colonization and infection are frequently associated with contaminated medical devices and instrumentation; however, *Enterobacter* spp. are commonly consumed in food and an endogeneous source should also be considered (71). *E. ludwigii* and the three subspecies of *E. hormaechei* are isolated from a variety of human sources including blood (63, 64). A nonhuman source for *E. ludwigii* has not been reported, but plants appear to be the natural habitat of *E. hormaechei* subspecies. A number of *Enterobacter* spp. were previously members of the genus *Erwinia* and are isolated from plants or trees (Table 1), where they may cause disease. The clinical microbiologist should be aware of these species as potential transients or commensals in clinical specimens.

Although *Serratia* spp. are seldom a cause of primary infections, they are notorious nosocomial pathogens and colonizers. The predominant mode of transmission is from person to person, but various medical apparatuses, intravenous fluids, and other solutions have often been implicated as well (71). Patients with indwelling catheters, particularly those for UTIs, serve as a primary reservoir for transmission via hospital personnel. For children, the gastrointestinal tract is a common source of infections. Outbreaks transmitted by hand are often insidious, occurring over long periods of time, and may subside and peak a number of times before recognition and infection control efforts can contain them. Pigment production in *S. marcescens* appears to be a marker that the strain is environmental in origin and of low virulence (11). Community-acquired infections are rare, except for *S. marcescens* contact lens-induced acute red eye (66). Most of the other species of *Serratia* have also been isolated from humans, where they too are usually transients or cause opportunistic infections.

The citrobacteria are considered inhabitants of the intestinal tract primarily, and their presence in the environment may reflect fecal excretion by humans and animals; however, the natural habitat of some newer *Citrobacter* species is unknown. Sepsis involving *Citrobacter* is often polymicrobic, with reported mortality rates as high as 48 to 50%; death is associated more often with polymicrobic than with monomicrobic infections (71). *Citrobacter* meningitis is almost exclusively associated with *C. koseri* and involves children less than 2 months of age, with the highest onset rates noted in neonates with a mean age of 7 days (71). An exceedingly high number (>75%) of these infants develop brain abscesses, and those who survive are generally afflicted with neurological defects. The most prominent risk factor is prior colonization; during outbreaks, colonization rates of 27% have been noted, in contrast to the normal rate of <1% (94, 142). Person-to-person spread from hospital personnel and, less often, mother-to-offspring infections are the most likely pathways; sampling of inanimate or environmental reservoirs in hospitals usually fails to yield *Citrobacter. Citrobacter* species previously belonging to the *Citrobacter freundii* complex, including *C. gillenii* and *C. murlinae,* have been found in human clinical, animal, food, and environmental specimens (21, 23). It is unclear what role many of these species may play in human infections; for some, the numbers of strains available are insufficient to either determine clinical significance in humans or establish a reservoir for infections. *C. rodentium* is the cause of murine colonic hyperplasia (97). This disease is self-limiting in adult mice but causes significant morbidity and mortality in infant mice when outbreaks occur in mouse colonies (97).

Proteus, Providencia, and *Morganella* are widespread in the environment and are normal inhabitants of the gut. While *Proteus* spp., especially *P. mirabilis,* are common causes of uncomplicated UTI, they frequently affect the upper urinary tract as well, causing renal scarring and kidney stone formation (102). Ammonia and CO_2 production from urine caused by the urease of *Proteus* lowers the pH, causing precipitation of soluble ions and the formation of stones, composed of struvite and apatite crystals, within the urinary tract. These crystals, which can be visualized within urine agar (filter-sterilized human urine solidified with agar) that has been inoculated with *Proteus,* are composed of magnesium ammonium phosphate or calcium phosphate (102). Since *P. mirabilis, P. penneri, Morganella,* and *P. alcalifaciens* have been isolated with greater frequency from diarrheal stools than from normal stools, it has been speculated that they may cause diarrhea. However, of these agents, only *P. alcalifaciens* plays a potential role in gastroenteritis. Studies by several groups have shown that *P. alcalifaciens* is invasive in HEp-2 cell assays (6, 77) and elicits diarrhea in the RITARD (reversible intestinal tie-adult rabbit diarrhea) model (6). However, some strains isolated in pure culture or in large numbers from diarrheal stools have failed to invade cell lines. The inability of non-invasive strains to penetrate epithelial cells may be explained by the fact that they appear unable to adhere to cell lines, a prerequisite to invasion (86).

Hafnia alvei has also been linked to gastrointestinal disease. *Hafnia* isolated from the diarrheal stool of a 9-month-old child in pure culture has been found to produce fluid in a rabbit intestinal tie model (5). A 1994 study of tourists returning to Finland found that 5% of patients with diarrhea had *Hafnia* in their stool whereas >300 asymptomatic individuals were negative for *Hafnia* (123). To date, putative virulence characteristics have not been demonstrated in hafniae. Prototypal *Hafnia alvei* isolates from Bangladesh originally identified as diarrheal strains, thought to possess the *eae* gene (attaching-effacing) and thus giving credence to *Hafnia*'s role in diarrhea, were eventually found to be a new species of *Escherichia, E. albertii* (7, 67, 73). Extraintestinal infections caused by *H. alvei* are unusual but can occur in immunocompetent individuals as well as those with underlying diseases, although the latter group is more likely to be affected (122). These infections are often community acquired and are believed to arise from the gastrointestinal tract. *Hafnia* appears to have a predilection for the biliary tree and may produce abscesses at the site of infection (122).

Averyella dalhousiensis is predominantly isolated from human wounds, where it is probably a contaminant since it appears to play no role in these infections (78). However, a recent isolation from blood indicates that it may be an opportunistic pathogen in compromised patients.

In humans, *Plesiomonas shigelloides* is primarily associated with diarrheal disease in infections occurring most frequently in individuals who live in or travel to tropical countries; a history of seafood consumption is common (76, 84). Bacteremia, which is primarily community acquired, is rare, with underlying biliary tract disease and advanced age (>75 years) as major risk factors (145). *Plesiomonas* bacteremias are polymicrobial; the gastrointestinal tract appears to serve as the primary source of infection since concomitantly isolated bacteria are all gastrointestinal agents. A retrospective study of *Plesiomonas* diarrheal infections undertaken in Hong Kong found an infection rate of 5.9% over a four-year period, with increasing numbers of isolations during three of the four years of the study (144). Most infections are self-limiting; however, hospitalization may be required for severe infections and/or for patients with underlying conditions. Symptoms associated with *Plesiomonas* diarrhea vary, and patients may present with either a secretory or a dysenteric type of disease. In the study of Wong et al. (144), 73% of cases had watery diarrhea and 25% had bloody diarrhea. Of 38 Bangladeshi children with monomicrobial *Plesiomonas* diarrhea, 76% were < 2 years of age and 74% were male (84). Vomiting was a significant feature (71%) of infections in these children, but in contrast to other reports, dehydration and fever were less prominent symptoms (23 and 8%, respectively), and the majority of infections were treated with oral rehydration alone. Patients may also report chronic diarrhea for 2 weeks or longer (76, 84). Association of *P. shigelloides* with diarrheal disease has been hampered by the inability to demonstrate putative virulence factors and the lack of an animal model. However, *Plesiomonas* has recently been observed both in membrane-bound vacuoles and free within the cytosol of cultured human colon cells examined by transmission electron microscopy (136). A beta-hemolysin described previously and now shown to be calcium and iron dependent probably plays a role in the infectious process by releasing *P. shigelloides* from intracellular vacuoles (12, 72). The dysenteric form of diarrhea seen with some patients may be explained by the ability to invade and multiply within human gastrointestinal cells. Association of *Plesiomonas* with secretory diarrheal infections is more tenuous, although cholera-like, heat-stable, and heat-labile toxins have been described (70). The lipopolysaccharide of *Plesiomonas* may also play a role in the infectious process in view of the fact that the O17 antigen belonging to the most common serogroup of *Plesiomonas* and the form 1 antigen of *S. sonnei* share almost identical gene regions (29). Although plesiomonads have an aquatic reservoir, wound infections associated with water contact and similar to those found with *Aeromonas* spp. are not encountered. *Plesiomonas* has been isolated from a wide range of mammals (other than humans),

birds, fish, and water-dwelling reptiles and amphibians, but with the possible exception of cats, there is no evidence to suggest that it plays a role in diarrheal disease in any of these species (69).

Edwardsiella tarda is typically associated with animals that inhabit water. It is an infrequent cause of gastroenteritis in humans, with most infections occurring after contact with fish or turtles. However, a low carriage rate in humans, except in tropical areas of the world, and the ability to produce a cell-associated hemolysin and invade HEp-2 cells suggest that *E. tarda* is a diarrheal agent (76). Serious wound infections, including myonecrosis, have been reported in immunocompetent individuals who had aquatic exposure (132). Systemic infections usually occur in patients with liver disease or iron overload conditions.

Except for *E. tarda*, the other *Enterobacteriaceae* listed in the left-hand column of Table 2 cause opportunistic infections and are not frequently encountered in clinical laboratories (71). Some, like *Cedecea* spp., *Leminorella* spp., *Moellerella,* and *Tatumella,* are rarely isolated from nonhuman sources, making it difficult to determine reservoirs for these organisms (53, 59, 60, 65, 71). Strains of *Ewingella, Leclercia,* and *Kluyvera* spp. have been found in a variety of foods, water, or animals (snails and slugs) and are probably, like many *Enterobacteriaceae,* ubiquitous in the environment (40, 54, 71, 135). Other genera that have been isolated from human specimens have more specific natural habitats. These include *Rahnella* (71), all of whose initially described isolates were recovered from water, which probably serves as the reservoir for human infections, or *Yokenella* and both subspecies of *Photorhabdus asymbiotica,* which are common in insects and have caused infections following insect bites (3, 41, 88).

ISOLATION

For the most part, none of the clinically relevant strains covered in this chapter present difficulties in isolation from sterile body sites. Cockerill et al. (31) did find, however, that in blood cultures both *E. cloacae* and *S. marcescens* grew significantly better in aerobic culture than in nonvented or anaerobic culture; no difference was noted for other major species of the genera included in this chapter. Isolation from nonsterile body or environmental sites may require specialized media.

A number of chromogenic media have been developed to isolate and differentiate urinary tract pathogens based on the colony color produced by the enzymatic action of the organism on chromogenic substrates in the medium. Of these, CHROMagar Orientation (Becton Dickinson, Sparks, Md.) and CPS ID2 (bioMérieux, Hazelwood, Mo.) perform similarly for the detection of the UTI pathogens covered in this chapter and can reliably replace commonly used media such as MacConkey and blood agars (BA) (26, 128). However, CHROMagar is more sensitive for the isolation of *E. coli* because it detects beta-galactosidase while CPS ID2 detects beta-glucuronidase (26, 128). Although *Proteus, Providencia, Morganella, Klebsiella, Citrobacter,* and *Enterobacter* are all easily identified on both of these media, further biochemical tests are required to identify them to the species level. Both media prevent swarming of *Proteus,* and mucoid colonies allowing for easier detection of multiple pathogens and antimicrobial susceptibility tests can be performed directly without the need for subculturing. Ohkusu (115) expanded the usage of CHROMagar Orientation to cover isolation of pathogenic organisms from wound, stool, and a variety of other specimens in addition to urine. Using colony color on CHROMagar in combination with indole, lysine, and ornithine decarboxylase tests and serology, 466 of 472 (98.7%) isolates were correctly identified.

Both of the diarrheal pathogens covered in this chapter are easily isolated. *E. tarda,* which is a lactose-negative, H_2S-positive organism, is indistinguishable from *Salmonella* on enteric plating media (opaque or opaque with black centers). A positive indole reaction and lack of agglutination in specific *Salmonella* antisera differentiate *E. tarda* strains. *Plesiomonas* grows as 2- to 3-mm, opaque, convex colonies on BA or as non-lactose-, non-sucrose-fermenting colonies on enteric plating media. It does not grow on thiosulfate-citrate-bile salts-sucrose medium but does grow well on, and can be isolated from, cefsulodin-irgasan-novobiocin (CIN) medium. Because *Plesiomonas* does not ferment mannitol, the colonies are opaque without a pink center on CIN medium. It must be distinguished from other oxidase-positive organisms *(Pseudomonas, Aeromonas)* that can also grow on this medium, although *Aeromonas* should have a pink center with an opaque apron. Inositol fermentation and a positive reaction in Moeller's lysine, arginine, and ornithine tests differentiate *Plesiomonas* from other organisms.

Other *Enterobacteriaceae* that are involved in opportunistic infections and that may be isolated from a variety of specimen types generally grow well on commonly used laboratory media (71). Some genera are lactose or sucrose fermenters and give the appearance of normal flora on enteric plating media, while others may produce H_2S and appear *Salmonella*-like. *Rahnella, Ewingella,* and *Tatumella* may require 48 h for growth. *Tatumella* also grows poorly on Mueller-Hinton agar, and a broth dilution method may be required for susceptibility testing. Strains of *Tatumella* do not survive well at room temperature and should be stored at −70°C.

K. (C.) granulomatis, as with many of the organisms in the third column of Table 2, does not grow on conventional laboratory media. Since it does not stain well with gram reagents, Giemsa- or Wright-stained Donovan bodies have been the method most commonly used to detect this organism. However, Donovan bodies, which are pleomorphic, bipolar staining bodies shaped like a closed safety pin, are not always present and are not reliable for diagnosis. Recently, growth in HEp-2 monolayers has been achieved (27). Primers to the *phoE* and *scrA* (sucrose regulon) genes have been used to identify and distinguish *K. (C.) granulomatis* (*phoE* positive) from *Klebsiella* species (*phoE* and *scrA* positive) (28).

IDENTIFICATION

The biochemical tests most useful for differentiating species within each genus are given in Tables 3 through 13. Full biochemical profiles for most species can be found in chapter 42. Correct identification to the species level is increasingly important in recognizing strains that are of high risk for carrying extended-spectrum beta-lactamases, cephalosporinases, or carbapenemases (95). Identification problems arising from the use of commercial systems vary with each genus. Of the *Klebsiella, K. ozaenae* and *K. rhinoscleromatis* do the poorest in commercial systems, probably as a result of slow growth. These species can be difficult to distinguish using conventional biochemicals as well. *R. (Klebsiella) planticola* and R. *(Klebsiella) terrigena* cannot be readily separated from other *Klebsiella* without temperature growth or carbon assimilation tests, neither of which are readily available in most clinical laboratories.

TABLE 3 Separation of members of the genus *Citrobacter*[a]

Species	Indole	ODC	Malonate	Acid[b] from:			
				Sucrose	Dulcitol	Melibiose	Adonitol
C. amalonaticus	+	+	−	−	−	−	−
C. braakii	V	+	−	−	V	+	−
C. farmeri	+	+	−	+	−	+	−
C. freundii (sensu stricto)	V	−	V	V	V	+	−
C. koseri	+	+	+	V	V	−	+
C. rodentium	−	+	+	−	−	−	−
C. sedlakii	V	+	+	−	+	+	−
C. werkmanii	−	−	+	−	−	V	−
C. youngae	V	−	−	V	V	+	−
C. gillenii	−	−	+	V	−	V	−
C. murlinae	+	−	−	V	+	V	−

[a] Abbreviations and symbols: ODC, ornithine decarboxylase; +, ≥85%; V, 15 to 85%; −, ≤15%.
[b] Fermentation reactions in commercial systems should be similar to reactions in conventional fermentation broths (1% carbohydrate in broth with indicator).

Growth at 10°C, utilization of histamine, and no gas production from lactose at 44.5°C can be used to separate *Raoultella* spp. from *K. pneumoniae* and *K. oxytoca; R. terrigena* can be distinguished from *R. planticola* by fermentation of β-gentibiose. The new species, *K. variicola*, is not in commercial systems; *K. variicola* strains do not ferment adonitol (some strains are L-rhamnose negative as well), and reportedly only this trait separates it from *K. pneumoniae* (124).

Members of the genus *Enterobacter* appear to confound commercial systems more often than other genera in the *Enterobacteriaceae,* probably because of the heterogeneity of several of the species (71). Most major commercial systems include at least nine *Enterobacter* species, and they all identify the three major *Enterobacter* species *(E. cloacae, E. aerogenes,* and *E. sakazakii)* at an acceptable accuracy rate of ≥90% (71). The percentage of correct identifications with many

TABLE 4 Differentation of *Pantoea agglomerans* and members of the genus *Enterobacter*[a,b]

Species	LDC	ADH	ODC	VP	Acid[c] from:							Yellow pigment
					Sucrose	Adonitol	D-Sorbitol	L-Rhamnose	α-Methyl-D-glucoside	Esculin	Melibiose	
Human species												
E. aerogenes	+	−	+	+	+	+	+	+	+	+	+	−
P. agglomerans	−	−	−	V	V	−	V	V	−	V	V	V
E. amnigenus biogroup 1	−	−	V	+	+	−	−	+	V	+	+	−
E. asburiae	−	V	+	−	+	−	+	−	+	+	−	−
E. cancerogenus	−	+	+	+	−	−	−	+	−	+	−	−
E. cloacae subsp. *cloacae*	−	+	+	+	+	V	+	+	V	V	+	−
E. cowanii[d]	−	−	−	+	+	−	+	+	−	+	+	V
E. gergoviae	+	−	+	+	+	−	−	+	−	+	+	−
E. hormaechei subsp. *hormaechei*	−	V	+	+	+	−	−	+	V	−	−	−
E. kobei	−	+	+	−	+	−	+	+	+	V	+	−
E. sakazakii	−	+	+	+	+	−	−	+	+	+	+	+
Environmental species												
E. amnigenus biogroup II	−	V	+	+	−	−	+	+	+	+	+	−
E. cloacae subsp. *dissolvens*	−	+	+	+	+	−	+	+	+	+	+	−
E. nimipressuralis	−	−	+	+	−	−	+	+	+	+	+	−
E. pyrinus[e]	NA	NA	NA	+	+	−	−	+	−	+	+	−
E. radicincitans	−	+	−	+	+	−	+	+	−	+	−	−

[a] See the text for *E. ludwigii* and *E. hormaechei* subsp. *steigerwaltii* and *oharae* identification.
[b] Abbreviations and symbols: LDC, lysine decarboxylase; ADH, arginine dihydrolase; ODC, ornithine decarboxylase; VP, Voges-Proskauer; +, ≥90%; V, 11 to 89%; −, ≤10%; NA, not available.
[c] See Table 3, footnote *b*.
[d] Separated from *P. agglomerans* by a negative malonate reaction and fermentation of D-sorbitol (68).
[e] Separated from *E. gergoviae* by positive reactions in potassium cyanide broth and *myo*-inositol.

TABLE 5 Separation of some members of the genera *Klebsiella* and *Raoultella*[a,b]

Species	Indole	ODC	VP	Malonate	ONPG	Growth at:		Acid[c] from D-melezitose
						10°C	44°C	
R. ornithinolytica	+	+	V	+	+	+	NA	NA
K. oxytoca	+	−	+	+	+	−	+	−
K. ozaenae	−	−	−	−	V	NA	NA	NA
K. pneumoniae[d]	−	−	+	+	+	−	+	−
R. planticola	V	−	+	+	+	+	−	−
R. terrigena	−	−	+	+	+	+	−	+
K. rhinoscleromatis	−	−	−	+	−	NA	NA	NA

[a] *K. singaporensis* biochemicals are not available; only a single strain is known.
[b] Abbreviations and symbols: ODC, ornithine decarboxylase; VP, Voges-Proskauer; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; NA, not available; +, ≥90%; V, 11 to 89%; −, ≤10%.
[c] See Table 3, footnote *b*.
[d] *K. variicola* is separated from *K. pneumoniae* by negative adonitol reaction; some strains are also L-rhamnose negative.

commercial systems increases significantly when "low probability" identifications are included as correct identifications or when additional biochemical tests are performed following initial testing results. Neither *E. ludwigii* nor the subspecies of *E. hormaechei* can be separated from other clinically relevant *Enterobacter* spp. by biochemicals readily available in most clinical laboratories. In the Biotype 100 system (bioMerieux, Marcy l'Etoile, France), *E. ludwigii* can be separated from *E. cloacae,* the most common species that it resembles phenotypically, by growth on 3-0-methyl-D-glucopyranose and putrescine (64). Likewise, *E. hormaechei* subspecies can be separated from *E. cloacae* in the Biotype 100 system by growth on L-fucose and 1-0-methyl-α-galactopyranoside for subsp. *hormaechei,* growth on D-arabitol and adonitol for subsp. *steigerwaltii,* and growth on 3-hydroxybutyrate for subsp. *oharae* (63).

Serratia spp. are generally easily identified by commercial systems, except for the *S. liquefaciens* group; differentiation of members within this group requires carbon assimilation tests (71). These tests can be performed using a combination of API 50 CH and API ZYM (carbohydrate and enzymatic panels, respectively) (bioMérieux, Hazelwood, Mo.) strips (50).

Most automated system databases now contain the newer *Citrobacter* spp., at least to subgroups *(C. braakii-C. freundii-C. sedlakii, C. werkmanii-C. youngae,* and *C. koseri-C. amalonaticus)*; however, subgroup identification requires further biochemical testing by standard methodologies to identify strains to the species level, and this delays final identification (71). A PYR (L-pyroglutamic acid to detect pyrrolidonyl peptidase [Oxoid PYR Test]) test may be useful for separating biochemically atypical strains of *Citrobacter* (positive) and *Salmonella* (negative) (15).

Two members of the genus *Proteus* can be rapidly identified with minimal testing. Gram-negative and oxidase-negative organisms with colonies that swarm on BA and appear flat with tapered edges on MacConkey agar may be reported as *Proteus*. *Proteus* spp. that are spot indole negative and ampicillin susceptible may be reported as *P. mirabilis* (13).

TABLE 6 Biochemical characterization of members of the genus *Serratia*[a,b]

Species	LDC	ODC	Mal	Acid[c] from:								Red pigment	Odor
				Arabinose	L-Rhamnose	D-Xylose	Sucrose	Adonitol	D-Sorb	Cello	D-Ara		
S. entomophila[d]	−	−	−	−	−	V	+	−	−	−	V	−	−
S. ficaria	−	−	−	+	V	+	+	−	+	+	+	−	V
S. fonticola	+	+	V	+	V	V	V	+	+	−	+	−	−
S. liquefaciens group	+	+	−	+	V	+	+	−	+	−	−	−	−
S. marcescens subsp. *marcescens*	+	+	−	−	−	−	+	V	+	−	−	V	−
S. marcescens biogroup 1	V	+	−	−	−	−	+	V	+	−	−	NA	−
S. odorifera biogroup 1	+	+	−	+	+	+	+	V	+	+	−	−	+
S. odorifera biogroup 2	+	−	−	+	+	+	−	V	+	+	−	−	+
S. plymuthica[e]	−	−	−	+	−	+	+	−	V	V	−	+	−
S. rubidaea	V	−	+	+	−	+	+	+	−	+	V	+	−

[a] *S. marcescens* subsp. *sakuensis* is reportedly a spore-forming organism; *S. ureilytica* is urea positive; only a single strain is known.
[b] Abbreviations and symbols: LDC, lysine decarboxylase; ODC, ornithine decarboxylase; Mal, malonate; D-Ara, D arabitol; D-Sorb, D-sorbitol; Cello, cellobiose; +, ≥90%; V, 11 to 89%; −, ≤10%; NA, information not available.
[c] See Table 3, footnote *b*.
[d] Growth at 37°C but biochemical characterization optimal at 30°C.
[e] May fail to grow at 37°C.

TABLE 7 Separation of members of the genera *Proteus, Providencia,* and *Morganella*[a]

Organism	Indole	H_2S	Urea	ODC	Acid[b] from:				
					Maltose	D-Adonitol	D-Arabitol	Trehalose	*myo*-Inositol
Proteus									
P. hauseri	+	V	+	−	+	−	−	+	−
P. mirabilis	−	+	+	+	−	−	−	+	−
P. penneri	−	V	+	−	+	−	−	V	−
P. vulgaris[c]	+	V	+	−	+	−	−	−	−
Providencia									
P. alcalifaciens	+	−	−	−	−	+	−	−	−
P. heimbachae	−	−	−	−	V	+	+	−	V
P. rettgeri	+	−	+	−	−	+	+	−	+
P. rustigianii	+	−	−	−	−	−	−	−	−
P. stuartii	+	−	V	−	−	−	−	+	+
Morganella									
M. *morganii* subsp. *morganii*	+	−[d]	+	+[e]	−	−	−	−	−
M. *morganii* subsp. *sibonii*	V	−[d]	+	+[e]	−	−	−	+	−

[a] Abbreviations and symbols: H_2S, hydrogen sulfide; ODC, ornithine decarboxylase; +, ≥90%; V, 11 to 89%; −, ≤10%.
[b] See Table 3, footnote *b*.
[c] *P. vulgaris* genomospecies 4, 5, and 6 cannot be differentiated phenotypically.
[d] Some members of some biogroups are H_2S positive.
[e] Some members of some biogroups are ornithine decarboxylase negative.

P. penneri, which is a rare clinical isolate, also fits the above description but can be separated from *P. mirabilis* by its negative reactions in ornithine decarboxylase and maltose. Spot indole-positive, ampicillin-resistant strains are reported as *P. vulgaris* (13). *P. hauseri,* previously a subgroup of *P. vulgaris,* can be differentiated from *P. vulgaris* by a negative salicin or esculin reaction (110). Although it is found in clinical specimens, *P. hauseri* is infrequently seen in the laboratory (75). If an organism does not fit any of the above qualifications, it must be fully identified by commercial or conventional biochemical methods (13). Most commercially available systems satisfactorily identify *Proteus,* with reports varying between 95 and 100% accuracy for different systems. *Providencia* spp., however, are not identified with the same level of accuracy in commercial systems, and the rates vary from 79 to 100% (110). When *Providencia* spp. are misidentified, they are usually called *Morganella* or *Proteus*. Urea-positive *P. stuartii* may be misidentified as *P. rettgeri,* or the system may require additional tests for identification. *P. heimbachae* is not in commercial system databases (110). M. *morganii* subsp. *morganii* is identified 100% of the time in commercial systems according to most studies, although 2-h identification methods misidentify it about 66% of the time. M. *morganii* subsp. *sibonii* is not included in most databases and may be distinguished from subsp. *morganii* only by a positive reaction in trehalose.

Hafnia alvei, which biochemically most closely resembles members of the genus *Enterobacter,* and *Yokenella regensburgei* (Table 11) are usually correctly identified in commercial systems. Biogroup 2 strains of *H. alvei* can be separated from *H. alvei* sensu stricto by a positive malonate reaction (74).

Averyella dalhousiensis is not included in commercial system databases; it is an indole-, VP-, H_2S-negative, lysine decarboxylase-positive organism that gives variable reactions in citrate, urea, and dulcitol (78). It most closely resembles *Kluyvera ascorbata* but differs from it by negative reactions in indole, melibiose, and mucate. It also may be misidentified as *Salmonella enterica* in commercial systems but can be differentiated from *Salmonella* subgroup 1 strains by a negative reaction in H_2S, a positive reaction for urease at 48 h, and fermentation of cellobiose. *Averyella* strains are *o*-nitrophenyl-β-D-galactopyranoside (ONPG)-, malonate-, and potassium cyanide-positive and ferment dulcitol and salicin, traits shared with *Salmonella* subgroups 2, 3, and 4.

The two agents of gastroenteritis, *Plesiomonas* (Table 13) and *E. tarda* (Table 12), are easily separated both from other

TABLE 8 Separation of *Cedecea* from selected *Enterobacter* species (VP, ADH, and ODC variable or positive)[a]

Organism	Acid[b] from:					
	D-Sorbitol	Raffinose	L-Rhamnose	Melibiose	D-Arabitol	Sucrose
C. davisae	−	−	−	−	−	+
C. lapagei	−	−	−	+	+	−
E. cloacae	+	+	+	+	V	−
E. sakazakii	−	+	+	+	−	+
E. cancerogenus	−	−	+	−	−	−

[a] Abbreviations and symbols: VP, Voges-Proskauer; ADH, arginine dihyrolase; ODC, ornithine decarboxylase; +, ≥90%; V, 11 to 89%; −, ≤10%.
[b] See Table 3, footnote *b*.

TABLE 9 Differentiation of *Kluyvera* from commonly seen indole-positive, VP-negative organisms[a]

Organism	Citrate	Urea	LDC	KCN
Kluyvera[b]	−	−	+	+
C. koseri	+	V	−	−
Morganella	−	+	−	+
Providencia	V	+	−	+
E. coli	−	−	+	−

[a] Abbreviations and symbols: VP, Voges-Proskauer; LDC, lysine decarboxylase; KCN, potassium cyanide; +, ≥90%; V, 11 to 89%; −, ≤10%. *Leclercia* and *E. tarda* are also indole positive and VP negative and can be found in Tables 10 and 12, respectively.

[b] Includes *K. intermedia* (*K. cochleae* and *Enterobacter intermedius* combined) (118).

enteric pathogens and from normal flora, and their identification presents no difficulty in either conventional or commercial systems. Sequencing data have shown that the somatic antigen that occurs in the most common serogroup of *Plesiomonas* (serogroup O17) and the form 1 antigen of *S. sonnei* share almost identical gene regions (29). Thus, if *Shigella* serotyping is mistakenly performed with strains of *Plesiomonas,* cross-reactions may occur.

Reactions to identify other enterobacteria isolated from clinical specimens can be found in Tables 8 through 12. The organisms are grouped with the genera with which they would most likely be confused and from which they must be differentiated. Many of the agents in these tables are in commercial system databases. Unfortunately, the number of strains available for use in challenge studies is very limited; therefore, the ability of these systems to accurately identify these organisms is really unknown. *Ewingella* and *Tatumella* are biochemically inactive, and the latter organism grows poorly in vitro. *Kluyvera* can be identified only to genus level by these systems; species determination requires an ascorbate test and irgasan susceptibility and/or gas-liquid chromatography profiles (71). *Photorhabdus asymbiotica* is not included in most commercial databases. *Pantoea dispersa, P. ananatis,* and *Erwinia persicinus* most closely resemble *P. agglomerans*. *P. dispersa* and *E. persicinus* may be distinguished from *P. agglomerans* by negative reactions for raffinose, salicin, and sucrose and by negative reactions for maltose and D-xylose, respectively. *P. ananatis* is more difficult to differentiate, and all suspected isolates of this organism, as with other rare *Enterobacteriaceae,* should be sent to a reference laboratory for confirmation.

At this time, 16S rRNA sequencing is not used extensively to identify this group of organisms and not all species are included in currently available databases. The use of molecular methods (see chapter 16) to determine the presence and/or identity of bacteria directly from clinical specimens or from isolates is increasingly reported but not universally available. These methods offer a great advantage when bacteria are difficult or impossible to culture, such as *K. (C.) granulomatis,* or in the case of diseases such as keratitis, where cultures are negative 40 to 60% of the time (87). Testing of specimens from sterile sites with universal primers based on conserved regions of the bacterial chromosome (with the exception of *K. granulomatis*) can be used to determine if a patient has a bacterial infection in 1 h (16). Normally sterile clinical specimens found negative by these assays would not then require culturing, saving considerably on laboratory resources. However, numerous problems remain to be worked out with these methods for bacterial identification. For rRNA sequencing these include, but are not limited to, DNA extraction protocols, ambiguous profiles arising from testing mixed cultures, the presence of too few species in the databases, and percent similarity cutoff guidelines for identification to the species or genus level (36).

The ability to trace the spread or involvement of nosocomial pathogens in outbreaks caused by the *Enterobacteriaceae* has become a major responsibility for the laboratory. Biotyping using commercially available identification systems is seldom suitably discriminating, unless an unusual marker or profile is present. Biotyping schemes using carbon assimilation tests have been developed, particularly for *Serratia,* but may be difficult and costly to perform (50). Typing all genera for which traditional typing methods are available (serotyping, bacteriocins, and bacteriophages) would necessitate multiple sets of reagents, which are not readily available and/or are economically prohibitive. Molecular techniques including plasmid analysis, ribotyping, pulsed-field gel electrophoresis (PFGE), and various PCR methodologies all appear satisfactorily discriminatory, some working better for a specific genus or species than others (see chapter 16). The variety of PCR techniques is proliferating at an astonishing pace, especially repetitive-element PCR methods for the *Enterobacteriaceae*. Care in the performance and interpretation of these assays is critical since many PCR techniques have not been standardized (38, 48). For now, since a single method applicable to the most strains is preferable, at least economically, PFGE remains the most universally accepted standardized technique for epidemiological studies.

TABLE 10 Separation of LDC-, ODC-, and ADH-negative unusual *Enterobacteriaceae* found in clinical specimens[a]

Organism	Motility	Gas from glucose	KCN	VP	Acid[b] from:		
					Sucrose	L-Arabitol	Trehalose
Ewingella	V	−	−	+	−	−	+
Leclercia	+	+	+	−	+	+	+
Moellerella	+	+	V	−	−	+	+
Rahnella	−	+	−	+	+	+	+
Tatumella	−	−	−	−	+	−	+
Photorhabdus asymbiotica[c]	+	−	−	−	−	−	−

[a] Abbreviations and symbols: LDC, lysine decarboxylase; ODC, ornithine decarboxylase; ADH, arginine dihydrolase; KCN, potassium cyanide; VP, Voges-Proskauer; +, ≥90%; V, 11 to 89%; −, ≤10%. *Budvicia* is also LDC, ODC, and ADH negative and can be found in Table 12.

[b] See Table 3, footnote *b*.

[c] *Photorhabdus* subsp. *australis* can be separated from subsp. *asymbiotica* by fermentation of maltose and glycerol.

TABLE 11 Separation of *Yokenella* from *Hafnia*[a]

Organism	VP	Malonate	Citrate	Acid[b] from:	
				Melibiose	Glycerol
Yokenella	−	−	+	+	−
Hafnia alvei	+	+	−	−	+
H. alvei biogroup 2	+	−	V	−	+

[a] Abbreviations and symbols: VP, Voges-Proskauer; +, ≥90%; V, 11 to 89%; −, ≤10%. Data from reference 74.
[b] See Table 3, footnote *b*.

The disadvantage of a long turn-around time (usually 4 days) has been partially overcome by a rapid PFGE protocol that is suitable for most enteric bacteria as well as other common clinical strains (45).

ANTIMICROBIAL SUSCEPTIBILITY

The number of strains harboring extended-spectrum β-lactamases (ESBL), cephalosporinases and carbapenemases that mediate resistance to the β-lactam antibiotics (Table 14) (for resistance mechanisms, see chapters 71, 74, and 78), and the increase in resistance to quinolones are major issues with members of the *Enterobacteriaceae* (10, 143). In one study, of >380,000 blood, respiratory, and urine isolates of *Enterobacteriaceae* tested against 14 antimicrobials, susceptibilities of <90% were recorded for eight (46 to 89%) and three (57 to 84%) drugs for intensive care unit (ICU) and non-ICU patients, respectively (83). Ceftazidime, cefotaxime, and ceftriaxone susceptibility rates were up to 10% lower for isolates from ICU patients than from non-ICU patients; however, only three isolates were carbapenem resistant, two of which were *E. aerogenes*. Only fluoroquinolone susceptibility decreased over a 4-year period, and fluoroquinolone-resistant *Enterobacteriaceae* were frequently resistant to extended-spectrum cephalosporins, aminoglycosides, and trimethoprim-sulfamethoxazole (SXT) (83). Ciprofloxacin resistance, influenced by antibiotic usage and clonal spread of strains containing ESBLs among patients, has been found in strains of *K. pneumoniae* and *K. oxytoca,* with rates as high as 7.2 and 3.4%, respectively (24). Imipenem resistance has been noted worldwide among the *Enterobacteriaceae;* it typically emerges during long therapy to treat ceftazidime- and aminoglycoside-resistant strains and appears reversible with cessation of therapy (1, 19). Cefepime resistance in *Enterobacter* spp. and *K. oxytoca* strains has been reported (126).

The spread of ESBL-producing strains can be controlled, without restricting antimicrobial usage, by using barrier precautions and cohorting patients after discharge from ICUs, since transmission occurs primarily on the hands of hospital staff (96, 117). However, Paterson and Yu (117) recommend a three-prong effort to control ESBL-producing organisms, which includes enhancing surveillance and control within the ICU, increasing laboratory capability in the detection of ESBLs, and limiting empirical use of antibiotics. Restriction of specific antimicrobials may reduce resistance in one or several species but select for resistance in others, substituting one problem for another (90). Further, resistance to one drug found in a multi-drug-resistant isolate will be maintained or accelerate in patient populations even if use of the drug is discontinued, as long as the other drugs to which the strain is also resistant are prescribed (83).

The effective infection control of organisms harboring ESBLs necessitates that the laboratory provides testing methods that will detect these strains. The question of whether it is cost-effective for laboratories to test for ESBL-producing organisms because their detection isn't likely to affect patient outcome has not been resolved (39). Studies to determine the outcomes of serious infections caused by organisms considered cephalosporin "susceptible" or "intermediate" in vitro found that 54% of patients who were treated with a cephalosporin experienced therapy failure (116). These patients either succumbed to their infections or required a change in therapy to achieve a cure. UTIs caused by ESBL-producing organisms can be successfully treated with cephalosporins, eliminating the need to test urine isolates for ESBL production for therapeutic consideration; however, testing may be required for infection control efforts to prevent horizontal transfer of these strains. Appropriate broth microdilution and disk diffusion testing procedures for ESBL-producing *Klebsiella* spp. are described in chapter 74.

Strains of *P. mirabilis* are resistant to nitrofurantoin but susceptible to trimethoprim-sulfamethoxazole, ampicillin, amoxicillin, piperacillin, cephalosporins, aminoglycosides, and imipenem. Although most strains are susceptible to ciprofloxacin, resistance occurs with unrestricted use of the drug (110). *P. penneri* and *P. vulgaris* have resistance profiles similar to that of *Morganella,* although *P. penneri* is more resistant to penicillin than *P. vulgaris*. All three organisms are susceptible to broad-spectrum cephalosporins, cefoxitin,

TABLE 12 Separation of *Enterobacteriaceae* that may be H_2S positive[a]

Organism	LDC	ODC	Urea	Acid[b] from L-arabinose	Citrate	KCN	ONPG
Leminorella spp.	−	−	−	+	V[c]	−	−
Edwardsiella tarda	+	+	−	−	−	−	−
Budvicia aquatilis[d]	−	−	+	V	−	−	+
Pragia fontium[d]	−	−	−	−	−	−	−
Trabulsiella guamensis[d]	+	+	−	+	V	+	+
Salmonella subgroup 1	+	+	−	+	+	−	−
Citrobacter	−	V	V	+	V	+	+
Proteus	−	V	+	−	V	+	−

[a] Abbreviations and symbols: LDC, lysine decarboxylase; ODC, ornithine decarboxylase; KCN, potassium cyanide; ONPG, *o*-nitrophenol-β-D-galactopyranoside; +, ≥90%; V, 11 to 89%; −, ≤10%.
[b] See Table 3, footnote *b*.
[c] *L. grimontii* is positive, *L. richardii* is negative.
[d] Found in clinical specimens but of questionable or no significance.

TABLE 13 Differentiation of *P. shigelloides* from other clinically significant members of the *Vibrionaceae* and the *Aeromonadaceae*[a]

Organism	LDC	ODC	ADH	Gas from glucose	Acid[b] from:		Growth in:		O/129 susceptibility
					Sucrose	Inositol	TCBS	0% NaCl	
Plesiomonas	+	+	+	−	−	+	−	+	+
Aeromonas spp.	V	−[c]	+	V	V	−	−	+	−
Vibrio spp.[d]	+	+	−	−	V	−	+	V	+

[a] Abbreviations and symbols: LDC, lysine decarboxylase; ODC, ornithine decarboxylase; ADH, arginine dihydrolase; TCBS, thiosulfate-citrate-bile salts-sucrose agar; O/129, 2,4-diamino-6,7-diisopropylpteridine; +, ≥90%; V, 11 to 89%; −, ≤10%.
[b] See Table 3, footnote *b*.
[c] Only *A. veronii* biotype *veronii* is positive.
[d] *V. hollisae* is LDC, ODC, and ADH negative; only *V. fluvialis* and *V. furnissii* are ADH positive; *V. furnissii* produces gas; *V. cincinnatiensis* ferments *myo*-inositol; *V. cholerae* and *V. mimicus* grow in 0% NaCl.

cefepime, aztreonam, aminoglycosides, and imipenem. They are resistant to piperacillin, amoxicillin, ampicillin, cefoperazone, cefuroxime, and cefazolin. *Providencia rettgeri* and *P. stuartii* are resistant to gentamicin and tobramycin but susceptible to amikacin. Urine isolates are susceptible to broad- and expanded-spectrum cephalosporins, ciprofloxacin, amoxicillin-clavulanic acid, imipenem, and SXT. *Providencia heimbachae,* although infrequently seen in humans, is resistant to tetracycline, most cephalosporins, gentamicin, and amikacin. Human isolates of *E. tarda* are susceptible to cephalosporins, aminoglycosides, imipenem, ciprofloxacin, aztreonam, and antibiotic-β-lactamase inhibitor combination agents (30). Isolates from fish and fish ponds may be more resistant because of the prophylactic use of antibiotics in fish farming. Most strains of *E. tarda* produce β-lactamases, even though they are susceptible to β-lactams. *P. shigelloides* is resistant to ampicillin, carbenicillin, piperacillin, and ticarcillin and is variably resistant to most aminoglycosides and tetracycline (70). Cephalosporins, quinolones, carbapenems, and SXT show good activity against *P. shigelloides*.

Freney et al. (44) performed susceptibility studies with 120 isolates of uncommonly seen species of *Klebsiella, Enterobacter,* and *Serratia* and found their susceptibilities to be similar to those of conventional species within each genus. Because they are infrequently seen in clinical laboratories, resistance profiles of many of the other *Enterobacteriaceae* are found only in individual case reports. Susceptibilities vary from isolate to isolate even within a genus, so that no empirical guidelines are available for therapy prior to susceptibility testing of the suspected strain.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

When any of the species included in this chapter are identified with a high level of accuracy (>90% probability) by a commercial system, the identification is probably reliable. However, if the organism is isolated from a source where it may be considered significant, such as blood or

Table 14 Extended-spectrum β-lactamases, cephalosporinases, and carbapenemases of the *Enterobacteriaceae*[a]

Ambler class	Bush class	Enzyme class	Substrate	Clavulanic acid	Organism	Location
Serine β-lactamase						
A	2b	Restricted-spectrum β-lactamase	Aminopenicillins, carboxypenicillins	S	*K. pneumoniae* Several other genera	Plasmid Chromosome
A	2be	Extended-spectrum β-lactamase	Extended-spectrum β-lactams	S	*Klebsiella* spp., *S. marcescens, Enterobacter* spp., *Proteus* spp., *C. freundii, M. morganii*	Plasmid
A	2f	Carbapenemase	Carbapenems, aztreonam	S	*Enterobacter* spp., *S. marcescens*	Chromosome
A	2e	Cefuroximase	Cephalosporins	S	*Proteus* spp., *C. koseri*	Chromosome
C	1	Cephalosporinase	Extended-spectrum β-lactam, cephamycins, aztreonam	R	*K. pneumoniae,* several other genera	Plasmid
C	1	Cephalosporinase	Extended-spectrum β-lactams, aztreonam	R	*Enterobacter* spp., *C. freundii, S. marcescens, Proteus* spp., *M. morganii*	Chromosome
Metallo-β-lactamase						
B	3	Carbapenemase	Oxyamino-cephalosporins, aztreonam	R	*K. pneumoniae* *S. marcescens*	Plasmid Chromosome

[a] Abbreviations: S, susceptible; R, resistant. Data from references 25 and 109.

cerebrospinal fluid, and the identification has a probability of <90%, the isolate should be identified by conventional methods or sent to a reference laboratory using these techniques. In the interim, the isolate may be reported to the physician with a presumptive identification. For those strains seen more commonly, the antimicrobial susceptibility profile may be a helpful adjunct for deciding if identifications with lower probabilities are reliable. Rare species that are identified with low probabilities should always be sent to a reference laboratory accompanied by a brief history. Colony appearance on MacConkey and blood agar plates, spot oxidase and indole reactions, and ampicillin susceptibility or resistance are sufficient for reporting *P. mirabilis* and *P. vulgaris* (13). At the very least, susceptibility testing of strains of *Klebsiella* that appear to contain ESBLs should be tested by the methods described in chapter 74. Any strain of *Enterobacteriaceae* that has been shown to have an ESBL or AmpC cephalosporinase should be reported as resistant to all penicillins, expanded-spectrum cephalosporins, and aztreonam (117).

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Aeromonas

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TAXONOMY

The genus *Aeromonas* resides within the family *Aeromonadaceae* (8) and the newly proposed order *Aeromonadales,* ord. nov., along with the genera *Oceanimonas* and *Tolumonas* (30). *Aeromonas* is the only one of these three genera that is pathogenic for humans. The frequent reclassifications and constant amended or extended descriptions within *Aeromonas* taxonomy can often be initially puzzling to microbiologists not working with these organisms on a daily basis; however, the information in this chapter should clarify the identification and significance of those species most often associated with human disease (Table 1). DNA hybridization group numbers, which no longer serve a meaningful purpose, and synonymous species designations for *Aeromonas veronii* bv. sobria *(A. ichthiosmia)* and *A. trota (A. enteropelogenes)* (7) are not included for simplicity. *Aeromonas* group 501, which is made up of *A. schubertii*-like organisms, and *Aeromonas* sp. DNA hybridization group 11 (31), which is made up of *A. eucrenophila*/*A. encheleia*-like organisms, are also not addressed in the table. These groups contain few strains, their taxonomic status has yet to be resolved and is still highly debated, and most importantly, neither group has been shown to be significant in human or animal disease. Newly proposed *Aeromonas* species and subspecies since the publication of the previous edition of this Manual include *A. hydrophila* subspecies *hydrophila,* sensu stricto (19); *A. hydrophila* subsp. *dhakensis,* isolated from pediatric diarrheal cases in Bangladesh (19); *A. hydrophila* subsp. *ranae,* isolated from septic frogs in Thailand (20); *A. culicicola,* isolated from mosquitoes and drinking water (11, 38); *A. simiae,* isolated from monkey feces (16); and *A. molluscorum,* isolated from bivalve mollusks (34). Because of its clinical significance, it should be noted that clinical strains formerly referred to as "*A. sobria*" are, in fact, *A. veronii* bv. sobria (esculin hydrolysis and ornithine decarboxylase negative and arginine dihydrolase positive) and should be reported as such. Nearly all rapid identification databases, excepting API 20E strips (BioMerieux, Hazelwood, Mo.), have converted their *A. sobria* identifications to *A. veronii* bv. sobria. This is especially important because of *A. veronii* bv. sobria's association with more severe, extraintestinal infections, such as septicemia, meningitis following leech therapy, and disseminated intravascular gas production (36, 43). It usually is not necessary to definitively separate members of the *A. hydrophila* complex (*A. hydrophila, A. bestiarum,* and *A. salmonicida)* or the *A. caviae* complex (*A. caviae, A. media,* and *A. eucrenophila),* especially when they are isolated from feces (see "Interpretation and Reporting of Results" below).

The type strain *Aeromonas hydrophila* ATCC 7966^T recently had its 4.7-megabase genome completely sequenced and is undergoing manual annotation at The Institute for Genomic Research (TIGR). Preliminary data can be accessed at the TIGR Homepage at http://www.tigr.org under the link for "Unfinished Genomes," and the entire genome will eventually be available in GenBank at NCBI.

DESCRIPTION OF THE GENUS

Members of the genus *Aeromonas* are gram-negative facultative anaerobes that are straight, coccobacillary to bacillary cells with rounded ends, 0.3 to 1.0 μm in diameter and 1.0 to 3.5 μm in length. They can occur singly, in pairs, or rarely in short chains. Most species are motile by a single, polar flagellum of 1.7-μm wavelength, but peritrichous flagella may be formed on solid media in young cultures and lateral flagella occur in some species. Aeromonads are usually oxidase positive and catalase positive and are generally resistant to 150 μg of the vibriostatic agent 2,4 diamino-6,7-diisopropylpteridine (O/129). They are chemoorganotrophic, displaying oxidative and fermentative metabolism of glucose. Acid and often acid with gas are produced from many carbohydrates, especially glucose, and nitrate is reduced to nitrite. A variety of exoenzymes such as arylamidases, amylase, DNase, esterases, peptidases, proteases, chitinase, chondroitinase, and hemolysins are produced. The main cellular fatty acids produced are hexadecanoic acid (16:0), hexadecenoic acid (16:1), and octadecenoic acid (18:1). Human (mesophilic) strains grow between 10 and 42°C, but occasional isolates may be more active in some biochemical assays at 22 to 25°C. Psychrophilic strains from fish and the environment *(A. popoffii* and *A. salmonicida)* seldom grow above 37°C and preferentially grow at 22 to 25°C. In brain heart infusion broth at 28°C, growth occurs between pH 4.5 and 9.0 and at salt concentrations between 0 and 4%. The mol% G+C of the DNA is 57 to 63%.

NATURAL HABITATS

Aeromonads are inhabitants of aquatic ecosystems worldwide. These include groundwater and drinking water at treatment plants and in distribution systems and reservoirs as well as

TABLE 1 Members of the genus *Aeromonas*[a]

Organism	Human isolation (extraintestinal/fecal)	Human pathogen (extraintestinal/fecal)	Frequency in humans	Pathogenic for animals, fish, reptiles
A. hydrophila complex				
A. hydrophila				
subsp. *hydrophila*	Yes	Yes	Yes	Yes
subsp. *dhakensis*	Yes	Yes	Rare	No
subsp. *ranae*	No	No	—	Yes
A. bestiarum	No/yes	—/no	One case	Yes
A. salmonicida[b]	No/yes	No/no	Rare	
subsp. *salmonicida*				Yes
subsp. *achromogenes*				Yes
subsp. *masoucida*				Yes
subsp. *smithia*				Yes
subsp. *pectinolytica*				No
A. caviae complex				
A. caviae	Yes	Yes	Common	Yes
A. media	No/yes	—/yes	Rare	No
A. eucrenophila	Yes	No/—	Very rare	No
A. veronii complex				
A. veronii bv. sobria	Yes	Yes	Common	Yes
A. veronii bv. veronii	Yes	Yes	Rare	No
A. jandaei	Yes	Yes/unknown	Rare	No
A. trota	Yes	Neither	Rare	No
A. schubertii	Yes/no	Yes/—	Rare	No
A. encheleia	Yes/no	No/—	One case	No
A. allosaccharophila	No/yes	—/no	Rare	Yes
A. sobria	Neither	—	—	No
A. popoffii	Yes	Yes	Rare	No
A. culicicola	No	No	—	No
A. simiae	No	No	—	No
A. molluscorum	No	No	—	No

[a] Abbreviations and symbols: bv., biovar; —, not applicable.
[b] There are motile strains of *A. salmonicida* that grow at 37°C and resemble clinical *A. hydrophila* strains that have been isolated from human feces; these can be distinguished using the tests in Table 3.

clean or polluted lakes and rivers. *Aeromonas* may also be found in marine environments but only in brackish water or water with a low saline content. Most *Aeromonas* species, particularly those associated with human infections, are found in a wide variety of fresh produce, meat (beef, poultry, and pork), and dairy products (raw milk and ice cream) (24). *A. veronii* bv. sobria is a symbiont in the gut of medicinal leeches, where it may grow as a pure culture (15). In fisheries, psychrophilic strains of *Aeromonas* cause severe infections resulting in considerable economic loss. Infections in frogs, pigs, cattle, birds, and marine animals have also been reported (24).

CLINICAL SIGNIFICANCE

Aeromonas gastroenteritis ranges from an acute watery diarrhea (most common form) to dysenteric illness to chronic illness. Stools from acute watery diarrhea are loose (take the shape of their container), and erythrocytes and fecal leukocytes are absent. Accompanying symptoms include abdominal pain (60 to 70%), fever and vomiting (20 to 40%), and nausea (40%) (22). Infections are usually self-limiting, but children may require hospitalization due to dehydration. *A. caviae* is the most common species associated with these infections and can even mimic inflammatory bowel disease in children (48). *A. veronii* bv. sobria strains may be associated with rare cholera-like disease characterized by abdominal pain (60%) and fever and nausea (20%) (22). In dysenteric diarrhea resembling shigellosis, patients suffer from severe abdominal pain and have bloody stools containing mucus and polymorphonuclear leukocytes. About 10 to 15% of patients with either cholera-like or dysenteric diarrhea are coinfected with another enteric pathogen(s).

A comprehensive study done in Bangladesh in 2000 found that the presence of loose stools was associated with *Aeromonas* strains possessing an *alt* gene encoding a heat-labile cytotonic enterotoxin (4). Patients with more severe disease and watery diarrhea had strains that possessed both the *alt* gene and a second gene, *ast,* which encodes a heat-stable cytotonic enterotoxin. A total of seven different *Aeromonas* species were associated with diarrhea in this study. This was followed by a large 2003 study of traveler's diarrhea associated with *Aeromonas* species in Spain, where the predominant species isolated were *A. veronii* bv. veronii and *A. caviae* (49). Finally, a large 2004 study completed in Kolkata, India, found seven different species of *Aeromonas* among hospitalized diarrheal cases, with *A. caviae* predominating, followed by *A. hydrophila* and *A. veronii* bv. sobria. They also found the *alt* and *act* genes in 71.9 and 20.1% of the isolates, respectively, and only 2.4% of the isolates carried the *ast* gene (44).

Complications from *Aeromonas* diarrheal disease include hemolytic uremic syndrome (5) or kidney disease requiring

kidney transplantation (12). These more severe infections are usually associated with *A. hydrophila* or *A. veronii* bv. sobria. Also, nonresolvable, intermittent diarrhea can occur months after the initial infection and may persist for months or several years.

Aeromonas can also be isolated from a variety of extraintestinal sites, although blood and wounds are the most common sources. *Aeromonas* septicemia occurs rarely in immunocompetent hosts; most cases involve patients with liver disease and hematological malignancies. The more commonly isolated species from septicemia are *A. hydrophila, A. veronii* bv. sobria, and *A. jandaei,* with a recent third case report (33) of septicemia with an *A. veronii* bv. veronii strain (arginine dihydrolase negative, esculin hydrolysis positive, and ornithine decarboxylase positive). Fatality rates in these infections range from 30 to 50%. Wound infections are usually preceded by traumatic injury that occurs in contact with water, where the predominant species is *A. hydrophila.* These infections range from uncomplicated cases of cellulitis to myonecrotic infections with a poor prognosis. Two such scenarios are the reported outbreak of wound infections with *A. hydrophila* associated with mud football (47) and wound infections among the 2004 Asian tsunami survivors (29). Surveys indicate that only 17 to 52% of *Aeromonas* wound infections are monomicrobic (24). Use of medicinal leeches postoperatively to enhance blood flow to surgical sites has resulted in wound infection rates of 20%, primarily with *A. veronii* bv. sobria (15). Other extraintestinal infections include ocular, respiratory, and urinary tract infections; meningitis; osteomyelitis; cholecystitis; endocarditis; and peritonitis (23). Two recent examples were the isolation of *A. caviae* from keratitis associated with contact lens wear (39) and the isolation of *A. popoffii* from a urinary tract infection (18).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Aeromonads survive well in specimens; any of the widely used transport media are acceptable for transport, including buffered glycerol in saline (chapter 20). Feces are always preferable to rectal swabs for isolation of enteric pathogens, and stools should be collected in the acute phase of disease.

ISOLATION PROCEDURES

Aeromonads generally grow well on a variety of enteric differential and selective agars, although sucrose- and/or lactose-fermenting strains usually resemble nonpathogens on these media. Blood agar (BA) with 20 μg of ampicillin per ml (ABA) is useful for isolating all *Aeromonas* species except *A. trota,* which is intrinsically susceptible to ampicillin, and a substantial percentage (15 to 57%) of *A. caviae* isolates (6, 27). Since most clinically relevant species are beta-hemolytic, including an increasing number of *A. caviae* strains, beta-hemolytic colonies on BA should be screened with oxidase and a spot indole test. Any colonies positive for both tests should be characterized further, although occasional indole-negative *A. caviae* isolates and nearly all known *A. schubertii* strains (which are generally associated with severe aquatic wounds) are indole negative (2). Modified cefsulodin-Irgasan-novobiocin (CIN) (which contains 4 μg of cefsulodin per ml versus 15 μg/ml in unmodified CIN) is also an excellent isolation medium for aeromonads. On this medium, *Aeromonas* colonies have a pink center with an uneven, clear apron and are indistinguishable from *Yersinia enterocolitica* morphologically. One can incubate CIN at 25°C to enhance the recovery of *Yersinia* and still be able to recover *Aeromonas* within 24 h at this temperature. For optimal isolation, use of both ABA and modified CIN is recommended (26). A xylose-galactosidase medium (XGM), containing novobiocin, bile salts, xylose, and two galactopyranosides, has been evaluated in Europe for the isolation of *Aeromonas, Salmonella, Shigella,* and *Yersinia* spp. (14). *Aeromonas* species, which form green colonies, were isolated more frequently from XGM agar (36%) than from any other medium except CIN (43%), but XGM had fewer false positives (11% for XGM versus 60% for CIN). Thiosulfate-citrate-bile salts-sucrose (TCBS) medium is usually inhibitory to aeromonads. Enrichment in alkaline peptone water enhances recovery of *Aeromonas* from populations that generally would be expected to shed low numbers of organisms (carriers, convalescent-phase patients, and those with subclinical infections). For patients with acute diarrhea, enrichment is probably unnecessary (40).

IDENTIFICATION

Aeromonas spp. are most easily confused in the laboratory with other oxidase-positive fermenters, i.e., *Vibrio* and *Plesiomonas* spp. *Plesiomonas* is easily differentiated from *Aeromonas* by positive reactions in Moeller's lysine, ornithine, and arginine tests and by fermentation of *m*-inositol. Vibrios may be more difficult to distinguish from aeromonads (1), which is particularly true for *Vibrio fluvialis* and *A. caviae,* and in laboratories where the sole means of identification is a rapid miniaturized system (21, 45). Resistance to the vibriostatic agent O/129 and the inability to grow in salt concentrations of ≥6% usually indicate the genus *Aeromonas*. *Vibrio cholerae* O139, a cholera toxin-positive, non-salt-requiring, O/129-resistant vibrio, is a major exception to this rule. However, the decarboxylase pattern (positive for lysine and ornithine) and negative reactions for arginine dihydrolase, production of gas from glucose, and fermentation of salicin separate this organism from most aeromonads. Unfortunately, strains of "ornithine decarboxylase-positive" *A. veronii* bv. veronii yield an "excellent to very good ID" for *V. cholerae* with a rapid identification kit, and serotyping and/or additional testing are required to resolve the issue. *A. veronii* bv. veronii would be String test negative, O/129 resistant, and able to produce gas from glucose fermentation, would not require additional salt for growth, and would be inhibited on TCBS agar, whereas *V. cholerae* strains would have the opposite reactions. Once it has been determined that you have a glucose-fermenting, oxidase-positive, motile gram-negative rod that is resistant to O/129, a small number of biochemical tests can be used for separating *Aeromonas* species into the three major species complexes (Table 2). If warranted, even more discriminatory tests for separating members of each complex can be found in boldface type in Table 3 (2), which should replace earlier published tests for species identification (3).

The sequencing of a single housekeeping gene 16S rRNA (32) and the development of an extended method using 16S rRNA restricted fragment length polymorphism analysis that followed (10) were both initially promising as methods to identify aeromonads to the species level. However, very recently published data on the intragenomic heterogeneity within the 16S rRNA gene in *Aeromonas* strains suggest caution in using this gene for anything beyond genus level identification (35). Therefore, the use of other housekeeping genes as multiple molecular markers, such as *gyrB* and *rpoD*

TABLE 2 Biochemical identification of *Aeromonas* to complex level

Test	No. of strains identified as belonging to[a]:		
	A. hydrophila complex (*A. hydrophila, A. bestiarum, A. salmonicida*)	*A. caviae* complex (*A. caviae, A. media, A. eucrenophila*)	*A. veronii* complex (*A. veronii* HG8,[b] *A. jandaei, A. schubertii, A. trota*)
Esculin	87 (92, 81, 85)	71 (76, 55, 78)	0
Voges-Proskauer	75 (88, 63, 62)	0	54 (88, 87, 17, 0)
Glucose (gas)	81 (92, 69, 77)	16 (0, 0, 78)	87 (92, 100, 0, 69)
L-Arabinose	93 (84, 100, 100)	96 (100, 100, 78)	4 (12, 0, 0, 0)

[a] The first number is the overall percent positive for each complex for a given trait; the numbers in parentheses are the percentages of positives for each species listed within that complex. Data are derived and modified from Table 5 in reference 2.

[b] Biovar sobria (DNA hybridization group 8); the separation of *A. veronii* biovar veronii (DNA hybridization group 10) from *A. veronii* biovar sobria is achieved with *A. veronii* bv. veronii having positive reactions for ornithine decarboxylase and esculin hydrolysis and a negative reaction for arginine dihydrolase.

(46), or an even broader approach using multilocus sequence typing seems to be the future avenue for accurate species identification.

Because isolates do not survive well at room or refrigerator temperature in the laboratory for long periods (>1 month), placing aeromonads in media such as Trypticase soy broth with 30% glycerol and deep freezing at −80°C are recommended for their long-term storage.

SEROLOGIC RESPONSE

Most serologic assays that have been used to detect antibodies to *Aeromonas* (tube agglutination, immunoblot, and enzyme-linked immunoassay) have low sensitivity and specificity and are not considered reliable. An immunoglobulin A (IgA fecal antibody) response to *Aeromonas* somatic lipopolysaccharides and exotoxins has been reported (9). Crivelli et al. (9) found secretory IgA to *Aeromonas* in 10 of 13 stools from patients when the stool was extracted with Jacalin, a lectin with high affinity for human IgA.

ANTIBIOTIC SUSCEPTIBILITIES

Two recent articles on *Aeromonas* antimicrobial susceptibilities (25, 37) included only strains well characterized to the species level and expand previously known susceptibility information on aeromonads isolated less frequently from clinical specimens. A general antimicrobial susceptibility profile for *Aeromonas* derived from both of these investigations as well as other studies (22, 28, 50) is given in Table 4. Ciprofloxacin, commonly used to treat gram-negative infections, remains active against all species of *Aeromonas,* with little or no resistance reported in studies in the United States and most of Europe (25, 37). However, a recent Spanish study of 43 strains, identified as *A. hydrophila, A. veronii* bv. sobria, and *A. caviae,* found 26 and 20% of the *A. caviae* and *A. hydrophila* strains, respectively, and 88% of the *A. veronii* bv. sobria strains to be resistant to nalidixic acid and pipemidic acid. This means that these organisms, though still susceptible to ciprofloxacin, are known to already have a mutation in the *gyrA* gene and could easily develop a second mutation resulting in resistance to ciprofloxacin (49). Two to three percent of *A. caviae, A. hydrophila,* and *A. veronii* bv. sobria strains in Asia have been reported to be ciprofloxacin resistant (28). Antimicrobial susceptibility testing of local isolates is necessary for the detection of species-related patterns, such as continued susceptibility to cephalothin in *A. veronii* bv. sobria isolates, and because susceptibilities may differ from one geographic area to another. *Aeromonas* species can express three chromosomal β-lactam-induced β-lactamases, including a group 1 molecular class C cephalosporinase, a group 2d molecular class D penicillinase, and a group 3 molecular class B metallo-β-lactamase (carbapenemase) (42). The presence of these β-lactamases in *Aeromonas,* in particular the carbapenemase, may not be detected by conventional susceptibility methods (42). It may be necessary to test strains of species known to potentially carry carbapenemases *(A. hydrophila, A. veronii* bv. sobria, *A. veronii* bv. veronii, and *A. jandaei)* with a higher-than-standard inoculum if imipenem or meropenem therapy is being considered. CphA, one of several enzymes responsible for resistance to carbapenems, hydrolyzes nitrocefin poorly or not at all, indicating that the nitrocefin test is not reliable for detecting carbapenemases (17, 42). A case of sepsis due to an extended-spectrum β-lactamase (ESBL)-producing *A. hydrophila* in a pediatric patient with diarrhea and pneumonia (41) and a case of *A. hydrophila* necrotizing fasciitis with probable "in vivo" transfer of a TEM-24 plasmid-borne ESBL gene from *Enterobacter aerogenes* have been reported (13).

INTERPRETATION AND REPORTING OF RESULTS

Regardless of the site of isolation (intestinal or extraintestinal), aeromonads should be identified either as belonging to the *A. hydrophila* or *A. caviae* complex or as *A. veronii* bv. sobria and not "*A. sobria.*" For routine isolates recovered from uncomplicated cases of gastroenteritis, this level of identification may be sufficient. Although there is strong evidence that some aeromonads are gastrointestinal pathogens, there is no convincing evidence at present that all fecal isolates of *Aeromonas* are involved in diarrheal disease. Thus, the significance of the recovery of aeromonads from stool specimens should be interpreted cautiously and must rely on both laboratory information and clinical interpretation. Because of this, the relative quantity of *Aeromonas* recovered on enteric media (few colonies, moderate growth, or predominant organism) should be reported in conjunction with the *Aeromonas* complex or species identification. For complicated cases of diarrhea, i.e., prolonged bloody diarrhea in pediatric patients or chronic gastroenteritis of >1-month duration or in cancer patients with positive fecal cultures in whom *Aeromonas* tends to disseminate, a definitive species identification is warranted.

For extraintestinal isolates (from blood or wounds), the same general rules should apply to species identification of aeromonads. Although it is clear that both the in vitro and in vivo pathogenic potentials of *Aeromonas* species and strains vary considerably, for the present, there are no universal markers or indicators available that dictate when

TABLE 3 Tests useful in the separation of members within the *Aeromonas* species complexes

Test	Result[a]									
	A. hydrophila	*A. bestiarum*	*A. salmonicida*	*A. caviae*	*A. media*	*A. eucrenophila*	*A. veronii*[b]	*A. jandaei*	*A. schubertii*	*A. trota*
Utilization of:										
Citrate	**+(92)**	**V(38)**	**+(85)**	**+(88)**	**V(82)**	**−(0)**	V(52)	**+(87)**	**V(58)**	**+(94)**
DL-Lactate	**V(84)**	**−(0)**	**−(0)**	**+(96)**	**V(56)**	**−(0)**	−(0)	−(7)	V (58)	+(88)
Urocanic acid	**V(16)**	**+(94)**	**+(100)**	**+(100)**	**+(100)**	**−(0)**	−(0)	−(7)	−(0)	V(75)
Gluconate oxidation	**V(64)**	**−(13)**	**−(0)**	−(0)	−(0)	−(0)	V(60)	V(60)	−(0)	−(0)
Gas from D-Glucose	+(92)	V(69)	V(77)	**−(0)**	**−(0)**	**V(78)**	**+(92)**	**+(100)**	**−(0)**	**V(69)**
PZA	V(24)	V(50)	V(31)	**+(88)**	**V(18)**	**+(100)**				
Indole	+(96)	+(100)	+(100)	V(84)	+(100)	+(89)	**+(100)**	**+(100)**	**V(17)**	**+(100)**
Voges-Proskauer	+(92)	V(63)	V(62)	−(0)	−(0)	−(0)	**+(92)**	**+(87)**	**V(17)**	**−(0)**
Lipase (corn oil)	+(100)	+(88)	+(92)	V(76)	V(82)	+(89)	**+(92)**	**+(100)**	**+(100)**	**−(0)**
Acid from:										
Cellobiose	**−(4)**	**V(38)**	**V(69)**	+(100)	+(100)	V(56)	**V(20)**	**V(20)**	**−(0)**	**+(100)**
Lactose	**V(64)**	**−(13)**	**+(92)**	V(60)	V(64)	−(11)	−(12)	−(0)	−(0)	−(0)
L-Rhamnose	**V(24)**	**V(69)**	**−(0)**	−(0)	−(0)	V(22)	−(0)	−(0)	−(0)	−(0)
D-Sorbitol	**−(0)**	**−(0)**	**+(85)**	−(4)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)
Glucose 1-phosphate	ND	ND	ND	**−(4)**	**+(100)**	**+(100)**	ND	ND	ND	ND
Glucose 6-phosphate	ND	ND	ND	**−(4)**	**+(100)**	**+(100)**	ND	ND	ND	ND
Lactulose	ND	ND	ND	**V(68)**	**V(55)**	**−(0)**	ND	ND	ND	ND
D-Mannose	+(100)	+(100)	+(100)	**V(32)**	**+(100)**	**+(100)**		+(100)	+(92)	+(100)
Glycerol	+(96)	+(100)	+(100)	V(68)	V(55)	−(11)	**+(100)**	**+(100)**	**−(0)**	**+(94)**
D-Mannitol	+(96)	+(100)	+(100)	+(100)	+(100)	+(100)	**+(100)**	**+(100)**	**−(0)**	**V(69)**
Sucrose	+(100)	+(94)	+(100)	+(100)	+(100)	V(33)	**+(100)**	**−(0)**	**−(0)**	**V(19)**
Amp^r	+(100)	+(94)	+(85)	+(100)	V(73)	+(100)	**+(100)**	**+(93)**	**+(92)**	**−(6)**

[a] +, ≥85% of the strains positive; −, <15% positive; V, 15 to 85% positive (results at 48 h); numbers in parentheses indicate the percentages of positives for the test at the final day of reading: gluconate, 2 days; DL-lactate and urocanic acid, 3 days; citrate, 4 days; carbohydrates, indole, and lipase, 7 days; PZA (pyrazinamidase), 2 days; Amp^r, resistance to 10 μg of ampicillin, 1 day; Voges-Proskauer, 3 days; ND, not done. For each of the three *Aeromonas* species complexes, the discriminatory reactions between the species within each complex are in boldface type.

[b] Biovar sobria (DNA hybridization group 8); the separation of *A. veronii* biovar veronii (DNA hybridization group 10) from *A. veronii* biovar sobria is achieved with *A. veronii* bv. veronii having positive reactions for ornithine decarboxylase and esculin hydrolysis and a negative reaction for arginine dihydrolase.

TABLE 4 *Aeromonas* species susceptibilities

Susceptibility[a]	Antibiotic agent
Resistant	Ampicillin (except *A. trota* [100% susceptible] and *A. caviae* [35% susceptible][b])
Variable	Ticarcillin or piperacillin (except *A. veronii* bv. veronii [100% resistant] and *A. trota* [100% susceptible])
	Cephalothin
	Cefazolin
	Cefoxitin (except *A. veronii* bv. veronii [100% susceptible])
	Cefuroxime
	Ceftriaxone
	Cefotaxime
Susceptible	Ciprofloxacin[c]
	Gentamicin
	Amikacin
	Tobramycin (*A. veronii* bv. veronii [42% resistant])
	Imipenem (*A. jandaei* [65% resistant], *A. veronii* bv. veronii [67% resistant])
	Trimethoprim-sulfamethoxazole

[a] Resistant or susceptible, ≥90% of all isolates are resistant or susceptible; variable, 10 to 90% of isolates are susceptible (data from reference 2).

[b] Data for *A. caviae* susceptibility are from references 6 and 27.

[c] Data for resistance to nalidixic acid and pipemidic acid in 26 and 20% of *A. caviae* and *A. hydrophila* isolates, respectively, and 88% in *A. veronii* clinical strains suggest possible future resistance to fluoroquinolones (49).

isolates should be definitively identified to the species level. Thus, for extraintestinal isolates, identification of aeromonads beyond complexes should be reserved for strains isolated from sterile body sites (blood or cerebrospinal fluid) and serious wound infections (cellulitis and necrotizing fasciitis); for strains exhibiting unusual resistance patterns, associated with nosocomial outbreaks; or for publications describing traditional species associated with new disease processes or newly described species isolated from new anatomic sites.

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Vibrio and Related Organisms*

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TAXONOMY

Vibrio is the type genus of the family *Vibrionaceae,* with *V. cholerae*, the causative agent of pandemic cholera, as the type species (25, 26). The genus is extremely diverse with greater than 75 validly published species to date (http://www.bacterio.cict.fr/). Of these 75 named species, 12 have been associated with or isolated from infections in humans. Phylogenetic investigations indicate that multiple clades (separate or distant groups in a phylogenetic sense) exist within this genus, indicating that many *Vibrio* species may eventually be reclassified to different genera (58). Several formal proposals have already been made over the years (26) including the classification of *V. hollisae* in the genus *Grimontia* as *G. hollisae* (77) and the classification of *V. damsela* in the genus *Photobacterium* as *P. damselae* (73).

Changes in Classification for This Edition

In this chapter we accept the taxon *Grimontia hollisae* and use it rather than *Vibrio hollisae*. Studies based on DNA-DNA hybridization, 16S rRNA and *rec*A sequencing, biochemical reactions, and other phenotypic characters (antibiotic susceptibility, poor growth on thiosulfate-citrate-bile salts-sucrose [TCBS], fastidious nature, metabolic inactivity, etc.) all indicate that *Vibrio hollisae* has diverged significantly from *Vibrio cholerae* and *V. mimicus*, the core species of the genus *Vibrio* (26). We suggest that clinical microbiology reports list both names to avoid confusion, with the reporter's choice listed first, i.e., *Grimontia hollisae (Vibrio hollisae)* or *Vibrio hollisae (Grimontia hollisae)*.

DESCRIPTION OF THE GENUS *VIBRIO*

A majority of *Vibrio* species have the following characteristics: gram-negative, facultatively anaerobic straight, curved, or comma-shaped rods, 0.5 to 0.8 μm in width and 1.4 to 2.6 μm in length, that are catalase and oxidase positive (26). Vibrios are motile by means of sheathed monotrichous or multitrichous polar flagella when grown in liquid media. Strains of some species, such as *V. parahaemolyticus* and *V. alginolyticus,* swarm on solid media by production of numerous lateral flagella (26, 51). All *Vibrio* species require Na^+ for growth, and the halophilic species usually require that NaCl be added to media (such as commercial decarboxylase broths) that do not include NaCl in their formulas. Most media formulated with peptone and meat extracts contain enough salt for *V. cholerae* and *V. mimicus* to grow. The minimal concentration for optimum growth varies from 0.029 to 4.1% NaCl (26). Vibrios ferment D-glucose but rarely produce gas, reduce nitrate to nitrite, and grow on TCBS medium. The G+C content of the DNA is 38 to 51 mol% (26). Key properties or characteristics useful in separating members of the genus *Vibrio* from phylogenetically or phenotypically related species are listed in Table 1. The description of the genus *Grimontia* is based on its single species and is given in Tables 2 and 4.

NATURAL HABITATS

Vibrios are primarily aquatic residents, and their relative distributions in such environs are typically dependent upon temperature, Na^+ concentration, available nutrients in the water column, and the presence of various plants and vertebrate and invertebrate animal species that inhabit such ecosystems (78). Vibrios that require small amounts of Na^+ for growth, such as *V. cholerae* and *V. mimicus,* can be found in freshwater rivers and lakes as well as estuarine and marine environments. Some *Vibrio* species, such as *V. fischeri* and *V. harveyi,* have evolved close symbiotic associations over thousands of years with marine inhabitants such as *Eupryema scolopes,* the Hawaiian small bobtail squid (59). In marine and estuarine environments, vibrios are commonly isolated from sediment, the water column, plankton, various bivalves (oysters, clams, and mussels), crabs, shrimp, and prawns (30, 47, 78). In temperate climates, *Vibrio* concentrations peak during the warmer months of the year (7). A "viable but nonculturable" state has been described for several *Vibrio* species including *V. cholerae* and *V. vulnificus* (9). It is unclear if the term "viable but nonculturable" represents a new idea or is just a subpart of the well-documented term "injured bacterial cell" that has been known for many decades, particularly in water microbiology. In either instance its significance is unknown.

CLINICAL SIGNIFICANCE

Vibrios are isolated from and actually cause a wide variety of human illnesses, both intestinal and extraintestinal. These

* This chapter contains information presented in chapter 46 by J. J. Farmer III, J. Michael Janda, and Karen Birkhead in the eighth edition of this Manual.

TABLE 1 Properties of the genus *Vibrio* and differentiation from other phenotypically similar genera

Test or property	Reaction or property of[a]:				
	Vibrio	*Photobacterium*	*Aeromonas*	*Plesiomonas*	*Enterobacteriaceae*
Associated with diarrhea and extraintestinal infections in humans	+	–[d]	+	+	+
Oxidase reaction	+	+	+	+	–
Na^+ is required for growth or stimulates growth	+	+	–	–	–
Sensitive to the vibriostatic compound O/129[b]	+	+	–	+	–
D-Mannitol fermentation	+	–	+	–	+
Growth on TCBS[c]	+	+	+	–	–

[a] Symbols: +, most species and strains are positive; –, most species and strains are negative.
[b] 2,4-diamino-6,7-diisopropylpteridine phosphate (150 μg per disk); resistance to O/129 has become common in *V. cholerae* from parts of India and Bangladesh.
[c] Relative growth of *Aeromonas* on TCBS is dependent on the commercial manufacturer.
[d] *Photobacterium damselae* is an exception if this classification is used.

include diarrhea, localized (cellulitis) and invasive (necrotizing fasciitis) wound infections, eye and ear infections, and septicemia (26). In individual cases of both diarrhea and extraintestinal infection it may be difficult to determine if a positive culture result represents true infection, colonization, or simply the transient presence of the organism because of its occurrence in sea- and estuarine water. Twelve vibrio species occur in human clinical specimens: *V. alginolyticus, V. cholerae, V. cincinnatiensis, V. damsela (P. damselae), V. fluvialis, V. furnissii, V. harveyi (= V. carchariae), Grimontia hollisae (V. hollisae), V. metschnikovii, V. mimicus, V. parahaemolyticus,* and *V. vulnificus* (26, 55). The clinical importance of some of these species in individual cases of infection is sometimes unclear. *V. cholerae* is the only species that causes endemic, epidemic, and pandemic cholera (68), but several other species are important causes of intestinal infections. *V. parahaemolyticus* is the foremost cause of food poisoning in Japan and Southeast Asia and is the leading cause of intestinal infections due to vibrios in the United States (81, 84).

Extraintestinal *Vibrio* infections are frequently associated with injuries or exposure to estuarine or marine waters and may result in severe tissue destruction and/or lead to systemic infection (63). Primary septicemia may occur after ingestion of raw seafood (oysters) or as a secondary bacteremia subsequent to a wound infection. Septicemia due to *V. vulnificus* tends to be fulminant with a fatality rate exceeding 50% (56) and is often accompanied by secondary skin lesions called bullae (63). *Vibrio vulnificus* is the most common cause of vibrio septicemia, but other vibrios may also cause bacteremia including *V. cholerae* non-O1.

V. cholerae

V. cholerae is the most important species in the genus *Vibrio*. It has caused many epidemics of cholera and millions of deaths (21, 68). It is now divided into three major subgroups: *V. cholerae* O1, *V. cholerae* O139, and *V. cholerae* non-O1.

V. cholerae O1

V. cholerae serogroup O1 is the organism responsible for seven pandemics of cholera (1816–1817, 1829, 1852, 1863, 1881, 1889, and 1961–present) (66, 68). While the majority of persons ingesting toxigenic *V. cholerae* O1 have asymptomatic infections or self-limiting diarrhea (≥75%), severe cholera ("cholera gravis") usually results in massive diarrhea and large volumes of "rice water stools" (clear fluid with flecks of mucus) passed painlessly; fluid loss can reach 200 ml/kg of body weight/day. If left untreated, the patient becomes prostrate with symptoms of severe dehydration, electrolyte imbalance, painful muscle cramps, watery eyes, loss of skin elasticity, and anuria. Dehydration subsequently leads to hypovolemic shock, acidosis, circulatory collapse, and death, even in healthy adults (42). Interestingly, there is a correlation between human blood types and susceptibility to *V. cholerae* infection with persons of O blood group presenting with more severe symptoms. In the United States, occasional cases of cholera are seen in travelers returning from regions of endemicity; rarely such illnesses are due to an O1 strain indigenous to the Gulf of Mexico. Treatment consists of fluid replacement by oral rehydration therapy and/or intravenous fluids.

The ability of *V. cholerae* serogroup O1 to uniquely cause this fulminant form of diarrhea is due to the presence of virulence "cassette" regions and pathogenicity islands on the bacterial chromosome. These regions encode a number of key virulence factors including cholera enterotoxin responsible for the large excretion of fluids and electrolytes into the lumen and the toxin coregulated pilus responsible for attachment of *V. cholerae* to the gastrointestinal epithelium (27). Traditional (classic) cholera can be produced by two different biotypes of *V. cholerae* O1, designated Classical and El Tor. These biotypes can be differentiated by a number of phenotypic tests including hemolysis of sheep erythrocytes, production of acetylmethylcarbinol (Voges-Proskauer test), and resistance to polymyxin B, all positive for the El Tor biotype (40). The first six pandemics were thought to be due to the Classical biotype, whereas the ongoing seventh pandemic that began in 1961 is caused by the El Tor biotype, which was first isolated in 1905 (68).

Although extremely rare, O1 strains have been known to cause severe extraintestinal infections. A 2001 report from Malawi describes three cases of *V. cholerae* O1 bacteremia in two adults and one neonate (34). All three patients died as a direct or indirect result of their infections.

V. cholerae O139

Until the last decade, only *V. cholerae* serogroup O1 was believed to cause epidemic cholera. In 1992, cholera cases due to a new serogroup of *V. cholerae*, O139 (synonym, *V. cholerae* O139 Bengal), appeared in India and Bangladesh and spread rapidly throughout Asia (4). This new serogroup probably resulted from the lateral transfer of a novel somatic antigen and capsule from an unknown bacterium to an El Tor strain (4). O139 strains carry cholera enterotoxin and other critical virulence factors that O1 strains harbor including the toxin coregulated pilus (54). The clinical diseases due to O1 and

O139 *V. cholerae* are strikingly similar, except that adults are more frequently affected than children since previous infection with O1 cholerae is not protective (2, 28). *V. cholerae* O139 replaced O1 as the cause of epidemic cholera between 1994 and 1995 in many areas of Southeast Asia, including Bangladesh. However, in this setting O1 reemerged in 1996. In 2002, O139 reemerged in Bangladesh, causing an estimated 30,000 cases of cholera, mostly in older patients than typically observed with O1 infections (28). Some researchers speculate that the emergence of O139 may be the beginning of the eighth cholera pandemic (4).

V. cholerae Non-O1

V. cholerae non-O1 strains do not agglutinate in O1 or O139 antisera but are otherwise phenotypically identical to O1 and O139 *V. cholerae* strains in their biochemical reactions. *V. cholerae* non-O1 strains are the third most commonly isolated vibrios in clinical laboratories in the United States, following *V. parahaemolyticus* and *V. vulnificus* (37). They typically do not produce cholera toxin and are usually isolated from patients with mild watery diarrhea, although the diarrhea is occasionally severe (57). However, unlike O1 strains, non-O1 isolates are commonly associated with extraintestinal infections such as septicemia. Persons at increased risk of developing non-O1 bacteremia include those with liver disease/cirrhosis or hematologic malignancies. The case fatality rate ranges from 47 to 65% (44, 69). Strains have also been isolated from ears, wounds, the respiratory tract, and urine (39, 56).

V. mimicus

V. mimicus is a nonhalophilic vibrio species that is biochemically similar to *V. cholerae* except that it is sucrose negative. It has been recovered from patients with diarrhea, which usually occurred after the consumption of uncooked seafood, particularly raw oysters (23). Rare strains carry the cholera toxin gene and can produce cholera-like symptoms. Human infections are uncommon. Symptoms include abundant watery diarrhea, vomiting, and severe dehydration. There has been one recent report of *V. mimicus* diarrhea in Costa Rica that involved 33 patients over a 3-year period (12).

V. parahaemolyticus

In Asia, *V. parahaemolyticus* is the leading cause of foodborne intestinal infections, almost always associated with the consumption of raw fish or shellfish (84). Fifty to 70% of the cases of foodborne diarrhea in Japan alone are due to *V. parahaemolyticus*. It is also the *Vibrio* species most frequently isolated from clinical specimens in the United States and is primarily associated with diarrhea, but it has occasionally been isolated from extraintestinal sites. *V. parahaemolyticus* causes gastroenteritis with nausea, vomiting, abdominal cramps, low-grade fever, and chills. The diarrhea is usually watery but can on rare occasions be bloody. Fatalities are extremely rare but can occur in cases of severe dehydration. Rehydration is usually the only treatment needed, but in some severe cases the patient requires hospital admission. Antimicrobial therapy may be beneficial.

A pandemic clone of *V. parahaemolyticus* serotype O3:K6 emerged worldwide in 1997 (62). Strains of this serotype caused an unusually high proportion of *V. parahaemolyticus* foodborne disease outbreaks in Taiwan from 1996 to 1999, suggesting something unusual in the organism's ecology, epidemiology, or virulence (17). This pandemic clone has continued to spread throughout Asia, to the United States, Canada, Russia, Chile, and Mozambique (6, 32). Recently, new pandemic serogroups have emerged that have been shown to be genetically closely related to the O3:K6 strain. These include O4:K68, O1:K25, O1:K41, and O1:KUT (UT, untypeable) (17).

V. vulnificus

V. vulnificus causes primary septicemia and wound infection and is responsible for more than 90% of deaths due to vibrios in the United States each year (63). Primary septicemia has a fatality rate that exceeds 50% even with hospitalization (15, 74) and occurs predominantly in men over 50. Cases generally have predisposing conditions such as liver disease, immunosuppression, increased serum iron, or other chronic diseases (74). Data from CDC showed that more than 95% of patients had consumed raw oysters within the last 7 days. Patients typically present with symptoms including a sudden onset of fever and chills, vomiting, diarrhea, and abdominal pain. Secondary skin lesions often appear, progressing to bulla formation and necrosis. Endotoxic shock often occurs and can rapidly lead to death. Blood cultures and biopsy specimens (scrapings) from skin lesions are usually positive.

V. vulnificus also causes severe wound infections usually after trauma and exposure to marine animals or the marine environment (63). Wound infections may progress to cellulitis with extensive necrosis (often requiring surgical debridement), myositis, necrotizing fasciitis that may mimic gas gangrene, and secondary septicemia. The fatality rate for wound infections ranges from 20 to 30%.

Three biogroups have now been defined for *V. vulnificus*. Most infections in the United States are due to biogroup 1 (see Table 5). *V. vulnificus* biogroup 2 was originally isolated from diseased eels, but in 1995 Amaro and Biosca (5) isolated it from a human wound infection from Rhode Island. To date, no other isolations of biogroup 2 from clinical specimens have been reported. *V. vulnificus* biogroup 3 was described in 1999 by Bisharat et al. (10), who isolated it from patients with wound infections and bacteremia. Cases have been limited to Israel and were acquired from exposure to live fish (tilapia) grown in aquaculture. One case report strongly suggests that *V. vulnificus* biogroup 3 can survive on fish skin for at least 24 h (19).

V. alginolyticus

V. alginolyticus is very common in the marine environment and is the fourth most commonly isolated *Vibrio* species in the United States. *V. alginolyticus* has most frequently been isolated from ear infections (otitis externa and otitis media) (29) and wound infections following exposure to seawater. *V. alginolyticus* has also been isolated from ocular infections and from infrequent cases of monomicrobic or polymicrobic bacteremia, mostly in immunocompromised persons (16). It is occasionally isolated from diarrheal stool (79), but there is no strong evidence that it can actually cause diarrhea or intestinal infections.

V. damsela (Photobacterium damselae)

V. damsela, originally isolated from wound infections in damselfish, is an important though infrequent cause of serious and aggressive wound infections (necrotizing fasciitis) and bacteremia (31, 33, 83). Risk factors for infection include puncture wounds (from a fish fin or a fish hook) and exposure of open wounds to seawater. Although the case fatality rate is unknown, many reports in the literature describe fatal *V. damsela* infections, suggesting a fairly high case fatality rate. The enhanced virulence of this species is thought to be related to the production of a damselysin (phospholipase-D).

V. fluvialis

V. fluvialis appears to cause sporadic cases of diarrhea worldwide (75). Although in the past it has only rarely been isolated from extraintestinal sites, several recent reports of cellulitis, cerebritis, peritonitis, and bacteremia have been attributed to *V. fluvialis* (38, 45, 67).

V. furnissii

V. furnissii is rarely isolated from human clinical specimens, but when it is recovered it is invariably from fecal specimens of patients with diarrhea (22). There is no convincing evidence that it can actually infect the intestinal tract or cause diarrhea, although this merits further investigation (22).

V. harveyi (originally known as *V. carchariae*)

A single case of a *V. harveyi* (the name *V. carchariae* was used in the report describing this organism [64]) wound infection, resulting from a shark bite, has been published (64). Subsequently, it was shown by DNA-DNA hybridization that the type strains of *V. carchariae* and *V. harveyi* are 88% related (65). The two organisms also have identical or almost identical 16S rRNA sequences. Since the two species appear to be synonyms (subjective), *V. harveyi,* being the older name, has priority.

Grimontia hollisae (Vibrio hollisae)

G. hollisae is a halophilic vibrio species that is primarily associated with moderate to severe cases of diarrhea (1), for which there is evidence for a causative role. It has been rarely isolated from extraintestinal sites such as bacteremia (35).

V. metschnikovii

V. metschnikovii has frequently been isolated from fresh, brackish, and marine waters. In 1981 Jean-Jacques et al. (41) reported that it caused peritonitis and bacteremia in a patient with an inflamed gallbladder. Subsequently, *V. metschnikovii* has been isolated from additional patients with bacteremia and rarely from wound infections (46). It has also been isolated from cases of cholecystitis, diarrhea, and pneumonia (46, 80). There is no convincing evidence that it can actually infect the intestinal tract or cause diarrhea, although this merits further investigation (46).

V. cincinnatiensis

V. cincinnatiensis was first reported by Brayton et al. (11) from a patient with bacteremia and meningitis. Subsequent isolates have been from feces (intestine), the ear, a foot or leg wound, animals, and water.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

As with all stools, specimens should be collected in the acute stage of disease before initiation of treatment (13). If stool is unavailable, rectal swabs (or vomitus) are reliable from acute cases but should not be used when the numbers of organisms present may be small, as occurs with contacts to known cases or for convalescing patients. Vibrios are particularly susceptible to desiccation; therefore, any specimen that cannot be inoculated onto plating media within 2 to 4 h should be placed in a transport medium. Cary-Blair, which maintains the viability of vibrios up to 4 weeks, or most commercially available transport media are satisfactory; but because some lots of glycerol may be toxic to vibrios, buffered glycerol in saline is unacceptable. Specimens collected in the field may be transported in tellurite-taurocholate-peptone or alkaline peptone water enrichment broths only if they can be plated within 12 to 24 h and are delivered by courier. If necessary, liquid stool may be placed on strips of blotting paper or gauze, inserted in airtight plastic bags with a few drops of saline to maintain moisture, and submitted to the laboratory. Detailed information on the collection and transport of specimens for vibrio isolation is available (13).

Direct microscopic detection of vibrios in stool is not routinely recommended, since it may not be possible to distinguish pathogenic vibrios from other enteric flora. Detection of *V. cholerae* directly from stool using an "O1 (or O139) serum immobilization" method is described in a subsequent section.

Special methods for the collection and processing of extraintestinal specimens (blood and wounds, etc.) for vibrio isolation are not required; vibrios are, as a rule, isolated in pure culture from these sites, and the concentration of salt in primary plating media is usually sufficient for their recovery. Once isolated, however, salt may need to be added to subsequent media to attain growth of salt-requiring vibrios.

ISOLATION PROCEDURES

Since it is not common practice to use special isolation media for vibrios, inclusion of pertinent clinical history (when known) should accompany specimens to alert the laboratory to include vibrio isolation techniques (media or reagents) in their stool workup (49). Such information includes consumption of seafood, any activity associated with marine or brackish water or wounds associated with such exposure, and hobbies associated with aquaria. Examination of blood agar plates for oxidase-positive colonies may also improve recovery of vibrios as well as *Aeromonas* spp. and *Plesiomonas shigelloides*.

Vibrios associated with human disease generally grow on MacConkey agar and when present appear as colorless colonies (with the exception of the lactose-fermenting species, *V. vulnificus*). Sucrose-fermenting vibrios associated with human disease such as *V. cholerae, V. fluvialis,* or *V. alginolyticus* cannot be differentiated from other sucrose-fermenting normal enteric flora on sucrose-containing agars such as Hektoen or xylose-lysine-desoxycholate. Additionally, *Grimontia hollisae* may grow poorly or not at all on any enteric isolation medium, including TCBS, and is most reliably isolated from blood agar. Plating efficiency on TCBS may be less for *V. cincinnatiensis* than that observed for other vibrio species, and it may be reduced for *V. metschnikovii* when the plate is incubated at 36°C. Although there are a number of selective media suitable for the isolation of vibrios, TCBS is generally used for the isolation of vibrios associated with human disease and is readily available from a number of commercial sources (26). Since this medium does not require autoclaving, powdered media may be kept available in the laboratory and readily prepared by boiling whenever needed. The use of TCBS is particularly useful in coastal areas, where vibrios are isolated with greater frequency. It is not cost-effective to use it for every stool specimen. The inclusion of sucrose in TCBS allows for preliminary differentiation of *Vibrio* species, with *V. cholerae*, *V. fluvialis*, and *V. alginolyticus* producing yellow colonies whereas *V. parahaemolyticus*, *V. mimicus*, and most strains of *V. vulnificus* produce green colonies, indicating sucrose was not fermented. Yellow colonies on TCBS may convert to green colonies if plates are refrigerated after incubation. It should be noted that oxidase testing is unreliable when performed directly on colonies growing on TCBS and should not be attempted. A chromogenic agar, CHROMagar

Vibrio (CHROMagar Microbiology, Paris, France), has been developed primarily for the recovery of *V. parahaemolyticus* from seafood and supports the growth of other vibrios as well (36). Vibrio colonies on this medium range in color from milk white to pale blue to violet; other enteric flora, with the exception of *Proteus mirabilis* and *Providencia rettgeri,* which also produce milk white colonies, do not grow. Marine agar (BD Biosciences, Sparks, Md.), which does not contain any inhibitory or selective ingredients, may be more appropriate for isolation of vibrios from the environment, especially salt-requiring vibrios. It is common for pure cultures of vibrios to produce multiple colony morphologies on any medium, but this is most readily noticeable on nonselective media such as blood or heart infusion agars. Morphologies can range from smooth, convex to flat, spreading colonies to rough; occasionally, rugose (extremely wrinkled) colonies are encountered.

Alkaline peptone water (1% NaCl, pH 8.5) is the most commonly used enrichment broth for human specimens and is available from a number of commercial sources. Alkaline peptone water is incubated at 36°C and subcultured at 18 h. Occasionally, vibrios are recovered only when subcultured after 6 h of incubation. Longer incubation times of such specimens may fail to yield a vibrio, probably due to overgrowth by other flora.

DIRECT DETECTION OF *V. CHOLERAE* O1 IN FECES

Direct detection of *V. cholerae* from stool requires experience to correctly interpret results and is typically done only in laboratories in areas where cholera is common or in situations where laboratory services are unavailable. One of the oldest assays, the microscopic immobilization test, detects loss of motility of *V. cholerae* O1 organisms in the presence of O1 antibody and can be used to detect *V. cholerae* O139 by using O139 antibody (66). Coagglutination, direct fluorescent-antibody (New Horizons Diagnostics Corp., Columbia, Md.; FDA approval pending), and latex agglutination (Denka Seiken, Tokyo, Japan; not FDA approved for human clinical specimens) assays are all commercially available.

IDENTIFICATION

Biochemical properties that separate members of the *Vibrionaceae* from the *Enterobacteriaceae* (including *Plesiomonas shigelloides*) and the *Aeromonadaceae* are listed in Table 1. Useful tests for separating *Vibrio* species are described in Tables 2 and 3, and comprehensive biochemical profiles of the 12 species that occur in human clinical specimens are given in Table 4. All vibrios are negative to 10% positive for H_2S in triple sugar iron agar, urea (except *V. parahaemolyticus* [15%]), phenylalanine deaminase (except *V. vulnificus* biogroup 1 [35%]), malonate, mucate production, yellow pigment production, and fermentation of D-adonitol, dulcitol, erythritol, melibiose (except *V. vulnificus* biogroup 1 [40%]), raffinose, L-rhamnose (except *V. furnissii* [45%]), D-sorbitol (except *V. metschnikovii* [45%]), and α-methyl-β-D-galactoside and D-xylose (except for 57 and 43% of *V. cincinnatiensis* isolates, respectively). Variable reactions are seen with methyl red, growth in potassium cyanide broth, D-galactose, glycerol, Jordan's tartrate, sodium acetate, DNase at 25°C, lipase, and tyrosine clearing. All vibrio species are 99 to 100% positive for growth in 1% NaCl, and fermentation of maltose (except *V. hollisae* [0%]) and D-mannose (except *V. cholerae* [78%] and *V. harveyi* [50%]). Many commercial standard tube tests have sufficient

TABLE 2 Key differential tests for the six groups of 12 *Vibrio* species that occur in clinical specimens

Test	Reactions of the species in[a]:											
	Group 1		Group 2,	Group 3,	Group 4,	Group 5		Group 6				
	V. cholerae	*V. mimicus*	*V. metschnikovii*	*V. cincinnatiensis*	*G. hollisae*	*V. damsela*	*V. fluvialis*[b]	*V. alginolyticus*	*V. parahaemolyticus*	*V. vulnificus*	*V. harveyi*
Growth in nutrient broth with:											
No NaCl added[c]	+	+	−	−	−	−	−	−	−	−	−
1% NaCl added	+	+	+	+	+	+	+	+	+	+	+
Oxidase production			−	+	+	+	+	+	+	+	+
Nitrate reduced to nitrite			−	+	+	+	+	+	+	+	+
myo-Inositol fermentation			V	+	−	−	−	−	−	−	−
Arginine dihydrolase					−/NG	+	+	−	−	−	−
Lysine decarboxylase					−/NG	V	−	+	+	+	+
Ornithine decarboxylase					−/NG						

[a] All data except those for oxidase production and nitrate reduction are for reactions that occur within 2 days at 35 to 37°C; oxidase production and nitrate reduction reactions are done only at day 1. Symbols: +, 90 to 100% positive; V, variable, 11 to 89% of strains are positive; −, negative, 0 to 10% positive; NG, no growth, possibly because the NaCl concentration is too low, even when 1% NaCl is added. See Table 3 for the exact percentages.

[b] Includes *V. furnissii,* which differs from *V. fluvialis* primarily by production of gas in D-glucose.

[c] Species that require salt should have salt added to each biochemical tested.

TABLE 3 Key differential biochemicals to separate species within Groups 1, 5, and 6

Test	% Positive for[a]:							
	Group 1		Group 5		Group 6			
	V. cholerae	*V. mimicus*	*V. damsela*	*V. fluvialis*[a]	*V. alginolyticus*	*V. parahaemolyticus*	*V. vulnificus*	*V. harveyi*
Voges-Proskauer (1% NaCl)	75	9	95	0	95	0	0	50
Motility	99	98	25	70–89	99	99	99	0
Acid production from:								
Sucrose	100	0	5	100	99	1	15	50
D-Mannitol	99	99	0	97	100	100	45	50
Cellobiose	8	0	0	30	3	5	99	50
Salicin	1	0	0	0	4	1	95	0

[a] The numbers indicate the percentages of strains that are positive after 48 h of incubation at 36°C (unless other conditions are indicated). Most of the positive reactions occur during the first 24 h.

salt to support growth without salt supplementation (0.5 to 1%), but the Microbial Disease Laboratory adds 1% salt to all biochemicals (except for the 0% salt broth) for all NaCl-requiring species. Voges-Proskauer, Moeller's decarboxylases and dihydrolase, and nitrate broth may contain no or insufficient NaCl to support growth of some NaCl-requiring strains, and these biochemicals should always have salt added (to a final concentration of 1%) to them when testing these species. Sensitivity to the vibriostatic compound O/129 (Remel, Lenexa, Kans.) should be used with caution, as many *V. cholerae* isolates from Bangladesh and surrounding areas are now resistant to O/129.

Correct identification of vibrios by commercial identification systems is problematic at best, and published evaluations of these kits are based upon specific lot numbers, software versions, and microbial databases. No commercial automated or manual identification system includes all 12 clinical vibrio species in their databases, and some manual systems do not contain any vibrio species (60, 61). Six commonly used identification systems claim that they are capable of identifying *V. cholerae, V. parahaemolyticus, V. vulnificus, V. alginolyticus,* and *V. damsela* (61). However, even when tested against only those species listed in their databases, the API 20E (bioMerieux Inc., Durham, N.C.), Crystal E/NF (BD Biosciences), MicroScan Neg ID type 2 and type 3 (MicroScan, West Sacramento, Calif.), and Vitek GNI+ and ID-GNB cards (bioMerieux) correctly identified only 63.1 to 80.9% of vibrios to the species level (61). Accurate identification by these systems for the three most commonly isolated species varied. Correct identification of *V. cholerae* ranged from 50.0 to 96.7%, with API 20E and Crystal being the least and most accurate, respectively; for *V. parahaemolyticus* the range was 40.0 to 96.6%, with Rapid Neg ID3 faring the worst and API 20E and GNI+ being the best; and for *V. vulnificus* (biogroup 1 strains) the range was 50.0 to 90%, with GNI+ and Crystal showing the lowest and highest correct identification rates, respectively. Only Crystal was able to correctly identify ≥90% of *V. cholerae* or *V. vulnificus* strains, and only API 20E and the two Vitek cards correctly identified ≥90% of *V. parahaemolyticus* strains. For *V. vulnificus* biotype 3 strains, the MicroScan (98.0%) and Phoenix (90.2%) systems did the best in identifying 51 well-characterized isolates to the correct species, while the identification rate obtained by Vitek (13.7%) was much less satisfactory (20). The manufacturer's instructions should be checked prior to testing salt-requiring vibrios to determine if salt supplementation is required. Identification of vibrios from seafood and environmental sources is problematic. Many newly identified "nonclinical" species are published with poor phenotypic descriptions, and identification is based on 16S rRNA.

In areas of the world where cholera is common, isolates of *V. cholerae* may be presumptively identified simply by agglutination with O1 or O139 antisera. In other areas of the world, complete biochemical testing should be performed, and cultures identified as *V. cholerae* should be sent to public health laboratories for O1 and O139 agglutination and cholera toxin testing. *V. cholerae* O1 isolates should be biotyped (see "*V. cholerae* O1" under "Clinical Significance" above) to determine whether they are El Tor or Classical biotypes. *V. cholerae* O139 strains are phenotypically similar to *V. cholerae* O1 El Tor. Strains of *V. cholerae* that fail to agglutinate in either O1 or O139 antisera are reported as *V. cholerae* non-O1. These strains may also be serotyped; however, this testing is available only in a limited number of reference laboratories. *V. cholerae* is distinguished from other vibrios, except *V. mimicus,* by Na^+ requirement (Table 2), and *V. cholerae* can be differentiated from *V. mimicus* by sucrose and Voges-Proskauer tests (Table 3). Strains of *V. mimicus* may produce cholera toxin.

Strains of *V. parahaemolyticus, V. alginolyticus,* and *V. damsela* may be urea positive. As with most vibrios isolated from humans, these species produce a buff or tan pigment; occasional strains of *V. parahaemolyticus* may produce a dark brown pigment. *G. hollisae* generally grows poorly, especially in Moeller's decarboxylases and dihydrolase broths even after salt supplementation, and produce extremely large zones of inhibition, often necessitating the use of two plates when performing antimicrobial susceptibility testing. Because it is oxidase negative, *V. metschnikovii* is the most difficult vibrio to detect, but it is easily separated from other vibrios by negative reactions for nitrate reduction, indole production (most strains), and ornithine decarboxylase and fermentation of sucrose. *V. fluvialis* and *V. furnissii* are frequently confused with *Aeromonas caviae,* especially as some strains are poorly halophilic and only moderately susceptible to O/129, and some strains of *A. caviae* grow on TCBS. *V. furnissii* is the only vibrio isolated from humans that is positive for gas production from glucose. Rapid, correct identification of *V. vulnificus* strains is critical because of the mortality associated with this organism. Occasionally, strains of *V. vulnificus* are sucrose positive, which may add to the confusion in identifying it; it is unique among human *Vibrio*

TABLE 4 Biochemical test results and other properties of the 12 *Vibrio* species that occur in human clinical specimens

Test[a]	% Positive for[b]:											
	V. cholerae	*V. mimicus*	*V. metschnikovii*	*V. cincinnatiensis*	*G. hollisae*	*V. damsela*	*V. fluvialis*	*V. furnissii*	*V. alginolyticus*	*V. parahaemolyticus*	*V. vulnificus* biogroup 1	*V. harveyi*
Indole production (HIB, 1% NaCl)*	99	98	20	8	97	0	13	11	85	98	97	100
Voges-Proskauer (1% NaCl)*	75	9	96	0	0	95	0	0	95	0	0	50
Citrate (Simmons)	97	99	75	21	0	0	93	100	1	3	75	0
Urea hydrolysis	0	1	0	0	0	0	0	0	0	15	1	0
Arginine (Moeller's; 1% NaCl)*	0	0	60	0	0	95	93	100	0	0	0	0
Lysine (Moeller's; 1% NaCl)*	99	100	35	57	0	50	0	0	99	100	99	100
Ornithine (Moeller's; 1% NaCl)*	99	99	0	0	100	0	0	0	50	95	55	0
Motility (36°C)	99	98	74	86	0	25	70	89	99	99	99	0
Gelatin hydrolysis (1% NaCl, 22°C)	90	65	65	0	0	6	85	86	90	95	75	0
D-Glucose, acid production	100	100	100	100	100	100	100	100	100	100	100	50
D-Glucose, gas production	0	0	0	0	0	10	0	100	0	0	0	0
Acid production from:												
L-Arabinose*	0	1	0	100	97	0	93	100	1	80	0	0
Lactose*	7	21	50	0	0	0	3	0	0	1	85	0
Sucrose*	100	0	100	100	0	5	100	100	99	1	15	50
ONPG test*	94	90	50	86	0	0	40	35	0	5	75	0
Growth in nutrient broth with:												
0% NaCl*	100	100	0	0	0	0	0	0	0	0	0	0
6% NaCl*	53	49	78	100	83	95	96	100	100	99	65	100
8% NaCl*	1	2	44	62	0	0	71	78	94	80	0	2
10% NaCl*	0	0	4	0	0	0	4	0	69	2	0	2
O/129, zone of inhibition[c]	99	95	90	25	40	90	31	0	19	20	98	100

[a] Symbols and abbreviations: *, the test is recommended as part of the routine set for *Vibrio* identification; 1% NaCl, 1% NaCl has been added to the standard media to enhance growth; HIB, heart infusion broth; TSI, triple sugar iron agar; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; a positive string test indicates that cells are lysed when they are suspended in a 0.5% sodium deoxycholate solution.

[b] The numbers indicate the percentages of strains that are positive after 48 h of incubation at 36°C (unless other conditions are indicated). Most of the positive reactions occur during the first 24 h.

[c] The content of the disk was 150 μg.

species because it ferments lactose, salicin, and cellobiose and is ONPG (*o*-nitrophenyl-β-D-galactopyranoside) positive. Table 5 gives biochemicals useful in separating the biogroups of *V. vulnificus* (10).

Although identification of vibrios by conventional methods is challenging, there is limited use of molecular methods for detection and identification of vibrios. Molecular methods are expensive, and vibrios are relatively rare pathogens in areas where cholera is not endemic. 16S sequencing has been used to identify clinically important *Vibrio* spp. The use of 16S sequencing alone is less than ideal for identification of vibrios, as the sequence differences between some species are very small and polymorphism has been shown to be fairly common in 16S rRNA genes of vibrios (53). There are few if any commercial molecular products with FDA approval for human clinical specimens. In-house molecular methods used in the United States for testing human clinical specimens will require extensive efforts to evaluate and implement in order to comply with all the Clinical Laboratory Improvement Amendments (CLIA) regulations. In a research setting, molecular methods for vibrios have proved to be very useful.

COMMERCIAL PRODUCTS FOR DETECTING CHOLERA TOXIN AND THE THERMOSTABLE DIRECT HEMOLYSIN OF *V. PARAHAEMOLYTICUS*

In reference laboratories, cholera toxin may be detected by fluid accumulation in animal assays or detection of a cytopathic effect in Y1 adrenal or Chinese hamster ovary cell cultures (40). However, a reverse passive latex agglutination assay produced by Denka Seiken, Tokyo, Japan, is commercially available (Oxoid, Inc., Ogdensburg, N.Y.).

The majority of human strains of *V. parahaemolyticus* produce a thermostable direct hemolysin (TDH) encoded by two genes, *tdh* and *tdh2x*. These toxins are rarely encountered in environmental strains of *V. parahaemolyticus* but have been detected in *V. cholerae* non-O1, *V. mimicus,* and *G. hollisae* strains. This hemolysin can be detected by observing hemolysis of red blood cells on Watgatsuma agar (Kanagawa test), a specialized agar, difficult to make. Like cholera toxin, TDH can be detected by a commercial latex assay (also available from Oxoid), but there are no commercial products that detect a second hemolysin seen in *V. parahaemolyticus* strains, i.e., thermostable-related hemolysin. PCR assays for TDH and thermostable-related hemolysin have been developed but are not commercially available (24).

TABLE 5 Differentiation of the three biogroups of *V. vulnificus*[a]

Test	Result for biogroup[b]:		
	1	2	3
Ornithine decarboxylase	V	−	+
Indole production	+	−	+
Acid produced by:			
D-Mannitol	V	−	−
D-Sorbitol	−	+	−
Cellobiose	+	+	−
Salicin	+	+	−

[a] Data from reference 10.
[b] V, variable (11 to 89% of strains are positive); +, positive (90 to 100% positive); −, negative (0 to 10% positive).

TYPING SYSTEMS

The usefulness of typing systems for determining strain relatedness among *Vibrio* isolates of the same species has very limited applicability for most clinical laboratories in the United States since, with the exception of very rare *V. parahaemolyticus* outbreaks, virtually all other *Vibrio* illnesses are sporadic in nature. Even then, state or federal reference laboratories will probably perform such extensive typing procedures. These typing schemes can basically be broken down into two groups, conventional (traditional) and molecular.

Among conventional techniques, serotyping is by far the most widely utilized procedure. Typing schemes have been described for a number of vibrio species including *V. cholerae, V. parahaemolyticus,* and *V. vulnificus* (40, 71, 72). However, commercial-grade typing sera are available only for *V. cholerae* and *V. parahaemolyticus*. *V. cholerae* O1 (polyclonal, serovars Inaba and Ogawa) and O139 antisera in one of several forms (slide, colorimetric) are available from Difco (Beckton Dickinson), Denka Seiken (Campbell, Calif.), Oxoid (Remel), and New Horizons (Columbia, Md.). *V. parahaemolyticus* antisera (O-group O1-O11 and K-group K1-K32) are available from Denka Seiken. *V. cholerae* isolates can also be typed by determining the sensitivity pattern to lytic bacteriophages (not commercially available), which may be another useful tool for tracking the spread of cholera. Phage (not commercially available) can be used for differentiating Classical from El Tor biotypes of *V. cholerae* O1 and O139 isolates (3, 14). The use of phage typing is limited to a few reference laboratories by the availability of typing phage and a lack of consensus in the typing schemes. Antibiograms, such as resistance to streptomycin, trimethoprim, and furazolidone, have been used to subtype *V. cholerae* O1 and O139 strains on a limited basis (8, 50). Such techniques are more useful in Southeast Asia, where unusual resistance patterns in isolates are observed more frequently than in the United States.

A large number of molecular typing methods have been successfully used in tracking the spread of *V. cholerae* epidemics and the clonal migration of *V. parahaemolyticus* strains and for phylogenetic analysis. However, these are primarily epidemiologic and taxonomic research techniques and not commercially available or easily adapted to clinical laboratories. Pulsed-field gel electrophoresis has been used extensively for both *V. cholerae* and *V. parahaemolyticus* (50, 52). Sequencing of single genes such as *ctx*A, *ctx*B, *hsp*60, and *rec*A shows promise as a molecular typing method (42).

SEROLOGIC TESTS

Serodiagnosis of cholera can be established with a high degree of certainty by titration of acute- and convalescent-phase sera in agglutination, vibriocidal, or antitoxin tests (48). The reagents are not commercially available, so this technique will normally be limited to a few reference laboratories.

ANTIMICROBIAL SUSCEPTIBILITY TESTING

Antibiotic resistance is more uncommon in *Vibrio* than in members of the family *Enterobacteriaceae*. However, all clinical *Vibrio* isolates should be tested against a number of therapeutically active compounds, since resistance can be acquired through plasmid transfer or exposure to antimicrobials and spread quickly through global travel. The Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) has interpretive guidelines only for *V. cholerae* limited to ampicillin, tetracyclines, folate pathway inhibitors,

and chloramphenicol (18). Because most vibrios grow rapidly and are similar to enteric bacteria in many ways, a first approximation might be to use interpretive guidelines for the *Enterobacteriaceae* for *Vibrio* species other than *V. cholerae* when testing agents that are not currently covered by the CLSI document.

There have been only limited susceptibility studies involving vibrios in recent times. Most strains of *V. cholerae* (O1, O139, and non-O1) are susceptible (>90%) in vitro to aminoglycosides, azithromycin, fluoroquinolones, extended-spectrum cephalosporins, carbapenems, and monobactams (70, 82). However, O1 El Tor and O139 strains from India and Bangladesh demonstrate moderate to high-level resistance to sulfamethoxazole, trimethoprim, and chloramphenicol (82). The fluoroquinolones alone or the synergistic combination of ciprofloxacin and cefotaxime shows excellent in vitro activity against *V. vulnificus* strains (43, 76). Most vibrios are also susceptible to tetracyclines, gentamicin, chloramphenicol (except *V. damsela*), monobactams, carbapenems, and fluoroquinolones.

INTERPRETATION AND REPORTING OF RESULTS

The isolation of *V. cholerae* O1 or O139 should be reported immediately to the attending physician because of the severe dehydration that cholera can produce. The case should also be reported by telephone to public health authorities, and the isolate should be sent to a public health laboratory for confirmation and toxin testing. Similarly, isolation of a vibrio from a sterile body site or a wound should be reported by telephone to the attending physician immediately so that rapid and appropriate antibiotic therapy can be initiated. *Vibrio* septicemia and/or meningitis have a high mortality rate associated with infection, and wound infections can frequently cause extensive tissue damage.

The clinical significance of *Vibrio* strains in many other specimens is more difficult to determine. Since physicians are not familiar with many *Vibrio* species, it would be helpful to provide a telephone consultation when a *Vibrio* isolate is identified. *Vibrio* isolates should also be submitted to public health laboratories, as they are monitored under the CDC emerging infections program and Vibrio Surveillance System; they may also be needed for confirmation and toxin testing. *Vibrio* species that are known to cause diarrhea should be considered clinically significant, particularly if they are present in large numbers and no other potential pathogens are present. Isolation of vibrios from stool in small numbers may reflect only transitory colonization. *V. cholerae*, *V. mimicus*, and *V. parahaemolyticus* have documented virulence factors that correlate with their ability to cause intestinal infections. Laboratory tests are helpful in determining pathogenic potential but are likely to be done only in reference laboratories. The same warning should be emphasized for *Vibrio* isolates from other specimens such as ears or wounds. The isolation of vibrios could represent infection, transient colonization, or merely the vibrio flora that is always present in seawater or brackish water.

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*Pseudomonas**

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TAXONOMY

Pseudomonas is a large and complex genus of gram-negative bacteria of importance, as it includes species with both clinical and environmental implications. The genus *Pseudomonas* first proposed by Migula in 1894 (91) has undergone many taxonomic revisions as methodologies of identification to the species level continue to improve. The genus *Pseudomonas* was comprised of five unrelated groups, as determined by ribosomal RNA-DNA hybridization studies in the early 1970s. *Pseudomonas* (sensu stricto) is rRNA homology group I (28), in the gamma subclass of the *Proteobacteria*. The other rRNA homology groups are II, *Burkholderia* species; III, *Comamonas, Acidovorax,* and *Hydrogenophaga* genera; IV, *Brevundimonas* species; and V, *Stenotrophomonas* and *Xanthomonas* genera (69). Currently there are 160 species within the *Pseudomonas* genus, with only 12 (described herein) of clinical interest. The closest phylogenetic neighbors of the *Pseudomonas* genus are the genera of *Azomonas, Azotobacter, Cellvibrio, Chrysomonas,* and *Flavimonas.*

Several of the clinically relevant *Pseudomonas* species demonstrate marked heterogeneity and have been subdivided into biovars or genomovars. Genomovars are genetically distinct groups that warrant species designation but lack phenotypically defining characteristics and that are determined by DNA-DNA reassociation experiments and 16S rRNA gene sequencing in combination with chemotaxonomic total fatty acid analysis and total protein pattern analysis (49).

The highest level of genetic diversity of any species known is found in *Pseudomonas stutzeri,* which has at least 18 genomovars (131), with clinical isolates being found in genomovars 1 and 2. There are no consistent phenotypic differences to justify splitting *P. stutzeri* into unique species (49).

Pseudomonas fluorescens was originally divided into biotypes A, B, C, D, E, F, and G (biotypes A to E are also referred to as biovars I, II, III, IV, and V). Biotype B was reclassified as *Pseudomonas marginalis.* Biotypes D and E *(Pseudomonas chlororaphis* and *Pseudomonas aureofaciens)* have now been combined into the single species *P. chlororaphis,* which is no longer considered a member of the fluorescent pseudomonad group.

Pseudomonas putida consists of biovars A and B. Biovar A should be regarded as the typical *P. putida* (34), while biovar B may have a closer affinity with *P. fluorescens.* More biovars of *P. putida* are warranted (34).

Great heterogeneity is found within the species of *P. stutzeri, P. fluorescens,* and *P. putida,* which are of interest mainly to plant, marine, soil, and biotechnical sciences. They are of limited importance in clinical medicine. As polyphasic taxonomy continues to advance, more changes will doubtlessly arise; the clinical laboratory must keep abreast of such changes in order to differentiate these isolates from the more clinically important *Pseudomonas* species.

GENERAL DESCRIPTION

Pseudomonas spp. are aerobic, non-spore-forming, gram-negative rods which are straight or slightly curved and 0.5 to 1.0 by 1.5 to 5.0 μm in size (63). They are usually motile, with one or several polar flagella. They possess a strictly aerobic respiratory metabolism with oxygen as the terminal electron acceptor; in some cases nitrate can be used as an alternative electron acceptor that allows anaerobic growth. Most species of clinical interest are oxidase positive (except *Pseudomonas luteola* and *Pseudomonas oryzihabitans*). *Pseudomonas* spp. are catalase positive and are chemolithotrophs.

NATURAL HABITATS

Most *Pseudomonas* species can reside in a wide variety of environmental niches. Since their preferred temperature requirements are between 4 and 36°C and they can utilize an extraordinarily varied range of nutrients, they can be found throughout nature, provided that a moist environment is available. For example, *P. putida* is commonly found in soil, water, plants, and hospital sources such as sinks and floors. When isolated from human specimens, they are usually of indeterminate clinical significance. Due to their opportunistic nature, *Pseudomonas* species such as *Pseudomonas veronii,* found in mineral water (32), may account for unusual isolates that may infect immunocompromised patients.

*This chapter contains information presented in chapter 47 by Deanna L. Kiska and Peter H. Gilligan in the eighth edition of this Manual.

EPIDEMIOLOGY AND TRANSMISSION

P. aeruginosa is hydrophilic and can be readily recovered from moist environments, such as sink drains, vegetables, river water, and even antiseptic solutions. The propensity of *P. aeruginosa* to colonize raw vegetables might pose a risk to immunocompromised (particularly neutropenic) patients (118). However, none of these environmental reservoirs poses a great risk to most individuals; ingestion does not appear to induce gastrointestinal colonization unless antibiotic therapy has altered the normal bacterial flora.

Pseudomonas species, and in particular the human pathogen *P. aeruginosa,* rarely colonize healthy humans (136). The throat, intact skin, or stools of healthy individuals are heavily colonized by normal flora, which does not include *Pseudomonas* species. Indeed, if *P. aeruginosa* (and in particular the mucoid variant) is recovered from a throat culture of an otherwise healthy individual, an explanation should usually be sought. The gastrointestinal tracts of healthy humans and mice are heavily colonized with other bacteria that likely provide colonization resistance against *P. aeruginosa*. Healthy mice fed 10^7 CFU of *P. aeruginosa* fail to sustain intestinal colonization unless they have been pretreated with antibiotics (72).

Individuals (such as neutropenic cancer patients) receiving frequent courses of antibiotic therapy are at risk of gastrointestinal colonization with *P. aeruginosa;* it is from this reservoir that they are then at risk for *P. aeruginosa* septicemia. Such autoinfection has been demonstrated by recovering *P. aeruginosa* from the stools of neutropenic hosts prior to a bout of sepsis with the same strain (149). Similar observations have been made for neutropenic mice (72).

P. aeruginosa can be recovered from body sites that remain moist, such as the outer ear of children who swim frequently or the endotracheal tubes of patients receiving mechanical ventilation (93). For reasons which remain poorly understood, *P. aeruginosa* can also colonize the upper respiratory tracts of patients in intensive care units; this may be due in part to the alteration of the buccal epithelial cells with loss of fibronectin and attendant loss of the cells' antiadhesive properties (163). Adults receiving mechanical ventilation or neutropenic patients are at high risk for developing ventilator-associated or other pneumonias caused by *P. aeruginosa* (17), particularly after or during treatment with broad-spectrum antimicrobial agents (121). Patients at greatest risk are adults undergoing cancer chemotherapy or marrow ablation for bone marrow transplantation; children with similar conditions are at lesser risk for *P. aeruginosa* bacteremia. Individuals with congenital neutropenia or cyclic neutropenia do not appear to be at much risk for invasive infection with *P. aeruginosa*. Normal skin does not support *P. aeruginosa* colonization, but burned skin is an attractive site for this bacterium, which is one of the leading causes of burn wound sepsis.

P. aeruginosa is the predominant respiratory tract pathogen in patients with cystic fibrosis (CF) (47), but its mode of acquisition is poorly understood (133). Several studies have each demonstrated a common clone in particular groups of patients who have received their care at the same center (7, 18, 68); this is most likely due to patient-to-patient spread. However, in most patients with CF, it appears that the infecting strain undergoes a switch from the environmental phenotype (lipopolysaccharide [LPS] smooth, nonmucoid, and motile) to the CF phenotype (LPS rough, mucoid, and nonmotile) during the course of infection (135). That patients each tend to carry a unique strain during the course of infection (101, 137) suggests that the infection was acquired from an environmental source. One large study performed in Vancouver, Canada, over more than 20 years failed to demonstrate patient-to-patient spread of *P. aeruginosa* except between siblings (137). Infection control policies applicable to CF patients for transmission prevention should be determined by local epidemiological experience (154).

CLINICAL SIGNIFICANCE

P. aeruginosa

Individuals with intact host defenses are not at risk for serious infection with *P. aeruginosa,* but those whose circulating neutrophil counts are profoundly depressed (such as patients with cancer receiving chemotherapy) are at risk for invasive infection (134). Neutropenic hosts who develop antibodies against specific serotypes of *P. aeruginosa* appear to be protected against infection with those types during periods of neutropenia (109), illustrating the important role for opsonization in protection against blood-borne infection. Individuals with thermal burns are at risk for invasive disease because the dermal barrier can be breached, and patients on mechanical ventilation are at risk for pneumonia because the normal respiratory mucociliary clearance is compromised.

Recovery of *P. aeruginosa* from respiratory tract cultures of patients receiving mechanical ventilation may not indicate a true infection, and the significance of its presence in the culture should be interpreted with caution.

P. aeruginosa has a particular tropism for CF epithelial cells and can resist normal respiratory tract host defenses. Once infection is established, it usually persists, and the bacteria undergo a transition to the CF phenotype consisting of the following: (i) a rough LPS (53), in which the O-polysaccharide is incompletely expressed, rendering the bacteria susceptible to the bactericidal effect of human serum; (ii) mucoid colonial morphology (80) resulting from the exuberant production of a mucoid expolysaccharide composed of O-acetylated guluronic and mannuronic acids; (iii) nonmotility (87), meaning that the bacteria lack normal functional flagellar function; and (iv) hypoexpression of various exotoxins and other exoproducts (11). Some of these changes may be under global regulation, but they can also be expressed individually. Transition of *P. aeruginosa* from nonmucoid to mucoid in the CF patient's lung is usually associated with an accelerated decline in pulmonary function and an adverse prognosis (106), perhaps because of the capacity of the mucoid exopolysaccharide to interfere with normal host phagocytic defenses (47, 136) and to facilitate the formation of biofilms (76). Biofilm formation may also be enhanced by another colonial form, small-colony variants (previously known as dwarf colonies) (56). Furthermore, CF patients receive frequent courses of antipseudomonas antimicrobial therapy, often rendering the bacterium with which they are chronically infected resistant to a wide range of antimicrobial agents (54).

Thermal burns of the skin inhibit an essential component of the body's defense against infection, the physical barrier of the intact dermis (113). The resulting damaged tissue is a rich culture medium and is at great risk for colonization and infection by *P. aeruginosa;* such infections have been one of the leading causes of morbidity and mortality in victims of burns. Topical therapy is designed to prevent *P. aeruginosa* and other pathogens from causing infection. Infections of burn wounds with gram-negative bacteria (in particular *P. aeruginosa*) typically occur about 1 week after the injury. The extent of the burn has a profound influence on the risk of infection and prognosis (113). Prevention of bacterial

burn wound infection has become so effective over the past decade that it is now very rare, and in many centers fungal infections predominate.

P. aeruginosa is the most common cause of osteochondritis of the dorsum of the foot following penetrating wounds (20). The typical scenario involves a child who has stepped on a nail which pierces the foot after passing through the sole of a running shoe. The prevalence of *P. aeruginosa* as the etiological agent may be due to its propensity to survive in the rubber of old running shoes (35).

Because *P. aeruginosa* can survive up to 42°C, hot tub users are at risk of *P. aeruginosa* folliculitis (48), a self-limiting condition for normal hosts that resolves rapidly. People who spend extended periods for time swimming are at risk for external ear infections ("swimmer's ear"), another self-limiting condition in immunocompetent people that responds readily to therapy with topical antimicrobial agents (9). The cornea is relatively resistant to infection except when its integrity has been broken. Users of contact lenses are at risk of *P. aeruginosa* conjunctivitis, especially if hygiene is poor or lenses are worn for extended periods of time (142).

P. aeruginosa can cause meningitis (usually following trauma or surgery) (36), malignant otitis externa in diabetics (122), sepsis and meningitis in newborns (145), endocarditis or osteomyelitis in intravenous drug users (126), community-acquired pneumonia in people with underlying lung disease such as bronchiectasis (43), urinary tract infections in patients with complex urinary tract abnormalities (120), and peritonitis (97). Each of these presentations is unusual and is superimposed on some inhibition of normal host defenses.

Other *Pseudomonas* Species

Healthy individuals are resistant to serious infections by all *Pseudomonas* species, including *P. aeruginosa*. However, immunocompromised hosts are occasionally infected with one of the many non-*P. aeruginosa* species (Table 1). Several of these species have been recovered from the respiratory secretions of patients with CF, but their role in pathogenesis of lung disease has not been determined. Some of these species have the capacity, like *P. aeruginosa,* to grow in hostile environments, such as antiseptic solutions; they can therefore be the cause of pseudobacteremia. Because of their low virulence, infections due to these species are often iatrogenic and are associated with the administration of contaminated solutions, medicines, and blood products or with the presence of indwelling catheters. *P. fluorescens* and *P. putida* have the ability to grow at 4°C, and *P. fluorescens* can be isolated from the skin of a small proportion of blood donors (128), resulting in occasional transfusion-associated septicemia in the recipient.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Pseudomonas spp. are able to survive in diverse environments. These organisms are easily recovered from clinical specimens by using standard collection, transport, and storage techniques as outlined in chapters 5 and 20.

DIRECT EXAMINATION

Pseudomonas organisms are gram-negative, non-spore-forming, straight or slightly curved bacilli measuring 0.5 to 0.8 μm by

TABLE 1 Non-*P. aeruginosa* isolates of *Pseudomonas* spp. recovered from human clinical specimens

Organism	Source	Reference(s)
P. fluorescens	Respiratory isolates from CF patients, transfusion-associated septicemia, pseudobacteremia	128
P. putida	Nosocomial infections including bacteremia, pneumonia, and urinary tract infections following external instrumentation/ catheterization. CF, elderly, and immunocompromised patients are most vulnerable.	74, 165
P. veronii	Associated with an intestinal inflammatory pseudotumor	19
P. monteilii	Recovered from stool, bile, placenta, bronchial aspirates, pleural fluid, and urine, but of uncertain clinical significance	33
P. mosseilii	Various clinical specimens of uncertain clinical significance	26
P. stutzeri	Respiratory isolates of CF patients, alcoholics with pneumonia; immunocompromised patients with bacteremia, meningitis, and pneumonia; and patients with osteomyelitis, iatrogenic endophthalmitis, pseudobacteremia, and bacteremia from contaminated hemodialysis fluid. Also recovered from respiratory tracts of intubated patients, from urine, and from wound specimens in which pathogenic role was uncertain.	15, 45, 67, 70, 99, 112, 117, 119
P. mendocina	Rare cases of endocarditis	6
P. alcaligenes	One reported case of catheter-related endocarditis in a bone marrow transplant recipient	90
P. pseudoalcaligenes	A case of meningitis. Also isolated from sputum and urine. May be coisolated with other pathogens in wounds in which clinical significance is uncertain.	24
P. luteola	Associated with bacteremia, cellulitis, osteomyelitis, peritonitis, endocarditis, and postsurgical meningitis	115, 116
P. oryzihabitans	Bacteremia in immunocompromised patients with central venous access devices, peritonitis in peritoneal dialysis patients, cellulitis, abscesses, wound infections, and postsurgical meningitis	89, 115

1.5 to 3.0 μm. The Gram stain morphology cannot easily distinguish *Pseudomonas* spp. from other nonfermenting bacilli, although they are usually thinner than *Enterobacteriaceae*. Among the pseudomonads, there is some variation in Gram stain morphology. Certain strains of *P. putida* can appear elongated. Organisms from older cultures may appear slightly pleomorphic. Mucoid strains may be distinguished on direct examination by the presence of clusters or long filaments of short gram-negative bacilli surrounded by darker pink staining material (alginate). It is important to note this on direct examination since the organisms may grow very slowly or not at all. The presence of these mucoid forms should be documented on clinical reports. Because *Pseudomonas* spp. may be colonizers, their isolation does not always link them to clinical disease. However, their intracellular presence in polymorphonuclear cells is clinically significant and should be documented and direct further workup. Flagellar stains reveal one or more polar flagella (Table 2). *P. aeruginosa* has a single polar flagellum.

NUCLEIC ACID DETECTION TECHNIQUES

P. aeruginosa and other *Pseudomonas* species are detected ordinarily by culture techniques; these methods are particularly important for determining antimicrobial susceptibility, as these organisms have a high degree of intrinsic and acquired resistance (54, 83). When a more rapid method is desired, such as for screening environmental niches or for rapidly evaluating the sputum of patients with CF (22, 138), methods that can be used include PCR amplification of various genomic regions, such as genes for ribosomal RNA (65), heat shock protein (22), or exotoxin A (71). Conventional and real-time PCR (RT-PCR) (65) are both useful, and the amplification of multiple targets is valuable in the identification of non-*aeruginosa Pseudomonas* species (114). Fluorescence in-situ hybridization (FISH) is very specific, although it lacks sensitivity below 10^4 CFU (62). Both RT-PCR and FISH are rapid, cost-effective methods, and FISH doesn't require costly technical equipment (159). Probes directed at species-specific 16S rRNA (50, 141) may have a role in the identification of clinically relevant, biochemically inactive *Pseudomonas* species including certain strains of *P. aeruginosa* (114). PCR amplification of 16S rRNA followed by restriction fragment length polymorphism (RFLP) analysis has been used to successfully identify and characterize members of the fluorescent pseudomonad group (75). Since RFLP of the 16S gene does not provide sufficient resolution among genomovars of a species, a more discriminatory test may be used, such as sequencing the internally transcribed 16S-23S rRNA spacer internal transcribed spacer 1 regions, believed to have more genetic variability among genomovars (49). It should be noted that commercial probes are not yet available.

CULTURE AND ISOLATION

Pseudomonas species have very simple nutritional requirements and grow well on standard broth and solid laboratory media such as tryptic soy or columbia agar with 5% sheep blood, chocolate agar, and MacConkey agar, which are recommended to isolate *Pseudomonas* spp. from clinical specimens. MacConkey agar is also a differential medium helpful in identifying different strains of *Pseudomonas* spp., including mucoid strains of *P. aeruginosa* from CF patients. Multiple selective media containing inhibitors such as acetamide, nitrofurantoin, phenanthroline, 9-chloro-9-[4-(diethyamino)phenyl]-9,10-dihydro-10-phenylacridine hydrochloride (C-390), and cetrimide (14, 58) have been used for the isolation and presumptive identification of *P. aeruginosa* from clinical and environmental samples. Inhibition of some strains of *P. aeruginosa* from CF sputum specimens has been reported to occur with the use of a selective agar containing cetrimide (200 mg/liter) and nalidixic acid (15 mg/liter) (37), emphasizing the need to use both selective and nonselective media for recovery of bacteria from these patients. Some of the non-*aeruginosa* pseudomonads may grow better at lower temperatures of 28 to 30°C. Good growth is usually achieved after 24 to 48 h of incubation. For cultures from CF patients, it is recommended that solid media plates be held at 35 to 37°C for 5 days.

IDENTIFICATION

Fluorescent Group

Members of the fluorescent pseudomonad group produce pyoverdin (fluorescein), a water-soluble yellow-green or yellow-brown pigment that fluoresces under short-wavelength UV light. Many strains of *P. aeruginosa* can produce the blue pigment pyocyanin. When pyoverdin combines with the blue water-soluble phenazine pigment pyocyanin, the bright green color characteristic of *P. aeruginosa* is created. This organism may also produce other water-soluble pigments such as pyorubrin (red) or pyomelanin (brown/black). Conditions of iron limitation enhance pigment production, as these pigments act as siderophores in iron uptake systems of the bacteria. Non-dye-containing media enhance visualization of pigments.

P. aeruginosa

Most *P. aeruginosa* are easily recognizable on primary isolation media on the basis of characteristic colonial morphology, production of diffusible pigments, and a grape-like odor. Older cultures may exhibit a corn taco-like odor. Colonies are usually flat and spreading and have a serrated edge and a metallic sheen that is often associated with autolysis of the colonies (166). Other morphologies exist, including smooth, mucoid, and dwarf (small-colony variants) (104, 155). Mucoid colonial variants are particularly prevalent in respiratory tract specimens from CF patients.

P. aeruginosa is distinct from the rest of the clinically relevant fluorescent pseudomonads in its ability to grow at 42°C (see Table 2 for other tests). In addition to pigment production, other tests that confirm its identification are positive oxidase and arginine tests and an alkaline over no-change reaction in a triple-sugar iron agar slant.

Microbiologists must be aware of certain variations in the phenotypes of *P. aeruginosa*. Isolates lacking oxidase activity have occasionally been reported, but they exhibit the other characteristic features; prior antibiotic therapy with agents that affect protein synthesis may cause the aberrant phenotype (52). Mucoid isolates of *P. aeruginosa* from CF patients may undergo several phenotypic changes including slow growth, loss of motility, and loss of pigment production. Small colony variants may require prolonged incubation, lack motility, be hyperpiliated, adhere to agar surface, and show autoaggregative properties in liquid medium (155).

P. fluorescens and *P. putida*

P. fluorescens and *P. putida* do not possess distinctive colony morphology or odor. Their inability to reduce nitrates to

TABLE 2 Characteristics of *Pseudomonas* species found in clinical specimens[a]

Test	*P. aeruginosa* (*n* = 201)	*P. fluorescens* (*n* = 155)	*P. putida* (*n* = 16)	*P. veronii* (*n* = 8)	*P. monteilii* (*n* = 10)	*P. mosseilii* (*n* = 12)	*P. stutzeri* (*n* = 28)	*P. mendocina* (*n* = 4)	*P. pseudoalcaligenes* (*n* = 34)	*P. alcaligenes* (*n* = 26)	*P. luteola* (*n* = 34)	*P. oryzihabitans* (*n* = 36)
Oxidase	99	97	100	100	100	100	100	100	100	96	0	0
Growth:												
MacConkey	100	100	100	ND[c]	ND	ND	100	100	100	96	100	100
Cetrimide	94	89	81 (6)	ND	90	100	4	75 (25)	56 (18)	15	0	25 (28)
6% NaCl	65	43	100	ND	0	100[e]	80 (16)	100	62 (6)	41	74	62
42°C	100	0	0	0	0	0	69	100	94	V[f]	94	33
Nitrate reduction	98	19	0	100	0	0	100	100	100	54	62	6
Gas from nitrate	93	3	0	100	0	0	100	100	0	0	0	0
Pyoverdin	65	96	93	100	100	100	0	0	0	0	0	0
Arginine dihydrolase	100	97	100	100	100	100	0[d]	100	78	12	100	14
Lysine decarboxylase	0	0	0	ND	0	0	0	0	0	0	0	7
Ornithine decarboxylase	0	0	0	ND	0	0	0	0	0	0	0	3
Hydrolysis:												
Urea	48 (9)	21 (31)	31 (44)	25	50	ND	33 (22)	50	3 (6)	0	26 (38)	77
Gelatin[g]	82	100	0	13	0	92	0	0	0	0	61	17
Acetamide	100	6 (12)	0	0	0	ND	0	0	ND	ND	ND	ND
Esculin	0	0	0	ND	0	0	0	0	0	0	100	0
Starch	0	0	0	ND	0	8	100	0	0	0	0	0
Acid from[b]:												
Glucose	97	100	100	100	100	100	96 (4)	100	9	0	100	100
Fructose	ND	ND	ND	100	100	100	ND	ND	79 (21)	0	ND	ND
Xylose	90	100	100	100	0	0	93 (7)	75 (25)	18 (12)	0	100	100
Lactose	<1	24	25 (13)	ND	0	0	0	0	0	0	3 (24)	14 (22)
Sucrose	0	48	0	100	0	17	0	0	0	0	12	25
Maltose	<1	2	31	ND	0	17	100	0	0	0	100	97
Mannitol	70	53	25	ND	0	75	89 (4)	0	0	0	76 (18)	100
Simmons citrate	95	93	94 (6)	ND	100	100	82 (14)	100	26 (9)	57 (8)	100	97
No. of flagella	1	>1	>1	1	ND	1	1	1	1	1	>1	1

[a] Results are given as percentages of positive strains; percentages in parentheses represent strains with delayed reactions. Data are from references 26, 32, 33, 63, and 160.
[b] Oxidative-fermentative basal medium with 1% carbohydrate.
[c] ND, no data.
[d] *P. stutzeri*-like organisms (formerly CDC group 3b) are arginine dihydrolase positive.
[e] Growth at 3 to 5% NaCl but not at 7% NaCl.
[f] V, variable, many strains can grow at 41°C; see comment in text under "Identification."
[g] Results are for 7-day incubation.

nitrogen gas and their ability to produce acid from xylose distinguish these two species from the other fluorescent pseudomonads. *P. fluorescens* can be differentiated from *P. putida* by its ability to grow at 4°C and to hydrolyze gelatin. *P. fluorescens* isolates may require 4 to 7 days of incubation for accurate detection of gelatin hydrolysis. The package insert for API 20NE (bioMérieux Vitek, Hazelwood, Mo.) states that only 39% of *P. fluorescens* isolates hydrolyze gelatin in 24 to 48 h in this test system.

P. veronii, P. monteilii, and *P. mosselii*

P. veronii can reduce nitrates to nitrogen gas but is unable to hydrolyze acetamide. The type strain of *P. veronii* (LMG 17761) is negative for acid from lactose and maltose and does not grow at 36°C (D. A. Henry, personal observation). *P. monteilii* can be distinguished from the other members of the fluorescent group by its inability to reduce nitrates to nitrites or nitrogen gas, to hydrolyze gelatin, or to produce acid from xylose. *P. mosselii* can reduce nitrates neither to nitrites nor to nitrogen gas, nor can it produce acid from xylose; but most isolates (92%) can hydrolyze gelatin (Table 2).

Other fluorescent pseudomonads are rarely encountered in clinical specimens. Many of these isolates are negative for arginine dihydrolase activity. Identification as "*Pseudomonas* species not *aeruginosa*" and susceptibility testing of the isolates, when appropriate, are sufficient in most circumstances. When necessary, these isolates can be referred to reference laboratories.

Nonfluorescent Group

P. stutzeri and *P. mendocina*

Most *P. stutzeri* isolates are easily recognized on primary isolation media by their distinctive dry, wrinkled colony morphology, similar to that of *Burkholderia pseudomallei. P. stutzeri* can be distinguished from the latter species by its lack of arginine dihydrolase activity and inability to produce acid from lactose. *P. stutzeri* colonies can pit or adhere to the agar and are buff to brown in color. The adherence can make removal of colonies from agar medium difficult. Because of the difficulty in making suspensions of specific turbidity, commercial susceptibility systems may not work well with this organism. Not all isolates of *P. stutzeri* produce wrinkled colonies; such strains can be distinguished from other pseudomonads by their ability to hydrolyze starch, a unique reaction for this species.

P. mendocina colonies are smooth and flat and produce a brownish yellow pigment. Key biochemical characteristics include the ability to reduce nitrates to nitrogen gas, positive arginine dihydrolase activity, and inability to hydrolyze acetamide.

P. alcaligenes and *P. pseudoalcaligenes*

P. alcaligenes and *P. pseudoalcaligenes* have rarely been encountered in clinical samples and do not have a distinctive colony morphology. Compared to other pseudomonads, they are biochemically inactive. Characteristics that distinguish them from other biochemically inactive gram-negative rods are a positive oxidase reaction, motility due to a polar flagellum, and growth on MacConkey agar. *P. alcaligenes* is distinguished from *P. pseudoalcaligenes* by its inability to oxidize fructose. Although growth at 42°C was thought to be a distinguishing feature between them, further studies now indicate that growth at 41°C (and probably 42°C) is also present in most strains of *P. alcaligenes* (N. Palleroni, personal communication). These organisms are difficult to identify by many commercial systems, and for most clinical situations they can simply be referred to as "*Pseudomonas* spp. not *aeruginosa*." If the clinical situation dictates a definitive identification, assistance from reference laboratories should be sought.

P. luteola and *P. oryzihabitans*

P. luteola and *P. oryzihabitans* (formerly CDCVe-1 and CDCVe-2, respectively) can be distinguished from other pseudomonads by their negative oxidase reaction and production of an intracellular, nondiffusible yellow pigment. Both organisms typically exhibit rough, wrinkled, adherent colonies or, more rarely, smooth colonies. *P. luteola* can be differentiated from *P. oryzihabitans* on the basis of its ability to hydrolyze *o*-nitrophenyl-β-D-galactopyranoside (ONPG) and esculin.

Use of Commercial Identification Systems

Commercial identification systems are used increasingly in many laboratories to identify *Pseudomonas* spp. Commercial products can be divided into manual and automated systems. The more frequently used manual systems are the API 20NE (bioMérieux Vitek), Crystal E/NF (Becton Dickinson, Sparks, Md.), and RapID NF Plus (Remel Inc., Lenexa, Kans.). The manual systems usually provide accurate identification of *P. aeruginosa,* including mucoid isolates as well as other *Pseudomonas* species, and are preferred over automated systems for isolates from CF patients.

Automated systems (see chapter 15) are commonly used in many medium-to-large clinical laboratories. As *P. aeruginosa* is easily identified by a few conventional biochemical tests, it is not necessary to use a more expensive commercial system. Several of the automated systems are not very accurate and may require additional testing for non-*P. aeruginosa* species; thus, their labor-, cost-, and time-saving benefits are lost. Automated systems can identify *P. aeruginosa* from non-CF patient sources with 90 to 100% accuracy (40, 103), but some systems such as the Autoscan-W/A (Dade Behring Microscan Inc., West Sacramento, Calif.) may require additional tests to achieve these results (102, 147). Most reviews focus on the evaluation of *P. aeruginosa* with only a few, if any, other *Pseudomonas* species represented in the organisms being tested. When other *Pseudomonas* species were included, the new Vitek 2GN panel performed well (39, 103) while the Autoscan and BD Phoenix (Becton Dickinson) often relied on additional testing to obtain an identification (30, 102, 140, 147). Hence, it is wise to consider carefully the clinical significance, colonial morphology, and other key features before accepting results from automated systems.

Identification of *Pseudomonas* species, especially those isolated from CF patients, is not always optimal with rapid systems. The Autoscan-W/A system (Negative Combo 15) performed poorly for CF isolates when they were incubated for 20 to 24 h according to the manufacturer's method, with only 57% of nonmucoid and 40% of mucoid *P. aeruginosa* isolates correctly identified (125). Extension of incubation to 48 h improved accuracy to 86 and 83%, respectively. Misidentified species were most commonly identified as either *Alcaligenes* spp. or *P. fluorescens/P. putida.* Other automated systems have not been evaluated to date specifically for the identification of CF patient isolates, so caution in interpreting results is advised.

TYPING SYSTEMS

Genotypic methods have generally supplanted conventional schemes based on phenotypic characteristics (reviewed in

reference 133) such as LPS serotyping and phage typing. Several different genotypic methods for typing *P. aeruginosa* for epidemiological purposes are useful, even for typing isolates from patients with CF, but they are not available in most clinical diagnostic laboratories, and their use is often dependent upon local availability.

RFLP

This method relies upon the genetic diversity that exists upstream of the gene for exotoxin A (*exoA*) in *P. aeruginosa* (101). In a study of different typing methods, *exoA* RFLP proved superior to all phenotypic methods for typing *P. aeruginosa* (151). Pilin gene RFLP has demonstrated that individual CF patients are durably infected with the same strain despite changes in pilin protein expression (101). The disadvantages of RFLP are its relatively weak discriminatory power compared to that of newer methods, its cumbersome nature, and its predominant use of radioactive probes.

PFGE

P. aeruginosa has substantial genetic plasticity, so there can be more than three band differences among isolates typed by pulsed-field gel electrophoresis (PFGE) (see chapter 11) and considered epidemiologically to be from the same strain, even though Tenover's criteria state that if there are three or fewer banding differences between two isolates, they should be considered to be from the same strain, as such differences are likely to be due to only one genetic event (150). The advantages of PFGE are its universal utility for typing virtually any bacterial species and its high discriminatory power. The major disadvantages are its requirement for specialized equipment and its inability to evaluate a large number of isolates rapidly.

PCR-Based Typing Methods

PCR-based methods used for typing *P. aeruginosa* are directed at known elements within the genome or against random but relatively frequently encoded sequences. The latter method, random amplified polymorphic DNA analysis (RAPD), has proved quite robust for typing *P. aeruginosa* (88), but it must be run consistently on the same equipment to yield reproducible results. Data from RAPD analysis usually are highly consistent with those from PFGE. PCR-amplified products can be digested with restriction enzymes to yield more discriminatory data (127).

Multilocus Sequence Typing

Multilocus sequence typing has only recently been employed for typing *P. aeruginosa*. It is likely to be the most highly discriminatory among the genetic typing tools, but it is extremely time-consuming and expensive to employ. The method entails PCR amplification of specific genes and then sequencing of the gene products. This can be done only in very specialized centers, but it has the power to provide highly reliable data on relatedness among isolates. Standards are currently being developed, and a large study is evaluating the suitability of this method for typing *P. aeruginosa* (25).

ANTIMICROBIAL SUSCEPTIBILITY

P. aeruginosa possesses intrinsic resistance to many antibiotic classes and has the ability to develop resistance by mutations in different chromosomal loci or by horizontal acquisition of resistance genes carried on plasmids, transposons, or integrons. The frequent acquisition of antimicrobial resistance in *P. aeruginosa* limits the utility of antimicrobial susceptibility patterns as a tool in epidemiologic typing.

Mechanisms of Resistance

Intrinsic Resistance

P. aeruginosa has two main mechanisms of intrinsic resistance: an inducible chromosomal AmpC β-lactamase that renders it resistant to ampicillin, amoxicillin, amoxicillin-clavulanate, narrow-spectrum and expanded-spectrum cephalosporins, cefotaxime, and ceftriaxone (81); and several efflux pump systems (83).

Acquired Resistance

Various antibiotics overcome the intrinsic resistance of *P. aeruginosa*. These include extended-spectrum penicillins (piperacillin and ticarcillin), certain expanded-spectrum cephalosporins (ceftazidime and cefipime), carbapenems (imipenem and meropenem), monobactams (aztreonam), fluoroquinolones (ciprofloxacin and levofloxacin), aminoglycosides (gentamicin, tobramycin, and amikacin), and colistin. Unfortunately, mutational resistance to all the antipseudomonal antibiotics can develop.

A mutation at the AmpD locus, selected by therapy with antipseudomonal penicillins or ceftazidime (82), can result in partial or total derepression of the AmpC enzyme, which may account for 30% of β-lactamase resistance in *P. aeruginosa* (16).

Target mutations to topoisomerases II (*gyrA* and *gyrB* subunits) and IV (*parC* and *parE* subunits) confer quinolone resistance more readily in *P. aeruginosa* than in *Enterobacteriaceae*. Selection of mutants occurs after exposure to quinolones, and evidence suggests that levofloxacin has a greater potential to induce these mutations than ciprofloxacin (44).

Although multidrug efflux pump systems play a significant role in the intrinsic resistance of *P. aeruginosa*, they also are critical to the development of multidrug resistance (98). MexAB-OprM is expressed constitutively in all strains of *P. aeruginosa*. Upregulation or a mutation in the *mexR* repressor gene (*nalB* mutant) results in efflux pump overproduction and significant increase in the MICs of multiple antibiotics (111).

Efflux pump mutants may appear under conditions favoring high bacterial density as found in abscesses, empyemas, diabetic foot infections, and chronic lung infections. In one study, efflux pump-overproducing mutants were found in isolates from 80% of CF patients who had received earlier treatment with ciprofloxacin (66). Overexpression of efflux pumps may also be selected by the use of antiseptics and biocides (79). Evidence of multiple resistance determinants including an efflux transport system encoded by a transmissible plasmid in environmental bacteria (148) would be of grave concern if these genetic elements were to be transferred to *Pseudomonas* species.

Impermeability mutations may result in resistance to carbapenem, aminoglycosides, colistin, and quinolones. They are important in carbapenem resistance and result from the loss of the OprD porin, which is associated with low-level (MIC, 8 to 32 μg/ml) imipenem resistance and decreased susceptibility to meropenem (73). Resistance by this mechanism depends on the continued expression of the chromosomal AmpC β-lactamase. Selection of imipenem resistance following imipenem therapy is more frequent than selection of resistance to any other β-lactam agent (152).

Mutational impermeability is also associated with reduced aminoglycoside transport into the cell and lack of

susceptibility to all aminoglycosides. Impermeability may be the major mechanism of aminoglycoside resistance, especially in CF patients (85), but it is clear that efflux systems also contribute (3).

Membrane changes most likely account for colistin resistance, which is rare but increasing, especially in CF patients who receive inhalational colistin (78). It appears that regulatory genes for the outer membrane protein OprH, specifically the LPS component of the outer membrane, may be involved in both aminoglycoside and colistin resistance (77, 110). Decreased uptake as a result of porin reduction contributes to quinolone resistance but seems to be synergistically linked to upregulation of efflux pumps (79).

The acquisition of β-lactamases is not as common for *P. aeruginosa* as it is for *Enterobacteriaceae* (81). Nevertheless, β-lactamases are being recognized increasingly and are very diverse in this organism. The most common β-lactamases are PSE-1 and PSE-4, which do not affect ceftazidime, cefipime, aztreonam, or carbapenems (83). Other enzymes have been found in limited geographic locations, suggesting a specific ecological niche. More recently, extended-spectrum β-lactamases (ESBLs) in *P. aeruginosa* have been described. Genes for ESBLs are carried on plasmids, integrons in plasmids, or in the bacterial chromosome (83, 157). This group of enzymes is inhibited by clavulanic acid and only marginally inhibited by tazobactam. With minor variations, substrates for most of these enzymes include antipseudomonal penicillins, ceftazidime, cefipime, and aztreonam (64).

The OXA family of enzymes (Ambler Molecular Class D) (4) are found most commonly in *P. aeruginosa* (96, 146). Genes for these enzymes are located in plasmids, on transposons, or on integrons, making their further dissemination likely. They confer resistance predominantly to antipseudomonal penicillins, ceftazidime, cefipime, and aztreonam but not carbapenems. Their activity is inhibited poorly by clavulanic acid or tazobactam (108).

With the exception of GES-2 (an ESBL that hydrolyzes carbapenems), all carbapenemases in *P. aeruginosa* belong to Ambler Class B, commonly referred to as metalloenzymes. Metalloenzymes are not inhibited by clavulanic acid but are inhibited by divalent ion chelators such as EDTA. They hydrolyze all β-lactam antibiotics, except aztreonam, and are associated with high-level (MIC, >32 μg/ml) carbapenem resistance. Underreporting of carbapenem resistance may occur, as expression of the carbepenemases varies, resulting in a wide range of MIC values (2 to 128 μg/ml) that may go undetected in clinical laboratories that rely only on automated systems. In many cases, carbapenem resistance, especially to meropenem, is derived from a synergistic combination of mechanisms (105), resulting in enhanced resistance. For example, diminished expression of OprD porin or activation of an efflux system enhances the activity of a β-lactamase by increasing extracellular accumulation of the antibiotic.

Although impermeability mutations can result in aminoglycoside resistance, especially in CF and intensive care patients, drug inactivation by plasmid or chromosomally encoded enzymes is the most common mechanism for resistance worldwide to aminoglycosides (110). Aminoglycoside-modifying enzymes have been detected in *P. aeruginosa* for over 30 years, resulting in various combinations of resistance to gentamicin, tobramycin, and/or amikacin. These enzymes are often encoded on transposons and/or integrons that carry resistance determinants for other classes of antibiotics such as sulfonamides, β-lactams, and chloramphenicol. Multiresistance genes for both aminoglycosides and extended-spectrum β-lactamases and metalloenzymes are of particular concern (110). Aminoglycoside-modifying enzymes can occur together with impermeability mutations (92), resulting in broad-spectrum aminoglycoside resistance.

The discovery of a plasmid-borne quinolone resistance determinant *(qnr)* in gram-negative organisms (156) is of significance for several reasons: it has been transferred by conjugation to multiple organisms including *P. aeruginosa;* it is associated with high-level quinolone resistance (up to 250-fold increase in MICs); it appears to be associated with integrons that carry resistance determinants to β-lactams and aminoglycosides; and it expands the spectrum of high-level plasmid-mediated resistance to quinolones.

Antibiotic Tolerance

Biofilm-producing *P. aeruginosa* isolates appear to be protected from killing by antibiotics (144). Although this is widely accepted to indicate antibiotic resistance, a more appropriate term is antibiotic tolerance. Although slower or stationary growth phase has classically been thought to account for relative antibiotic tolerance, many other mechanisms have been proposed. These include quorum sensing (130), decreased diffusion of antibiotics through the matrix polysaccharide alginate (59), synthesis of glucans that specifically bind antibiotics (86), phenotypic variability (31, 38), presence of persister cells (139), and anaerobic growth of biofilm bacteria, which affects the activity of multiple antibiotics (10, 55).

Multidrug Resistance

Worldwide, antimicrobial resistance, including multidrug (three or more antimicrobial classes) resistance among *P. aeruginosa,* is widespread and increasing. In 2003, the European MYSTIC study group reported considerable country-to-country variation in the proportion of multidrug-resistant *P. aeruginosa* isolates within Europe, ranging from 50% to less than 3% (46). The SENTRY Antimicrobial Surveillance Program confirmed geographic variation in Latin America but emphasized the rapid increase in multidrug-resistant strains with rates approaching 35% (123). From 1993 to 2002, in the United States, the rates of multidrug resistance increased from 4 to 14%, with highest rates of increase reported for ciprofloxacin, imipenem, tobramycin, and aztreonam (100). Globally, multidrug resistance was found in 10% of *P. aeruginosa* strains analyzed (41).

Antimicrobial Susceptibility Testing

It may be difficult to estimate the true prevalence of antimicrobial resistance in *P. aeruginosa* because detection of resistance by routine tests agrees poorly with MIC data (5, 60). Susceptibility testing of *P. aeruginosa* is challenging due to multiple mechanisms of resistance, both intrinsic and acquired, which are frequently expressed concurrently, often at low levels.

In clinical laboratories, susceptibility testing for *Pseudomonas* species may be performed by disk diffusion, agar or broth dilution, E test (AB Biodisk, Solna, Sweden), or automated susceptibility systems using broth microdilution. Disk diffusion tests are standardized in North America and perform satisfactorily for most clinical isolates of *P. aeruginosa* (23). Limitations of this method include the lack of a quantitative result (MIC) and the potential to miss low-level resistance. Agar dilution is a well-accepted, reliable MIC method, especially for mucoid isolates, but it is time-consuming and too expensive for most routine clinical

laboratories. The E test (see chapter 73) has been shown to correlate well with agar dilution for isolates from CF (13) and non-CF patients (29).

Good correlation with reference methods has been reported for most automated systems (57, 140) when testing *Pseudomonas* isolates from non-CF patients. Results evaluating the performance of various automated systems must be interpreted with caution, as the numbers of isolates tested is often limited, especially for non-*P. aeruginosa* strains. Whereas most *P. aeruginosa* isolates grow well on agar media, growth of some isolates in broth is variable and may pose difficulties for laboratories that rely solely on automated systems. Alternatively, a liquid medium improves the detection of the efflux resistance phenotype, which may not be detected by solid-media-based testing (2, 30). This may account for some of the discrepancies reported when comparing different susceptibility methods.

Several antibiotics pose specific challenges to susceptibility testing. Carbapenem susceptibility testing results are difficult to interpret due to several factors that include rapid imipenem degradation (153), variable levels of efflux pump expression, and unstable impermeability mutations. Carbapenemase detection is especially challenging because it is associated with a wide range of MICs and lacks a simple test for detection. Susceptibility testing of imipenem with and without EDTA (disks or E-test strips) may be used but has been associated with variable results (164). Meropenem or ceftazidime, with or without EDTA, may be better substrates than imipenem, and testing these combinations may increase the sensitivity of the test (107). Reproducibility of carbapenem resistance results by using various susceptibility methods is poor, and it is recommended that initial carbapenem resistance be confirmed by a second antimicrobial susceptibility method (143). Although still restricted to reference laboratories, there are PCR-based methods for detection of carbapenemase production (158).

Colistin is being used more frequently in the treatment of multidrug-resistant *P. aeruginosa*. Disk diffusion testing does not correlate well with MIC results, and underreporting of resistance has been reported (42). Susceptibility testing of colistin should be performed by an MIC method such as agar dilution, E test, or broth microdilution. Prolonged incubation of 48 h is recommended for broth microdilution (61).

Isolates of *P. aeruginosa* from CF patients pose specific difficulties for microbiology laboratories. Isolates from these patients often exhibit mixed morphotypes including mucoid phenotypes, small-colony variants, and bacterial microcolonies in biofilms. Susceptibility testing is complicated by several factors including lack of correlation between susceptibility results and clinical response (132), different susceptibility patterns within a morphotype (38), lack of reproducibility of susceptibility tests, falsely positive susceptibility results, and the presence of hypermutable strains (51, 104). Mucoid and nonmucoid phenotypes of *P. aeruginosa* are often coisolated in specimens from patients with CF. Mucoid isolates tend to be more susceptible and have lower β-lactamase activity than nonmucoid isolates (21). One explanation may be that these isolates are protected from selective antibiotic pressure. Selective antibiotic pressure, notably from inhalational tobramycin or colistin therapy, gives rise to small-colony variants of *P. aeruginosa* with properties of increased antimicrobial resistance, autoaggregative growth behavior, and enhanced ability to form biofilms (155). In turn, bacterial cells in biofilms adapt into symbiotic bacterial communities in which the mucoid alginate-producing bacterial cells provide physical protection to the biofilm, while the highly antibiotic-resistant nonmucoid cells protect against antibiotic killing (21). The increased ability of biofilm bacteria to acquire resistance phenotypes (27) and the selection of hypermutable strains following antimicrobial therapy (51, 104) may explain the lack of eradication of *P. aeruginosa* from chronically infected CF patients. Since for bacteria found in biofilms MICs are 100- to 1,000-fold greater than for free-living, planktonic bacteria (95), routine susceptibility testing may underestimate resistance and may contribute to treatment failures. In a study of 597 CF isolates (13), both disk diffusion and E test were found to be generally acceptable as routine susceptibility testing methods. However, poor correlation was found with disk diffusion testing of mucoid isolates for piperacillin, piperacillin-tazobactam, and meropenem. Underreporting of resistance was more frequent with disk diffusion than with E test, especially when ceftazidime, piperacillin, and piperacillin-tazobactam were tested.

Hypermutable strains may be detected using either disk diffusion or E-test methods by the presence of resistant subpopulations within the inhibition zones of three or more antibiotics (84).

Mucoid isolates pose a specific challenge for automated systems (8, 12, 30). Overestimation of susceptibility may occur, as mucoid isolates often demonstrate insufficient growth at 24 h. Automated systems that allow for longer incubation may be preferable. On the other hand, overcalling resistance may result from the presence of large amounts of exopolysaccharide, resulting in turbidity without adequate bacterial growth. These limitations have led many microbiologists who routinely work with mucoid isolates of *P. aeruginosa* to choose alternative methods for susceptibility testing.

Isolating and individually testing all the morphotypes of *P. aeruginosa* is labor-intensive and time-consuming and may not provide clinically relevant susceptibility results. Mixed-morphotype testing using phenotypically different colonies directly from sputum cultures or from subcultures of isolated colonies has been shown to correlate well with disk diffusion and MIC susceptibility methods (162) and may provide clinically useful susceptibility data with significant time and cost savings. However, the correlation appears to be better for susceptible strains than for resistant strains (94). Direct sputum susceptibility testing using the E-test method has been suggested as an alternative to morphotype testing in assessing the in vivo situation by evaluating bacterial population susceptibility as well as potential interactions with other organisms, including commensal flora (129).

Other methods have been recommended in an attempt to better predict susceptibility results. Biofilm susceptibility assays which confirm that biofilm inhibitory concentrations are much higher than conventionally determined MICs for multiple antibiotics have been developed (95). Synergy testing, using microtiter checkerboard, time-kill test, broth macrodilution breakpoint combination sensitivity test, or E-test methods (129, 161), has been used to assess the activity of antibiotic combinations in vitro in order to predict in vivo synergistic activity. This testing is labor-intensive, time-consuming, and difficult to reproduce and does not result in better clinical and bacteriological outcomes than those obtained with therapy directed by standard culture and sensitivity techniques (1).

Susceptibility testing of *Pseudomonas* species other than *P. aeruginosa* is rarely indicated, and clinical correlation is required before susceptibility testing is performed. These

organisms are generally susceptible to most antipseudomonal antibiotics as well as to trimethoprim-sulfamethoxazole (except most *P. fluorescens* or *P. putida* strains), a property that differentiates them from *P. aeruginosa. P. fluorescens, P. putida,* and *P. oryzihabitans* may be more resistant to aztreonam and ticarcillin-clavulanic acid. *P. stutzeri* is usually very susceptible to all antipseudomonal agents (124).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Pseudomonas species represent a diverse group of organisms widely distributed in nature. *P. aeruginosa* may be associated with colonization or clinically significant infections. It is a major pathogen in CF patients and represents an important nosocomial and opportunistic pathogen. Interpretation of the Gram stain often directs the further workup of this organism. The presence of small clusters of gram-negative organisms surrounded by amorphous material is indicative of biofilm formation compatible with a chronic infection. This finding should be reported to physicians, and incubation should be prolonged because these strains usually exhibit slower-growth characteristics. The intracellular presence of these organisms in polymorphonuclear cells is clinically significant. Isolation of *P. aeruginosa* from sterile body sites should always be interpreted as indicative of probable infection. Pseudoinfection from contaminated skin disinfectant solutions can occur and should be considered if the patient is not severely ill and especially if there is a cluster of infections with the same strain of *Pseudomonas* spp. Disinfection solutions should be cultured using the same methods as those recommended for *Pseudomonas* species. Isolation in mixed culture requires correlation with the direct smear, other organisms isolated, and clinical history. Identification of this organism requires only a few simple tests, and commercial tests are not usually needed. Isolates from sites of chronic infection often exhibit multiple morphotypes, frequently with altered characteristics, which can make identification more difficult. Molecular methods increasingly are finding a role in the identification of this organism, especially for epidemiological studies. Susceptibility testing of these organisms is difficult, especially for mucoid isolates, due to increasing resistance, lack of reproducibility of results, and lack of clinical correlation. A basic understanding of the multiple mechanisms of resistance, both intrinsic and acquired, is essential to interpret susceptibility testing results and give therapeutic recommendations to physicians. Optimal treatment to eradicate infection and prevent resistance, especially in chronic infections, remains controversial. Judicious use of antibiotics is necessary to eradicate infections and avoid resistance. Combination therapy is prudent in serious infections such as endocarditis, septicemia, nosocomial pneumonia, central nervous system and prosthetic material-related infections, and bacteremia in neutropenic patients. Strict adherence to infection control measures, including handwashing, is essential to prevent the spread of these organisms within hospitals. Other *Pseudomonas* species are infrequently isolated in the laboratory and are usually not clinically significant. Clinical correlation and correlation with the Gram stain are essential before further workup is undertaken.

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Burkholderia, Stenotrophomonas, Ralstonia, Cupriavidus, Pandoraea, Brevundimonas, Comamonas, Delftia, and *Acidovorax**

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TAXONOMY

In 1973, the taxonomic heterogeneity of the genus *Pseudomonas* was revealed by the work of Palleroni and coworkers, who identified five major species clusters (referred to as rRNA homology groups) among the pseudomonads (169). DNA-rRNA hybridization experiments led to the gradual dissection of the genus during the following decades (131). The name *Pseudomonas* was confined to rRNA homology group I organisms because they constituted the type species, *Pseudomonas aeruginosa* (see chapter 48).

The nomenclatural rearrangements of the genus *Pseudomonas* entailed the creation of several new genera. Some of these encompassed complete rRNA homology groups (e.g., both rRNA homology group IV species were reclassified into the genus *Brevundimonas*), whereas others encompassed only partial groups. rRNA group II pseudomonads belong to the class of the *β-Proteobacteria* and were reclassified into the genera *Burkholderia* and *Ralstonia* (233, 234). The rRNA group II pseudomonads form a remarkable group of primary and opportunistic human, animal, and plant pathogens, as well as environmental species with considerable potential for biological control, remediation, and plant growth promotion. During the past decade, the interest in several peculiar characteristics of these organisms led to the discovery and description of a multitude of novel species. The genus *Burkholderia* now contains more than 35 validly named species, most of which have been isolated from soil and water samples. Some other novel *Burkholderia*-like species were found to represent a distinct phylogenetic lineage with a position intermediate between those of the genera *Burkholderia* and *Ralstonia* and were classified into the novel genus *Pandoraea* (41).

More recently, Vaneechoutte et al. (205) reported that results of comparative 16S rRNA gene sequence analysis, further supported by phenotypic differences, indicated that two distinct sublineages existed within the genus *Ralstonia*. It was proposed that species of the *Ralstonia eutropha* lineage be classified into a novel genus named *Wautersia*. The name *Ralstonia* was preserved for the sublineage comprising *Ralstonia pickettii,* the type species. Shortly thereafter, Vandamme and Coenye (201) reported that *Wautersia eutropha*, the name of the type species of the genus *Wautersia,* was a junior synonym of *Cupriavidus necator*, the name of the type (and only) species of the genus *Cupriavidus,* an environmental organism which was validly named in 1987, i.e., long before 16S rRNA gene sequence studies were performed routinely (152). Thus, to conform to the International Code of Nomenclature of Bacteria (191), the name *Wautersia* was replaced by *Cupriavidus* and all species of the genus *Wautersia* became species of the genus *Cupriavidus*. Although renaming and subsequent further renaming of bacterial species cause confusion and irritation in the wider microbiological community, adhering to the rules of nomenclature is essential for establishing a truly systematic taxonomy.

Several *Burkholderia* species have been isolated from human clinical samples, but only the *Burkholderia cepacia* complex, *B. mallei*, and *B. pseudomallei* are generally recognized as human or animal pathogens. Recent taxonomic studies using 16S rRNA and *recA* sequence analysis, DNA-DNA hybridization experiments, whole-cell protein and fatty acid analyses, and biochemical characterization revealed that *B. cepacia*-like bacteria belong to at least nine distinct genomic species (genomovars), referred to collectively as the *B. cepacia* complex (54, 204). Ongoing surveys of the diversity of *B. cepacia*-like bacteria recovered from specimens from cystic fibrosis (CF) patients and other specimens revealed the presence of several additional groups in the *B. cepacia* complex which cannot be assigned to one of the established species within this complex by using traditional or molecular identification approaches (178). Further polyphasic taxonomic analyses are needed to determine if these groups represent additional novel species within the *B. cepacia* complex or if they represent new variants of established species. All *B. cepacia* complex species have been recovered from human clinical samples, but primarily *B. multivorans* and *B. cenocepacia* are important opportunistic pathogens in CF patients (99, 178).

Apart from the *B. cepacia* complex species, *B. mallei,* and *B. pseudomallei*, the genus *Burkholderia* now comprises an additional 26 validly described species. Most of these organisms are not associated with human disease and are not discussed further here. Organisms associated with human infections include *B. fungorum, B. gladioli* (including strains previously classified as *B. cocovenenans*[45]), and *B. thailandensis* (*20, 46*). A complete overview of validly named species can be obtained through Internet sites such as http://www.bacterio.cict.fr/ and http://www.dsmz.de/bactnom/genera1.htm.

*This chapter contains information presented in chapter 48 by Peter H. Gilligan, Gary Lum, Peter A. R. Vandamme, and Susan Whittier in the eighth edition of this Manual.

There are now five species in the genus *Ralstonia*. The human pathogens include *Ralstonia pickettii*, *R. mannitolilytica* (previously known as *R. pickettii* biovar 3/'*thomasii*') (72), and *R. insidiosa* (43). *R. paucula* (previously known as Centers for Disease Control group IVc-2) (202), *R. gilardii* (42), *R. respiraculi* (56), *R. taiwanensis* (31), and five additional species, which occur primarily in environmental samples, are now all classified as *Cupriavidus* species (201).

Five distinct species of *Pandoraea*, *Pandoraea apista* (the type species), *P. pulmonicola*, *P. pnomenusa, P. sputorum*, and *P. norimbergensis*, were distinguished by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of whole-cell proteins, amplified fragment length polymorphism fingerprinting, DNA-DNA hybridization, and 16S rRNA gene sequence analysis. In addition, four strains, each representing a distinct novel *Pandoraea* species, presently remain unnamed (41, 70).

Organisms in the *Pseudomonas* rRNA homology group III also belong to the *β-Proteobacteria* and are now classified in the family *Comamonadaceae*, which includes the genera *Comamonas*, *Delftia*, and *Acidovorax* (216, 223). The genus *Comamonas* was originally created in 1985 and included a single species, *Comamonas terrigena*. Two years later, *Pseudomonas acidovorans* and *Pseudomonas testosteroni* were reclassified as members of the genus *Comamonas*. *Comamonas acidovorans* was subsequently again reclassified as *Delftia acidovorans* (216). *Comamonas terrigena* encompassed three strain clusters on the basis of DNA-rRNA and DNA-DNA hybridization data and data from protein electrophoretic patterns and immunotyping (226). Wauters et al. (214) reported biochemical differences between these three clusters and consequently described them as separate species. They proposed to rename *Comamonas terrigena* DNA groups 2 and 3 as *Comamonas aquatica* and *Comamonas kerstersii,* respectively. *Comamonas terrigena, Comamonas aquatica,* and *Comamonas kerstersii* all occur in human clinical samples. Additional novel species have been isolated from environmental samples (27, 83, 102, 196).

Originally, *Acidovorax facilis* was classified as *Hydrogenomonas facilis* based on its ability to oxidize hydrogen. Poly-β-hydroxybutyrate metabolism studies resulted in the transfer of this species to the genus *Pseudomonas*, along with a new species called *Pseudomonas delafieldii*. A new genus, *Acidovorax*, was proposed which included three species, *A. facilis*, *A. delafieldii*, and *A. temperans*, all members of rRNA homology group III (224). An additional five plant-pathogenic pseudomonads and novel environmental species have been classified as *Acidovorax* species (89, 90, 185, 225).

The genus *Brevundimonas,* consisting of the species *Brevundimonas diminuta* and *Brevundimonas vesicularis,* was proposed for bacteria originally classified as members of *Pseudomonas* rRNA homology group IV (186) and is a member of the *α-Proteobacteria*. Phylogenetic studies by Abraham et al. (2) revealed that the taxonomy of the genera *Brevundimonas* and *Caulobacter* was intertwined. Six *Caulobacter* species and two novel environmental species were therefore described as novel *Brevundimonas* species (2, 87, 139).

Finally, *Pseudomonas maltophilia* represented *Pseudomonas* rRNA homology group V (115). Based on genotypic and phenotypic characteristics, including DNA-rRNA hybridizations, cellular fatty acid composition, and growth parameters, its transfer to the genus *Xanthomonas*, a member of the *γ-Proteobacteria,* was proposed (195). However, many differences were also noted, including flagellum number, nitrate reduction characteristics, fimbriation, and plant pathogenicity. Therefore, the organism was once again reclassified into a novel genus, *Stenotrophomonas* (168). More recently, a novel species, *Stenotrophomonas africana,* was proposed (81). However, Coenye et al. (57) demonstrated that *S. maltophilia* and *S. africana* are the same species and nomenclatural priority was given to the former. Three additional environmental *Stenotrophomonas* species were described recently (11, 85, 228).

DESCRIPTION OF THE AGENTS

Burkholderia, Ralstonia, Cupriavidus, Pandoraea, Brevundimonas, Comamonas, Delftia, and *Acidovorax* spp. are aerobic, non-spore-forming, straight or slightly curved gram-negative rods. They are 1 to 5 μm in length and 0.5 to 1.0 μm in width (113). *Stenotrophomonas* spp. are straight rods and tend to be slightly smaller than members of the other genera (0.7 to 1.8 μm in length and 0.4 to 0.7 μm in width) (113). With the exception of *B. mallei*, these organisms are motile due to the presence of one or more polar flagella (167). These bacteria are catalase positive, and most, with the exception of *Stenotrophomonas* and *B. gladioli*, are either weakly or strongly oxidase positive. All grow on MacConkey agar, except for certain strains of *Brevundimonas vesicularis*, and appear as nonfermenters. The majority of species degrade glucose oxidatively, and most degrade nitrate into either nitrite or nitrogen gas. Certain species have distinctive colony morphologies or pigmentation. They are nutritionally quite versatile, with different species being able to utilize a variety of simple and complex carbohydrates, alcohols, and amino acids as carbon sources. Certain species can multiply at 4°C, but most are mesophilic, with optimal growth temperatures of between 30 and 37°C (167). For some genera, growth at higher temperatures (i.e., up to 42°C) can be useful for species identification.

EPIDEMIOLOGY AND TRANSMISSION

Burkholderia, Ralstonia, Cupriavidus, Pandoraea, *Comamonas, Delftia, Acidovorax, Brevundimonas,* and *Stenotrophomonas* spp. are environmental organisms found in water, soil, and the rhizosphere and in and on plants including fruits and vegetables. They have a worldwide distribution. Members of these genera are widely recognized as phytopathogens, and many species were first described in that context. Because of their ability to survive in aqueous environments, these organisms have become particularly problematic as opportunistic nosocomial pathogens in hospitals and health care settings.

The natural distribution of *B. cepacia* complex species is being intensively studied because of interest in their biotechnological properties and their pathogenicity in persons with CF (99, 144). *B. cepacia* complex bacteria often have antifungal, antinematodal, or plant growth-promoting properties, which makes them attractive as biological pesticides and fertilizers (170). Because of their nutritional versatility, *B. cepacia* complex bacteria also have applications for bioremediation of contaminated soils. Unlike *P. aeruginosa*, *B. cepacia* complex bacteria are rarely recovered from environmental sites such as sinks, swimming pools, showers, and salad bars (99, 157). However, they are frequently recovered from soil and environmental water samples (12, 176), provided that appropriate growth conditions are used to inhibit the growth of vast numbers of other environmental bacteria. Studies of a variety of foodstuffs and bottled water have shown that *B. cepacia* complex bacteria have been found in unpasteurized dairy products (17, 156). Due to their intrinsic resistance to antibiotics and disinfectants, *B. cepacia* complex bacteria are also notorious contaminants of pharmaceutical preparations and medical equipment such as nebulizers, which may be sterilized with contaminated anti-infectives (116, 163).

Genotypic and conventional epidemiologic investigations provide compelling evidence for interpatient transmission of common or epidemic *B. cepacia* complex strains among persons with CF (142). One such strain, referred to as the ET12 (for electrophoretic type 12) lineage, is common among CF patients in eastern Canada and the United Kingdom (124, 174). This organism is a *B. cenocepacia* strain that is characterized by the presence of a distinctive cable-like pilus and an associated adhesin that mediates adherence to the respiratory epithelium (181). *B. cenocepacia* strain PHDC dominates among infected CF patients in the mid-Atlantic region of the United States and has recently been identified in agricultural soil as well as in CF patients in several European countries (30, 53, 148).

B. pseudomallei and *B. thailandensis* are found primarily in tropical and subtropical areas. *B. pseudomallei* is endemic in rodents and is found in moist soil, on vegetables, and on fruit. Both species are particularly prevalent in the rice-growing regions of northern Thailand and southern and central Vietnam because of high concentrations of the organisms in rice paddy surface water (171). Reports have suggested that *B. pseudomallei* can also be found with some frequency on the Indian subcontinent but goes unrecognized (69).

Because of the increasing frequency of nosocomial infections due to *S. maltophilia*, its presence in hospital environments is being more closely examined. Like *P. aeruginosa*, *S. maltophilia* is ubiquitous in aqueous environments and can be readily cultured from water sources in homes and hospitals (77).

Unlike that of certain *B. cepacia* complex strains, evidence for person-to-person transmission of *B. gladioli*, *B. pseudomallei*, *B. mallei*, *S. maltophilia,* and the other species discussed in this chapter is lacking.

CLINICAL SIGNIFICANCE

B. cepacia Complex and *B. gladioli*

B. cepacia has long been recognized as an occasional opportunistic human pathogen capable of causing a variety of infections, including bacteremia, urinary tract infection, septic arthritis, peritonitis, and pneumonia in persons with underlying illness (144, 166). Persons with chronic granulomatous disease (CGD), a primary immunodeficiency in which white blood cells are unable to generate the superoxide and reactive oxidants necessary for intracellular microbicidal activity, are particularly susceptible to infection (227). *B. cepacia* also has a history as a nosocomial pathogen, causing infections associated with contaminated hospital equipment, medications, and disinfectants including povidone-iodine and benzalkonium chloride (143). Nosocomial outbreaks of respiratory tract infections in patients on mechanical ventilation in intensive care units have been attributed to contamination of nebulizers or nebulized medications such as albuterol (177). Contamination of blood culture systems or disinfectants resulting in pseudobacteremia has been described following the isolation of *B. cepacia* from the blood of multiple patients over a short period (60).

During the 1980s, *B. cepacia* emerged as a life-threatening pathogen in persons with CF, which is the most common inherited lethal disease in Caucasians in North America and Europe, affecting about 1 in 2,750 live births (144). Early reports of infection in CF described patients with acute pulmonary deterioration and sepsis (referred to as cepacia syndrome) or chronic respiratory tract infections associated with an accelerated decline in lung function (121, 198). Clinical outcome studies consistently identified *B. cepacia* infection as a significant independent risk factor for morbidity and mortality in CF (59, 140).

The recognition that several closely related species can be distinguished from among organisms previously identified as *B. cepacia* has stimulated interest in the clinical significance of each of these species (141). Approximately 3% of CF patients in the United States are infected with *B. cepacia* complex species, although rates of infection vary from 0 to 20% among CF treatment centers (68). Rates of infection increase with increasing patient age; approximately 6 to 7% of adults with CF are infected (68). Most strains are inherently resistant to currently available antimicrobial agents (see below), and pulmonary infection is generally refractory to therapy. Furthermore, due to the poor postoperative prognosis associated with *B. cepacia* complex infection, most CF treatment centers consider infection to be an absolute contraindication for lung transplantation, which at present is the only therapeutic option for successful intermediate-term survival of persons with end-stage pulmonary disease (142). Thus, respiratory tract infection with these species is a cause of great concern to CF patients and their caregivers.

Although all nine species of the *B. cepacia* complex have been recovered from persons with CF, the distribution of species in this patient population is disproportionate. In the United States, *B. multivorans* and *B. cenocepacia* together account for approximately 85% of infections (178). In Canada and some European countries, *B. cenocepacia* alone accounts for as much as 80% of infections (4, 192). Some *B. cepacia* complex species are recovered only rarely. In a recent survey, *B. stabilis, B. ambifaria, B. anthina,* and *B. pyrrocinia* each accounted for less than 1% of *B. cepacia* complex infections among 1,218 infected CF patients (178).

Emerging data suggest that *B. cepacia* complex species also vary with respect to their virulence levels and clinical impacts in CF. Studies with lung transplant recipients, for example, indicate that rates of postoperative mortality are greater for persons infected preoperatively with *B. cenocepacia* than for patients infected with other *B. cepacia* complex species (8, 80). However, although it is almost certainly true that *B. cenocepacia* is the species most frequently associated with cepacia syndrome, it remains to be shown whether this species, in general, is disproportionately associated with poor outcome; recent reports document fatal infection associated with other *B. cepacia* complex species, including *B. multivorans*, *B. stabilis*, and *B. dolosa* (18, 73, 164). Thus, although a positive correlation between species frequency and poor clinical outcome seems likely, firm conclusions regarding the relative virulence of *B. cepacia* complex species must await more definitive study. Recent studies also suggest that epidemic strains, particularly ET12, are relatively more virulent in CF (125, 136), but again, further comparative outcome studies are needed before firm conclusions about relative virulence can be drawn.

B. gladioli is most notable as a plant pathogen but is also well recognized to be capable of causing infection in persons with CF or CGD and, occasionally, other immunocompromised patients (101, 180). Anecdotal reports describe acute pulmonary deterioration and recurrent soft tissue abscesses, as well as severe post-lung transplantation infections due to *B. gladioli* in CF patients (15, 126, 132). A more complete appreciation of the epidemiology and clinical significance of *B. gladioli* infection in CF has been confounded by difficulty with accurate identification of this species, which typically is capable of growth on selective media used to isolate the *B. cepacia* complex (38) and is frequently misidentified as a member of the *B. cepacia* complex by commercial test systems (40, 189).

B. pseudomallei and *B. mallei*

B. pseudomallei is the causative agent of the human and animal disease melioidosis, which is endemic in Southeast Asia and tropical northern Australia and is being increasingly recognized on the Indian subcontinent and in Central and South America (32). In locations where the disease is endemic, infection is seasonal, with up to 85% of cases occurring during the monsoon wet season. As travel to Southeast Asia and northern Australia has become more frequent, reports of melioidosis in travelers returning to Europe and the United States are becoming more common (69), including those of infections in persons with CF (162, 184, 211). Infection with this organism should be considered in the differential diagnosis of any individual with a fever of unknown origin or a tuberculosis-like illness who has a history of travel to a region where *B. pseudomallei* infection is endemic.

B. pseudomallei is present in soil and surface water and is acquired either by inoculation through cut or abraded skin or by inhalation. Zoonotic disease is described but is exceedingly uncommon, as are person-to-person transmission and laboratory-acquired infection (32). The association of severe weather events with respiratory infection and high mortality rates has been attributed to a shift from percutaneous inoculation to inhalation (65). This idea supports the potential of *B. pseudomallei* as a bioterrorism agent; its isolation from patients who do not give a history of travel to an area where melioidosis is endemic should be immediately reported to local or state public health authorities. For further details, see chapter 9 or http://www.bt.cdc.gov.

The majority of persons infected with *B. pseudomallei* remain asymptomatic, with rates of seropositivity of over 20% in some locations (128). Latent infection with subsequent reactivation is well recognized, with a recent description of disease onset in the United States, where melioidosis is not endemic, 62 years after presumed infection in Thailand (159). Nevertheless, the vast majority of cases of melioidosis are from recent infection, with an incubation period of 1 to 21 days (mean, 9 days) (64). Risk factors for clinical disease following infection with *B. pseudomallei* include diabetes, excessive alcohol consumption, chronic renal disease, and chronic lung disease (66). Around 20% of patients have no identified risk factor, and mortality in this group is usually low. Disease in children is also uncommon, although parotid abscesses are well recognized as an important manifestation of melioidosis in children in Thailand. Overall rates of mortality from melioidosis vary from 15% in centers where state-of-the-art intensive care therapy is available to over 50% in locations with poor resources (32, 222). Fifty percent of cases present with pneumonia, which can be part of a fatal septicemia or a less severe unilateral infection indistinguishable from other community-acquired pneumonias or a chronic illness mimicking tuberculosis (29, 61). Chronic melioidosis, defined as illness present for over 2 months, occurs in only 10% of cases. Overall, 50% of cases are bacteremic; the presence of >100 CFU/ml of blood and a blood culture showing growth in the first 24 h of incubation are markers for high mortality (199). Other common presentations with or without bacteremia are genitourinary infections, septic arthritis, and osteomyelitis (32, 63, 222). Prostatic abscesses are especially common (63). Abscesses can also occur in the spleen, liver, kidneys, and adrenal glands. Parotitis, lymphadenitis, sinusitis, orchitis, myositis (especially psoas abscesses), mycotic aneurysms, and pericardial and mediastinal collections have all been described. Lesions can be frankly purulent and may include microabscesses or granulomas or a combination of these features. Clinical meningitis is rare, but melioidosis encephalomyelitis syndrome (62, 63, 229) and brain abscesses have also been reported (135). The one presentation that has yet to be described is *B. pseudomallei* endocarditis.

B. mallei is the etiologic agent of glanders, a highly communicable disease of livestock, particularly horses, mules, and donkeys. It can be transmitted to humans and is also identified as a potential agent of bioterrorism. Unlike *B. pseudomallei*, *B. mallei* is a host-adapted pathogen that does not persist in the environment outside its host. Glanders has been eradicated from most countries, but enzootic foci persist in the Middle East, Asia, Africa, and South America. The only human case of glanders in the past 50 years in the United States was a recent laboratory-acquired case in a biodefense scientist (26). Like melioidosis, human glanders can be acute or chronic, with the clinical presentation and course depending on the mode of infection, the inoculation dose, and host risk factors. Respiratory inoculation can result in pneumonia with potential for dissemination to internal organs and septicemia. Cutaneous inoculation can result in skin nodules and regional lymphadenitis, also with potential for disseminated disease. Involvement of lymph nodes, both mediastinal and peripheral, is much more common in glanders than in melioidosis, often with suppurative abscesses in untreated cases.

S. maltophilia

S. maltophilia, although typically not pathogenic for healthy persons, is a well-known opportunistic human pathogen. It is among the most common causes of wound infection due to trauma involving agricultural machinery (3). It is also an important nosocomial pathogen associated with substantial morbidity and mortality, particularly in debilitated or immunocompromised patients and patients requiring ventilatory support in intensive care units (5, 88, 107, 158). The incidence of human infection appears to have increased in recent years, and a variety of clinical syndromes have been described, including bacteremia, pneumonia, urinary tract infection, ocular infection, endocarditis, meningitis, soft tissue and wound infection, mastoiditis, epididymitis, cholangitis, osteochondritis, bursitis, and peritonitis (76, 183). Septicemia can be accompanied by ecthyma gangrenosa, a skin lesion more commonly associated with *P. aeruginosa* and *Vibrio* spp. (206).

The incidence of *S. maltophilia* respiratory tract infection in persons with CF also appears to be increasing (74, 197); however, the unreliability of historical data limits firm conclusions. Approximately 11% of CF patients included in the CF Foundation's patient registry were culture-positive for *S. maltophilia* in 2003 (68). In large multicenter clinical trials, however, *S. maltophilia* was found in a larger proportion of CF patients, being second only to *P. aeruginosa* in frequency of isolation from study subjects (25). Infection or colonization was most frequently transient, with 30% of subjects having at least one sputum culture positive for *S. maltophilia* during the course of 6 months (100). Several case control studies have drawn conflicting conclusions regarding the role that *S. maltophilia* plays in contributing to pulmonary decline in CF (74, 98).

Ralstonia and *Cupriavidus* spp.

As described above, the taxonomy of the genus *Ralstonia* has been recently revised, with several species being assigned to the genus *Cupriavidus* (201). Among the species in these two genera, *R. pickettii* is best known with respect to human infection. Older reports describe this species as being recovered

from a variety of clinical specimens (179) and as causing various infections including bacteremia, meningitis, endocarditis, and osteomyelitis (217). *R. pickettii* also has been identified in cases of pseudobacteremias and nosocomial outbreaks due to contamination of intravenous medications, sterile water, saline, chlorhexidine solutions, respiratory therapy solutions, and intravenous catheters (19, 36, 84). This species has also been recovered from the respiratory tracts of persons with CF (25). However, *R. pickettii* is easily confused with *Pseudomonas fluorescens* and members of the *B. cepacia* complex (25, 72, 111). Furthermore, several newly recognized *R. pickettii*-like species are also now known to be involved in human infection, particularly in CF (52). Thus, the role of *R. pickettii* as a human pathogen is difficult to assess based on historical data.

R. mannitolilytica (formerly known as *R. pickettii* biovar 3/'*thomasii*') was recently described as causing nosocomial outbreaks and a case of recurrent meningitis (72). This species accounts for the majority of *Ralstonia* infections in CF patients, being found in more than twice as many CF patients as *R. pickettii* (52). *R. insidiosa* and *Cupriavidus respiraculi* are recently described species recovered from persons with CF (43, 56). *Cupriavidus gilardii* has been recovered from cerebrospinal fluid (42), and cases of *Cupriavidus paucula* bacteremia, peritonitis, and tenosynovitis have been reported (202). Both of these species may be found in sputa from patients with CF (52). Although *Cupriavidus metallidurans* and *Cupriavidus basilensis* are not known to cause human infection, they too have been recovered recently from sputum cultures from patients with CF (52). Despite these observations, the roles of *Ralstonia* and *Cupriavidus* species in human infection, particularly in persons with CF, require further elucidation.

Other Genera

In general, *Brevundimonas*, *Comamonas*, *Delftia*, *Acidovorax*, and *Pandoraea* spp. infrequently cause human infection. Interest in these species focuses primarily on their roles as plant pathogens or on studies of microbial biodiversity and biodegradation.

Brevundimonas spp. are occasionally recovered from clinical specimens (37). *Brevundimonas vesicularis* bacteremia in patients with various underlying illnesses has been reported (92), and the organism has been recognized in cervical specimens because of its ability to produce bright orange colonies on Thayer-Martin agar (165). *Brevundimonas diminuta* has been recovered from blood, urine, and pleural fluid from patients with cancer (105).

Among the *Comamonas* species, *Comamonas testosteroni* has been implicated most often in human infection, with recent reports describing endocarditis, meningitis, and catheter-associated bacteremia due to this species (7, 58, 137). *D. acidovorans* has similarly been reported to cause infection, being identified in cases of bacteremia, endocarditis, ocular infection, and suppurative otitis (82). *Acidovorax* spp. have been isolated from a variety of clinical sources (224), including blood from a patient with a hematological malignancy (232). *Acidovorax* spp., *Comamonas testosteroni,* and *D. acidovorans* have also been recovered from sputa of persons with CF (44; J. J. LiPuma, unpublished data); however, the roles of these species in contributing to lung disease in CF have not been established.

In addition to causing infections in CF (123, 127), *Pandoraea* spp. have been recovered from blood and from patients with chronic obstructive pulmonary disease or CGD (70). Although the roles of these species in contributing to poor outcomes in persons with underlying diseases are unclear, a recent report describes sepsis, multiple organ failure, and death in a patient who underwent lung transplantation due to sarcoidosis (194).

COLLECTION, TRANSPORT, AND STORAGE

The genera described in this chapter include organisms that can survive in a variety of hostile environments and at temperatures found in clinical settings. Therefore, standard collection, transport, and storage techniques as outlined in chapters 5, 6, and 20 are sufficient to ensure the recovery of these organisms from clinical specimens.

DIRECT EXAMINATION

Members of these genera have similar morphologies and, with the exception of *B. pseudomallei*, are not easily distinguished from one another on the basis of Gram staining. *B. pseudomallei* often appears as small, gram-negative bacilli with bipolar staining, making the cells resemble safety pins (Fig. 1). It is not uncommon for the presumptive laboratory diagnosis of *B. pseudomallei* infection to be made upon the examination of the initial Gram-stained smear.

Although PCR-based assays have been described for the identification of *B. cepacia* complex species, *B. pseudomallei, B. gladioli*, several *Ralstonia* and *Cupriavidus* species, *Pandoraea* species, and *S. maltophilia* following culture and isolation (see "Identification" below), the use of PCR for direct detection of these species in clinical specimens remains a research tool (71, 153, 220). Studies of CF sputum samples have indicated that some specimens may be PCR positive but culture negative for certain *B. cepacia* complex species, raising important questions about the natural history of infection in CF. However, the sensitivities and specificities of such PCR assays for the intended target species are difficult to determine in the absence of reliable "gold standards." The development of assays employing real-time PCR technology may yield reliable approaches to direct detection of these species in clinical specimens in the near future.

Because septicemia with *B. pseudomallei* is frequently fatal, several rapid direct detection methods have been developed in research laboratories, including urinary antigen detection using latex agglutination (LA) and enzyme immunoassay (EIA), direct fluorescent-antibody (DFA) staining, and PCR (78, 103, 190, 212). The EIA for the detection of urinary antigens is more sensitive than LA, with an overall sensitivity of 71% for patients with melioidosis compared with an LA sensitivity of 62% (with concentrated urine) or only 17.5% (with unconcentrated urine). The EIA has higher sensitivity (84%) with samples from septicemic patients. Cross-reactions with other urinary tract pathogens including *Klebsiella pneumoniae* and *Escherichia coli* have been reported with EIA but not LA; therefore, EIA results must be interpreted cautiously (78, 190).

Antibodies raised against heat-killed whole cells of *B. pseudomallei* have been used to prepare a reagent for DFA staining. When this DFA reagent was used to stain clinical specimens from patients with suspected melioidosis, it showed a sensitivity of 73%, similar to those of other bacterial DFA stains. The reagent (not available commercially) apparently does not cross-react with other organisms, although the number of isolates tested for cross-reaction was small (212).

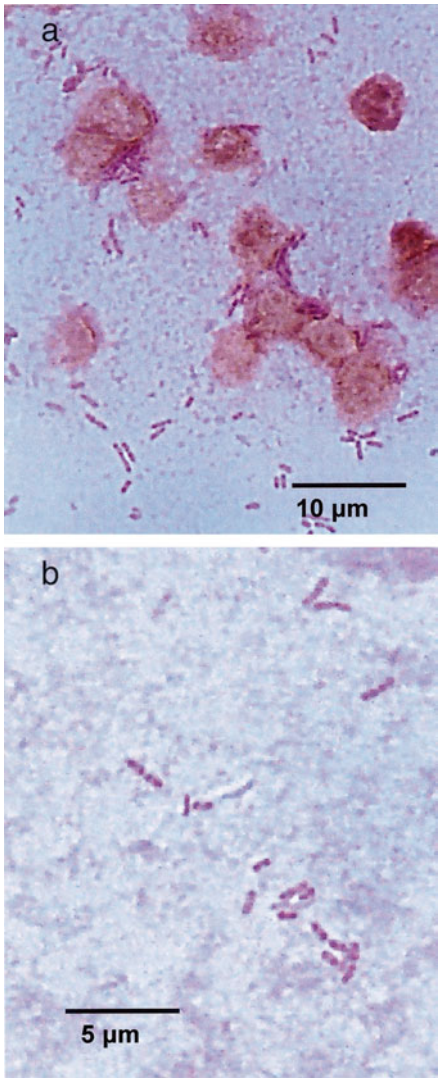


FIGURE 1 (a) Gram stain of *B. pseudomallei* in a blood culture; (b) Gram stain of *B. pseudomallei* from a colony on blood agar.

Reports on the use of PCR for the direct detection of *B. pseudomallei* in clinical specimens indicate that the currently used primer sets and assay conditions are sensitive but lack specificity, resulting in positive predictive values of only 70% (103).

CULTURE AND ISOLATION

These species grow well on standard laboratory media such as 5% sheep blood and chocolate agars. Such media can be used to recover the organisms from sterile fluid or tissue where a mixed flora is not anticipated (see chapter 20). All species that have been reported to be recovered from blood, including *B. pseudomallei* (199), grow in broth-based blood culture systems within the standard 5-day incubation period, so special blood culture techniques such as lysis-centrifugation and extended incubation periods are not required. The use of selective media facilitates the isolation of these organisms from specimens with mixed microbiota. With the exception of *Brevundimonas vesicularis*, MacConkey agar can be used to isolate most species of these genera.

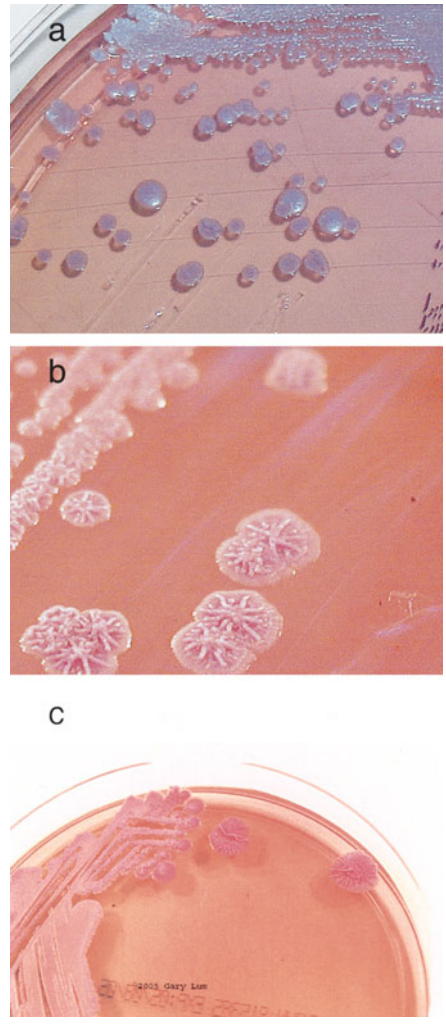


FIGURE 2 (a) *B. pseudomallei* colonies on MacConkey agar; (b) *B. pseudomallei* colonies on blood agar; (c) *B. pseudomallei* colonies on Ashdown medium agar.

Burkholderia species grow on MacConkey agar (Fig. 2), but the use of specific selective media with the ability to inhibit *P. aeruginosa* is preferred for the isolation of *B. cepacia* complex species and *B. pseudomallei*. Several selective media have been described, and some are commercially available. A multicenter comparison of three media, PC (for *Pseudomonas cepacia*) agar (BD Diagnostics, Franklin Lakes, N.J.) (94), OFPBL (for oxidation-fermentation base-polymyxin b-bacitracin-lactose) agar (BD Diagnostics) (215), and BCSA (for *B. cepacia* selective agar; Hardy Diagnostics, Santa Maria, Calif.) (109), showed that BCSA was superior, being both more sensitive (more *B. cepacia* isolates were recovered) and more specific (fewer other types of organisms grew) than PC or OFPBL agar (109, 110). More recently, the sensitivities of TB-T (for trypan blue-tetracycline) (104), PC-AT (for *Pseudomonas cepacia* azelaic acid) (23), and BCSA (109, 110) were compared with those of three commercial media, i.e., *B. cepacia* media from MAST Diagnostics (Bootle, Merseyside, United Kingdom), LAB M Ltd. (Bury, United Kingdom), and Oxoid Ltd. (Basingstoke, United Kingdom), through the analysis of 142 clinical and environmental isolates representing all species within the *B. cepacia* complex (209).

BCSA and MAST *B. cepacia* medium supported the growth of *B. cepacia* complex isolates most efficiently. The latter two media were also compared in a study to evaluate the sensitivities and specificities for the isolation of *B. cepacia* complex species from sputum specimens from CF patients (230). BCSA was reported to be equally sensitive as MAST agar but more selective.

Ashdown medium is effective for the isolation of *B. pseudomallei* (Fig. 2); crystal violet and gentamicin act as selective agents. It has been shown to be superior to MacConkey agar or MacConkey agar supplemented with colistin for the recovery of *B. pseudomallei* from clinical specimens containing mixed bacterial microbiota, such as throat, rectal, and sputum specimens (231). An enrichment broth consisting of Ashdown medium supplemented with 50 mg of colistin per liter allowed the recovery of 25% more *B. pseudomallei* isolates than direct plating of clinical specimens on Ashdown agar (213).

For the recovery of *B. pseudomallei* from rectal and throat swabs, selective broth should be inoculated at the bedside and incubated for up to 7 days at 35 to 37°C. The broth can be subcultured to selective media in the laboratory, and solid-phase agar can be kept for an additional 7 days at 35 to 37°C. During the monsoon season, specimens such as sputa, urine (male patients), and wound swabs should be inoculated onto Ashdown agar (213, 229). Subculture from broth should be done earlier if a pellicle at the liquid-air interface is observed or if the broth changes from a deep purple to a deep pink hue due to a change in pH affecting the neutral red indicator.

Recently, a new selective agar, BPSA (for *B. pseudomallei* selective agar), was reported to improve the recovery of *B. pseudomallei* over that with other media (114). BPSA was more inhibitory to *P. aeruginosa* and *B. cepacia* complex species and made recognition of *Burkholderia* species easier due to their distinctive colony morphology. However, in clinical practice this medium may offer only a modest advantage over current commercially available media.

The use of selective media (130) increases the isolation rates for *S. maltophilia* from clinical and environmental samples (75). Denton et al. (75) studied the sensitivity of a selective medium incorporating vancomycin, imipenem, and amphotericin B as selective agents (VIA medium) for isolating *S. maltophilia* from sputum samples collected from children with CF. This study compared the use of VIA medium to an existing in-house method that utilized an imipenem disk placed upon bacitracin-chocolate agar (BC medium) and reported improved detection using VIA medium as a selective medium.

IDENTIFICATION

B. cepacia Complex and *B. gladioli*

Accurate identification of *B. cepacia* complex species presents a challenge (154). Commercial bacterial identification systems are not able to reliably distinguish among the species of the *B. cepacia* complex and often fail to differentiate these species from other closely related species such as *B. gladioli* and *Ralstonia, Cupriavidus,* and *Pandoraea* spp. (21, 111, 133, 189). This failure presents a serious problem for CF patients and their caregivers as detailed in "Clinical Significance" above. The identification of *B. cepacia* complex species from CF sputum culture has a dramatic impact on patient management and is a cause of considerable anxiety for patients with CF (142, 143). Consequently, when *Burkholderia, Ralstonia, Cupriavidus,* or *Pandoraea* species are tentatively identified in a patient with CF by using a commercial system, the identity of the isolate should be confirmed by conventional biochemical testing (111) and, if necessary, molecular techniques. To aid clinical microbiologists in the United States, the CF Foundation has established a *B. cepacia* reference laboratory, which uses a combination of phenotypic and genotypic methods (described below) to confirm the identity of suspected *B. cepacia* complex isolates (146). Further information concerning the *B. cepacia* reference laboratory can be found on the CF Foundation website (http://www.cff.org).

B. cepacia complex species may require 3 days of incubation before colonies are seen on selective media. On MacConkey or Mueller-Hinton agar, these colonies may be punctate and tenacious, and on blood agar or a selective medium such as BCSA, PC agar, or OFPBL agar, the colonies are smooth and slightly raised; occasional isolates are mucoid. On MacConkey agar, colonies of the *B. cepacia* complex frequently become dark pink to red due to oxidation of lactose after extended incubation (4 to 7 days). Most clinical isolates are nonpigmented, but on iron-containing media such as a triple sugar iron slant, many strains produce a bright yellow pigment. *B. cepacia* complex species have a characteristic dirt-like odor.

The species of the *B. cepacia* complex are phenotypically very similar, making their differentiation, even with an extended panel of biochemical tests, rather difficult (Table 1) (111). *B. multivorans*, *B. stabilis*, and *B. dolosa* rarely oxidize sucrose. *B. stabilis* is ornithine decarboxylase positive, as are most *B. cenocepacia* strains, but is distinctive in that more than two-thirds of strains are *o*-nitrophenyl-β-D-galactopyranoside (ONPG) negative. *B. stabilis* and most *B. ambifaria* strains show poor growth at 42°C. *B. dolosa* is usually lysine decarboxylase negative, whereas only approximately half of *B. multivorans* strains are negative. Other *B. cepacia* complex species are usually lysine decarboxylase positive. *B. vietnamiensis* and most *B. anthina* strains do not oxidize adonitol. *B. anthina* strains show a distinctive creamy morphology on BCSA, which also turns pink (i.e., alkaline) despite the ability of this species to utilize sucrose (203).

Phenotypic differentiation of *B. cepacia* complex species from *B. gladioli* and *Pandoraea* spp. is also difficult (Table 1). Cellular fatty acid analysis is unable to differentiate *B. cepacia* complex species from *B. gladioli* (193). However, in contrast to *B. cepacia* complex species, most *B. gladioli* strains are oxidase negative and whereas most *B. cepacia* complex strains oxidize maltose and lactose, *B. gladioli* typically oxidizes neither. *Pandoraea* spp. do not oxidize maltose, lactose, xylose, sucrose, or adonitol, and most are ONPG negative. *B. cepacia* complex species also may be difficult to differentiate from *Ralstonia* and *Cupriavidus* species. However, several of the latter species show a fast and strong oxidase reaction whereas *B. cepacia* complex species produce a slow, weak-positive oxidase reaction. Further, in contrast to most *B. cepacia* complex species, *Ralstonia* and *Cupriavidus* species are lysine decarboxylase negative and most often ONPG negative.

The difficulty in differentiating *B. cepacia* complex species has prompted the development of molecular genetic diagnostic tests capable of identifying these species individually and distinguishing them (as a group) from biochemically similar species. DNA sequence differences in 16S and 23S rRNA genes have been used to develop species-specific PCR assays for the identification of several *B. cepacia* complex species (16, 146, 207), as well as *B. gladioli* (221). *B. multivorans*, *B. vietnamiensis*, and *B. dolosa* can be reliably identified with 16S rRNA-targeted assays, but insufficient sequence variation in rRNA genes exists to enable reliable separation of the remaining *B. cepacia* complex species. Fortunately, species-specific

TABLE 1 Characteristics of the *B. cepacia* complex, *B. gladioli,* and *Pandoraea* spp.

Test	% of strains positive										
	B. cepacia complex (genomovar)									*B. gladioli*	*Pandoraea* spp.
	B. cepacia (I)	*B. multivorans* (II)	*B. cenocepacia* (III)	*B. stabilis* (IV)	*B. vietnamiensis* (V)	*B. dolosa* (VI)	*B. ambifaria* (VII)	*B. anthina* (VII)	*B. pyrrocinia* (IX)		
Oxidase[a]	100	98	98	96	99	98	100	100	86	14	57
Growth:											
MacConkey[b]	83	96	84	93	83	100	100	100	100	96	100
BCSA[a]	100	100	100	100	98	100	100	100	100	69	83
42°C[b]	43	100	84	0	100	100	22	60	66	4	89
Yellow pigment[b]	78	2	3	0	0	0	0	0	33	44	0
Brown pigment[b]	4	2	14	0	0	0	6	0	0	33	0
Lysine decarboxylase[a]	98	50	97	100	99	0	98	87	100	1	4
Ornithine decarboxylase[b]	30	0	71	100	0	0	0	0	67	0	0
Acid from[c]:											
Glucose[b]	100	100	96 (95)	100	100	100	100	100	100	100	89 (11)
Maltose[b]	70 (39)	99 (98)	86 (78)	93	100 (97)	100	100	100	100	0	0
Lactose[a]	91	99	92	86	99	98	96	100	100	8	1
Xylose[b]	100 (87)	99 (98)	92 (88)	78 (44)	86 (75)	100	100	100 (94)	100	96	0
Sucrose[a]	84	2	90	6	92	0	94	59	79	1	1
Adonitol[b]	78 (70)	92 (91)	87 (79)	96 (78)	0	100	100	20	100	96 (93)	0
PNPG or ONPG [a,d]	98	99	99	29	99	100	100	83	93	95	11
Nitrate to nitrite reduction[b,e]	4	94	31	4	47	100	67	88	0	33	11
Gelatin liquefaction[b,e]	74	2	55	93	0	0	94	0	66	70	0
Esculin hydrolysis[b,e]	56	2	33	0	0	0	56	6	33	11	0

[a] The number of strains of each species tested was as follows: *B. cepacia*, 122; *B. multivorans*, 715; *B. cenocepacia*, 768; *B. stabilis*, 49; *B. vietnamiensis*, 145; *B. dolosa*, 57; *B. ambifaria*, 51; *B. anthina*, 24; *B. pyrrocinia*, 14; *B. gladioli*, 280; and *Pandoraea* spp., 75. Data are from references 111 and 203 and J. J. LiPuma (unpublished data) and D. A. Henry (unpublished data).

[b] The number of strains of each species tested was as follows: *B. cepacia*, 23; *B. multivorans*, 109; *B. cenocepacia*, 139; *B. stabilis*, 27; *B. vietnamiensis*, 36; *B. dolosa*, 9; *B. ambifaria*, 18; *B. anthina*, 16; *B. pyrrocinia*, 3; *B. gladioli*, 27; and *Pandoraea* spp., 9. Data are from references 111 and 203 and D. A. Henry (unpublished data).

[c] Oxidation test results were recorded after 7 days of incubation (data in parentheses were recorded after 3 days of incubation).

[d] PNPG, *p*-nitrophenyl-β-D-glucoside.

[e] Results presented are from the API 20 NE test strip.

sequence variation does exist in the *recA* gene, and PCR assays targeting this locus enable the reliable identification of *B. multivorans, B. cenocepacia*, and *B. ambifaria* (49, 150, 208). Other 16S rRNA- and *recA*-based PCR assays identify all *Burkholderia* spp. (i.e., at the genus level) or all species within the *B. cepacia* complex (i.e., as a group) (146, 150, 172).

Another molecular genetic approach to identifying *B. cepacia* complex species involves restriction fragment length polymorphism (RFLP) analysis of either 16S rRNA or *recA* genes (150, 187). Again, insufficient sequence variation in the 16S rRNA gene limits the use of RFLP analysis of this locus, even when multiple restriction enzymes are used (86, 187, 210). In contrast, *recA* RFLP analysis has proved quite useful in reliably distinguishing all species within the *B. cepacia* complex (150, 172, 208).

Other genomic approaches, including amplified fragment length polymorphism typing, ribotyping, and whole-cell protein profiling, have been proposed for the differentiation of *B. cepacia* complex species (22, 50, 204). However, these methods are time-consuming and expensive and require an extensive validated database before isolates can be reliably identified. These limitations render them impractical for use in a routine diagnostic laboratory. Cellular fatty acid methyl ester analysis is useful for identification of *Burkholderia* strains at the genus level but is not reliable for identification of individual *B. cepacia* complex species and does not differentiate *B. gladioli* (40, 202).

B. pseudomallei and *B. mallei*

If *B. pseudomallei* is suspected, all efforts should be made to confirm or exclude a positive oxidase reaction, production of gas from nitrate, multitrichous polar flagella, and arginine dihydrolase and gelatinase activities (Table 2). The bacterium gives off a distinctive earthy odor such that a clue to its presence can be gleaned upon opening a petri dish (even with a mixed culture). However, active sniffing of cultures is strongly discouraged in order to prevent infection (10). Cellular fatty acid profiles may be useful for differentiating *B. pseudomallei* from other genera, but reports vary on their utility in differentiating *B. thailandensis* and *B. pseudomallei* (117) or other pathogenic *Burkholderia* species including *B. mallei*, *B. cepacia* complex species, and *B. gladioli*.

B. pseudomallei must be differentiated from *Pseudomonas stutzeri* and *B. cepacia* complex species in clinical specimens. *Pseudomonas stutzeri* appears very similar to *B. pseudomallei* after a few days of incubation, and both *B. pseudomallei* and *B. cepacia* complex species may be isolated from persons with CF (162, 184, 211). Whereas *B. pseudomallei* produces gas from nitrate and is arginine dihydrolase positive, most *B. cepacia* complex isolates are negative for both characteristics. *Pseudomonas stutzeri* is negative for arginine dihydrolase, oxidation-fermentation glucose, and gelatin hydrolysis. *Pseudomonas stutzeri* also has only one flagellum, and *B. pseudomallei* has more than one.

There have been reports linking a lack of virulence of *B. pseudomallei* with the ability to assimilate arabinose (6). Environmental strains tend to be arabinose assimilators, and those that do not assimilate arabinose are almost always found as clinical isolates. The name *B. thailandensis* is used to describe arabinose-utilizing strains with low virulence (20).

The Microbact 24E strip (MedVet, Adelaide, Australia) appears to more accurately identify *B. pseudomallei* than does the API 20NE system (bioMérieux, Hazelwood, Mo.) (118). Limited experience with automated systems such as Vitek-1 and Vitek-2 (bioMérieux, Durham, N.C.) and MicroScan WalkAway (Dade International Inc., West Sacramento, Calif.) indicates that they reliably identify *B. pseumomallei*. However, further evaluation of commercially available identification systems is required (96, 119). Because of the difficulty with accurate laboratory identification, referral to a reference laboratory is advised when isolation of *B. pseudomallei* is suspected. This is especially important with the advent of emerging bioterrorism legislation in many countries (see chapter 9).

TABLE 2 Characteristics of *B. mallei*, *B. pseudomallei,* and *B. thailandensis*[a]

Test	*B. mallei*	*B. pseudomallei*	*B. thailandensis*
Oxidase	v	+	+
Growth:			
MacConkey	+	+	+
42°C	−	+	+
Nitrate reduction	+	+	+
Gas from nitrate	−	+	+
Arginine dihydrolase	+	+	+
Lysine decarboxylase	−	−	−
Ornithine decarboxylase	−	−	−
Hydrolysis of:			
Urea	v	v	v
Citrate	−	v	v
Gelatin	−	v	v
Esculin	−	v	v
Acid from:			
Glucose	+	+	+
Xylose	v	+	+
Lactose	v	+	+
Sucrose	−	v	v
Maltose	−	+	+
Mannitol	−	+	+
Arabinose	ND	−	+
Motility	0	100% +	100% +
No. of flagella	0	≥2	≥2

[a]Data from references 20 and 218. +, >90% positive; −, >90% negative; v, variable; ND, not determined.

Ralstonia and *Cupriavidus* spp.

Although *R. pickettii* was considered to be the *Ralstonia* species most frequently isolated from clinical specimens (179), the recent recognition that several other *Ralstonia* and *Cupriavidus* species can be identified from among *R. pickettii*-like isolates limits previous observations. As is the case with *B. cepacia* complex species, *Ralstonia* and *Cupriavidus* species are phenotypically similar, requiring rather extensive biochemical testing to reliably differentiate them; species-level identification with standard biochemical testing is difficult (Table 3). These species may grow slowly on primary isolation media, requiring ≥72 h of incubation before colonies are visible. They are lysine decarboxylase negative and generally catalase positive, although catalase-negative *R. pickettii* strains have been described (42, 202). Most species show a fast and strong oxidase reaction; however, the intensity of the oxidase reaction varies for *R. mannitolilytica*, *R. pickettii,* and *Cupriavidus gilardii*, with some strains showing a weakly positive reaction (42, 202; LiPuma, unpublished). *R. pickettii, R. mannitolilytica,* and *R. insidiosa* grow on BCSA; growth of other species is strain dependent. These species do not produce acid from sucrose.

TABLE 3 Characteristics of *Ralstonia* and *Cupriavidus* spp.[a]

Test	*R. pickettii*	*R. mannitolilytica*	*R. insidiosa*	*Cupriavidus respiraculi*	*Cupriavidus gilardii*	*Cupriavidus paucula*
Catalase	v	+	+	+	+	+
Oxidase	+	+	+	+	+	+
Growth:						
BCSA	+	+	+	−	v	v
42°C	v	+	ND	ND	+	v
Colistin resistance	+	+	ND	ND	ND	−
Nitrate reduction	+	−	+	v	−	−
Tween 80 hydrolysis	+	+	ND	ND	−	+
Urease	+	+	v	−	−	+
Lysine decarboxylase	−	−	−	−	−	−
ONPG	−	v	v	−	v	ND
Acid from:						
L-Arabinose	+	+	ND	ND	−	−
D-Arabitol	−	+	ND	ND	ND	−
Glucose	+	+	−	−	−	−
Inositol	−	−	ND	ND	ND	−
Lactose	v	+	−	−	−	−
Maltose	v	+	ND	ND	ND	−
Mannitol	−	+	ND	ND	ND	−
Sucrose	−	−	−	−	−	−
Xylose	+	+	ND	ND	−	−
Motility	+	+	ND	ND	+	+
Flagella	1 polar	1 polar	ND	ND	1 polar	Peritrichous

[a]Data from references 42, 43, 72, and 202, and J. J. LiPuma (unpublished data). +, >90% positive; −, >90% negative; v, variable; ND, not determined.

Most *R. mannitolilytica* strains acidify lactose, whereas most strains from other species do not. *R. insidiosa, Cupriavidus respiraculi*, *Cupriavidus gilardii,* and *Cupriavidus paucula* are differentiated from *R. pickettii* and *R. mannitolilytica* in failing to acidify glucose. *Cupriavidus gilardii* has a characteristic cellular fatty acid profile different from that of other *Ralstonia* species (42, 72).

Molecular genetic tests have proved quite helpful in differentiating these species. A recently described 16S rRNA-directed PCR assay reliably identifies all *Ralstonia* and *Cupriavidus* species (as a group), allowing their differentiation from the phenotypically similar species in the genera *Burkholderia* and *Pandoraea* (52). Species-specific 16S rRNA-based PCR assays have also been developed recently; these enable the accurate identification of *R. pickettii*, *R. mannitolilytica*, *R. insidiosa*, and *Cupriavidus respiraculi* (52, 55, 56).

Pandoraea spp.

Overall, the biochemical profiles of *Pandoraea* strains are similar to those of *Burkholderia* and *Ralstonia* strains isolated from clinical specimens (Table 1) (41, 70, 111). A lack of saccharolytic activity is indicative of *Pandoraea* but is also seen with some *Ralstonia* species. Definitive identification of putative *Pandoraea* isolates requires molecular confirmation. Coenye et al. (48) described 16S rDNA-based PCR assays for the identification of these bacteria. A PCR assay was developed for the identification of *Pandoraea* isolates to the genus level. PCR assays for the identification of *P. apista* and *P. pulmonicola* (as a group), *P. pnomenusa*, *P. sputorum*, and *P. norimbergensis* were also developed. *Pandoraea* strains can be differentiated from *Burkholderia* and *Ralstonia* strains by their specific 16S rDNA restriction profile (111, 188) and can be identified at the species level through MspI restriction analysis of the *gyrB* gene (47). A quantitative comparison of the whole-cell fatty acid profiles of the members of these three genera allows the differentiation of *Pandoraea* strains from the others (42, 70). However, with the use of the commercially available microbial identification system database (Microbial ID, Inc., Newark, Del.), these organisms are mostly identified with low identification scores as *Burkholderia* or *Ralstonia* species (42, 111) due to a lack of discriminatory fatty acids.

S. maltophilia

Key features for identifying *S. maltophilia* include a negative oxidase reaction, oxidation of glucose and maltose with a more intense reaction with the latter, positive reactions for DNase and lysine decarboxylase, and a tuft of polar flagella (Table 4) (218). Detection of extracellular DNase activity by *S. maltophilia* is a key to differentiating this species from most other glucose-oxidizing, gram-negative bacilli. It can be detected in tube-based or plated DNase medium with a methyl green indicator. DNase-positive organisms produce a zone of clearing around the colonies on this medium. Care must be taken in interpreting the DNase reaction, since one report documented the misidentification of *S. maltophilia* as *B. cepacia* based partially on false-negative DNase reactions that were finalized with 48 h of incubation rather than 72 h (24). Selected isolates of *Flavobacterium* and *Shewanella* spp. may also be DNase positive (see chapters 14 and 21). On sheep blood agar, colonies appear rough and lavender-green and have an ammonia-like odor. *S. maltophilia* has a characteristic cellular fatty acid profile with large amounts (>30%) of 13-methyl tetradecanoic acid ($C_{15:0\ iso}$) and lesser amounts (>10%) of 12-methyl tetradecanoic acid ($C_{15:0\ anteiso}$) and *cis*-9-hexadecanoic acid ($C_{16:1\ cis9}$) (218). To overcome the problems associated with definitive identification of *S. maltophilia*, Whitby et al. (219) developed a species-specific PCR assay targeting the 23S rRNA gene and reported sensitivity and specificity of 100%. This PCR test was recently

TABLE 4 Characteristics of *Acidovorax*, *Brevundimonas*, *Delftia*, *Comamonas*, and *Stenotrophomonas* spp. found in clinical specimens[a]

Test	*A. delafieldii* (*n* = 2)	*A. facilis* (*n* = 2)	*A temperans* (*n* = 2)	*Brevundimonas diminuta* (*n* = 68)	*Brevundimonas vesicularis* (*n* = 94)	*D. acidovorans* (*n* = 69)	*Comamonas* spp. (*n* = 28)	*S. maltophilia* (*n* = 228)
Oxidase	100	100	100	100	98	100	100	0
Growth:								
MacConkey	100	0	100	100	43	100	100	100
Cetrimide	0	0	0	0	0	4	0	2
6.0% NaCl	0	0	0	21	23	6	0	22
42°C	50	0	100	38	19	29	68	48
Nitrate reduction	100	100	100	3	5	99	96	39
Gas from nitrate	0	0	100	0	0	0	0	0
Pigment	Yellow, soluble	None	Yellow, soluble	Brown-tan, soluble	52% yellow-orange, insoluble[d]	26% fluorescent, 44% yellow-tan, soluble	27% yellow-brown, soluble	Brown-tan, soluble
Arginine dihydrolase	100	100	0	0	0	0	0	0
Lysine decarboxylase	0	0	0	0	0	0	0	93
Ornithine decarboxylase	0	0	0	0	0	0	0	0
Indole	0	0	0	0	0	0	0	0
Hemolysis	0	0	0	0	0	0	0	1
Hydrolysis of:								
Urea	100	100	50	13	2	0	7	3
Citrate	100	0	0	1	1	94	47	34
Gelatin	0	100	0	68	25	11	0	93
Esculin	0	0	0	5	88	0	0	39
Acid from:								
Glucose[b]	100	100	100	21	87	0	0	85
Xylose	85	100	0	0	27	0	0	35
Lactose	0	0	0	0	0	0	0	60
Sucrose	0	0	0	0	0	0	0	63
Maltose	0	0	0	0	94	0	0	100
Mannitol	50	100	50	0	0	100	0	0
H_2S[c]	100	100	100	34	49	57	0	95
Motility	100	100	100	100	100	100	100	100
No. of flagella	1–2	1–2	1–2	1–2	1–2	>2	>2	>2

[a] Data are from reference 218. Results are expressed as the percentage of strains positive.
[b] Oxidation-fermentation basal medium with 1% carbohydrate.
[c] Lead acetate paper.
[d] Pigment observed on Thayer-Martin agar.

used as a standard to evaluate the identification of *S. maltophilia* using the API 20NE strip and the Vitek-2 ID-GNB card (95). Both systems showed good reliability compared to PCR. A multiplex PCR assay to identify *P. aeruginosa*, *B. cepacia* complex species, and *S. maltophilia* directly in sputum and oropharyngeal specimens from CF patients has been reported, but only a limited number of *S. maltophilia* isolates were examined (71).

Acidovorax, Brevundimonas, Delftia, and *Comamonas* spp.

Characteristics of *Acidovorax*, *Brevundimonas*, *Delftia*, and *Comamonas* spp. are given in Table 4.

Acidovorax species, rarely encountered in clinical and environmental samples, are straight to slightly curved gram-negative bacilli which occur either singly or in short chains. They are oxidase positive and nonpigmented and have a single polar flagellum. Urease activity varies among strains (218, 224).

Brevundimonas diminuta and *Brevundimonas vesicularis*, infrequently encountered in clinical and environmental samples, have growth requirements for specific vitamins, including pantothenate, biotin, and cyanocobalamin. An additional growth requirement for *Brevundimonas diminuta* is cysteine. Most strains of *Brevundimonas diminuta* grow on MacConkey agar, while only approximately 25% of *Brevundimonas vesicularis* strains do so. On primary isolation media, *Brevundimonas diminuta* colonies are chalk white whereas many strains of *Brevundimonas vesicularis* are characterized by an orange intracellular pigment. These organisms are oxidase positive, have a single polar flagellum, and weakly oxidize glucose *(Brevundimonas vesicularis* more so than *Brevundimonas diminuta),* and the vast majority fail to reduce nitrate to nitrite. The most reliable method for differentiating these two species is the test for esculin hydrolysis. Almost all strains of *Brevundimonas vesicularis* (88%) are reported to hydrolyze this substrate, while *Brevundimonas diminuta* strains rarely do (5%) (Table 4) (218).

Comamonas spp. are straight to slightly curved gram-negative bacilli which occur singly or in pairs. The organisms are catalase and oxidase positive and have a single tuft of polar flagella. All human clinical *Comamonas* species reduce nitrate to nitrite. Phenotypic differentiation of *Comamonas terrigena* from *Comamonas testosteroni* is difficult, and as a result isolates are typically reported as *Comamonas* spp. (Table 4).

D. acidovorans is phenotypically similar to *Comamonas*. Key characteristics of the species include abilities to oxidize fructose and mannitol. One-quarter of the strains produce a fluorescent pigment, and approximately half of the strains may produce a soluble yellow to tan hue (218, 223).

TYPING SYSTEMS

Several molecular genetic methods are available to assess the relatedness of isolates of these genera during nosocomial outbreak investigations. These methods are preferred over phenotypically based systems, which are less discriminatory and reproducible. Analysis of whole-genome macrorestriction profiles with pulsed-field gel electrophoresis (PFGE) has gained acceptance as a preferred genotyping method and has proved useful in numerous studies of *Burkholderia*, *Ralstonia,* and *S. maltophilia* (36, 51, 200). The endonucleases XbaI and SpeI are most frequently used and typically yield a dozen or more DNA fragments for analysis. Care must be taken in interpreting PFGE profiles of *Burkholderia* species, however. These species have unusually large and dynamic multichromosome genomes that are prone to large-scale alterations in content and arrangement (138). Consequently, epidemiologically irrelevant genomic polymorphisms may arise in the short term and confound outbreak investigations (51).

Ribotyping, which relies on polymorphisms in and around rRNA operons, has been used to investigate the epidemiology of the *B. cepacia* complex and *B. pseudomallei* (120, 145, 147). Both PFGE and ribotyping are relatively time-consuming and expensive to perform and are therefore not particularly well suited for routine analysis in clinical microbiology laboratories. A variety of PCR-based methods, including randomly amplified polymorphic DNA typing and repetitive-sequence PCR typing, offer attractive alternatives for genotyping *S. maltophilia* and *Burkholderia*, *Ralstonia,* and *Pandoraea* spp. (30, 44, 134, 151, 188). These methods are inexpensive and can provide rapid, reliable results. Multilocus sequence typing, which assesses DNA sequence variation at several chromosomal loci, has been developed for numerous bacterial species, including the *B. cepacia* complex, *B. pseudomallei,* and *B mallei* (13, 34, 97). This genotyping strategy provides robust, reproducible, and portable results and is quickly becoming the preferred method for investigating bacterial epidemiology, evolution, and population structure. Despite these attributes, however, multilocus sequence typing carries considerable expense and at present is largely a research tool. Typing methods have not been reported for *Brevundimonas*, *Delftia*, *Comamonas*, or *Acidovorax* spp.

SEROLOGIC TESTS

Of the organisms discussed in this chapter, serologic tests have been used clinically to diagnose only infections with *B. pseudomallei.* The indirect hemagglutination assay (IHA), although not available commercially, is the most widely used test in regions where infection is endemic (67). It is performed by using a prepared antigen from strains of *B. pseudomallei* sensitized to sheep cells and includes unsensitized cells as a control. This assay can be adapted to a microtiter plate test system. Because of high antibody background levels in healthy individuals, cross-reactions with other organisms including the *B. cepacia* complex, and the rapid onset of septicemic disease, IHA is of limited clinical value. Interpretation is difficult in areas where infection is endemic, and all results must be viewed in a clinical context. IHA titers may rise to high levels in culture-negative individuals, and a specific titer cutoff that indicates disease has not been established. In regions where infection is endemic, single titers may be reported without interpretation. The need for acute- and convalescent-phase titers along with relevant clinical information to aid in interpretation is paramount in attempting to establish serologically the diagnosis of subacute or chronic *B. pseudomallei* infection (160). Evaluation of recently developed rapid immunochromatographic test kits (Pan-Bio, Windsor, Queensland, Australia) suggests that the immunoglobulin G test may be useful for investigating travelers presenting with possible melioidosis after returning from regions where the disease is endemic but the immunoglobulin M test has low specificity (161).

ANTIMICROBIAL SUSCEPTIBILITY

Specific susceptibility testing interpretative criteria are not available for all of the species discussed in this chapter. For some species, such as the *B. cepacia* complex and *S. maltophilia,*

interpretive criteria for disk diffusion testing are available for only a limited number of antibiotics. In general, MIC broth microdilution tests or E tests are preferred for this group of organisms.

B. cepacia complex species are among the most antimicrobial-resistant bacteria encountered in the clinical laboratory. These species are intrinsically resistant to aminoglycoside and polymyxin antibiotics and are often resistant to β-lactam antibiotics due to inducible chromosomal β-lactamases and altered penicillin-binding proteins (106). Antibiotic efflux pumps may mediate resistance to chloramphenicol, fluoroquinolones, and trimethoprim (175). Clinical strains may be susceptible to only a handful of agents, including trimethoprim-sulfamethoxazole (TMP-SMX), ceftazidime, chloramphenicol, minocycline, imipenem, meropenem, and some fluoroquinolones (1, 112, 173). Clinical and Laboratory Standards Institute (formerly NCCLS) interpretative criteria for disk diffusion susceptibility testing are available only for ceftazidime, meropenem, minocycline, and TMP-SMX (39). MIC broth microdilution tests or E tests are preferred methodologies for susceptibility testing of these species. Because isolates that are initially susceptible may become resistant during the course of therapy, susceptibility testing of repeat isolates may be warranted. Furthermore, strains recovered from patients with CF who have received repeated courses of antibiotic therapy are frequently resistant to all currently available antimicrobial agents (1, 93). Combinations of antimicrobial agents may provide synergistic activity against resistant strains; however, antagonism with combinations is also observed in vitro (1). Testing for synergy with both double and triple combinations of agents is available in reference laboratories (http://synergy.columbia.edu) (1). The value of such testing is controversial, however; studies have yet to correlate in vitro synergy testing results with patient response to therapy (1, 155).

B. pseudomallei is resistant to penicillin, aminoglycosides, and macrolides, but several agents have antimicrobial activity in vitro, including ceftazidime, meropenem and imipenem, cefoperazone, amoxicillin-clavulanate, ampicillin-sulbactam, ticarcillin-clavulanate, chloramphenicol, and doxycycline. TMP-SMX is often used clinically; although reported in vitro susceptibility results are variable, most isolates are susceptible by agar dilution testing (122, 129). Resistance to fluoroquinolones is common, and their use has been associated with higher relapse rates (28). E tests have proved satisfactory for determining in vitro resistance or susceptibility, especially for TMP-SMX (158). Disk diffusion techniques have been disappointing, and inaccurate results suggest that they should probably be avoided (149).

Current trends in the management of melioidosis involve an initial 10- to 14-day intensive therapy phase with ceftazidime with or without TMP-SMX, followed by eradication therapy with TMP-SMX with or without doxycycline for at least 3 months (32, 222). Amoxicillin-clavulanate is recommended for eradication therapy in pregnancy and is an alternative to TMP-SMX in children. In critically ill patients requiring intensive care, meropenem or imipenem may be superior to ceftazidime, and granulocyte colony-stimulating factor is being used in some centers (33, 35, 122). According to molecular genotyping studies, relapse following antimicrobial therapy has been observed in as many as 15% of patients (79).

Because of the potential role of *B. mallei* as a bioterrorism agent, studies have been done recently to determine the activities of a variety of agents against this species. *B. mallei* has a susceptibility profile similar to that of *B. pseudomallei*, except that *B. mallei* is susceptible to aminoglycosides and newer macrolides such as clarithromycin and azithromycin whereas *B. pseudomallei* is resistant (108, 129). Current recommended treatment and duration of therapy for glanders are the same as those for melioidosis.

S. maltophilia is intrinsically resistant to many classes of antibiotics. Resistance can also develop rapidly during infection (91). Resistance to β-lactam agents is mediated by at least two β-lactamases, one of which is zinc dependent and resistant to β-lactamase inhibitors and confers resistance to imipenem. Aminoglycoside and quinolone resistance results from mutations in outer membrane proteins. In a recent study of isolates recovered from patients with CF, doxycycline was the most active agent in vitro (182). TMP-SMX is usually active and is often used in combination with ticarcillin-clavulanate, minocycline, or piperacillin-tazobactam (182). Other combinations that may be effective include ciprofloxacin paired with ticarcillin-clavulanate, ciprofloxacin and piperacillin-tazobactam, or doxycycline and ticarcillin-clavulanate. Clinical and Labaratory Standards Institute interpretive criteria for disk diffusion susceptibility testing are available for minocycline, levofloxacin, and TMP-SMX (39). However, broth microdilution, E test, or agar dilution methods are the preferred susceptibility testing methods (9, 235). Many U.S. laboratories comment only on the activity of TMP-SMX but will test additional antibiotics such as minocycline, ceftazidime, ticarcillin-clavulanate, and ciprofloxacin or levofloxacin upon request.

In general, *Comamonas testosteroni* is susceptible to extended- and broad-spectrum cephalosporins, carbapenems, quinolones, and TMP-SMX (14). *D. acidovorans* is frequently resistant to the aminoglycosides.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

These species are found in the natural environment and may occasionally contaminate clinical specimens. Nevertheless, they are increasingly recognized as nosocomial and opportunistic pathogens, especially in certain patient populations, such as persons with CF. They are also frequently misidentified by commercial microbial identification systems. Therefore, their recovery in the clinical laboratory must be given careful consideration. In particular, species of the *B. cepacia* complex are not reliably differentiated by phenotypic analyses and their recovery from persons with CF has serious consequences with respect to patient management and psychosocial well-being (141). Identification of these species should be confirmed by genotypic analyses at a reference laboratory and should promptly be reported to the CF care team. Similarly, identification of *B. pseudomallei*, *B. mallei,* or *B. thailandensis* should be confirmed by a reference laboratory with experience with these species. Care must be given to ensure that culture handling and shipping comply with current biosafety regulations (see chapters 8 and 9). Identification of these species must be reported to public health officials due to the potential of these species as agents of bioterrorism (see chapter 9).

Interpretive criteria for disk diffusion antimicrobial susceptibility testing of most of these species are lacking; MIC broth microdilution and the E test are, therefore, the preferred methodologies for susceptibility testing. For multidrug-resistant strains, consideration should be given to testing for synergy with double or triple combinations of antimicrobial agents in reference laboratories.

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Acinetobacter, Achromobacter, Chryseobacterium, Moraxella, and Other Nonfermentative Gram-Negative Rods*

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DESCRIPTION OF GENERA

The organisms covered in this chapter belong to a group of taxonomically diverse, nonfermentative gram-negative rods. They all share the common phenotypic features of failing to acidify the butt of Kligler iron agar (KIA) or triple sugar iron (TSI) agar or of oxidative-fermentative (OF) media and grow significantly better under aerobic than under anaerobic conditions; many strains fail to grow anaerobically. Most of the organisms covered in this chapter, with the exception of *Neisseria elongata* subspecies *elongata* and *nitroreducens,* are catalase positive. Oxidase and growth on MacConkey agar are variable. They either are nonmotile or, if motile, often have peritrichous flagella.

The methods used for growth and identification of these organisms are those used for *Pseudomonas* spp. (see chapter 48). Initial incubation should be at 35 to 37°C, although many pink-pigmented strains grow better at ≤30°C and may be detected only on plates left at room temperature after the initial readings. In such cases, all identification tests should be carried out at that temperature. In fact, some of the commercial kits, such as the API 20 NE, are designed to be incubated at 30°C. Growth on certain selective primary media (e.g., MacConkey or Salmonella-Shigella [SS] agar) is variable; and there can be significant lot-to-lot variations in the media. Nonfermenters that grow on MacConkey agar generally form colorless colonies, although some form lavender or purple colonies due to uptake of crystal violet contained in the agar medium.

When laboratory identification of this group of organisms is deemed necessary, a simplified approach is recommended whereby unknown isolates are initially characterized and placed into one of seven groups based on microscopic morphology, oxidase reaction, motility, acidification of carbohydrates, indole production, and production of pink-pigmented colonies (Fig. 1). Further characterization is made on the basis of the biochemical reactions (see Tables 1 through 7) that are examined after 1, 2, and 7 days of incubation. Additional differential tests can be found in other publications (96, 207, 256, 257). Carbohydrate utilization and mode of utilization are based on the King formulation of OF medium (256). In the authors' experience, indole production is best demonstrated by inoculation of heart infusion broth and incubation at 35 to 37°C for 48 h followed by extraction with xylene and addition of Ehrlich's reagent. The oxidase test is performed after overnight incubation using *N*, *N*, *N*, *N*-tetramethyl-*p*-phenylenediamine dihydrochloride (0.5%). Motility is easily determined by performing a wet mount preparation of a young colony from a blood agar plate. For some strains, motility can best be demonstrated after incubation of cultures at room temperature.

Traditional diagnostic systems, e.g., those based on OF media, aerobic low-peptone media, or buffered single substrates, have now been replaced in many laboratories by commercial kits. The ability of commercial kits to identify this group of nonfermenters is variable and often results in identification to the genus or group level only, necessitating the use of supplemental biochemical testing for species identification (10, 123, 135, 147, 175, 223). If such kits are used, the laboratory must be familiar with the extent of the database; organisms not included will be unidentified or identified incorrectly. Because assimilation test results often depend on the basal medium used, most of those results are not included in the tables presented here. Identification of nonfermenters by automated fatty acid analysis has also been attempted (243). In view of the difficulties inherent in this approach (176), it is recommended that fatty acid profiles be used only in conjunction with traditional or commercial diagnostic systems. The fatty acid profiles for the most common species of nonfermenting bacilli have been published by Weyant and colleagues (256) and are included in the tables presented here. Furthermore, rRNA gene sequencing is likely to become the standard for identification of many organisms that are difficult to identify using conventional methods, such as those described in this chapter, and laboratories are encouraged to develop such capabilities. The impact of this technology has already been noted in several publications (38, 63, 180); this method was used to confirm the identification of several new nonfermenting species included in this chapter.

TAXONOMY, NATURAL HABITATS, AND CLINICAL SIGNIFICANCE

Since a large and diverse group of organisms are covered in this chapter, the taxonomy, natural habitats, and clinical significance of the individual species are covered under the individual organism headings.

* This chapter contains information presented in chapter 49 by Paul C. Schreckenberger, Maryam I. Daneshvar, Robbin S. Weyant, and Dannie G. Hollis in the eighth edition of this Manual.

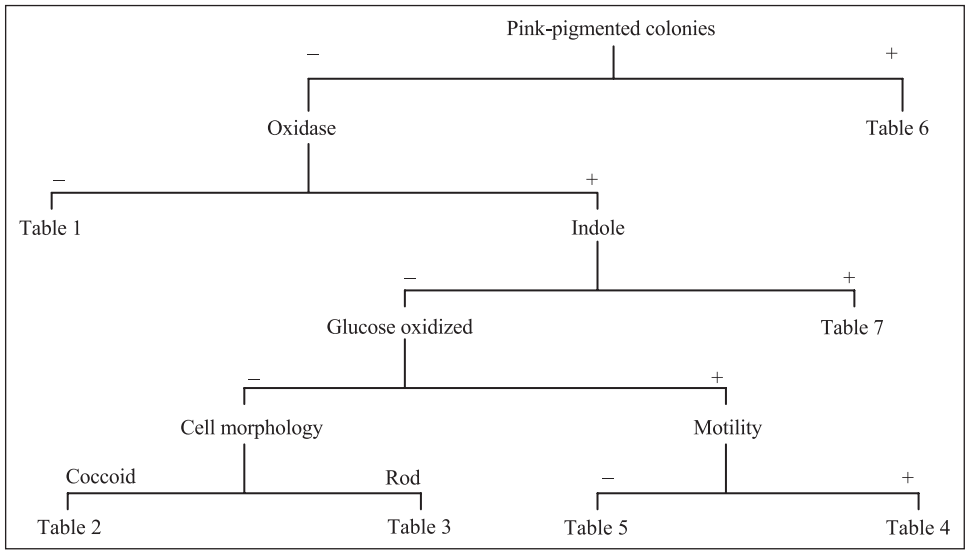


FIGURE 1 Identification of miscellaneous gram-negative nonfermenters.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Standard methods for collection, transport, and storage of specimens as detailed in chapters 5 and 20 are satisfactory for this group of organisms.

OXIDASE-NEGATIVE GROUP

See Table 1.

Acinetobacter spp.

General Description

The genus *Acinetobacter* consists of strictly aerobic, gram-negative coccobacillary rods that are oxidase negative, nonmotile, usually nitrate negative, and nonfermentative. Individual cells are 1 to 1.5 by 1.5 to 2.5 μm in size, sometimes difficult to decolorize, and frequently arranged in pairs. Clinical microbiologists should be alert to the fact that *Acinetobacter* species may initially appear as gram-positive cocci in direct smears prepared from positive blood culture bottles (83). In the stationary growth phase and on nonselective agars, coccobacillary forms predominate, whereas early growth in fluid media and growth on plates containing cell wall-active antimicrobial agents yield mostly rods. Colonies are smooth, opaque, and slightly smaller than those of members of the family *Enterobacteriaceae*. Many strains grow on MacConkey agar as either colorless or slightly pinkish colonies. Some strains are fastidious, showing punctate colonies on blood agar, and fail to grow in nutrient broth (256). Certain glucose-oxidizing acinetobacters may also cause a unique brown discoloration of heart infusion agar with tyrosine or blood agar into which glucose is incorporated (213, 256). We have also observed this phenomenon on MacConkey and Mueller-Hinton agars with a clinical isolate of *A. baumannii.* Differential and selective media have been described for isolation of *Acinetobacter* spp. from contaminated specimens. (103, 118).

Taxonomy

The genus was originally placed within the family *Neisseriaceae* but more recently moved to the family *Moraxellaceae* (203). Studies based on DNA-DNA hybridization have resulted in the description of 25 DNA homology groups (also called genomospecies) within the genus *Acinetobacter* (18, 20, 75, 115, 169, 171, 229). While only 11 species have been named (18, 169, 170, 171), differential biochemical and growth tests have been published for at least 19 species (18, 20, 76, 169, 170, 171). Genomospecies 1, 2, 3, and 13 of Tjernberg and Ursing (229) may be difficult to separate in the clinical laboratory and have been referred to as the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex (76). Because of problems in the clinical laboratory separating the DNA groups by phenotypic tests, we have chosen to separate the *Acinetobacter* species in Table 1 into two groups, saccharolytic and asaccharolytic. Most glucose-oxidizing nonhemolytic clinical strains are *A. baumannii,* most glucose-negative nonhemolytic ones are *Acinetobacter lwoffii,* and most hemolytic ones are *Acinetobacter haemolyticus*.

Natural Habitat and Clinical Significance

Acinetobacter species are widely distributed in nature and in the hospital environment, are the second most commonly isolated nonfermenters in human specimens (*Pseudomonas aeruginosa* being the first), are able to survive on moist and dry surfaces (77), and may be present in foodstuffs (105) and on healthy human skin (208). *Acinetobacter* spp. are generally considered to be nonpathogenic to healthy individuals but may cause infections in debilitated individuals. The species most frequently isolated from humans is *A. baumannii* (with 19 biotypes identified by assimilation tests [19] and 34 serovars [230]).

A. baumannii is the species most often responsible for hospital-acquired infections (152, 244). A biotyping system for differentiating 17 biotypes of *A. baumannii* based on the utilization of six substrates has been established and may be useful for epidemiologic studies (19). Turton and colleagues have reported that integrons are useful markers for epidemic strains of *A. baumannii* and that integron typing provides valuable information for epidemiological studies (232). The ability of this microorganism to acquire antimicrobial multiresistance and its high capacity for survival on most environmental surfaces has led to an increased concern

TABLE 1 Oxidase-negative group[a]

Test	*Acinetobacter* species[b]		CDC group EO-5 (*Acetobacter*-like)[c] (10)	CDC group NO-1 (22)	*Bordetella ansorpii*[d] (1)	*Bordetella holmesii* (13)	*Bordetella parapertussis* (12)	*Bordetella trematum* (1)	*Kerstersia gyiorum*[e] (2)	*Pseudomonas oryzihabitans*[f] (36)	*Pseudomonas luteola*[f] (34)
	Asaccharolytic (270)	Saccharolytic (77)									
Motility; flagella	0	0	0	0	100; ND	0	0	100; pe	50; pe	100; p, 1–2	100; p, >2
Acid from[g]:											
D-Glucose	0	95(5)	100	0[h]	0	0	0	0	0	100	100
D-Xylose	<1	98	100	0	0	0	0	0	0	100	100
D-Mannitol	0	0	0	0	0	0	0	0	0	100	76(18)
Lactose	0	62(13)	0	0	0	0	0	0	0	14(22)	3(24)
Sucrose	0	0	0	0	0	0	0	0	0	25	12
Maltose	0	17(37)	0	0	0	0	0	0	0	97(3)	100
Catalase	>99	100	100	100	ND	38	100	100	100	94	100
Growth on:											
MacConkey	74(4)	96	10w	5(15)	100	77(23)	100	100	100	100	100
SS	9(2)	10(2)	0	0	ND	0	0	100	100	22	68
Cetrimide	<1	2	0	0	ND	0	0	0	ND	25(28)	0
Simmons citrate	30(3)	63(3)	0	0	100	0	67(8)	100w	100	97	100
Urea, Christensen's	8(18)	22(24)	(10w)	5w	0	0	100	0	0	77	26(38)
Nitrate reduction	6	8	0	100	0	0	0	0[i]	0	6	62
H_2S (lead acetate paper)	51	38	50	5, 45w	ND	62	17	100	ND	97	12
Gelatin hydrolysis[j]	2	6	0	0	100	0	0	0	0	17	61
Pigment:											
Insoluble	0	0	0	0	ND	0	0	0	0	100 yel	97 yel
Soluble	12 yel/tan	22 br-yel-tan	20 yel	0	ND	100 br	100 br	0	ND	0	0
Growth at:											
25°C	98	100	90	20	ND	67	60	100	ND	100	100
35°C	98	100	90	100	100	100	100	100	100	100	100
42°C	48	72	0	15	ND	0	18	0	100	33	94
Esculin hydrolysis	0	0	0	0	0	0	0	0	0	0	100
Lysine decarboxylase	0	10	0	0	ND	0	ND	0	0	7	0
Arginine dihydrolase	9, 6w	19	0	0	0	0	ND	0	0	14	100
Ornithine decarboxylase	0	5	0	0	ND	0	ND	0	0	3	0

Nutrient broth, 0% NaCl	86(1)	99	90	10(5)	ND	46(15)	92	100	100	100	100
Nutrient broth, 6% NaCl	19	13	0	0	ND	0	0	100	50	62	74(3)
Major CFAs[k]	16:0,[l]	16:0,[l]	16:0,	16:0,	16:0,	16:0,	16:0,	16:0,	16:0,	16:0,	16:0,
	16:1ω7c,	16:1ω7c,	18:1ω7c	16:1ω7c,	16:1ω7c,	17:0cyc	17:0cyc	17:0cyc,	16:1ω7c,	16:1ω7c,	16:1ω7c,
	18:1ω9c	18:1ω9c		18:1ω7c	17:0cyc,			18:1ω7c	18:1ω7c	18:1ω7c	18:1ω7c
					18:1ω7c						

[a]Unless otherwise indicated, data are from the CDC Special Bacteriology Reference Laboratory (256). All taxa were negative (<10% positive) for gas from nitrate, nitrite reduction, indole production, acid in TSI butt, and H_2S in TSI. Numbers indicate the percentage positive at 2 days of incubation; parentheses indicate a delayed reaction (3 to 7 days of incubation); w, weak reaction; pe, peritrichous; p, polar; br, brown; yel, yellow; ND, not determined or not available. Numbers in parentheses after organisms indicate the number of strains.

[b]A total of 25 *Acinetobacter* genomospecies have been described. See the text.

[c]Probable *Acetobacter* species based on three phenotypically similar strains that were identified by 16S rRNA gene sequencing.

[d]Data from reference 138.

[e]Data from reference 41.

[f]Included in table only; see description in chapter 48.

[g]Oxidative-fermentative basal medium with 1% carbohydrate.

[h]Usually does not grow in oxidative-fermentative medium.

[i]Nitrate reduction reported variable by other authors (see chapter 51).

[j]14 days of incubation.

[k]CFA, cellular fatty acid. The number before the colon indicates the number of carbons; the number after the colon is the number of double bonds; **ω**, the position of the double bond counting from the hydrocarbon end of the carbon chain; c, *cis* isomer; cyc, a cyclopropane ring structure.

[l]Cannot distinguish most *Acinetobacter* species by CFA.

regarding hospital-acquired infections. Corbella et al. have shown that the digestive tract of intensive care unit patients is an important epidemiologic reservoir for multiresistant *A. baumannii* infections in hospital outbreaks, and they suggest that a fecal surveillance program might be considered for early implementation of patient isolation precautions in an outbreak setting (48). Hospital-acquired infections are most likely to involve the respiratory tract (most often related to endotracheal tubes or tracheostomies), urinary tract, and wounds (including catheter sites) and may progress to septicemia (9, 37, 258). Sporadic cases of continuous ambulatory peritoneal dialysis peritonitis, endocarditis, meningitis, osteomyelitis, arthritis, and corneal perforation have also been reported (9). There are an increasing number of reports of *Acinetobacter* species as agents of nosocomial pneumonia, particularly ventilator-associated pneumonia in patients confined to hospital intensive care units (9). Risk factors are antibiotic treatment and/or surgery, instrumentation, mechanical ventilation, and stay in intensive care units; clinical isolates, however, are more often colonizers than infecting agents (9, 37, 258). Hospital outbreaks have been investigated by various typing methods (139, 150, 232). Community-acquired *Acinetobacter* pneumonias in which a fatal outcome was strongly associated with inappropriate initial antibiotic therapy have also been reported (2). Most recently, American troops wounded in Iraq and Afghanistan have suffered severe wound infections and osteomyelitis from multidrug-resistant *A. baumannii* (30, 56).

Other species, such as *Acinetobacter johnsonii, A. lwoffii,* and *Acinetobacter radioresistens,* seem to be natural inhabitants of the human skin and may also be commensals in the oropharynx and vagina (21). *A. lwoffii* has been more commonly associated with meningitis than other *Acinetobacter* species (214). *Acinetobacter ursingii* has been shown to cause bloodstream infections in hospitalized patients (153, 169). *Acinetobacter junii* is a rare cause of ocular infection (190) and bacteremia, particularly in pediatric patients (126, 146). A case of community-acquired *A. radioresistens* bacteremia in a human immunodeficiency virus-positive patient has also been reported (245). *Acinetobacter schindleri* has been recovered from a variety of human specimens (vaginal, cervical, throat, nasal, ear, conjunctiva, and urine) but is mostly regarded as clinically nonsignificant (169).

Antibiotic Susceptibility

Cephalothin is ineffective against *Acinetobacter* spp., but trimethoprim-sulfamethoxazole, imipenem, imipenem-cilastatin, ampicillin-sulbactam, ticarcillin-clavulanate, piperacillin-tazobactam, amoxicillin-clavulanate, doxycycline, and quinolones are effective against most strains (9, 37, 119), but susceptibility testing is required for each clinically significant strain. Multiply resistant strains including carbapenem-resistant *Acinetobacter* species have been reported in nosocomial outbreaks (17, 108, 157, 158). Although the carbapenems are still the most active antimicrobials against *Acinetobacter* species, carbapenem resistance is now becoming common (47, 50, 158, 269).

Antimicrobial susceptibility testing for *Acinetobacter* species is problem prone. Swenson and colleagues at the CDC have shown that results obtained using standardized broth microdilution do not agree with results obtained with the standardized disk diffusion method for certain antibiotics. Very major errors were frequent with the β-lactam and β-lactam inhibitor combination antibiotics, with the broth microdilution method typically showing greater resistance (225). At present, there are no data to

indicate which method provides more clinically relevant information.

Treatment

Combined treatment with an aminoglycoside and ticarcillin or piperacillin is synergistic and may be effective in serious infections. For multiply resistant *Acinetobacter* infections, several studies have demonstrated clinical efficacy of sulbactam in combination with ampicillin or cefoperazone (46, 85, 119, 143). The only other antibacterial agent that has been shown to be active against multiply resistant *Acinetobacter* is colistin (78, 121, 144). For a review of the clinical features, molecular epidemiology, and antimicrobial susceptibility of nosocomial *Acinetobacter* infections, consult the reviews by Bergogne-Berezin and Towner (9) and Wisplinghoff et al. (258).

CDC Group EO-5

CDC Eugonic Oxidizer (EO) Group 5 organisms (M. I. Daneshvar, D. G. Hollis, C. W. Moss, J. G. Jordan, J. P. MacGregor, and R. S. Weyant, *Abstr. 98th Gen. Meet. Am. Soc. Microbiol.,* abstr. C-204, 1998) are glucose-oxidizing gram-negative rods that have a biochemical profile similar to that of *A. baumannii*. Based on 16S rRNA sequencing, these are probably *Acetobacter* species (M. I. Daneshvar, unpublished data). They are nonmotile and oxidase negative and fail to grow on MacConkey agar. Some strains produce a yellow soluble pigment. Cellular fatty acid (CFA) analysis is also useful in differentiating EO-5 strains from *A. baumannii*. Other characteristics are given in Table 1. Isolates have been recovered from blood, peritoneal fluid, transtracheal aspirate, gall bladder, and an arm wound. Antibiotic susceptibility data are not available.

CDC Group NO-1

Group NO (nonoxidizer)-1 bacteria (87) are oxidase negative, asaccharolytic, nonmotile, coccoid to medium-sized gram-negative rods forming small colonies on sheep blood agar (SBA). Other differential features are shown in Table 1. Nitrate, but not nitrite, is reduced; however, since approximately 6% of asaccharolytic *Acinetobacter* spp. reduce nitrate, CFA profiles and 16S rRNA gene sequencing are useful for making a definitive identification. Most strains have been isolated from human wounds resulting from dog or cat bites, suggesting that these animals are a reservoir for NO-1 infections (87, 125). They are susceptible to antibiotics used for infections caused by gram-negative organisms, including aminoglycosides, β-lactam antibiotics, tetracyclines, quinolones, and sulfonamides (87).

Bordetella spp.

The *Bordetella* species are discussed in detail in chapter 51 of this Manual. There are eight *Bordetella* spp. that are nonfastidious and will grow on ordinary culture media (i.e., sheep blood and MacConkey agars) and biochemically resemble either *Acinetobacter* spp. or *Alcaligenes/Achromobacter* spp. Four species *(B. ansorpii, B. holmesii, B. parapertussis,* and *B. trematum)* are oxidase negative and are included in Table 1, and four species are oxidase positive *(B. avium, B. bronchiseptica, B. hinzii,* and *B. petrii)* and are included in Table 3. *B. holmesii* (formerly CDC group NO-2) is nitrate negative (differentiating it from NO-1 strains) and produces a brown soluble pigment on heart infusion-tyrosine agar. Other differential tests are given in Table 1. *B. bronchiseptica* is rapid urease positive and must be differentiated from *Cupriavidus pauculus* (Table 3) and *Oligella ureolytica* (Table 2).

Genus *Kerstersia*

Kerstersia is a newly described novel genus that consists of a single species, *K. gyiorum* (Greek *gyion,* meaning "limb," referring to the fact that the majority of strains were isolated from human leg wounds) (41). They appear as gram-negative, small (1 to 2 μm long), coccoid cells that occur singly, in pairs, or in short chains. On nutrient agar, colonies are flat or slightly convex, with smooth margins and color ranging from white to light brown. Growth occurs at 28 and 42°C, motility is strain dependent, and all strains are asaccharolytic. All strains are catalase positive but are negative for oxidase, arginine, lysine and ornithine decarboxylase, β-galactosidase, gelatinase, amylase, urease, DNase, reduction of nitrate and nitrite, and hydrolysis of esculin (Table 1). Strains have been isolated from various human specimens, including feces, sputum, and leg and ankle wounds. Pathogenicity is unknown (41).

OXIDASE-POSITIVE, INDOLE-NEGATIVE, ASACCHAROLYTIC COCCOID NONFERMENTERS

See Table 2.

Psychrobacter phenylpyruvicus, Moraxella catarrhalis, and Other *Moraxella* spp.

Members of the genus *Moraxella* are oxidase-positive, nonmotile, asaccharolytic coccobacilli that are often plump, occur predominantly in pairs and sometimes in short chains, and have a tendency to resist decolorization (54).

Moraxellae are parasitic on human skin and mucous membranes. *M. catarrhalis, M. osloensis, M. nonliquefaciens,* and *M. lincolnii* are part of the normal flora of the human respiratory tract, while most *M. canis* strains have been found in the upper respiratory tracts of dogs and cats. These and other moraxellae are rare agents of infections (conjunctivitis, keratitis, meningitis, septicemia, endocarditis, arthritis, and otolaryngologic infections) (122, 127, 209, 241).

M. catarrhalis has been reported to cause sinusitis and otitis media by contiguous spread of the organisms from a colonizing focus in the respiratory tract (127). However, isolation of *M. catarrhalis* from the upper respiratory tract (i.e., a throat culture) of children with otitis media or sinusitis does not provide evidence that the isolate is the cause of these infections. Rates of upper respiratory tract colonization by *M. catarrhalis* in children vary widely and are influenced by many factors (both environmental and genetic). The presence of the organism in the oropharynx or nasopharynx is not necessarily predictive of infection in contiguous anatomic sites. Isolates from sinus aspirates and middle ear specimens obtained by tympanocentesis should be identified and reported. Similarily, little is known about the pathogenesis of lower respiratory tract infection in adults with chronic lung diseases. Examination of Gram-stained smears of sputum specimens from patients with exacerbations of bronchitis and pneumonia due to *M. catarrhalis* usually reveals an abundance of leukocytes, the presence of many gram-negative diplococci as the exclusive or predominant bacterial morphotype, and the presence of intracellular gram-negative diplococci. Such specimens may yield *M. catarrhalis* in virtually pure culture, and the organism should be identified and reported.

Colonies of *M. catarrhalis* grow well on both blood and chocolate agars, and some strains also grow well on Modified Thayer-Martin and other selective media. Colonies are generally gray to white, opaque, and smooth and measure about

1 to 3 mm after 24 h of incubation. Characteristically, the colonies may be nudged across the plate intact with a bacteriological loop like a "hockey puck." *M. catarrhalis* is strongly oxidase positive and catalase positive. It does not produce acid from glucose or other carbohydrates. Most strains reduce nitrate and nitrite and produce DNase. *M. catarrhalis* may be easily distinguished from *Neisseria* species by its ability to hydrolyze ester-linked butyrate groups (butyrate esterase) (179, 219). A very rapid (2.5-min) and reliable indoxyl-butyrate hydrolysis spot test has been described and is commercially available (Remel, Inc., Lenexa, Kans.) (58).

M. nonliquefaciens is the second most frequently isolated species after *M. catarrhalis*. It forms smooth, translucent to semiopaque colonies 0.1 to 0.5 mm in diameter after 24 h and 1 mm in diameter after 48 h of growth on SBA plates. Occasionally, these colonies spread and pit the agar. The colonial morphologies of *M. lincolnii* (237), *M. osloensis*, and *Psychrobacter phenylpyruvicus* (formerly *M. phenylpyruvica*) are similar, but pitting is rare. On the other hand, pitting is common with *M. lacunata,* whose colonies are smaller and form dark haloes on chocolate agar. Colonies of *M. atlantae* are small (usually 0.5 mm in diameter) and show pitting and spreading (22). Most *M. canis* colonies resemble those of the *Enterobacteriaceae* (large, smooth colonies) and may produce a brown pigment when grown on starch-containing Mueller-Hinton agar (117). Some strains may also produce very slimy colonies resembling colonies of *Klebsiella pneumoniae* (117). Microscopically, *M. canis* resembles *M. catarrhalis*, both appearing as gram-negative diplococci measuring 0.5 to 1.5 μm in diameter. Animal species include *M. bovis*, isolated from healthy cattle and other animals, including horses, *M. boevrei* and *M. caprae* (goats), *M. caviae* (guinea pigs), *M. cuniculi* (rabbits), and *M. ovis* and *M. oblonga* (sheep).

Biochemical reactions for the human isolates are listed in Table 2. Most laboratories do not determine the species of moraxellae, other than *M. catarrhalis*, because of the similarity in pathogenic significance of the species and because many strains are somewhat fastidious and biochemical reactions are often negative or equivocal. *M. atlantae, M. lacunata*, and *M. nonliquefaciens* are similar in many of their features. Growth of *M. atlantae* is stimulated by bile salts and sodium desoxycholate, while *M. lacunata* and *M. nonliquefaciens* are not. Only *M. lacunata* liquefies gelatin, whereas both *M. lacunata* and *M. nonliquefaciens* reduce nitrate to nitrite (22, 182) Separation of *M. lacunata* and nonspreading *M. nonliquefaciens* may prove difficult, because gelatin hydrolysis (with any method) and liquefaction of Loeffler slants may take more than 1 week. In some instances, fatty acid analysis may help determine the species (256); in other cases, quantitative transformation of a high-level streptomycin resistance marker can be used (124). The differential diagnosis of *P. phenylpyruvicus* and *Brucella* spp. is of great practical importance (see the discussion of agents of bioterrorism in chapter 9) and requires microscopy (*Brucella* organisms are tiny coccobacilli) and tests for acidification of xylose and glucose (183, 184). *P. phenylpyruvicus* is asaccharolytic, whereas *Brucella* spp. utilize xylose and usually glucose when a sufficiently sensitive method for detecting acidification of glucose is employed (183). Microbiologists should be aware that *Brucella* species that are unwittingly inoculated into certain commercial identification systems may be misidentified as *P. phenylpyruvicus* or *Haemophilus influenzae* (5, 6, 178). The tributyrin test may be positive for several *Moraxella* spp. and therefore cannot be used to separate them from *M. catarrhalis* (179). Likewise, gamma-glutamyl aminopeptidase occurs not only in *M. canis* but also in some strains of other moraxellae (117).

Most *Moraxella* strains are susceptible to penicillin and its derivatives, cephalosporins, tetracyclines, quinolones, and aminoglycosides (70, 201, 218). Production of beta-lactamase has been only rarely reported in *Moraxella* species other than *M. catarrhalis*, which commonly produces an inducible, cell-associated beta-lactamase (122, 201). Isolates of *M. catarrhalis* are generally susceptible to amoxicillin-clavulanate, expanded-spectrum and broad-spectrum cephalosporins (i.e., cefuroxime, cefotaxime, ceftriaxone, cefpodoxime, ceftibuten, and the oral agents cefixime and cefaclor), macrolides (e.g., azithromycin, clarithromycin, erythromycin), tetracyclines, and rifampin. While most isolates are susceptible to the fluoroquinolones, resistance to these agents has emerged in isolates recovered from patients who were receiving long-term therapy with such agents.

Oligella spp.

The genus *Oligella* consists of two species: *O. urethralis* (formerly *Moraxella urethralis* and CDC group M-4) and *O. ureolytica* (formerly CDC group IVe) (202). *O. urethralis* is nonmotile, while most strains of *O. ureolytica* are motile by peritrichous flagella. Biochemical features that help differentiate *Oligella* spp. from *Moraxella* spp. are shown in Table 2. *O. urethralis* is similar to *Moraxella* spp. in that isolates are coccobacillary, oxidase positive, and nonmotile. Colonies are smaller than those of *M. osloensis* and are opaque to whitish. *O. urethralis* and *M. osloensis* share additional biochemical similarities, e.g., accumulation of poly-β-hydroxybutyric acid and failure to hydrolyze urea, but can be differentiated on the basis of nitrite reduction and alkalinization of formate, itaconate, proline, and threonine (all positive for *O. urethralis* and negative for *M. osloensis*) (185). CFA analysis can also be used to differentiate these two species (256).

Colonies of *O. ureolytica* are slow growing on blood agar, appearing as pinpoint colonies after 24 h but large colonies after 3 days of incubation. Colonies are white, opaque, entire, and nonhemolytic. *O. ureolytica* strains are both phenylalanine deaminase and rapid urease positive, with the urease reaction often turning positive within minutes after inoculation. In this regard, *O. ureolytica* is similar to *Bordetella bronchiseptica* and *Cupriavidus pauculus* from which it must be differentiated. CFA analysis is useful since the CFA profiles are different for each of these species (256).

Both *Oligella* spp. have been isolated chiefly from the human urinary tract, and both have been reported to cause urosepsis (191, 200). A case of septic arthritis due to *O. urethralis* has also been reported (160). *O. urethralis* is generally susceptible to most antibiotics, including penicillin, while *O. ureolytica* exhibits variable susceptibility patterns (70).

Haematobacter spp. (Proposed)

Haematobacter is a proposed new genus of aerobic gram-negative rods that phenotypically most closely resembles *Psychrobacter phenylpyruvicus* (L. O. Helsel, D. Hollis, A. G. Steigerwalt, R. E. Morey, J. Jordan, T. Aye, J. Radosevic, D. Jannat-Kahah, D. R. Lonsway, J. B. Patel, M. I. Daneshvar, and P. N. Levett, *Abstr. 105th Gen. Meet. Am. Soc. Microbiol.*, abstr. C-404, 2005). The genus consists of two named *(H. cherryi* and *H. missouriensis)* and one unnamed genomospecies. All strains are nonmotile and asaccharolytic. They produce catalase, oxidase, urease, and H_2S (lead acetate paper) but do not produce indole, reduce nitrate or nitrite, or hydolyze gelatin or esculin. Colonies of *Haematobacter* are nonpigmented, and most strains grow on MacConkey agar. Additional phenotypic properties are given in Table 2. Strains received at the CDC have been mainly from patients

TABLE 2 Oxidase-positive, indole-negative, asaccharolytic, coccoid nonfermenters[a]

Test	*Moraxella atlantae*[b] (73)	*Moraxella canis* (1)	*Moraxella catarrhalis* (74)	*Moraxella lacunata*[b] (66)	*Moraxella lincolnii* (1)	*Moraxella nonliquefaciens*[b] (243)	*Moraxella osloensis* (163)	*Oligella ureolytica* (37)	*Oligella urethralis* (22)	*Psychrobacter phenylpyruvicus* (50)	*Psychrobacter immobilis* (5)	*Haematobacter* species (proposed) (12)
Motility; flagella	0	0	0	0	0	0	0	100[c]; pe	0	0	0	0
Growth on MacConkey	80 (20)	100	5	2	0	8 (2)	70	62 (27)	96	80 (6)	40	58
Simmons citrate	0	0	0	0	0	0	0	14 (16)	46	0	20	0
Urea, Christensen's	0	0	68	0	0	0	0	97	0	100	(20)	100
Nitrate reduction	0	100	92	98	0	95	24	100	0	68	40 (20)	0
Gas from nitrate	0	0	0	0	0	0	0	60	0	0	0	0
Nitrite reduction	3	ND	86	0	0[d]	0	0	100	100	0	ND	0
H_2S (lead acetate paper)	61	100	73	34	0	83	74	38	9	47	20 (20)	92
Gelatin hydrolysis[e]	0	0	0	42	0	0	0	0	0	0	0	0
Growth at:												
25°C	51	100	85	33	100	93	96	67	50	85	100	100
35°C	99	100	97	73	100	88	98	88	100	100	40	100
42°C	46	100	23	0	0	15	51	18	59	29	20	0
Phenylalanine deaminase	0	100w	ND	17	ND	ND	14	100[f]	100	97	ND	ND
Penicillin sensitivity[g]	100	ND	ND	95	ND	99	92	ND	100	73	ND	ND
Loeffler slant digestion	ND	ND	ND	100	ND	0	0	ND	ND	ND	ND	ND
Sodium acetate alkalinization	ND	ND	ND	0	0	0	100	ND	84	43	ND	ND
Nutrient broth, 0% NaCl	0	100	47	5	0	22	98	19 (3)	96	53	100	67
Nutrient broth, 6% NaCl	0	100w	ND	2	0	0	12	15 (5)	59	19	60	42
DNase[h]	0	100	100	0	0	0	0	0	0	0	ND	ND

Major CFAs[i]	16:0, 18:0, 18:1ω9c, 18:1ω9c, 18:2	16:1ω7c, 17:1ω8c, 18:1ω9c	16:0, 16:1ω7c, 16:0alc, 17:1ω8c, 18:0, 18:1ω9c, 18:2, 18:0alc[j]	16:0, 16:1ω7c, 18:1ω9c	16:0, 16:1ω7c, 18:1ω9c	16:1ω7c, 18:1ω9c	16:0, 18:1ω7c	16:0, 16:1ω7c, 18:1ω9c, 18:2	16:1ω7c, 17:1ω8c, 18:1ω9c	16:0, 18:1ω7c, 19:0 cyc

[a] Unless otherwise indicated, data are from the CDC Special Bacteriology Reference Laboratory (256). All taxa were positive (≥90%) for catalase. All were negative (<10% positive) for acid from D-glucose, D-xylose, D-mannitol, lactose, sucrose, and maltrose; growth on SS and cetrimide agars, esculin hydrolysis, acid production in TSI agar, and H_2S production in TSI agar. Numbers indicate the percentage positive at 2 days of incubation; numbers in parentheses indicate a delayed reaction (3 to 7 days of incubation); w, weak reaction; pe, peritrichous; ND, not determined or not available. Numbers in parentheses after the organism indicate the number of strains.

[b] Usually does not grow in OF medium.

[c] Motility may be delayed or difficult to demonstrate.

[d] Nitrite-positive strains have been described (237).

[e] 14 days of incubation.

[f] Data from P. Schreckenberger.

[g] Based on results obtained by streaking a blood agar plate with growth from an 18- to 36-h culture and then placing a 10-U penicillin disk on the streaked area. A positive reaction is indicated by the appearance of a zone of inhibition.

[h] Data from reference 117.

[i] The number before the colon indicates the number of carbons; the number after the colon is the number of double bonds; ω, the position of the double bond counting from the hydrocarbon end of the carbon chain; c, *cis* isomer; alc, alcohol.

[j] Two profiles exist. Most strains of *M. lacunata* II differ from *M. lacunata* I by lower amounts of 17:1ω8c, 16:1ω7c, and 18:1ω9c, higher amounts of 18:0 (16 versus 3%), and higher amounts of 16:0 alc and 18:0 alc. However, there is some overlap in relative amounts of these acids in some strains of both groups which prohibit their differentiation solely on the basis of CFA data (256).

with septicemia. *Haematobacter* strains have low MIC values for amoxicillin, fluoroquinolones, aminoglycosides, and carbapenems but variable MICs for cephalosporins, monobactams, and piperacillin.

OXIDASE-POSITIVE, INDOLE-NEGATIVE, ASACCHAROLYTIC, ROD-SHAPED NONFERMENTERS

See Table 3.

Alcaligenes faecalis and Asaccharolytic *Achromobacter* spp.

Based on taxonomic studies, Yabuuchi et al. transferred the species *Alcaligenes ruhlandii* and *Alcaligenes piechaudii* to the genus *Achromobacter* and proposed the transfer of *Alcaligenes denitrificans* to the genus *Achromobacter* as *Achromobacter xylosoxidans* subsp. *denitrificans* (263), thus automatically creating a second subspecies, *Achromobacter xylosoxidans* subsp. *xylosoxidans*. However, the reclassification of *Alcaligenes denitrificans* as a subspecies of *Achromobacter xylosoxidans* contradicted previous work that showed that the two taxa are distinct species (239). Accordingly, Coenye and colleagues have proposed that *Alcaligenes denitrificans* be reclassified as *Achromobacter denitrificans* (41). In this text we treat these organisms as separate species, referring to them as *Achromobacter xylosoxidans* and *Achromobacter denitrificans*, respectively. *Alcaligenes faecalis* remains the only *Alcaligenes* species of clinical importance.

Members of these genera are rods (0.5 by 1 to 0.5 by 2.6 μm) with peritrichous flagella. Both phylogenetically and biochemically, they are closely related to members of the genus *Bordetella*. They occur mainly in the environment and show limited action on carbohydrates. Colonies are nonpigmented and similar in size to those of *Acinetobacter* spp. Medically important species are divided into the asaccharolytic species (Table 3) including *Alcaligenes faecalis*, *Achromobacter piechaudii* (263), and *Achromobacter denitrificans* (41, 263) and the saccharolytic species (see Table 4) *Achromobacter xylosoxidans* (41, 263) as well as the unnamed Achromobacter groups B, E, and F (89, 90). The asaccharolytic species are rarely observed as human pathogens (14, 134, 248). *A. faecalis* is the most frequently isolated species and characteristically produces colonies with a thin, spreading, irregular edge. Some strains (previously named "*A. odorans*") produce a characteristic fruity odor (sometimes described as the odor of green apples) and cause a greenish discoloration of blood agar medium. A key biochemical feature of this species is its ability to reduce nitrite but not nitrate. It is often found in mixed cultures, particularly in diabetic ulcers of the feet and lower extremities, and its clinical significance is difficult to determine. *Achromobacter piechaudii* has been recovered from blood, recurrent ear discharge, nose, pharynx, and soil (129, 134, 177). In one instance the blood isolate was associated with an infected Hickman catheter in a patient with hematological malignancy (129). *A. faecalis* and *A. piechaudii* have been reported to be resistant to ampicillin, aztreonam, and gentamicin and of variable susceptibility to other antimicrobials (13, 129). The clinical significance of *Achromobacter denitrificans* remains to be elucidated; however, an organism that is biochemically similar, known as *Alcaligenes*-like group 1, has been recovered from blood, urine, knee joint, brain abscess, and bronchial washings, suggesting that it may have greater potential to cause human infection. It can be differentiated from *A. denitrificans* by CFA composition, failure to grow on

TABLE 3 Oxidase-positive, indole-negative, asaccharolytic, rod-shaped nonfermenters[a]

Test	*Achromobacter denitrificans* (4)	*Achromobacter piechaudii* (5)	*Advenella incenata*[b] (6)	*Alcaligenes faecalis* (49)	*Alishewanella fetalis*[c] (1)	CDC *Alcaligenes*-like group 1 (8)	*Aquaspirillum* species (5)
Motility; flagella	100; pe	100; pe	v; ND	100; pe	0	100; pe	60; m; p, 1–2
Acid from D-glucose	0	0	v	0	0	0	0
Catalase	100	100	100	98	ND	100	100
Growth on:							
MacConkey	100	100	ND	100	100	100	0
SS	100	100	ND	100	100	13	0
Cetrimide	25 (25)	80 (20)	0	59	ND	0	0
Simmons citrate	100	100	100	100	0	100	0
Urea, Christensen's	0	0	v	2	0	75	0
Nitrate reduction	100	100	0	0	100	100	20
Gas from nitrate	100	0	0	0	ND	100	0
Nitrite reduction	ND	0	ND	100	100	100	0
H_2S (lead acetate paper)	25w	100[l]	ND	8	ND	13	100
Gelatin hydrolysis[m]	0	0	0	22	100	0	0
Pigment:							
Insoluble	0	0	Lt br	0	ND	0	0
Soluble	25 yel	0	ND	22 yel	ND	0	0
Growth at:							
25°C	100	100	ND	100	ND	100	40
35°C	100	100	100	100	ND	100	100
42°C	25w	60	v	18	100	50	0
Alkalinization of:							
Acetamide	33 (33)	0	0	83	ND	0	ND
Serine	33 (33)	40 (40)	ND	39	ND	(75)	ND
Tartrate	75 (25)	80 (20)	ND	6	ND	0	ND
Arginine dihydrolase	0	0	0	0	0	(33)	ND
Nutrient broth, 0% NaCl	100	100	100	100	ND	100	60
Nutrient broth, 6% NaCl	25	100[o]	v	98 (2)	100	13	0
Major CFAs[p]	16:0, 16:1ω7c, 17:0cyc	16:0, 16:1ω7c, 17:0cyc	ND	3–OH–14:0, 16:0, 16:1ω7c, 17:0cyc	16:1ω7c, 17:1ω8c	16:0, 16:1ω7c, 17:0cyc, 18:1ω7c	ND

[a] Unless otherwise indicated, data are from the CDC Special Bacteriology Reference Laboratory (256). All taxa were negative (<10% positive) for acid production from D-xylose, D-mannitol, lactose, sucrose, and maltose; acid in TSI agar, H_2S production in TSI agar, esculin hydrolysis, lysine decarboxylase and ornithine decarboxylase activity. Numbers indicate the percentage positive at 2 days of incubation; numbers in parentheses indicate a delayed reaction (3 to 7 days of incubation); w, weak reaction; pe, peritrichous; p, polar; ND, not determined or not available; yel, yellow; amb, amber; Lt br, light brown; v, strain dependent. Numbers in parentheses after the organisms indicate the number of strains.

[b] Data from reference 43.

[c] Data from reference 247. Carbohydrate results are from assimilation tests.

[d] Data from references 73 and 252.

[e] *Myroides* genus consists of two phenotypically similar species: *M. odoratus* and *M. odoratimimus*. The type strain of *M. odoratus* did not grow on MacConkey agar; in contrast, the type strain of *M. odoratimimus* grew heavily.

[f] Data from reference 268.

SS agar, failure to alkalinize tartrate and acetamide, and a positive urease reaction (Table 3) (256).

Advenella incenata

Advenella incenata is a gram-negative, small (1- to 2-μm), rod-shaped or coccoid bacterium that occurs singly, in pairs, or in short chains (43). On nutrient agar, colonies are flat or slightly convex with smooth margins and appear light brown in color (43). They are oxidase and catalase positive. Motility and oxidation of OF glucose are strain dependent. Additional characteristics are given in Table 3. Isolates have been recovered from human blood, sputum, and wound specimens (43).

Alishewanella fetalis

Alishewanella fetalis is a halophilic gram-negative rod that grows at temperatures between 25 and 42°C with optimum growth at 37°C. NaCl is required for growth. It can withstand NaCl concentrations of up to 8% but does not grow at 10% NaCl, which helps differentiate this species from *Shewanella algae*, which can grow in 10% NaCl (247). Also, unlike *S. algae*, it is esculin hydrolysis positive. It is oxidase and catalase positive and asaccharolytic. It does not produce H_2S in the butt of TSI and KIA. Other reactions are given in Table 3. It has been isolated from a human fetus at autopsy; however, its association with clinical infection is unknown (247).

Bordetella avium (3[j])	*Bordetella bronchiseptica* (85)	*Bordetella hinzii* (2)	*Bordetella petrii*[d] (2)	*Myroides* species[e] (74)	*Laribacter hongkongensis*[f] (1)	*Neisseria weaveri* (132)	*Neisseria elongata* subsp. *elongata* (15)
100; pe	100; pe	100; pe	0	0	0	0	0
0	0	0	0	0	0	0	0
100	100	100	100	100	100	100	0
100	100	100	100	91 (5)	100	27 (18)	13 (53)
100	99	100	ND	30 (11)	ND	0	0
0	0	0	ND	0	100	0	0
33w	98 (1)	100	100	0	0	0	0
0	99	0	0	100	100	0	0
0	92	0	0	0	100	0	0
0	0	0	0	0	0	0	0
0	ND	0	100	83	ND	100	92
67	74	50	ND	16	ND	86	67
0	0	0	0	96	0	0	0
0	0	0	0[n]	85 yel	0	0	0
33 yel	0	0	ND	0	0	24 yel	13 yel
100	99	100	ND	100	100	94	67
100	100	100	100	100	100	100	100
100	78	100	ND	31	100	41	27
0	0	0	ND	(2w)	0	2	0
(50w)	30 (61)	50	ND	5, 2w	ND	0	0
0	5	0	ND	(2w)	ND	0	0
0	0	50 (50)	ND	(9)	100	0	ND
100	100	100	ND	100	100	85	100
67	82 (5)	100	ND	20 (5)	0	18	0
16:0, 17:0cyc	16:0, 16:1ω7c, 17:0cyc	16:0, 17:0cyc	ND	i15:0	ND	16:0, 16:1ω7c, 18:1ω7c	16:0, 16:1ω7c, 18:1ω7c

[g] Data from reference 39.
[h] Data from reference 42.
[i] Data from reference 32.
[j] All from avian sources.
[k] Acid production may be detected in rapid sugar test base.
[l] H_2S (TSI butt), the line of the stab became dark in 3 to 7 days with three strains.
[m] 14 days of incubation.
[n] Slightly red when grown anaerobically on selenate-containing media (252).
[o] Light growth at 48 h, heavier at 7 days.
[p] The number before the colon indicates the number of carbons; the number after the colon is the number of double bonds; ω, the position of the double bond counting from the hydrocarbon end of the carbon chain; c, *cis* isomer; i, *iso;* 3–OH, a hydroxyl group at the 3 (β) position from the carboxyl end; cyc, a cyclopropane ring structure.

Aquaspirillum Species

The genus *Aquaspirillum* is a heterogeneous group that at present includes 13 species and two subspecies. All species are aerobic and helical and do not grow in the presence of 3% NaCl (60, 188). Colonies on nutrient agar are generally pinpoint in size at 48 h but become larger (up to 2.0 mm in diameter) at 7 days. They are usually convex or umbonate, glistening, opaque, pale yellow, and butyrous (188). They are oxidase and catalase positive and asaccharolytic. Additional reactions are given in Table 3. Aquaspirillae are found in a wide variety of freshwater sources, especially stagnant water sources such as ditch water, canal water, and ponds. They have also been isolated from storage tanks of distilled water in laboratories (188). Three of the isolates characterized at the CDC have been from human blood cultures, one from a patient with diabetes, one from a patient with pneumonia, and one from an individual whose clinical condition is unknown. All three were shown by 16S rRNA sequencing to be *Aquaspirillum* species.

Laribacter hongkongensis

Laribacter hongkongensis belongs to the family *Neisseriaceae* and is a facultatively anaerobic, nonsporulating bacillus. On Gram stain the organisms appear as gram-negative gull-shaped or spiral rods. It grows on sheep blood agar as non-hemolytic, gray colonies 1 mm in diameter after 24 h of

TABLE 3 Oxidase-positive, indole-negative, asaccharolytic, rod-shaped nonfermenters[a] *(Continued)*

Test	*Neisseria elongata* subsp. *glycolytica* (2)	*Neisseria elongata* subsp. *nitroreducens* (26)	*Shewanella algae* (26)	*Cupriavidus pauculus* (36)	*Cupriavidus gilardii*[g] (8)	*Cupriavidus respiraculi*[h] (5)	*Cupriavidus taiwanensis*[i] (9)	Gilardi rod group 1 (15)
Motility; flagella	0	0	100; p, 1–2	100; pe	100; p, 1–2	pe	pe	0
Acid from D-glucose	0[k]	23	0	0	0	0	0	0
Catalase	100	0	ND	100	100	100	100	100
Growth on:								
MacConkey	50 (50)	19 (35)	100	94 (6)	ND	ND	ND	93
SS	0	0	92 (4)	3 (6)	ND	ND	ND	80
Cetrimide	0	0	8	0 (3)	0	ND	0	0
Simmons citrate	0	0	8	100	0	0	100	0
Urea, Christensen's	0	0	42	100	0	0	0	0
Nitrate reduction	0	100	100	11	0	v	100	0
Gas from nitrate	ND	0	ND	0	0	ND	ND	0
Nitrite reduction	50	100	0	ND	0	ND	ND	0
H_2S (lead acetate paper)	100	100	100	51	ND	ND	ND	87
Gelatin hydrolysis[m]	0	0	100	0	0	0	ND	0
Pigment:								
Insoluble	0	0	0	0	0	0	0	60 amb
Soluble	50 yel	15 yel	0	22 yel	0	ND	ND	0
Growth at:								
25°C	100	62	ND	94	ND	100	100	100
35°C	100	100	ND	100	100	100	100	100
42°C	0	23	23	86	100	0	0	80
Alkalinization of:								
Acetamide	0	0	ND	ND	0	ND	ND	0
Serine	0	0	ND	ND	100	ND	ND	0
Tartrate	0	0	ND	100	0	ND	ND	0
Arginine dihydrolase	ND	0	100	ND	0	0	0	0
Nutrient broth, 0% NaCl	100	88	0	100	100	100	100	100
Nutrient broth, 6% NaCl	0	4	100	11	0	0	0	7 (13)
Major CFAs[p]	16:0, 16:1ω7c, 18:1ω7c	16:0, 16:1ω7c, 18:1ω7c	i-15:0, 16:1ω7c, 17:1ω8c	16:0, 16:1ω7c, 17:0cyc, 18:1ω7c	16:0, 16:1ω7c, summed feature 7	14:0, 3–OH 14:0, 16:1ω7c, 16:0, 17:0cyc, 18:1ω7c	ND	14:0, 16:0, 18:1ω7c, 19:0cyc

incubation at 37°C in ambient air. Growth also occurs on MacConkey agar and at 25 and 42°C. No enhancement of growth is observed with 5% CO_2. Most strains are motile with bipolar flagella. All strains are oxidase, catalase, urease, and arginine dihydrolase positive and reduce nitrate but do not ferment, oxidize, or assimilate any carbohydrates (Table 3) (259, 268). *L. hongkongensis* has been isolated from the blood and pleural fluid of a 54-year-old cirrhotic patient (268) and from the stools of patients with community-acquired diarrhea (141, 259, 260, 261). Woo and colleagues have reported an association between *L. hongkongensis* and community-acquired diarrhea, eating fish, and travel (260); however, a causative role has not been shown (69). *L. hongkongensis* has been reported from countries in Asia (China and Japan), Europe (Switzerland), Africa (Tunisia), and Central America (Cuba), suggesting that the bacterium is found worldwide (260). However, as of this writing, no isolates have been reported from North America.

Myroides spp.

Vancanneyt et al. (235) determined that the organism formerly classified as *Flavobacterium odoratum* consisted of a heterogenous group that comprised two distinct species for which they proposed the names *Myroides odoratus* and *Myroides odoratimimus*. Cells of both species are gram-negative rods 0.5 μm in diameter and 1 to 2 μm long. Various colony types may occur, but most colonies are yellow pigmented and form effuse, spreading colonies that may be confused with the colony morphology of a *Bacillus* species. A characteristic fruity odor (similar to the odor of *A. faecalis*) is produced by most strains. *Myroides* grows on most media including MacConkey agar. Growth occurs at 18 to 37°C but not at 42°C. They are asaccharolytic but are oxidase, catalase, urease, and gelatinase positive. Indole is not produced, and nitrite (but not nitrate) is reduced (Table 3). There are no routine phenotypic tests for differentiating the two *Myroides* species, their differences being confined to assimilation tests and CFA profiles (235).

Organisms identified as *M. odoratus* have been reported mostly from urine but have also been found in wound, sputum, blood, and ear specimens (98, 265). Clinical infection with *Myroides* spp. is exceedingly rare; however, cases of rapidly progressive necrotizing fasciitis and bacteremia (112) and recurrent cellulitis with bacteremia (3) have been reported. Most strains are resistant to penicillins, cephalosporins, aminoglycosides, aztreonam, and carbapenems (98).

Neisseria weaveri and *Neisseria elongata*

Although assigned to the genus *Neisseria*, the rod-shaped *Neisseria weaveri* and *Neisseria elongata* morphologically resemble nonfermenting gram-negative bacilli and therefore are placed in Table 3 to help in the differentiation of these phenotypically similar bacteria. The *Neisseria* species are covered in detail in chapter 39.

Shewanella Species

The organism formerly called *Pseudomonas putrefaciens*, *Alteromonas putrefaciens*, *Achromobacter putrefaciens*, and CDC group Ib has now been placed in the genus *Shewanella* (154). Colonies on SBA are convex, circular, smooth, and occasionally mucoid; produce a brown to tan soluble pigment; and cause green discoloration of the medium. Cells are long, short, or filamentous. Motility is due to a single polar flagellum. Ornithine decarboxylase, nitrate reductase, and DNase are always produced, and with few exceptions, hydrogen sulfide (H_2S) is produced in KIA and TSI agar (shewanellae are the only nonfermenters that produce H_2S in these media). The CDC recognizes two biotypes based upon the requirement of NaCl for growth, oxidation of sucrose and maltose, and the ability to grow on SS agar (256). CDC biotype 2 was subsequently assigned to a new species, *S. alga* (173), later corrected to *S. algae* (231). Khashe and Janda (132) have reported that *S. algae* is the predominant human clinical isolate (77%), while *S. putrefaciens* (CDC biotype 1) represents the majority of nonhuman isolates (89%). *S. algae* is halophilic (requires NaCl for growth) and asaccharolytic (Table 3), whereas *S. putrefaciens* is nonhalophilic and saccharolytic (see Table 4). Although infrequently isolated in the clinical laboratory, *S. putrefaciens* and *S. algae* have been recovered from a wide variety of clinical specimens and are associated with a broad range of human infections including skin and soft tissue infection (33), otitis media (102), ocular infection (27), osteomyelitis (16), peritonitis (51) and septicemia (24, 116). Many of these infections were probably caused by *S. algae*. The habitat for *S. algae* is saline habitats, whereas *S. putrefaciens* has been isolated mostly from fish, poultry, and meats as well as fresh water and marine samples. *Shewanella* species are generally susceptible to most antimicrobial agents effective against gram-negative rods except penicillin and cephalothin (70, 248). The mean MICs of *S. algae* to penicillin, ampicillin, and tetracycline are higher than the corresponding MICs of *S. putrefaciens* (132, 246).

Cupriavidus Species

Cupriavidus pauculus was formerly designated CDC Group IVc-2 and *Ralstonia paucula* (236, 238). It is a short to medium-sized gram-negative rod that is asaccharolytic and motile by peritrichous flagella. Cells may stain irregularly. It is rapid urease positive and can be differentiated from the phenotypically similar organisms *B. bronchiseptica* and *O. ureolytica* by a usually negative nitrate reduction test and its CFA composition (Table 3).

Cupriavidus gilardii (formerly *Ralstonia gilardii*) is the new designation for an *Alcaligenes faecalis*-like organism that has been isolated from human clinical sources and the environment (39, 236). *C. gilardii* can be differentiated from *A. faecalis* by the absence of nitrite reduction, failure to utilize acetamide, the presence of polar rather than peritrichous flagella, and CFA composition (Table 3).

Cupriavidus respiraculi (formerly *Ralstonia respiraculi*) has been recovered from the respiratory tract of cystic fibrosis (CF) patients, although the isolates do not grow on *B. cepacia*-selective agar (42, 236). Characteristics that differentiate *C. respiraculi* from other *Cupriavidus* species are given in Table 3.

C. taiwanensis (formerly *Ralstonia taiwanensis*) has also been isolated from the sputum of a CF patient (32, 236). It is a nonsaccharolytic gram-negative rod that is oxidase, catalase, nitrate, and esculin positive. Additional biochemical characteristics are given in Table 3. The *Cupriavidus* species are discussed in more detail in chapter 49.

Gilardi Rod Group 1

Gilardi rod group 1 consists of oval to medium-length asaccharolytic gram-negative rods that resemble *N. weaveri* in many respects except that Gilardi rod group 1 isolates do not reduce nitrite and are strongly phenylalanine deaminase positive, producing a dark green slant after addition of $FeCl_3$ (10%), whereas *N. weaveri*, when positive, produces a weak to moderate reaction. Additional reactions are given in Table 3. There is >98.7% 16S rRNA gene similarity between Gilardi rod group 1 and *Schineria larvae* (K. Bernard, personal communication); however, no formal proposal to name Gilardi rod group 1 has been made as of this writing. Isolates of Gilardi rod group 1 have been recovered from a variety of human sources including leg, arm, and foot wounds, an oral lesion, urine, and blood; however, their pathogenic potential has yet to be determined (166). They are susceptible to many antimicrobial agents including various penicillins, cephalothin, and chloramphenicol (166).

OXIDASE-POSITIVE, INDOLE-NEGATIVE, SACCHAROLYTIC, MOTILE, ROD-SHAPED NONFERMENTERS

See Table 4.

Achromobacter xylosoxidans

Achromobacter xylosoxidans is a relatively frequent agent of infection in immunocompromised patients, causing both local and systemic infections in nosocomial settings (34, 64, 254). *A. xylosoxidans* colonizes the respiratory tract of intubated children and patients with CF, leading to exacerbation of pulmonary symptoms (65). Epidemiologic typing of *A. xylosoxidans* by restriction fragment length polymorphism and PCR has been described (34, 145). Susceptibilities are unpredictable, which requires testing of individual isolates. Strains are frequently resistant to aminoglycosides, ampicillin, narrow- and expanded-spectrum cephalosporins, chloramphenicol, and fluoroquinolones but are usually susceptible to antipseudomonal broad-spectrum cephalosporins, piperacillin, ticarcillin-clavulanic acid, imipenem, and trimethoprim-sulfamethoxazole (13, 218, 248). Panresistance has been reported in at least one clinically significant infection (254).

TABLE 4 Oxidase-positive, indole-negative, saccharolytic, motile, rod-shaped nonfermenters[a]

Test	"*Achromobacter*" group B[b] (3)	"*Achromobacter*" group E[b] (2)	"*Achromobacter*" group F[b] (2)	*Achromobacter xylosoxidans* (135)	*Agrobacterium* yellow group (3)	CDC group Ic (34)	CDC group O-1 (62)	CDC group O-2 (66)	CDC group O-3 (13)
Motility; flagella	100; p, L	100; p, L	100	100; pe	100; p, 1–2[g]	100; p, 1–2	100; p, 1–2[g]	100; p, 1–2 or p, L[g]	100; p, 1–2[g]
Acid from:									
D-Glucose	100	100	100	78	33 (67)	97	69 (31)	73 (11)	100
D-Xylose	100	100	100	99	67 (33)	0	0	2	100
D-Mannitol	(67)	0	100	0	0	0	0	2	0
Lactose	(33)	(100)[h]	ND	0	(100)	0	0	2	0
Sucrose	(100)	(100)	100	0	(100)	0	0	64 (36)	100
Maltose	33 (67)	100	100	0	100	100	0	71 (27)	100
Catalase	100	100	ND	98	100	97	98	91	23, 62w
Growth on:									
MacConkey	100	100	0	100	(33)	100	6 (40)	5 (5)	(38)
SS	100	100	ND	98	0	100	0	0	0
Cetrimide	0	50 (50)	ND	95 (1)	0	94	0	0	0
Simmons citrate	100	100	0	95	0	41 (15)	0	0	0
Urea, Christensen's	100	100	100	0	(33)	18 (15)	2	12	0
Nitrate reduction	100	(100)[j]	100	100	0	100	0	15	8
Gas from nitrate	100	100	0	60	0	0	0	0	0
Nitrite reduction	100[k]	100[k]	0	ND	0	0	0	ND	0
TSI slant, acid	0	0	0	0	0	0	0	18	0
TSI butt, acid	0	0	0	0	0	0	0	20	0
H_2S (TSI butt)	0	(50)	ND	0	0	3	0	0	0
H_2S (lead acetate paper)	(100)	100	ND	0	100	100	93	91	15
Gelatin hydrolysis[j]	0	0	ND	0	0	0	25	38	0
Pigment:									
Insoluble	0	0	ND	0	100 yel	9w pk	100 yel	100 yel	0
Soluble	67 yel	50 tan	ND	5 br	0	32 tan-br	0	0	0
Growth at:									
25°C	100	100	ND	98	100	100	90	89	92
35°C	100	100	ND	100	100	100	100	100	100
42°C	100	100	ND	84	0	97	24	31	40
Esculin hydrolysis	100	100	100	0	67 (33)	0	93 (2)	64	92 (8)
Lysine decarboxylase	0	0	ND	0	0	0	0	0	0
Arginine dihydrolase	100	100	0	13	0	100	0	22	0
Ornithine decarboxylase	0	0	ND	0	0	0	0	6	0
Nutrient broth, 0% NaCl	100	100	ND	100	67 (33)	100	34	92	100
Nutrient broth, 6% NaCl	100	100	ND	69	0	91	0	22	0
3-Ketolactonate	0	0	ND	ND	100	ND	ND	ND	ND
ONPG	100[l]	100[l]	0[l]	0[m]	ND	ND	ND	ND	ND
Major CFAs[n]	18:1ω7c	18:1ω7c	ND	16:0, 16:1ω7c, 17:0cyc	16:0, 18:1ω7c	ND	16:0, 18:1ω7c	ND[o]	16:0, 16:1ω7c, 18:1ω7c

[a] Unless otherwise indicated, data are from CDC Special Bacteriology Reference Laboratory (256). Numbers indicate percentage positive at 2 days of incubation; parentheses, delayed reaction (3 to 7 days of incubation); w, weak reaction; ND, not determined or not available; pe, peritrichous; p, polar; L, lateral; br, brown; yel, yellow; pk, pink. Numbers in parentheses after organisms indicate numbers of strains.

[b] The following tests are useful in identifying to the species level "A*chromobacter*" group B, E, and F: L-rhamnose, D-sorbitol, and cellobiose: B (+, +, +); E (+, −, +), and F (−, ND, +) (89).

[c] Data from reference 249. Carbohydrate results are from assimilation tests.

[d] Data from reference 148.

[e] Colistin resistance has been suggested to differentiate *O. intermedium* (colistin resistant) from *O. anthropi* and *O. tritici* (colistin sensitive), whereas acid production from melibiose is useful in identifying *O. grignonense* (142).

[f] Glycerol and rhamnose oxidation are absent in *S. paucimobilis* and present in *S. parapaucimobilis*.

[g] Motility may be difficult to demonstrate.

Halomonas venusta[c] (15)	*Herbaspirillum* species 3 (1)	*Ingulinus linosus* (3)	*Massilia timonae*[d] (4)	*Ochrobactrum* species[e] (14)	OFBA-1 (6)	*Pseudomonas*-like group 2 (11)	*Rhizobium radiobacter* (66)	*Shewanella putrefaciens* (24)	*Sphingomonas paucimobilis*[f] (1)	*Sphingomonas parapaucimobilis*[f] (2)
100; pe	100; >2p	67; p, 1–2	100; 1p, L	100; pe	100; p, 1–2	100; p, >2	100; pe	100; p, 1–2	100; p, 1–2	100; p, 1–2
100	100	33 (67)	(100w)	93 (7)	100	100	94 (6)	17 (33)	100w	(100)
0	100	33 (67)	100	100	100	100	97 (3)	0	100	100
80	100	33 (67)	0	43 (14)	67 (33)	100	94 (6)	0	0	0
0	100w	33 (67)	0	0	67 (33)	100	79 (21)	0	100	50 (50)
80	0	(100)	0	50	67 (33)	0	95 (5)	96 (4)	100	100
100	0	(100)	100	64	67 (33)	0	97 (3)	92 (8)	100	50 (50)
100	100	67	100	100	100	100	98	100	100	100
ND	100	67 (33)	100	100	100	100	100	100	0	0
ND	0	0	0	100	100	0	20 (5)	(8)	0	0
100	0	67	ND	100	100	0	0	4	0	0
100	100	67	75 (25)	64	33 (33)	100	97 (3)	4 (4)	0	0[g]
100	100	33 (33)	0	100	33 (17)	91 (9)	88 (9)	4 (8)	0	0
ND	67	33	25	86	100	18	83	100	0	0
ND	0	0	0	43	100	0	5	0	0	0
ND	0	0	0	ND	ND	ND	38	ND	ND	ND
ND	0	0	0	0	100	0	0	0	0	100w
0	0	0	0	0	33 (67)	0	0	0	0	0
ND	0	0	0	43	0	0	11 (3)	96	0	0
ND	100	100	ND	100	50	89	100	100	0	100
0	0	0	100	0	50	0	2	65	0	0
ND	0	0	100 straw	0	0	0	0	0	100 yel	100 yel
ND	0	0	0	21 yel	0	0	21 yel	71 br	0	0
ND	100	33	100	100	100	80	100	100	100	100
ND	100	100	100	100	100	100	100	96	100	100
ND	100	33	0	64	100	20	32	38	100w	0
100	0	100	100	29 (7)	0	0	100	0	100	100
0	ND	0	0	0	0	0	0	0	ND	ND
0	ND	0	0	71	100	30	8	0	ND	ND
0	ND	0	0	0	0	0	0	100	ND	ND
ND	100	100	100	100	100	100	100	100	100	100
100	0	0	0	60	75	0	18	43	0	50
ND	ND	ND	ND	0	ND	ND	100	ND	0	0
ND	ND	ND	ND	0[m]	ND	ND	100[m]	ND	100[m]	ND
16:0, 18:1ω9c	16:0, 16:1ω7c, 18:1ω7c	18:1ω7c, 18:1-20H, 18:0-30H, 19:0cyloω8c	3–OH–10:0, 16:1ω7c, 16:0, 18:1ω7c	18:0, 18:1ω7c, 19:0cyc	16:0, 19:0cyc	16:0, 16:1ω7c, 18:1ω7c	16:0, 18:1ω7c, 19:0cyc	i-15:0, 16:1ω7c, 17:1ω8c	16:0, 18:1ω7c	16:0, 17:1ω6c, 18:1ω7c

[h] Acid production was detected only after 21 days of incubation.
[i] Reported to be positive (264).
[j] 14 days of incubation.
[k] When tested at 48 h of incubation, nitrite reduction may be observed only in media containing <0.01% nitrite.
[l] Data from reference 96.
[m] Data from P. Schreckenberger.
[n] The number before the colon indicates the number of carbons; the number after the colon is the number of double bonds; ω, the position of the double bond counting from the hydrocarbon end of the carbon chain; c, *cis* isomer; cyc, a cyclopropane ring structure; i, *iso;* ND, not determined.
[o] Fatty acid profile analysis, performed on six strains in our laboratory, indicates that this group is heterogeneous.

Ochrobactrum Species and *Achromobacter* Groups B, E, and F

Ochrobactrum anthropi (97) comprises the urease-positive *Achromobacter* species formerly designated CDC group Vd (biotypes 1 and 2) and *Achromobacter* groups A, C, and D described by Holmes et al. (96). Subsequent studies showed, however, that biogroup C and some strains belonging to biogroup A constitute a homogeneous DNA-DNA hybridization group separate from *O. anthropi,* named *Ochrobactrum intermedium* (242). Both *Ochrobactrum* species are closely related to *Brucella* spp., with *Ochrobactrum intermedium* occupying a phylogenetic position that is intermediate between *O. anthropi* and *Brucella* (242). The two species share identical phenotypic properties. They appear as medium-length rods with peritrichous flagella, but individual cells may have a single flagellum only. Colonies on SBA resemble those of *Enterobacteriaceae*, except that those of *Ochrobactrum* are smaller. Colonies measure about 1 mm in diameter and appear circular, low convex, smooth, shining, and entire.

O. anthropi has been isolated from various environmental and human sources, predominantly from patients with catheter-related bacteremias (97, 131, 204) and rarely with other infections (155) including one case of meningitis (29). Pulsed-field gel electrophoresis and PCR genome fingerprinting based on repetitive chromosomal sequences have both been used successfully for epidemiologic typing of outbreak strains (240). *O. anthropi* strains are usually resistant to β-lactams, such as broad-spectrum penicillins, broad-spectrum cephalosporins, aztreonam, and amoxicillin-clavulanate, but are usually susceptible to aminoglycosides, fluoroquinolones, imipenem, tetracycline, and trimethoprim-sulfamethoxazole (12, 131, 204).

There are no biochemical tests currently available that separate *O. intermedium* from *O. anthropi;* however, it has been suggested that colistin (polymyxin E) and polymyxin B resistance can be used to separate *O. intermedium* (resistant) from *O. anthropi* (susceptible) (242). One case of pyogenic liver infection due to *O. intermedium* has been reported (162), but because of the close phenotypic similarity of *O. anthropi* and *O. intermedium*, it is possible that certain infections thought to be caused by *O. anthropi* were actually caused by *O. intermedium.*

Achromobacter groups B and E constitute biotypes of a single new species that has yet to be named (89, 90, 92). *Achromobacter* group F is genetically distinct from groups B and E (89, 90). *Achromobacter* group B strains susceptible to chloramphenicol, ciprofloxacin, gentamicin, imipenem, tobramycin, and trimethoprim-sulfamethoxazole have been isolated from patients with septicemia (91, 120). Isolates of *Achromobacter* groups E and F have also been recovered from blood (89, 90).

Rhizobium radiobacter

The former genus *Agrobacterium* contained several species of plant pathogens occurring worldwide in soils. Four distinct species of *Agrobacterium* were recognized: *A. radiobacter* (formerly *A. tumefaciens* and CDC group Vd-3), *A. rhizogenes* (subsequently transferred to the genus *Sphingomonas* as *S. rosa*), *A. vitis*, and *A. rubi* (206). More recently, Young and colleagues (267) proposed an emended description of the genus *Rhizobium* to include all species of *Agrobacterium*. Following this proposal the new combinations are *Rhizobium radiobacter, R. rhizogenes, R. rubi,* and *R. vitis* (267). Cells measure 0.6 to 1.0 by 1.5 to 3.0 μm and occur singly and in pairs. Colonies of *R. radiobacter* grow optimally at 25 to 28°C but grow at 35°C as well. They appear circular, convex, smooth, and nonpigmented to light beige on SBA with a diameter of 2 mm at 48 h. Colonies may appear wet looking and become extremely mucoid and pink on MacConkey agar with prolonged incubation (Fig. 2).

R. radiobacter has been most frequently isolated from blood, followed by peritoneal dialysate, urine, and ascitic fluid (66, 114). The majority of cases have occurred in patients with transcutaneous catheters or implanted biomedical prostheses, and effective treatment often requires removal of the device (67). *R. radiobacter* septicemia has also been reported in hospitalized patients with advanced human immunodeficiency virus disease (156). One case of endophthalmitis caused by a *Rhizobium radiobacter*-like (3-ketolactonate-negative) organism has also been reported (161). Most strains are susceptible to broad-spectrum cephalosporins, carbapenems, tetracyclines, and gentamicin but not to tobramycin (66, 248). Testing of individual isolates is recommended for clinically significant cases.

Agrobacterium Yellow Group

Organisms in the *Agrobacterium* yellow group are represented by slender, medium to long gram-negative rods that produce a yellow insoluble growth pigment and most closely resemble *Sphingomonas paucimobilis* and CDC group O-1 and O-2 organisms. Growth on MacConkey agar is variable, motility occurs via a single polar flagellum, oxidase and catalase are positive, and glucose, xylose, lactose, sucrose, and maltose are oxidized, but not mannitol. Only a positive 3-ketolactonate reaction differentiates this organism from *S. paucimobilis* (Table 4). Isolates have been recovered from blood and peritoneal fluid (224, 256).

CDC Group Ic

Members of CDC group Ic are gram-negative, slender, short to long, motile rods with one or two polar flagella. The organisms grow well on MacConkey, SS, and usually cetrimide agars; oxidize glucose and maltose; reduce nitrate to nitrite without gas; produce H_2S on lead acetate paper (usually strong); they are arginine dihydrolase positive, urease and citrate variable, and esculin and gelatin hydrolysis negative; and they grow well at 25, 35, and usually 42°C. Other characteristics are listed in Table 4. Most isolates have come from human sources including urine, sputum, blood, and other sites (256). Antibiotic susceptibility data are not available.

CDC Groups O-1, O-2, and O-3

CDC groups O-1, O-2, and O-3 are phenotypically similar, motile, and usually oxidase-positive, gram-negative rods. Groups O-1 and O-2 are yellow pigmented and most closely resemble *Agrobacterium* yellow group and *Sphingomonas* species. These organisms grow poorly or not at all on MacConkey agar and usually hydrolyze esculin but are otherwise inactive. All are motile, although motility may be difficult to demonstrate. O-1 organisms appear as uniformly short gram-negative rods, O-2 organisms appear as slightly pleomorphic rods with some cells appearing thin in the central portion with thickened ends, and O-3 cells appear as thin, medium to slightly long curved rods with tapered ends (sickle-like) (52, 256). O-3 is the only group in which yellow growth pigment is not produced. Most isolates of O-3 grow well on CAMPY CVA (Campylobacter agar with cefoperazone, vancomycin, and amphotericin B) plates under microaerophilic conditions, thus creating the potential for misidentification of

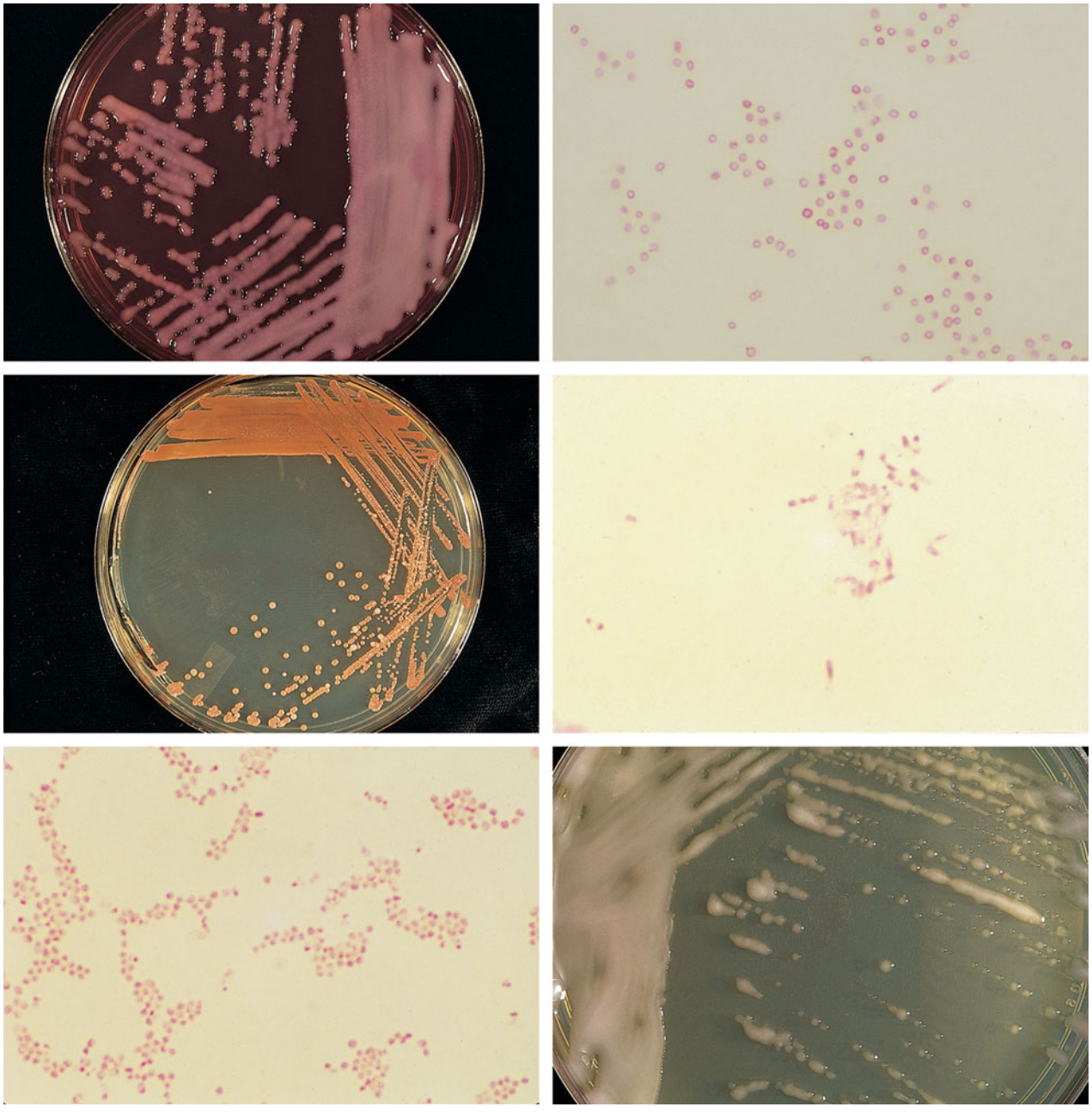


FIGURE 2 (top left) *Rhizobium radiobacter* on MacConkey agar after 48 h of incubation.

FIGURE 3 (top right) Gram stain of *Paracoccus yeei* (EO-2) showing characteristic donut-shaped morphology.

FIGURE 4 (middle left) *Methylobacterium* on Sabouraud's dextrose agar showing coral-pigmented colonies.

FIGURE 5 (middle right) Gram stain of *Methylobacterium* showing pleomorphic gram-negative rods with vacuoles.

FIGURE 6 (bottom left) Gram stain of *Roseomonas* showing gram-negative coccoid rods.

FIGURE 7 (bottom right) *Roseomonas* on Sabouraud's dextrose agar showing pink mucoid colonies.

O-3 organisms as *Campylobacter* (52). 16S rRNA gene sequencing and cell wall fatty acid analysis performed at the CDC show a close match of CDC group O-1 with *Hydrogenophaga palleroni* and of CDC group O-2 with *Caulobacter vibrioides* (Daneshvar, unpublished). Isolates of all three O groups have been recovered from a variety of clinical sources. One case of group O1-associated pneumonia complicated by bronchopulmonary fistula and bacteremia has been reported (192). Antibiotic susceptibility data have been reported only for group O-3. All isolates tested were susceptible to the aminoglycosides, trimethoprim-sulfamethozaxole, and imipenem. Resistance to most β-lactams was noted, and variable susceptibility was noted for chloramphenicol, tetracycline, ciprofloxacin, and amoxicillin-clavulanate (52).

Halomonas venusta and CDC Halophilic Nonfermenter Group 1

Halomonas venusta was originally described as *Alcaligenes venustus* (7) but later transferred to the new genus *Deleya*, as *Deleya venusta* (8). In 1996 Dobson and Franzmann proposed combining the genus *Deleya* into a more broadly defined genus, *Halomonas* (62). von Graevenitz and colleagues were the first to report a human infection caused by *H. venusta* in a wound that originated from a fish bite. It was reported to be susceptible to most antibiotics (249).

CDC Halophilic Nonfermenter Group 1 consists of six phenotypically similar isolates received by the CDC between 1971 and 1998 that are similar to *H. venusta* except for esculin hydrolysis and CFA composition. Five of these are from human blood cultures and the sixth is from a hip wound culture (unpublished data from CDC). Growth requirements suggest exposure to brackish water.

Herbaspirillum Species 3

Herbaspirillum is a gram-negative, generally curved, and sometimes helical bacillus. Individual cells are 0.6 to 0.7 μm wide and 1.5 to 5.0 μm long and have one to three or more flagella on one or both poles (4). A group of clinical isolates, previously described as EF-1, was shown by molecular hybridization to belong to the genus *Herbaspirillum* and was designated as a new unnamed species, *Herbaspirillum* species 3 (4). The organism is oxidase and urease positive; catalase is weak or variable. Other reactions are given in Table 4. Isolates have been recovered from the respiratory tract, feces, urine, ear, eye, and wound sites (4). Antibiotic susceptibility data are not available.

Inquilinus limosus

Inquilinus limosus is a rod-shaped gram-negative bacterium that measures 1.5 to 2 μm in width by 3.5 μm in length, grows at 35 and 42°C but poorly at 25°C, and is either nonmotile or motile with one or two polar flagella. It forms very slimy, nonpigmented colonies on nonselective media. Growth on MacConkey agar is very slight after 3 days. It is polymyxin B resistant and lipase positive, making it appear phenotypically similar to the *B. cepacia* complex (40, 186). The original description of *Inquilinus* stated that there is no utilization of glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin, or arabinose (40); however, all three isolates tested at CDC showed oxidative utilization of glucose, xylose, mannitol, lactose, sucrose, and maltose either within 2 days or between 3 and 7 days of incubation (Table 4). All strains are positive for catalase, beta-glucosidase, phosphatase, proline aminopeptidase, pyrollidonyl aminopeptidase, and acetoin production and negative for lysine, arginine, ornithine, denitrification, and indole (40). Additional reactions are given in Table 4. All strains have been recovered from respiratory secretions of CF patients and are very mucoid. Identifying the species is difficult because it is not contained in the databases of commercial identification kits and its mucoid appearance may lead to confusion with mucoid *P. aeruginosa* strains (186, 255). Isolates can be recovered on colistin containing *B. cepacia* selective media, but are inhibited on *Burkholderia cepacia* selective agar, which also contains gentamicin (35). The natural habitat of *Inquilinus* is unknown to date, and the clinical impact of chronic colonization with *Inquilinus* sp. remains unclear. Chiron and colleagues reported that for one patient, *Inquilinus* sp. was the only potential pathogen recovered from the sputum and *Inquilinus* acquisition was followed by a worsening of his lung function (35). All isolates are reported to be resistant to penicillins and cephalosporins, kanamycin, tobramycin, colistin, doxycycline, and trimethoprim-sulfamethoxazole and susceptible to imipenem and ciprofloxacin (35, 255).

Massilia timonae

M. timonae is an actively motile (with lateral flagella as well as a single polar flagellum), strictly aerobic gram-negative rod that is oxidase positive, catalase positive, and weakly saccharolytic. Additional phenotypic characteristics are given in Table 4. Colonies appear pale yellow and are distinctively tenacious on agar media and have a tendency to form flocs and films in liquid medium (140). Isolates have been recovered from a surgical wound, cerebrospinal fluid (CSF), the femur of a patient with osteomyelitis, and the blood of several patients (140, 148, 216). *M. timonae* is susceptible to most antibiotics active against gram-negative bacteria with resistance only to ampicillin, cephalothin, and aztreonam (140, 216).

OFBA-1

OFBA-1 is an unclassified medium to long gram-negative, motile rod with one or two polar flagella and has the unusual property of producing acid in OF base medium without carbohydrate, thus the acronym OFBA for OF base acid. The organism most closely resembles *P. aeruginosa* biochemically due to beta-hemolysis, growth at 42°C, presence of arginine dihydrolase, nitrate reduction to gas, and utilization of most carbohydrates (251, 256). Unlike *P. aeruginosa*, it is negative for pyocyanin and pyoverdin production and acetamide hydrolysis. Isolates have been recovered from blood, leg ulcer, abdominal wound, bronchial wash, and a catheter tunnel infection in a patient on continuous ambulatory peritoneal dialysis (251, 256).

Pseudomonas-Like Group 2

The organisms in *Pseudomonas*-like group 2 were previously included in a heterogeneous group of organisms designated CDC group IVd (59). 16S rRNA gene sequencing and cell wall fatty acid analysis performed at the CDC show a close match between *Pseudomonas*-like group 2 and *Herbaspirillum rubrisubalbicans* (Daneshvar, unpublished). Strains are oxidase positive and motile with polar tufts of flagella and are urea, ONPG (*o*-nitrophenyl-β-D-galactopyranoside), and phenylalanine deaminase positive (59). Isolates are similar to *Burkholderia gladioli* but do not oxidize dulcitol or inositol. Other characteristics are given in Table 4. Colonies tend to stick to the agar. Human clinical isolates have come from the respiratory tract, blood, spinal fluid, feces, urine, and dialysate (136).

Sphingomonas spp.

On the basis of its 16S rRNA sequence and the presence of unique sphingoglycolipid and ubiquinone types, the genus

Sphingomonas was created for the organism formerly known as *Pseudomonas paucimobilis* and CDC group IIk-1 (93, 264). Since the original proposal, numerous novel species originating from various environments have been added to the genus *Sphingomonas*.

Members of this genus are known to be decomposers of aromatic compounds and are being developed for use in bioremediation. The genus *Sphingomonas* can be divided into four phylogenetic groups, each representing a different genus. Consequently, three new genera, *Sphingobium, Novosphingobium,* and *Sphingopyxis,* in addition to the genus *Sphingomonas* have been created to accommodate the four phylogenetic groups (226). The emended genus *Sphingomonas* contains at least 12 species, of which only *S. paucimobilis,* designated the type species, and *S. parapaucimobilis* are thought to be important clinically.

S. paucimobilis is characterized by medium to long motile rods with a polar flagellum. However, few cells are actively motile in broth culture, thus making motility a difficult characteristic to demonstrate. Motility occurs at 18 to 22°C but not at 37°C. The oxidase reaction is weakly positive, although occasional strains may be oxidase negative. Colonies are slow growing on blood agar medium, with only small colonies appearing after 24 h of incubation. Older colonies demonstrate a deep yellow (mustard color) pigment. Growth occurs at 37°C but not at 42°C, with optimum growth occurring at 30°C. Isolates are strongly esculin hydrolysis positive and produce a zone of growth inhibition around a vancomycin disk (30 μg) placed on a blood agar plate inoculated with a pure culture isolate (P. Schreckenberger, personal observation). *S. paucimobilis* is widely distributed in the environment, including water, and has been isolated from a variety of clinical specimens, including blood, CSF, peritoneal fluid, urine, wounds, the vagina, the cervix, and from the hospital environment (110, 165, 194). Most strains are susceptible to tetracycline, chloramphenicol, trimethoprim-sulfamethoxazole, and aminoglycosides; susceptibility to other antimicrobial agents including fluoroquinolones varies (70, 110, 194).

The cellular and colonial characteristics of *S. parapaucimobilis* are similar to those of *S. paucimobilis*. It is differentiated from *S. paucimobilis* by blackening of lead acetate paper suspended over KIA, ability to grow and alkalinize Simmons' citrate medium, and a negative extracellular deoxyribonuclease reaction (264). Clinical isolates have been obtained from sputum, urine, and the vagina (264). Antibiotic susceptibility data are not available.

Thermophilic Bacteria

Rabkin and colleagues studied 31 bacterial isolates from clinical specimens received by the CDC that were described as "thermophilic," because they had the unusual feature of growing better when incubated at 42 or 50°C than at 35°C and none grew at 25°C in 18 to 24 h. All were slow growers even at optimum temperatures with colonies appearing circular, usually 0.5 mm or less in diameter after 48 to 72 h at 35°C, and smooth, convex, semitranslucent, and slightly glossy (193). Biochemically, they are oxidase positive, fail to grow on MacConkey agar, and do not ferment glucose. Most strains are oxidative in OF glucose and OF maltose. Isolates have been recovered from blood, CSF, urine, nasopharynx, abscess, wound, and liver biopsy specimens. Two cases of meningitis occurred in previously healthy children. In four other cases, infections occurred in compromised hosts (193). The thermophiles were uniformly susceptible to penicillins and cephalosporins and, with few exceptions, to the aminoglycosides (193).

Ko and colleagues recovered a novel asaccharolytic thermophilic gram-negative bacillus from the bone marrow of a patient who developed febrile neutropenia after induction chemotherapy for treatment of myelogenous leukemia. The isolate grew poorly at 37°C but grew optimally at 50°C. Based on 16S rRNA gene sequencing and fatty acid composition, the isolate was classified as a new species, *Tepidimonas arfidensis* (137).

OXIDASE-POSITIVE, INDOLE-NEGATIVE, SACCHAROLYTIC, NONMOTILE, COCCOID OR ROD-SHAPED NONFERMENTERS

See Table 5.

Sphingobacterium and *Pedobacter*

Sphingobacterium spp. are oxidase-positive, indole-negative gram-negative rods that form yellow-pigmented colonies. They have no flagella but may exhibit sliding motility. They are nonproteolytic and produce acid from carbohydrates. The currently described species of *Sphingobacterium* are *S. multivorum* (formerly *Flavobacterium multivorum* and CDC group IIk-2), *S. spiritivorum* (includes species formerly designated *Flavobacterium spiritivorum, F. yabuuchiae,* and CDC group IIk-3), *S. antarcticum, S. faecium, S. thalpophilum,* and unnamed species *Sphingobacterium* genomospecies 1 and 2 (101, 211, 227, 262). The former *Sphingobacterium* species *S. heparinum* and *S. piscium* have been placed in a new genus, *Pedobacter,* as *P. heparinus* and *P. piscium* (220). The genus *Pedobacter* contains several species of heparinase-producing bacteria found in soil, activated sludge, or fish, but not from human sources. Steyn and colleagues have shown that all these organisms constitute a separate rRNA branch in the rRNA superfamily V for which they have proposed a new family called the *Sphingobacteriaceae* (220).

S. multivorum and *S. spiritivorum* have been most frequently recovered from human clinical specimens. They can be distinguished from the similar organism *Sphingomonas paucimobilis* (formerly IIk-1) by lack of motility, urease production, and resistance to polymyxin B (*S. paucimobilis* is usually motile, urease negative, and usually susceptible to polymyxin B). *S. multivorum* has been isolated from various clinical specimens but has only rarely been associated with serious infections (peritonitis and sepsis) (72, 95, 189). Blood and urine have been the most common sources for the isolation of *S. spiritivorum* (94). *S. mizutaii* has been isolated from blood, CSF, and wound specimens and can be differentiated from *S. multivorum* by its failure to grow on MacConkey agar and usual lack of urease (256). *S. thalpophilum* has been recovered from wounds, blood, eye, abscess, and abdominal incision (256). A positive nitrate test and growth at 42°C differentiate *S. thalpophilum* from other *Sphingobacterium* species.

The organism formerly known as *Sphingobacterium mizutaii* has been transferred to the genus *Flavobacterium* (101) but is included with the sphingobacteria in Table 5 because it is indole negative and phenotypically resembles the *Sphingobacterium* species. *F. mizutaii* has been isolated from blood, CSF, and wound specimens and can be differentiated from *Sphingobacterium* species by its failure to grow on MacConkey agar and usual lack of urease activity (256).

Sphingobacterium species are generally resistant to aminoglycosides and polymyxin B while susceptible in vitro to the quinolones and trimethoprim-sulfamethoxazole. Susceptibility to β-lactam antibiotics is variable, requiring testing of individual isolates (218).

TABLE 5 Oxidase-positive, indole-negative, saccharolytic, nonmotile, coccoid or rod-shaped nonfermenters[a]

Test	*Flavobacterium mizutaii* (6)	*Sphingobacterium multivorum* (22)	*Sphingobacterium spiritivorum* (13)	*Sphingobacterium thalpophilum* (10)	CDC group EF-4b (34)	*Paracoccus yeei* (formerly CDC group EO-2) (11)	CDC group EO-3 (7)	CDC group EO-4[b] (8)	*Pedobacter* spp.[c] (14)	*Psychrobacter immobilis* (saccharolytic strains) (7)
Acid from:										
D-Glucose	67 (33)	100	100	100	70 (26)	91 (9)	100	100	43	57 (43)
D-Xylose	(100)	100	92 (8)	100	0	91 (9)	100	100	ND	57 (43)
D-Mannitol	0	0	100	0	0	0	57 (43)	0	7	0
Lactose	100	100	92 (8)	100	0	45 (55)	71 (29)	0	21	57 (43)
Sucrose	50 (50)	100	100	100	0	0	0	0	21	0
Maltose	50 (50)	100	92 (8)	100	0	(9)	14 (14)	0	ND	0
Catalase	100	100	100	100	100	82	100	100	100	100
Growth on MacConkey	0	100	(46)	100	65 (6)	64 (18)	100	87 (13)	0	40
Simmons citrate	0	0	0	0	14 (6)	64 (36)	29 (71)	87	0	20
Urea, Christensen's	0	95	62 (38)	90 (10)	0	36 (55)	14 (86)	62 (38)	0	(43)
Nitrate reduction	0	0	0	100	97	100	0	0	0	86
Gas from nitrate	0	0	0	0	0	18	0	0	0	0
Nitrite reduction	100[d]	0	0	0	71	0[e]	0[e]	0	ND	0[e]
TSI slant, acid	17	55 (5)	0	100	0	0	0	0	0	0
TSI butt, acid	17	5 (76)	0	10 (70)	6	0	0	0	0	0
H_2S (lead acetate paper)	100	86 (5)	56	100	88	64 (9)	100	100	ND	43 (14)
Gelatin hydrolysis[f]	0	0	15	40	9	0	0	0	0	0
Pigment:										
Insoluble	33 yel[g]	57 light yel	54 pale yel	50 pale yel	50 yel	0	100 yel	75 yel	93 yel	0
Soluble	0	0	0	0	0	55 yel	0	0	0	0
Growth at:										
25°C	100	100	100	100	88	73	100	100	100	100
35°C	100	100	100	100	100	100	100	100	36	57
42°C	0	0	9	100	69	36	14	0	ND	0
Esculin hydrolysis	100	100	100	100	0	0	0	0	100	0
Arginine dihydrolase	25	0	25	0	0	ND	ND	14	0	29
Nutrient broth, 0% NaCl	100	100	100	100	89 (7)	64	71	87	100	43 (57)
Nutrient broth, 6% NaCl	0	25	0	10	0	36	43	13	ND	100
Major CFAs[h]	i-15:0, i-2-OH-15:0, 16:1ω7c	i-15:0, i-2-OH-15:0, 16:1ω7c	i-15:0, i-2-OH-15:0	i-15:0, i-2-OH-15:0, 16:1ω7c	16:0, 16:1ω7c, 18:1ω7c	16:0, 18:1ω7c	18:1ω7c	18:1ω7c	i-15:0, i-2-OH-15:0, 16:1ω7c, 16:0, i-3-OH-17:0	16:1ω7c, 18:1ω9c

[a] Most data are from the CDC Special Bacteriology Reference Laboratory (256). All taxa were negative (≤10% positive) for motility, growth on SS and cetrimide agars, production of H_2S in TSI, lysine decarboxylase, and ornithine decarboxylase activity. Numbers indicate percentage positive at 2 days of incubation; parentheses, delayed reaction (3 to 7 days of incubation); w, weak reaction; ND, not determined or not available; yel, yellow. Numbers in parentheses following organisms indicate number of strains.

[b] Data from Weyant et al., *Abstr. 99th Gen. Meet. Am. Soc. Microbiol.*

[c] Data from reference 220.

[d] A partial reduction may be observed with 0.1% nitrite. A full reduction is observed with 0.01% nitrite.

[e] Fewer than five strains tested.

[f] 14 days of incubation.

[g] Pigment production may be enhanced by room temperature incubation.

[h] The number before the colon indicates the number of carbons; the number after the colon is the number of double bonds; ω, the position of the double bond counting from the hydrocarbon end of the carbon chain; c, *cis* isomer; i, *iso;* 2-OH, a hydroxyl group at the 2 (β) position from the carboxyl end; 3-OH, a hydroxyl group at the 3 (β) position from the carboxyl end.

CDC Group EF4b

EF-4b and EF-4a were originally designated Eugonic Fermenter group 4 (EF-4); however, EF-4b does not ferment glucose, hydrolyze arginine, or produce gas from nitrate, which separates it from the glucose-fermenting strains now designated CDC group EF-4a (see chapter 40). 16S rRNA studies indicate that EF-4b is closely related to *Neisseria canis* (Daneshvar, unpublished). EF-4b strains are coccoid to short rods that are nonmotile and oxidase and catalase positive. Colonies on culture plates are nonpigmented and reported to smell like popcorn. Most isolates have been recovered from human infections following dog and cat bites (256). Antibiotic susceptibility resembles that of EF-4a.

Paracoccus yeei (EO-2), CDC Groups EO-3, EO-4, and *Psychrobacter immobilis*

The classification of the eugenic oxidizers (EO) and the saccharolytic strains of *Psychrobacter immobilis* is incomplete. All are strongly oxidase-positive, nonmotile, saccharolytic coccobacilli and grow, sometimes poorly, on MacConkey agar. In contrast to the EO groups, *P. immobilis* grows best at 20°C and only occasionally at 37°C. EO-3 and many EO-4 strains have a yellow, nondiffusible pigment that is not observed with either *P. yeei* or *P. immobilis*. Daneshvar et al. (53) proposed the name *Paracoccus yeeii,* the epithet being later changed to *yeei* (68), for the former CDC group EO-2. CDC group EO-3 has been shown by 16S rRNA sequencing to closely match *Fulvimarina pelagi* (Daneshvar, unpublished), and CDC EO-4 remains unnamed.

Microscopically, *P. yeei* is characterized by distinctive "O-shaped" cells (Fig. 3) upon Gram stain examination due to the presence of vacuolated or peripherally stained cells, and *P. immobilis* is characterized by paired, coccoid organisms. *P. immobilis* is divided into saccharolytic and asaccharolytic strains. Saccharolytic *P. immobilis* strains (Table 5) share all of the characteristics of the asaccharolytic strains (Table 2) except that glucose, xylose, and lactose, but not sucrose and maltose, are oxidized. Asaccharolytic strains are phenotypically similar to *P. phenylpyruvicus*. The diagnosis of *P. immobilis* can be confirmed by transformation studies, CFA profile, and optimal growth at temperatures of <35°C (167, 256). Many strains of *P. immobilis* have an odor resembling phenylethyl alcohol agar (roses) and are resistant to penicillin but susceptible to most other antibiotics (79, 151). All four groups have been recovered from clinical specimens. *P. yeei* has been isolated from various human wound infections (53). EO-3 has been reported to cause peritonitis in a patient on continuous peritoneal dialysis (49). EO-4 has been recovered from blood, urine, and a nasal sinus, but the clinical significance of these isolates is unknown (R. S. Weyant, M. I. Daneshvar, J. G. Jordan, J. P. MacGregor, and D. G. Hollis, *Abstr. 99th Gen. Meet. Am. Soc. Microbiol.,* abstr. C-196, 1999). One case of ocular infection and one case of infant meningitis have been reported to be caused by *P. immobilis* (79, 151).

PINK-PIGMENTED NONFERMENTERS

See Table 6.

Asaia spp.

Asaia is a recently described genus consisting of two members, *A. bogorensis* (266) and *A. siamensis* (128). The natural habitats of *Asaia* spp. are reported to be in the flowers of the orchid tree, plumbago, and fermented glutinous rice, all originating in hot tropical climates, particularly in Indonesia and Thailand. *Asaia bogorensis* has been reported as a cause of peritonitis in a patient on automated peritoneal dialysis (217). Daneshvar and colleagues identified 14 isolates of a novel *Asaia* species, of which 3 were from a dialysis-associated outbreak (M. I. Daneshvar, L. W. Mayer, A. G. Steigerwalt, A. M. Whitney, R. E. Morey, L. O. Helsel, P. N. Levett, B. J. Paster, F. E. Dewhirst, R. S. Weyant, and D. J. Brenner, Int. Conf. Emerg. Infect. Dis., Atlanta, 2004). *Asaia* species have also been isolated from a batch of fruit-flavored bottled water, which had spoiled as a result of bacterial overgrowth (164). Isolates characterized at the CDC are pink-pigmented, oxidase-negative, motile rods that are oxidative in OF glucose, xylose, and mannitol. Additional characteristics are given in Table 6. Moore reported that *Asaia* spp. were resistant to ceftazidime, meropenem, imipenem, trimethoprim, amikacin, vancomycin, aztreonam, penicillin, and ampicillin by disk diffusion (164). The *A. bogorensis* strain reported by Snyder et al. was susceptible to aminoglycosides (amikacin, tobramycin, and gentamicin) and resistant to ceftazidime and meropenem by disk diffusion (217).

Methylobacterium spp.

Members of the genus *Methylobacterium* are pink-pigmented bacteria able to utilize methanol as a sole source of carbon and energy, although this characteristic may be lost on subculture. They occur mostly on vegetation but may also be found in the hospital environment. The genus currently consists of 20 named species plus additional unassigned biovars recognized on the basis of carbon assimilation type, electrophoretic type, and DNA-DNA homology grouping (74, 80, 81, 233). *Methylobacterium mesophilicum* (formerly *Pseudomonas mesophilica, Pseudomonas extorquens, and Vibrio extorquens*) and *Methylobacterium zatmanii* have been the two most commonly reported species isolated in clinical samples. Methylobacteria are oxidase positive and motile by one polar or lateral flagellum, although motility is often difficult to demonstrate. Isolates are slow growing on ordinary media, producing 1-mm-diameter colonies in 4 to 5 days on SBA, modified Thayer-Martin, Sabouraud, buffered charcoal-yeast extract, and Middlebrook 7H11 agars, with best growth occurring on Sabouraud agar, and usually no growth on MacConkey agar. Optimum growth occurs from 25 to 30°C. Colonies are dry and appear pink or coral in incandescent light (Fig. 4). Under UV light, *Methylobacterium* species appear dark due to absorption of UV light (199). On Gram stain the cells appear as large, vacuolated, pleomorphic rods that stain poorly and may resist decolorization (Fig. 5). Oxidation of sugars (xylose and sometimes glucose) is weak; urea and starch are hydrolyzed.

Methylobacterium species have been reported to cause septicemia, continuous ambulatory peritoneal dialysis-related peritonitis, skin ulcers, synovitis, and other infections often in immunocompromised patients, as well as pseudoinfections (104, 130, 149, 205). Tap water has been implicated as a possible agent of transmission in hospital environments, and methods for monitoring water systems for methylobacteria have been described (197). Active drugs include aminoglycosides and trimethoprim-sulfamethoxazole, whereas β-lactam drugs show variable patterns (26). They are best tested for susceptibility by agar or broth dilution at 30°C for 48 h (26).

Roseomonas and *Azospirillum* spp.

Members of the genus *Roseomonas* (199) are also pink pigmented but differ in morphologic and biochemical characteristics from *Methylobacterium* spp. (Table 6). They are

TABLE 6 Pink-pigmented nonfermenters[a]

Test	*Asaia* spp.[b] (8)	*Methylobacterium* species[c] (90)	*Roseomonas cervicalis* (7)	*Roseomonas gilardii*[d] (21)	*Roseomonas mucosa* (22)	*Roseomonas* genomospecies 4 (3)	*Roseomonas* genomospecies 5 (3)	*Azospirillum* sp. (*Roseomonas* genomospecies 6) (1)	*Azospirillum* sp. (*Roseomonas fauriae*) (5)
Motility; flagella	87; p, 1–2, L	100; p, 1	100; p, 1–2	33; p, 1–2[e]	100; p, 1	67; p, 1–2[f]	0	100; p, 1–2	100; p, 1–2
Acid from:									
D-Glucose	100	40	0	(43)	0	0	0	0	20
D-Xylose	100	94	43	19 (57)	0	100	67	0	80 (20)
D-Mannitol	100	2	0	14 (38)	0 (27)	0	0	0	0
Oxidase	0	96	100	52	27	100	100	100	100
Growth on:									
MacConkey	13 (37)	15	100	43 (52)	50 (50)	100	67(33)	100w	60 (40)
SS	0	0	0	0	0	0	0	0	20
Simmons citrate	0	2 (3)	86 (14)	100	91 (5)	0	33	(100)	60 (20)
Urea, Christensen's	0	29 (26)	86 (14)	71 (29)	95 (5)	67(33)	100	100	100
Nitrate reduction	0	25	0	5	5	100	0	100	100
Gas from nitrate	0	0	0	0	0	0	0	0	20
H_2S (lead acetate paper)	0	47	100	100	100	100	100	100	100
Growth at 42°C	0	12	100	67	100w	100	67	100	100
Esculin hydrolysis	13	0	0	0	0	0	0	100	100
Nutrient broth, 0% NaCl	87	93	100	100	100	100	100	100	100
Nutrient broth, 6% NaCl	13	0	0	24	0	33	0	0	20
Major CFAs[g]	ND	18:1ω7c	16:0, 18:1ω7c	16:0, 18:1ω7c, 19:0cyc, 2–OH–19:0cyc	16:0, 18:1ω7c, 19:0cyc	16:0, 18:1ω7c	16:0, 3–OH–16:0, 18:1ω7c	3–OH–14:0, 16:1ω7c, 18:1ω7c	16:1ω7c, 18:1ω7c

[a] Unless otherwise indicated, data are from the CDC Special Bacteriology Reference Laboratory (256). Numbers indicate the percentage positive at 2 days of incubation; parentheses, delayed reaction (3 to 7 days of incubation); w, weak reaction; ND, not determined or not available; L, lateral; p, polar. All taxa were positive (≥90%) for catalase, growth at 25 and 35°C. All were negative for growth on cetrimide agar, indole production, acid in TSI agar, and H_2S production in TSI agar.

[b] Data from Daneshvar et al. Int. Conf. Emerg. Infect. Dis. Atlanta, 2004.

[c] At least 20 species and additional biovars have been described (see the text).

[d] Two subspecies are proposed: *R. gilardii* subsp. *gilardii* and *R. gilardii* subsp. *rosea* (82).

[e] Motility was more easily demonstrated in OF medium than in motility medium. Motile strains demonstrated either 1 or 2 polar flagella or detached flagella.

[f] Motile strains demonstrated 1 or 2 flagella.

[g] The number before the colon indicates the number of carbons; the number after the colon is the number of double bonds; ω, the position of the double bond counting from the hydrocarbon end of the carbon chain; c, *cis* isomer; OH, a hydroxyl group at the 2(α) or 3(β) position from the carboxyl end; cyc, a cyclopropane ring structure.

nonvacuolated and rather plump and coccoid and form mostly pairs and short chains (Fig. 6). They grow on SBA, modified Thayer-Martin, and usually on MacConkey agars at 37°C, but best growth is observed on Sabouraud's agar. Colonies are mucoid and runny (Fig. 7). They are separated from *Methylobacterium* by their inability to oxidize methanol and assimilate acetamide and by lack of absorption of long-wave UV light (199). All strains are weakly oxidase positive (often after 30 s) or oxidase negative, catalase positive, and urease positive. The original description of the genus *Roseomonas* included three named species, *Roseomonas gilardii* (genomospecies 1), *Roseomonas cervicalis* (genomospecies 2), *Roseomonas fauriae* (genomospecies 3), and three unnamed species, *Roseomonas* genomospecies 4, 5, and 6 (199). More recently, Han et al. proposed a new species, *Roseomonas mucosa,* and a new subspecies, *R. gilardii* subspecies *rosea* (to differentiate from *R. gilardii* subspecies *gilardii*) (82). 16S rRNA gene sequencing of all six *Roseomonas* genomospecies suggests that *Roseomonas* genomospecies 1, 2, 4, and 5 are valid taxa while genomospecies 3 and 6 are not. The invalid *Roseomonas* genomospecies 3 and 6 belong to the genus *Azospirillum,* a nitrogen-fixing plant symbiont that is in a different order of bacteria (44, 82, 228).

Clinical isolates have been recovered from blood, wounds, exudates, abscesses, genitourinary sites, continuous ambulatory peritoneal dialysis fluid, and bone (11, 57, 159, 168, 198, 212, 221, 222). In a review of the laboratory, clinical, and epidemiologic data on 35 patients from whom *Roseomonas* was isolated, Struthers and colleagues reported that *Roseomonas* spp. appear to have a low pathogenic potential for humans but that some species, particularly *R. gilardii,* may be significant pathogens in persons with underlying medical complications (221). In multiple-case reports about 60% of the isolates recovered have been from blood, with about 20% from wounds, exudates, and abscesses and about 10% from genitourinary sites (57, 221).

De et al. summarized susceptibility data from three published reports on a combined 80 strains of *Roseomonas*. All strains were susceptible to amikacin (100%); frequently susceptible to imipenem (99%), ciprofloxacin (90%), and ticarcillin (83%); less susceptible to ceftriaxone (38%), trimethoprim-sulfamethoxazole (30%), and ampicillin (13%); and rarely susceptible to ceftazidime (5%). All strains were resistant to cefepime (57). In catheter-related infections, eradication of the organism has proven difficult unless the infected catheter is removed (198).

OXIDASE-POSITIVE, INDOLE-POSITIVE, NONMOTILE OR MOTILE, YELLOW-PIGMENTED NONFERMENTERS

See Table 7.

Chryseobacterium, Elizabethkingia, Empedobacter, and Unnamed CDC Groups IIc, IIe, IIg, IIh, and IIi

The natural habitats of *Chryseobacterium, Elizabethkingia, Empedobacter,* and the unnamed CDC groups are soil, plants, foodstuffs, and water sources, including those in hospitals. Species in these genera are oxidase positive, indole positive, and nonmotile. The indole reaction is often weak and difficult to demonstrate; therefore, the more sensitive Ehrlich method should be used. Pigment formation with these organisms is variable. Colonies of *Elizabethkingia meningoseptica,* formerly *Chryseobacterium meningosepticum* (133), are smooth and fairly large (1 to 2 mm in diameter after 24 h) but show only weak (if any) production of yellow pigment. In contrast, colonies of *Chryseobacterium indologenes* are deep yellow due to the production of the water-insoluble pigment flexirubin (181). Colonies of *Empedobacter brevis* are pale yellow. Microscopically, cells of *E. meningoseptica, C. indologenes,* and groups IIe, IIh, and IIi are thinner in their central than in their peripheral portions and include filamentous forms; IIh cells are significantly smaller than those of other species. It should be emphasized that test results (e.g., DNase, indole, urea, and starch hydrolysis) in this group are dependent on the choice of medium, reagents, and length of incubation (181). *Chryseobacterium indologenes, C. gleum,* and CDC group IIb are tabulated individually in Table 7. Group IIb is genetically heterogeneous and includes strains of *C. indologenes, C. gleum,* and probably additional genomospecies. Phenotypic separation between *C. indologenes* and *C. gleum* has been difficult; however, acid production from xylose and growth at 41°C are consistently positive in DNA groups clustering around the type strain of *C. gleum* (234).

Chryseobacterium indologenes is the most frequent human isolate, although it rarely has clinical significance (248). It causes bacteremia in hospitalized patients with severe underlying disease, although the mortality is relatively low even among patients who were administered antibiotics without activity against *C. indologenes* (107, 215). Nosocomial infections due to *C. indologenes* have been linked to the use of indwelling devices during hospital stay (109, 111, 174).

Elizabethkingia meningoseptica (133) is most often associated with significant disease in humans, causing neonatal meningitis and nosocomial miniepidemics (15, 36, 215) verifiable by ribotyping (45) and random amplified polymorphic DNA fingerprinting (36), and rarely, adult pneumonia and septicemia (15, 210, 248). A case of respiratory colonization and infection following aerosolized polymyxin B treatment has also been described (25).

Empedobacter brevis and the unnamed CDC groups IIc, IIe, IIg, IIh, and IIi are rarely recovered from clinical material, and little is known about their involvement in clinical disease. One case of meningitis caused by CDC group IIe has been reported (253), and the phenotypic characteristics of several clinical isolates of CDC groups IIc and IIg have been described (86, 88).

The appropriate choice of effective antimicrobial agents for treatment of chryseobacterial infections is difficult. *Chryseobacterium* spp. and *E. meningoseptica* are inherently resistant to many antimicrobial agents commonly used to treated infections caused by gram-negative bacteria (aminoglycosides, β-lactam antibiotics, tetracyclines, and chloramphenicol) but are often susceptible to agents generally used for treating infections caused by gram-positive bacteria (rifampin, clindamycin, erythromycin, sparfloxacin, trimethoprim-sulfamethoxazole, and vancomycin) (70, 218, 248). Although early investigators recommended vancomycin for treating serious infection with *E. meningoseptica* (84, 187), subsequent studies showed greater in vitro activity of minocycline, rifampin, trimethoprim-sulfamethoxazole, and quinolones (15, 71, 218). Among the quinolones, sparfloxacin and levofloxacin are more active than ciprofloxacin and ofloxacin (218). Di Pentima et al. (61) suggested that the combination of intravenous vancomycin and rifampin is an appropriate regimen for initial empirical therapy of *E. meningoseptica* meningitis in newborns. *C. indologenes* is reported to be uniformly resistant to cephalothin, cefotaxime, ceftriaxone, aztreonam,

TABLE 7 Oxidase-positive, indole-positive, nonmotile or motile, nonpigmented or yellow-pigmented nonfermenters[a]

Test	*Elizabethkingia meningoseptica* (149)	*Chryseobacterium gleum* (type strain)	*Chryseobacterium indologenes* (type strain)	*Empedobacter brevis* (7)	*Weeksella virosa* (87)	*Bergeyella zoohelcum* (41)	*Balneatrix alpica* (1)	CDC group IIb[b] (155)	CDC group IIc (20)	CDC group IIe (30)	CDC group IIg (12)	CDC group IIh (21)	CDC group IIi (23)
Motility; flagella	0	−	−	0	0	0	100; p, 1−2	0	0	0	0	0	0
Acid from:													
D-Glucose	95 (4)	(+)	(+)	85 (15)	0	0	100	92 (6)	100	83 (17)	0	85 (15)	91 (9)
D-Xylose	2 (1)	(+)	−	0	0	0	0	30 (1)	0	0	0	5	87 (13)
D-Mannitol	91 (8)	−	−[b]	0	0	0	(100)	10	0	0	0	0	0
Lactose	42 (15)	−	−	0	0	0	0	0	0	0	0	0	91 (9)
Sucrose	0	−	−	0	0	0	0	13 (1)	100	0	0	0	91 (9)
Maltose	93 (7)	(+)	(+)	85 (15)	0	0	100	92 (6)	100	97 (3)	0	95	91 (9)
Starch	0	−	(+)	75	ND	ND	(100)	100	ND	ND	ND	ND	ND
Trehalose	100	(+)	(+)	0	ND	ND	0	100	ND	ND	ND	ND	ND
ONPG	100	ND	−	0	ND	ND	ND	57	ND	ND	ND	ND	ND
Catalase	100	+	+	100	98	100	100	99	100	100	92	100	100
Oxidase	99	+	+	100	100	100	100	96	100	100	100	100	100
Growth on MacConkey	89 (3)	+	(+)[b]	100	(10)	2	0	54 (9)	0	3	100	0	0
Citrate	9 (3)	+[b]	+	0	0	0	100	2 (1)	0	0	0	0	0
Urea, Christensen's	3 (5)	(+)	−	0	0	100	0	14 (28)	0	0	0	0	14 (18)
Nitrate reduction	0	+	−[b]	0	0	0	100	22	90	0	0	0	0
Nitrite reduction	50[c]	+	−	0[c]	0	0	0	20	90	ND	100	ND	ND
TSI slant, acid	0	−	−	0	0	0	0	1	60 (20)	0	0	0	0
TSI butt, acid	(3)	−	−	0	0	0	0	5 (5)	10 (70)	0	0	5	0
H_2S (lead acetate paper)	98	+	+	100	95	59	100	99	100	87	50	100	70
Gelatin hydrolysis[d]	91	+	+	100	100	98	0	78	20	3	0	7	0
Yellow insoluble pigment	0	+	+	85w	0	0	0	99	0	7w	0	0	22
Growth at:													
25°C	100	+	+	100	58	30	100	100	100	90	100	100	100
35°C	100	+	+	100	100	95	100	100	100	100	100	100	100
42°C	45	+	−	0	70	10	100	42	5	0	90	5	36
Esculin hydrolysis	99	+	+	0	0	0	0	70	100	0	0	100	96

Lysine decarboxylase	0[c]	ND	0	0	0	0	12	0	0	0	0
Arginine dihydrolase	33[c]	ND	0	0	100	0	24	(20)	0	ND	0
Nutrient broth, 0% NaCl	100	+	100	99	15	100	100	100	97	100	86	100
Nutrient broth, 6% NaCl	7	–	0	7	0	0	0	10	3	0	5	9
CFAs[e]	i-15:0, i-2-OH-15:0, i-3-OH-17:0	i-15:0, i-2-OH-15:0, i-17:1ω8c	i-15:0, i-2-OH-15:0, i-17:1ω8c	i-15:0, 16:1ω7c	i-15:0, i-2-OH-15:0	i-15:0	16:0, 16:1ω7c, 18:1ω7c	i-15:0, i-2-OH-15:0, i-17: i-17: 1ω8c	i-15:0, i-2-OH-15:0, i-3-OH-17:0, i-17:1ω8c	3-OH-14:0, i-2-OH-16:0, 16:1ω7c, 18:1ω7c	i-15:0, a-15:0	i-15:0, i-2-OH-15:0, i-3-OH-17:0

[a] Unless otherwise indicated, data are from the CDC Special Bacteriology Reference Laboratory (256). All taxa were negative (<10% positive) for growth on SS agar, H_2S production in TSI, and ornithine decarboxylase activity. Numbers indicate the percentage positive at 2 days of incubation; parentheses, delayed reaction (3 to 7 days of incubation); w, weak reaction; +, positive; –, negative; ND, not determined or not available; p, polar.

[b] The original description of *C. gleum* lists the type strain as citrate negative. In the original description of *C. indologenes*, 30% of strains (4/13) oxidized mannitol, 46% of strains (6/13) grew on MacConkey agar, and 38% of strains (5/13) reduced nitrate to gas. CDC group IIb includes *C. indologenes* and *C. gleum* (262).

[c] Fewer than five strains tested.

[d] 7 to 14 days of incubation.

[e] The number before the colon indicates the number of carbons; the number after the colon is the number of double bonds; ω, the position of the double bond counting from the hydrocarbon end of the carbon chain; c, *cis* isomer; i, *iso*; a, anteiso; OH, a hydroxyl group at the 2 (α) or 3 (β) position from the carboxyl end; cyc, a cyclopropane ring structure.

aminoglycosides, erythromycin, clindamycin, vancomycin, and teicoplanin, while susceptibility to piperacillin, cefoperazone, ceftazidime, imipenem, quinolones, minocycline, and trimethoprim-sulfamethoxazole is variable, requiring testing of individual isolates (107, 111, 218, 250). Further complicating the choice of appropriate antimicrobial therapy is the fact that MIC breakpoints for resistance and susceptibility of chryseobacteria have not been established by the Clinical and Laboratory Standards Institute (CLSI) and the results of disk diffusion testing are unreliable in predicting antimicrobial susceptibility to *Chryseobacterium* species (1, 31, 71, 250). The E test is a possible alternative to the standard agar dilution method for testing cefotaxime, ceftazidime, amikacin, minocycline, ofloxacin, and ciprofloxacin but not piperacillin (106). Definitive therapy for clinically significant isolates should be guided by individual susceptibility patterns determined by an MIC method.

Weeksella and *Bergeyella*

Weeksella virosa and *Bergeyella zoohelcum* are morphologically similar organisms measuring 0.6 by 2 to 3 μm, with parallel sides and rounded ends. Both species are oxidase positive and indole positive, fail to grow on MacConkey agar, are nonpigmented, and are nonsaccharolytic. Both species have the unusual feature of being susceptible to penicillin, a feature that allows them to be easily differentiated from the related genera of *Chryseobacterium* and *Sphingobacterium*. *W. virosa* colonies are mucoid and adherent to the agar and develop tan to brown pigmentation; *B. zoohelcum* colonies are sticky and tan to yellow. *W. virosa* is urease negative and polymyxin B susceptible; *B. zoohelcum* is rapid urease positive and polymyxin B resistant. *W. virosa* occurs mainly in urine and vaginal samples (99, 196), whereas *B. zoohelcum* is isolated mainly from wounds caused by animal (mostly dog) bites (100, 195). Meningitis or septicemia due to *B. zoohelcum* has occurred in patients either bitten by a dog (23, 163) or with continuous contact with cats (172). Both organisms are susceptible to most antibiotics; however, at present no specific antibiotic treatment is recommended; therefore, antibiotic susceptibility testing should be performed on significant clinical isolates.

Balneatrix

The genus *Balneatrix* contains a single species, *B. alpica* (55), that was first isolated in 1987 during an outbreak of pneumonia and meningitis among persons who attended a hot (37°C) spring spa in southern France (28, 55, 113). Isolates from eight patients were recovered from blood, CSF, and sputum, and one was recovered from water. The bacterium is described as a gram-negative, straight or curved rod, motile by a single polar flagellum, and strictly aerobic. Growth occurs at 20 to 46°C, producing colonies that are 2 to 3 mm in diameter, convex, and smooth. The center of the colonies is pale yellow after 2 to 3 days and pale brown after 4 days. Growth occurs on chocolate and tryptic soy agars but not on MacConkey agar. It is oxidase positive and nonfermentative but oxidizes glucose, mannose, fructose, maltose, sorbitol, mannitol, glycerol, and inositol. Indole is produced and nitrate is reduced to nitrite (Table 7). Gelatin is weakly hydrolyzed and lecithinase is positive. It is similar to *E. meningoseptica* but can be differentiated by positive motility and nitrate and negative ONPG reactions. *B. alpica* is reported to be susceptible to penicillin G and all other β-lactam antibiotics and to all aminoglycosides, chloramphenicol, tetracycline, erythromycin, sulfonamides, trimethoprim, ofloxacin, and nalidixic acid. It is resistant to clindamycin and vancomycin (28).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Although certain nonfermenting bacilli (NFBs) can on occasion be frank pathogens, e.g., *Pseudomonas aeruginosa, Burkholderia pseudomallei,* and *Elizabethkingia meningoseptica,* NFBs are generally considered to be of low virulence and often occur in mixed cultures, making it difficult to determine when to work up cultures and when to perform susceptibility studies. Decisions regarding the significance of NFBs in a clinical specimen must take into account the clinical condition of the patient and the source of the specimen submitted for culture. In general, the recovery of an NFB in pure culture from a normally sterile site warrants identification and susceptibility testing, whereas predominant growth of an NFB from a nonsterile specimen, such as an endotracheal culture of a patient with no clinical signs or symptoms of pneumonia, would not be worked up further. Because many NFBs exhibit multiple antibiotic resistance, patients who are on antibiotics often become colonized with NFBs. NFB species isolated in mixed cultures can usually be reported by descriptive identification, e.g., "growth of *P. aeruginosa* and two varieties of nonfermenting gram-negative bacilli not further identified." The Gram stain made from the clinical material should be used to guide the laboratory decision on how far to work up the specimen.

Decisions about performing susceptibility testing are complicated by the fact that the CLSI interpretive guidelines for disk diffusion testing of the nonfermenting gram-negative bacilli are limited to *Pseudomonas* spp., *Burkholderia cepacia, Stenotrophomonas maltophilia,* and *Acinetobacter* spp. and therefore, except for *Acinetobacter* species, do not include the organisms covered in this chapter. Furthermore, results obtained with certain organisms (e.g., *Acinetobacter* species) by using disk diffusion do not correlate with results obtained by conventional MIC methods (see discussion elsewhere in this chapter). In general, laboratories should try to avoid performing susceptibility testing on the organisms included in this chapter. When clinical necessity dictates that susceptibility testing be performed, an overnight MIC method is recommended.

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Bordetella

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TAXONOMY

The genus *Bordetella,* in the family *Alcaligenaceae,* is named for J. Bordet, who, with O. Gengou, described the bacterium in 1906 (9). A total of eight *Bordetella* species have been described: *B. pertussis*, *B. bronchiseptica*, *B. parapertussis*, *B. avium*, *B. hinzii*, *B. holmesii*, *B. trematum* (5, 61), and the recently described species *B. petrii* (96). An additional organism, *B. ansorpii* sp. nov. (50), currently has no standing in nomenclature (http://www.bacterio.cict.fr/nonvalid.html). Phylogenetic analysis based on 16S rRNA gene sequencing has demonstrated that *B. pertussis, B. parapertussis, B. bronchiseptica,* and *B. holmesii* are very closely related (48). *B. pertussis* and *B. parapertussis* may be recent derivatives of *B. bronchiseptica,* developing unique host ranges and pathogenicity due to gene loss (72). In spite of considerable genetic relatedness among some *Bordetella* species, current literature still refers to distinct species. Evidence suggests that the genetic diversity of *B. pertussis* is decreasing, due to selective pressure of vaccination (84). This may be partially responsible for the reemergence of pertussis, as described later in this chapter.

DESCRIPTION OF THE GENUS

Bordetella organisms are small gram-negative coccobacilli. Some species are motile. With the exception of the recently described *B. petrii,* they are strictly aerobic, with optimal growth at 35 to 37°C. All species possess catalase activity. All species oxidize amino acids, but none ferment carbohydrates. While all species have relatively simple nutritional requirements, fastidiousness varies depending on the degree of sensitivity to toxic substances and metabolites found in common laboratory media. *B. pertussis* is the most fastidious species and is inhibited by constituents present in many media, including fatty acids, metal ions, sulfides, and peroxides. Isolation of *B. pertussis* requires media containing protective substances such as charcoal, blood, or starch. The other *Bordetella* species are less fastidious and will grow on routine agars containing blood and on MacConkey agar. Growth rates of *Bordetella* species are generally inversely related to fastidiousness; *B. pertussis* grows slowly, while *B. avium* and *B. bronchiseptica* grow rapidly. *B. petrii* is unique among the *Bordetella* in that it is capable of growth under both aerobic and anaerobic conditions. Table 1 lists some of the common characteristics used to differentiate *Bordetella* species.

B. pertussis produces a number of virulence factors that are responsible for pathogenesis (61). These virulence factors include toxins (pertussis toxin, adenylate cyclase toxin, and tracheal cytotoxin) and components that mediate adherence to ciliated epithelial cells of the respiratory tract (pertussis toxin [PT], fimbriae, filamentous hemagglutinin [FHA], and pertactin). Other virulence factors include dermonecrosis (heat-labile) toxin, lipopolysaccharide (endotoxin), and tracheal colonization factor. *Bordetella* species other than *B. pertussis* also express virulence factors. *B. parapertussis* and *B. bronchiseptica* produce pertactin and FHA (61). Promoter and structural genes for PT are present in both *B. parapertussis* and *B. bronchiseptica* but are not expressed.

NATURAL HABITATS

With the exception of *B. petrii, Bordetella* species are isolated from humans and warm-blooded animals. *B. petrii,* the sole environmental species, was isolated from an anaerobic culture enriched from river sediment (96). *B. pertussis* produces disease only in humans, who also serve as the sole reservoir. *B. parapertussis,* once thought to be strictly a human pathogen, is also found in sheep (61). Genotypic strain analysis showed that human and ovine *B. parapertussis* strains are distinct (93). *B. pertussis* has historically been considered a strict respiratory pathogen, causing localized infection of the ciliated epithelium of the bronchial tree. However, its isolation from blood indicates the potential for invasive infection (13, 47). *B. bronchiseptica* is a respiratory tract pathogen of a variety of animals, including dogs, swine, cats, and rabbits (61). *B. avium* is found in wild and domesticated birds and causes coryza in turkeys (75). *B. hinzii* colonizes the respiratory tracts of poultry, and recent evidence suggests that it may cause disease (78). An organism isolated from a cancer patient undergoing chemotherapy has been designated *B. ansorpii* sp. nov., based on 16S rRNA gene sequence, cellular fatty acid composition, G+C content, and biochemical testing. Although recovered from the exudate from an epidermal cyst, a pathogenic role for this isolate was not established (50). On rare occasions *B. bronchiseptica* and *B. hinzii* cause disease in humans (19, 102). *B. avium* is strictly a veterinary pathogen. *B. holmesii* and *B. trematum* are infrequently associated with nonrespiratory infections in humans (88, 90). Additionally, *B. holmesii* has been linked to respiratory infections with pertussis-like symptoms (103).

TABLE 1 Differential characteristics of *Bordetella* spp.[a]

Characteristic	*B. pertussis*	*B. parapertussis*	*B. bronchiseptica*	*B. avium*	*B. hinzii*	*B. holmesii*	*B. trematum*	*B. petrii*[b]
Oxidase	+	−	+	+	+	−	−	+
Nitrate reduction	−	−	+	−	−	−	V	−
Urease production	−	+ (24 h)	+ (4 h)	−	V	−	−	−
Motility	−	−	+	+	+	−	+	−
Growth on:								
Blood agar	−	+	+	+	+	+	+	+[c]
MacConkey agar	−	V (delayed)	+	+	+	+ (delayed)	+	+

[a] Modified from references 19, 90, and 96 and earlier editions of this Manual. Responses: + , activity or growth present; − , not present; V, variable.
[b] Data from a single strain.
[c] Ulf Göbel, personal communication.

CLINICAL SIGNIFICANCE

The incubation period of pertussis is usually 7 to 10 days, with a range of approximately 4 to 21 days (12). The symptoms that develop following the incubation period can be classified as either typical (classical) or atypical. Classical pertussis consists of a catarrhal stage lasting 1 to 2 weeks, a paroxysmal stage lasting 1 to 6 weeks (as long as 10 weeks), and a convalescent stage lasting 2 to 4 weeks (as long as several months). Patient symptoms during the catarrhal stage are nonspecific and include rhinorrhea, sneezing, low-grade fever, and occasional mild cough. Pertussis is frequently unsuspected during this period. The paroxysmal stage of pertussis is characterized by the presence of one or more of the pathognomonic signs of pertussis: episodes of paroxysmal cough, whoop, and posttussive vomiting. The severity of respiratory symptoms gradually decreases during the convalescent stage. Atypical symptoms in older children and adults consist of a prolonged, nondescript cough. The differential diagnosis of atypical pertussis includes bronchitis and upper respiratory tract infections caused by adenovirus, parainfluenza virus, respiratory syncytial virus, *Chlamydophila* (formerly *Chlamydia*) *pneumoniae,* and *Mycoplasma pneumoniae* (29, 30). Infants with pertussis may present with choking and apnea, while cough may be absent. Morbidity and mortality rates are higher in infants than in other age groups (61). Adults experience more complications from pertussis than do adolescents (21). *Bordetella* infections have recently been shown to play a major role in acute exacerbation of chronic bronchitis (8).

B. pertussis is responsible for the vast majority of pertussis cases and causes more-severe respiratory symptoms than other *Bordetella* species associated with pertussis syndrome. Worldwide, *B. pertussis* causes an estimated 20 million to 40 million cases of pertussis and 200,000 to 400,000 deaths each year, primarily among children in countries lacking organized vaccination programs (http://www.who.int/vaccines/en/pertussis.shtml#summary). *B. pertussis* is transmitted from person to person via respiratory droplets and is highly contagious, infecting 80 to 90% of susceptible contacts (12). Relatively close contact (within several feet) is required for transmission. Transmission is not uncommon in health care facilities and can be associated with high rates of morbidity in this high-risk setting (14).

A carrier state is not recognized for *B. pertussis.* Sensitive nonculture tests such as PCR can detect organisms in asymptomatic immune persons (35), but this is more likely to represent a transient colonization or infection rather than a prolonged carrier state. The disease is endemic, with epidemics consistently occurring every 3 to 5 years (12). The number of reported pertussis cases in the United States has increased steadily since the 1980s, in spite of continued high levels of vaccination coverage. Explanations offered for the increased incidence include heightened awareness and reporting of disease (particularly the atypical presentation, and disease in older persons), and the use of more sensitive laboratory diagnostic tests.

Immunity to pertussis following natural infection or vaccination wanes after 5 to 12 years. As a result, *B. pertussis* is a significant cause of respiratory disease in older children and adults (15, 61, 87) who, in countries with vaccination programs, have replaced young children as the primary reservoir of *B. pertussis.* Serologic evidence indicates that as much as one-third of chronic cough illness in adults is due to *B. pertussis* (15). However, in some studies in which culture was performed, *B. pertussis* was not isolated from any seropositive subjects (64). Therefore, the public health significance of this high seroprevalence is unclear.

Vaccines have dramatically reduced the public health impact of pertussis. The initial heat-killed, whole-cell vaccines have been largely replaced by multicomponent acellular vaccines, which are associated with fewer side effects (73). The efficacy of whole-cell and acellular vaccines is estimated to be between 60 and 90% (12). Until recently, available pertussis vaccines had been formulated and licensed for children younger than 7 years. However, two aspects of recent pertussis epidemiology have stimulated evaluations of the immunogenicity and safety of acellular vaccines in older persons (10). The first is the recognition of the importance of *B. pertussis* as a cause of respiratory disease in adolescents and adults (61, 87); the second is the accumulating evidence for the predominant role of adolescents and adults in transmission of pertussis to infants (7). Acellular booster doses are licensed in Australia, Austria, Canada, France, and most recently, the United States (http://www.cdc.gov/nip/vaccine/tdap/tdap_acip_recs.pdf) for administration to adolescents or adults. Vaccination (with either acellular or whole-cell vaccine) may be selecting for *B. pertussis* strains distinct from those from the prevaccination era (66, 84). The role of the divergence between vaccine and recently circulating strains in the changing epidemiology of pertussis over the last 20 years is still uncertain. There is no epidemiological evidence to suggest that vaccine efficacy in the United States has been compromised by the divergence of circulating strains. Vaccines directed against *B. pertussis* do not confer protection from infections by other *Bordetella* spp.

Septicemia caused by *B. pertussis* has been reported only rarely, but blood cultures showing gram-negative bacilli that

are not recovered on routine subculture media should be subcultured on media supporting *B. pertussis* (13, 47).

B. parapertussis causes a pertussis syndrome similar to but usually less severe than that caused by *B. pertussis* (61). While *B. parapertussis* may be quite prominent in isolated outbreaks (56), overall it accounts for 2 to 20% of *Bordetella* isolates (61). Symptomatic *B. parapertussis* infections more commonly present as a nonspecific cough illness or bronchitis. Mixed infections by *B. pertussis* and *B. parapertussis* have been reported, and this should be considered when examining primary cultures from clinical specimens (56). *B. bronchiseptica* (23, 102) and *B. holmesii* (81, 103) have been implicated as infrequent causes of pertussis syndrome and other respiratory illnesses. *B. holmesii* is also a rare cause of bacteremia in asplenic patients (85). Most reported cases of *B. bronchiseptica* respiratory disease have been associated either with underlying conditions such as immunosuppression or with exposure to animals. *B. bronchiseptica* has been associated with pleural effusion in a patient with AIDS (94) and pneumonia in lung transplant recipients (71). *Bordetella* species isolated from cystic fibrosis patients include *B. bronchiseptica* (99) and *B. hinzii* (18).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Excellent summaries of specimen collection and handling for optimal detection of *B. pertussis* are available (70), including several earlier editions of this Manual. Preferred specimens for laboratory diagnosis of pertussis are nasopharyngeal (NP) aspirates and posterior NP swabs. When properly collected, these specimens contain the ciliated respiratory epithelial cells for which *B. pertussis* exhibits tropism. NP aspirates yield more positive culture results than do NP swabs (31). Aspirates offer additional advantages over swabs; there is usually sufficient specimen for multiple analyses (important when verifying new test procedures), and the specimen collection technique may be preferred by clinicians and parents of young patients (31). Throat swabs are inferior specimens for culture; they do not sample the ciliated epithelium, and they contain large numbers of members of the normal flora, which can result in overgrowth of isolation media. In one study, significantly fewer infections were detected by culture using throat swabs than by culture using NP swabs (60). However, throat swabs may be suitable for PCR diagnosis of *B. pertussis* infection (25).

To obtain an NP aspirate, a narrow catheter or infant feeding tube is inserted through the nostril to the posterior nasopharynx. A mucous trap and hand-operated vacuum pump are connected to the other end, and suction is applied while the tube is in place and while slowly withdrawing it back through the nostril. Any secretions remaining in the tube should be flushed into the trap by aspirating *Bordetella* transport medium or phosphate-buffered saline. Additionally, the catheter tip can be cut off and placed into suitable transport medium for shipping. Swab specimens are collected by inserting a small swab on a flexible (usually aluminum wire) shaft through the nostril. The placement of the swab is important. Figure 1 depicts the correct positioning of the patient's head and placement of the swab. The swab should then be rotated for several seconds before being withdrawn. It is generally recommended that two NP swab specimens be collected, one through each nostril. This provides separate swabs if multiple laboratory tests are to be performed. Specimens are then either directly plated or placed in a suitable medium for transport.

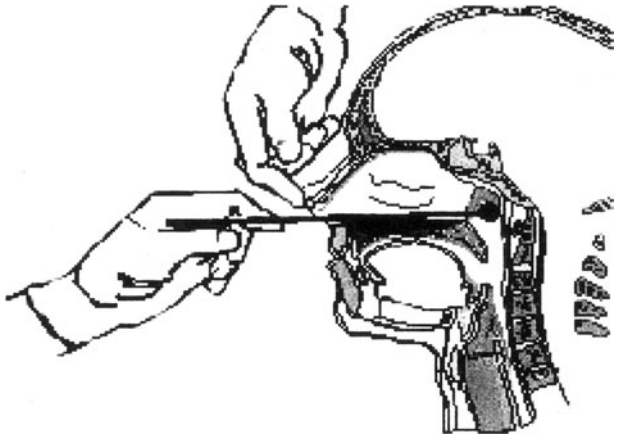


FIGURE 1 Correct positioning of patient's head and placement of the swab for collection of NP specimens (reprinted from reference 12). (Courtesy of Kris Bisgard, Centers for Disease Control and Prevention.)

Several transport media are readily available for NP specimens, including 1% acid-hydrolyzed casein (Casamino Acids; Becton Dickinson, Sparks, Md.) and Amies medium with charcoal. Survival of *B. pertussis* at 4°C in Casamino Acids was substantially greater than in water or phosphate-buffered saline (11). Regan-Lowe (RL) transport medium contains half-strength charcoal agar and horse blood, with cephalexin added to suppress growth of the normal NP flora. Substitution of 2.5 μg of methicillin per ml or 0.625 μg of oxacillin per ml for cephalexin reportedly allows the growth of *B. holmesii* (62). Unlike Casamino Acids and Amies medium, RL transport medium also functions as an enrichment medium for *B. pertussis*. Preincubation of specimens in RL transport medium is controversial. Preincubation at 36°C may enhance the recovery of *B. pertussis* due to multiplication of organisms (76). Some recommend against preincubation because of overgrowth of cephalexin-resistant members of the flora (12). Transport at 4°C provides better recovery of *B. pertussis* than does transport at room temperature (33, 68) but adds additional costs due to packaging and weight.

Swab material should be calcium alginate, Dacron, or rayon if culture isolation is to be performed (17, 44). Cotton contains inhibitors that will decrease isolation rates of *B. pertussis* (44). Dacron or rayon is recommended if PCR testing is performed, since calcium alginate can inhibit PCR (17, 97). The inhibitory effect of calcium alginate on PCR amplification may depend in part on the specimen extraction procedure. Crude proteinase K extracts were completely inhibitory to PCR (97).

Because of the lability of *B. pertussis* outside its normal host environment, proper handling and shipping conditions are critical for optimal culture sensitivity. For culture testing, direct plating of NP specimens onto agar plates provides optimal sensitivity. Kurzynski et al. showed that 14% more NP swab specimens were positive by a plate inoculated bedside than by a plate inoculated after enrichment and transport (54). However, this requires clinician participation and training and is not practical for off-site reference and public health laboratories.

For direct fluorescent-antibody (DFA) testing, the specimen collection kit should contain a glass slide, on which a smear is prepared from an NP swab immediately after collection. The slide is allowed to air dry and is then transported

to the laboratory. Alternatively, smears can be prepared at the laboratory from liquid transport medium containing an NP swab or from NP aspirates.

For PCR testing, swabs can be transported dry (57), in transport medium, or in saline. Transport in liquid medium provides a specimen from which multiple aliquots can be tested, whereas processing a dry swab for PCR testing leaves no unextracted specimen material available for repeat analysis if required. Detailed consensus recommendations for managing specimens for PCR are available elsewhere (80).

DIRECT DETECTION

DFA Testing

The direct detection of *B. pertussis* in NP secretions by using fluorochrome-conjugated antibody provides the most rapid and simple diagnosis of pertussis. Commercially available antibodies are polyclonal (Becton Dickinson) or monoclonal, recognizing a lipooligosaccharide epitope (Accu-Mab; Altachem Pharma, Edmonton, Alberta, Canada) (1). The monoclonal antibodies are available as a dual-fluorochrome reagent for the detection of both *B. pertussis* and *B. parapertussis* in a single smear. A head-to-head comparison of the polyclonal and monoclonal reagents showed that they had similar sensitivity (89). Factors to consider when choosing an antibody source include cost and performance in the laboratory's own hands.

Regardless of the antibody used, the direct detection of *B. pertussis* by DFA lacks both sensitivity and specificity. Compared to that of culture, DFA sensitivity in several published studies ranged from 30 to 71% (32, 57). DFA-positive, culture-negative specimens are not unusual. When DFA was compared to an expanded diagnostic standard (e.g., two positive nonculture tests or a clinical diagnosis), its sensitivity ranged from one-half that of culture (32) to threefold greater than that of culture (57). The large range in DFA performance reflects variation in the sensitivities of both the DFA procedure and the culture procedures against which it is compared. The specificity of DFA is highly variable, due to the cross-reactivity of immunological reagents and the subjectivity of fluorescence interpretation. Weak cross-reactivity of the Accu-Mab monoclonal antibody with *B. bronchiseptica* has been reported (63). Polyclonal antibodies cross-react with a number of organisms, including unencapsulated *Haemophilus influenzae* and diphtherioids (61). In a study conducted by Loeffelholz et al., several specimens positive only by DFA were considered to be true positives because of strong clinical or epidemiological evidence of infection (57). Other studies have shown extremely poor DFA specificity (32). Notwithstanding the variable performance, DFA testing can provide valuable information when appropriate quality assurance and quality control measures are implemented to maximize specificity. DFA testing should be performed only as an adjunct to culture or PCR, and the results should be considered presumptive.

Antibody for DFA testing must be appropriately titrated before use. To control for staining specificity, control slides should contain separate smears of *B. pertussis* and *B. parapertussis*. Smears should contain 10 to 100 organisms per oil immersion field. Suspensions of control organisms can be prepared in phosphate-buffered saline with a turbidity equivalent to a McFarland standard of 1. Stained smears are examined under a fluorescent microscope for the presence or absence and degree of fluorescence. Slides are viewed initially at a magnification of ×400. Any fluorescence is examined at a magnification of ×1,000 to confirm morphology. *Bordetella* organisms appear as small coccobacillary rods with pronounced peripheral apple green fluorescence and dark centers. Fluorescence must be intense, and other staining characteristics and morphology must be ignored.

Nucleic Acid Detection

Nucleic acid detection methods such as PCR can provide a test response time similar to that of DFA, while offering enhanced sensitivity and objectivity in results interpretation. These advantages, together with the continuing loss of expertise in interpreting DFA results, have contributed to the ascension of PCR over DFA as the routine nonculture test method in many clinical laboratories. Although the Centers for Disease Control and Prevention recommended in 2000 that PCR be used as a presumptive assay in conjunction with culture (12), this test is rapidly moving from presumptive to diagnostic status and becoming more available.

PCR clinical sensitivity suffers from the same constraints associated with other detection methods, including specimen quality and timing in the disease course and antimicrobic treatment. However, PCR has consistently been reported to be more sensitive than DFA or culture (25, 27, 28, 34, 35, 57, 91, 98). The superiority of PCR performance over that of culture is due in part to variation in the sensitivity of culture. PCR tests themselves vary in sensitivity, depending on the nucleic acid extraction method, the amount of specimen amplified, the amplification conditions and efficiency, the sequence targeted, and the DNA detection format. Presumably because of its greater sensitivity and ability to detect dead organisms, PCR remains positive longer during the course of disease than does culture (91, 98) and is more likely to remain positive following antibiotic therapy (24, 98). Longitudinal analysis of erythromycin-treated infants showed that after 4 days of treatment, 56 and 89% of NP swab specimens were positive by culture and PCR, respectively (24), whereas after 7 days of treatment, no specimens were positive by culture but 56% were still positive by PCR. The higher sensitivity of PCR is also marked among persons with mild or atypical symptoms (83) and among older persons (91, 98).

Despite the increasing use of PCR methods to diagnose pertussis, these assays continue to demonstrate significant shortcomings. There is no standardized or Food and Drug Administration (FDA)-cleared, commercially available PCR test for *B. pertussis* and *B. parapertussis*. In fact, many assays fail to test for *B. parapertussis*, which remains a significant cause of pertussis in some outbreaks (56). Validation criteria vary by regulatory program, but extensive method validation on the part of the laboratory is required before the test can be offered for diagnostic use. Unfortunately, the validation process is easily compromised by insufficient availability of positive specimens and inadequate resolution of discrepant results between the PCR test and the diagnostic standard, especially when the PCR is positive and the comparative standard is negative. The validation criteria specified by the Clinical and Laboratory Standards Institute (http://www.nccls.org) are helpful in designing a validation program and would likely satisfy all state criteria. The laboratory must also develop and implement quality control and an ongoing quality assurance program; these requirements also vary by regulatory program. A widely available and independently certified proficiency testing program is lacking, so it is currently difficult to assess interlaboratory performance.

Because PCR detects nonviable cells and DNA, defining the clinical sensitivity and specificity of PCR tests for pertussis

is critical. However, these predictive values for pertussis PCR remain incompletely defined, especially regarding the effect on these values of pertussis prevalence in the host community, host vaccination status and age, and disease severity. Interpretation standards should include relevance of unpredicted results such as multiple consecutive positives. Similarly, results reporting formats also must reflect these limitations and the clinical community must be educated regarding test limitations and reporting criteria. It is strongly recommended that positive test results be confirmed with an additional laboratory test or clinical pertussis ascertainment, until PCR clinical sensitivity and specificity values are more completely defined.

Consensus recommendations for the diagnosis of *B. pertussis* and *B. parapertussis* infections by PCR have been proposed (80). These recommendations focus heavily on quality assurance and control, verification of assay performance (clinical sensitivity and specificity and analytical specificity), and ongoing validation studies and also describe appropriate sample collection, transport, pretreatment, and DNA extraction. Chapter 16 of this Manual describes good laboratory practices to avoid false-positive PCR results due to contamination. Positive and negative controls must be able to monitor the entire PCR assay procedure, including specimen extraction. Internal specimen amplification controls should be included for each specimen; ideally these would be amplified by the same primers as the diagnostic target (36). While there are no established guidelines about the number of negative controls to include in each run, some laboratories routinely include a negative control for approximately every five specimens.

Chromosomal regions targeted by PCR include, but are not limited to, the pertussis toxin promoter region (28, 45), a region upstream of the porin gene (26), repetitive insertion sequences IS*481* of *B. pertussis* (27, 34, 92) and IS*1001* of *B. parapertussis* (92), the adenylate cyclase gene (22), and a region upstream of the flagellin gene (46). A set of primers targeted to the toxin promoter was shown not to detect *B. parapertussis* or *B. bronchiseptica* (28, 45). Primers targeting IS*481* have been shown to detect *B. holmesii* (58) and may cross-react with other *Bordetella* spp. (80). Carriage of IS*481* by strains of several *Bordetella* species makes monitoring the specificity of IS*481* necessary (80). Nested-PCR assays for the detection of *Bordetella* have been described (25, 79). While these assays were reported to be sensitive and specific, nested PCR is inherently more susceptible to contamination than unnested PCR. It is our opinion that when PCR conditions are properly optimized, nesting of PCR is not required for the accurate, sensitive diagnosis of most infectious diseases, including pertussis.

ISOLATION

Culture provides the most specific diagnosis of pertussis and produces isolates for genotypic analysis and antimicrobial susceptibility testing. The sensitivity of culture varies greatly, depending on patient factors (including prior antibiotic therapy, duration of symptoms, age, and vaccination status), specimen transport conditions, the type and quality of media used, and other conditions (38). While PCR testing is also affected by these factors, culture is affected to a greater degree because of its requirement for viable organisms. The highest isolation rates are achieved when specimens are inoculated onto media immediately after collection from the patient. Since this is often not possible, transport media should be used.

Several media for the isolation of *B. pertussis* have been described. Traditional media consist of bases such as potato infusion (Bordet-Gengou [BG] medium) or charcoal (RL medium) supplemented with glycerol, peptones, and horse or sheep blood. An antimicrobial agent is added to reduce the growth of the normal flora. Cephalexin is the agent of choice, yet growth of resistant members of the NP flora is still quite common (70). RL medium provides better isolation of *B. pertussis* than does BG medium (68, 70). The shelf life of media ranges from 5 days for BG medium to 4 to 8 weeks for RL medium (76).

Plates are incubated in ambient air at 35 to 36°C and examined daily for suspicious colonies. In one study, *B. pertussis* colony development was more rapid in ambient air than in air containing 5 to 10% carbon dioxide (43). The humidity must be sufficient to prevent the desiccation of agar plates during the long incubation period. While most *B. pertussis* colonies become visible after 3 to 4 days of incubation (*B. parapertussis* becomes visible after 2 to 3 days), an incubation time of 7 days has historically been recommended. In one study, 12 days of incubation recovered more isolates than did 7 days of incubation (49).

IDENTIFICATION

Phenotypic

On RL agar, young *B. pertussis* colonies are round, domed, mercury-silver colored, and shiny. *B. parapertussis* colonies are similar but are grayer and less domed (Fig. 2). On BG agar, *B. pertussis* and *B. parapertussis* produce zones of hemolysis.

Bordetella organisms appear as gram-negative coccobacilli or short rods. Poly- or monoclonal antibodies are available to confirm identity (Fig. 3). Suspensions of colonies are prepared in phosphate-buffered saline and should be dilute to avoid dulling of fluorescence. Both *B. pertussis* and *B. parapertussis* controls should be included. Fluorescence is interpreted as described earlier in this chapter. For identification by slide agglutination, a heavier suspension is prepared, equivalent to a McFarland standard of 3. Uniform suspensions are best prepared from cultures no more than 3 days old and harvested with a Dacron or rayon swab instead of a bacteriological loop. Cell aggregates can be dispersed by repeatedly forcing the suspension through a pipette and using aerosol precautions. Colonies grown on antibiotic-free RL agar produce more homogeneous suspensions for easier interpretation of agglutination. In addition to identification using specific antisera, *Bordetella* species can be identified by biochemical tests based on differential phenotypic characteristics (Table 1). Oxidase-positive, nonfermentative gram-negative rods, particularly *Oligella ureolytica* and *Ralstonia paucula,* can be misidentified as *B. bronchiseptica. B. bronchiseptica* differs from *R. paucula* by having a positive nitrate reduction test and from *O. ureolytica* by having a negative nitrite reduction test and by showing penicillin susceptibility.

Commercially obtained strains (American Type Culture Collection, Manassas, Va.) or previously characterized clinical isolates can serve as controls for quality control of media and reagents. Clinical isolates should be frozen as soon as possible at −70°C in glycerol. Once plated, cultures lose viability quickly and must be replaced regularly. As per Clinical Laboratory Improvement Amendment regulations, laboratories must maintain thorough documentation of control strains, including strain source/origin and storage conditions. Standard operating procedures must be developed for the maintenance of control strains.

Biosafety level 2 practices are recommended when working with *Bordetella* spp. Routine manipulations of specimens and isolates can be conducted on open bench tops.

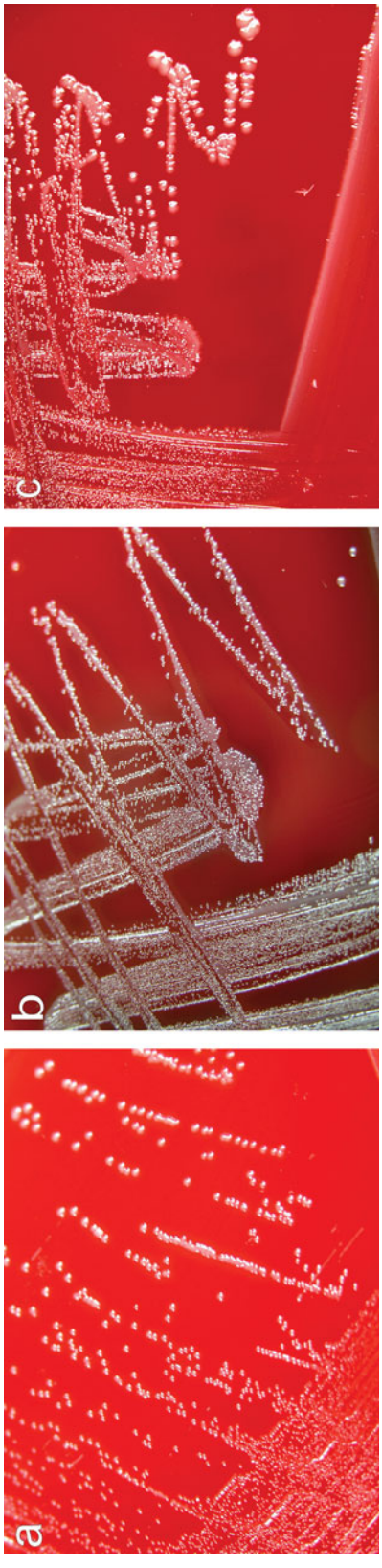


FIGURE 2 Colony morphologies of *Bordetella* after 3 to 5 days of incubation on BG agar. (a) *B. pertussis*; (b) *B. parapertussis*; (c) *B. bronchiseptica*.

Genotypic Strain Typing

Pulsed-field gel electrophoresis (PFGE) methods for the identification of *B. pertussis* strains have been described previously (12, 67). PFGE has been proven to be valuable in tracking outbreak transmission patterns (12, 65). It has also been used to identify *B. parapertussis* (74) and *B. bronchiseptica* isolates (6). The rare-cutting restriction enzyme XbaI is used in most PFGE procedures for typing *B. pertussis, B. parapertussis,* and *B. bronchiseptica* (6, 12, 74). Other genotypic methods used to discriminate *Bordetella* isolates include restriction enzyme analysis (82), arbitrarily primed PCR (randomly amplified polymorphic DNA) (104), PCR targeting a specific repetitive element (65), ribotyping (77), sequence analysis of genes encoding pertactin and the S1 fragment of pertussis toxin (67), and multilocus variable-number repeat analysis (84). Comparative analysis of PFGE and PCR-based typing methods for discrimination of *B. pertussis* isolates showed PFGE to be more discriminatory (65). Standardized approaches to genotypic analysis of *B. pertussis* isolates have focused on PFGE, largely due to the wide availability and demonstrated epidemiological relevance of this approach (12, 67).

SEROLOGIC TESTS

Serologic diagnostic methods are generally limited to the detection of immune responses to *B. pertussis*. In the past, these methods included enzyme-linked immunosorbent assay (ELISA), agglutination, complement fixation, immunoblotting, indirect hemagglutination, and toxin neutralization (70). Assays other than ELISAs, immunoblotting, and toxin neutralization are not common in clinical laboratories. The toxin neutralization assays are generally limited to reference and research institutions to measure functional serum antibodies. Because of ease of use and sensitivity, ELISAs in microwell plate format have become the method of choice.

ELISAs have been used to measure immunoglobulin A (IgA), IgG, and IgM levels to PT, FHA, pertactin, fimbriae, and sonicated *B. pertussis* cells. As measured by ELISAs, at least 90% of infected persons develop IgG to PT and FHA after ⩾3 to 4 weeks following onset of symptoms (70). IgG and IgA responses to these antigens are most often used as indicators of *B. pertussis* infection. Responses to PT are specific for *B. pertussis,* since no other *Bordetella* species expresses PT. FHA is expressed by *B. pertussis*, *B. parapertussis,* and *B. bronchiseptica.* Other bacteria, including *Haemophilus influenzae,* possess epitopes that cross-react with FHA (95). Clinical sensitivity of IgA-specific responses is not as great as for IgG responses, but IgA is rarely detected in uninfected vaccines, and therefore, such responses enhance clinical specificity when present (70, 91). IgM responses do not increase clinical sensitivity and specificity sufficiently over those for IgA and IgG to be widely tested.

The specific strategy used for the serodiagnosis of pertussis is often a function of patient age and vaccination status, and sensitivity and specificity may vary. Currently, the demonstration of seroconversion or a significant rise in the concentration of IgG against PT is widely accepted as the most sensitive and specific serodiagnostic test. Consequently, a suitable strategy for serologic diagnosis in unvaccinated children is the demonstration of increases in the concentrations of IgG or IgA to PT or FHA, although young infants may not produce IgA (91). However, the appropriate collection of acute and convalescent serum specimens is often problematic in pertussis patients of any age (61, 86). True acute-phase specimens are often

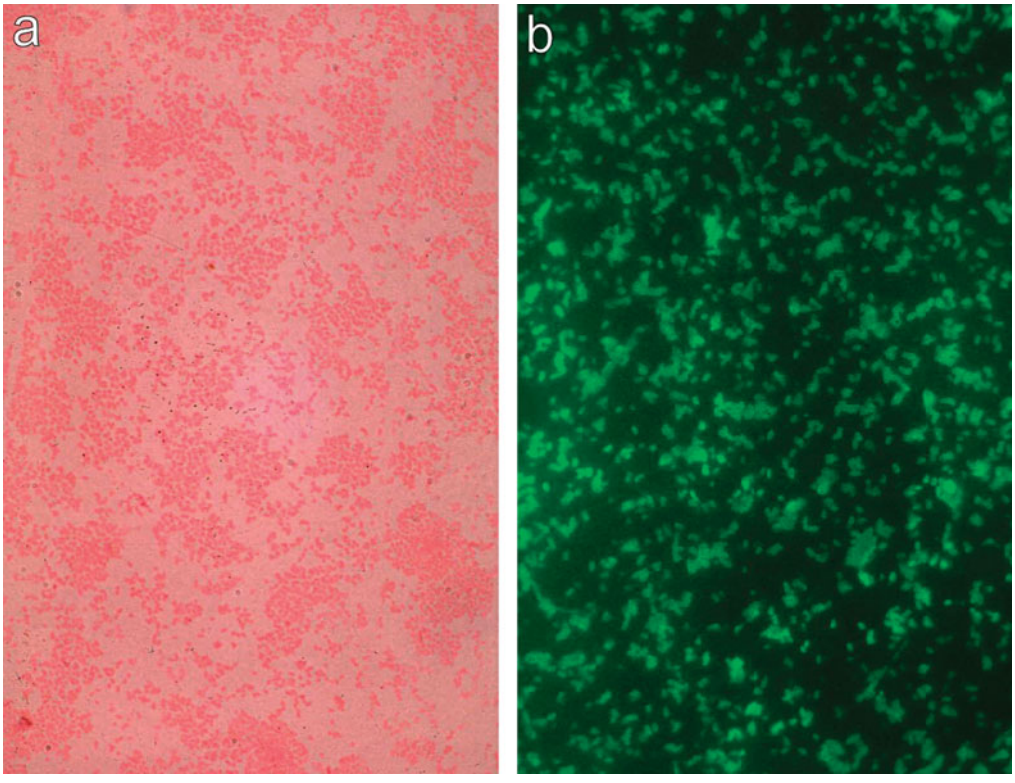


FIGURE 3 (a) Gram stain morphology of *B. pertussis*. (b) Immunofluorescent staining of *B. pertussis*. (Photographs courtesy of Charlotte Parker.)

excluded by the clinical presentation of patients late in the illness due to the nonspecific symptoms early in disease, and convalescent-phase specimens can be compromised by poor patient compliance with recall. Testing of single specimens collected early in the disease (1 to 3 weeks after onset of cough) was reportedly compromised by low sensitivity (101).

Serodiagnosis of pertussis in vaccinated children is even more problematic due to rapid increases in antibody concentration and the consequent difficulty in detecting significant rises in these responses. The tendency to collect "acute"-phase specimens late in this population further contributes to this problem. These conditions among vaccinees suggest that a significant decrease in specific antibodies between late acute- and convalescent-phase specimens may be a valid diagnostic criterion, and one report has demonstrated the feasibility of this approach (86).

Since obtaining acute- and convalescent-phase specimens is problematic, serodiagnosis of pertussis in adolescents and adults based on high antibody concentrations in a single specimen is an attractive strategy (70). The general waning of vaccine-induced serum antibodies in these populations further promotes this approach. Until recently, results from single-serum specimens required interpretation in the context of control values determined for a specific patient population. Epidemiologic studies can stringently identify case-patient controls by age and vaccination status in order to derive serodiagnostic control (cutoff) values related to their study population (16). Although these diagnostic values are limited to specific study populations, potential single-serum IgG anti-PT diagnostic levels with more general diagnostic relevance are emerging from longitudinal studies of the kinetics of IgG anti-PT responses and seroepidemiologic studies of representative samples of populations (4, 20). The specificity of this approach depends on the particular assay and cutoff used. Diagnostic sensitivity has not been widely evaluated for any of the currently proposed diagnostic cutoffs (20).

IgA-, IgG-, and IgM-specific ELISAs are available from a number of manufacturers on several continents, and the immunoblot format is also commercially available (MarBlot; Trinity Biotech, Wicklow, Ireland). Although there is currently no FDA-cleared serodiagnostic test, ELISAs are widely used outside the United States, particularly for testing adolescents and adults. In the United States, the lack of an FDA-cleared test and inadequate supply of standard reference serum specimens severely restrict the use of diagnostic ELISAs. Comparative analyses of serologic testing, culture, DFA, and PCR have generally shown serologic testing to be the most sensitive method for diagnosis of *B. pertussis* infections (28, 32, 91). In one study, PCR was positive for only 21% of patients with positive serology (either high IgG or IgA concentrations in a single specimen or significant increases in antibody concentrations in paired specimens) (91). The authors attributed the low sensitivity of PCR to the collection of specimens relatively late in disease. The same study demonstrated that the sensitivity of both PCR and culture decreases rapidly after the paroxysmal phase. An evaluation of several commercial IgG-based ELISAs showed that individual assay reproducibility was acceptable, but results between tests and between test and reference assays were not concordant (52). Sensitivities with challenge paired serum specimens were 60 to 95%, but none of the assays identified all cases correctly.

ANTIMICROBIAL SUSCEPTIBILITY

Erythromycin, azithromycin, and clarithromycin are the antimicrobial agents of choice for treatment and prophylaxis of pertussis (3, 69). Trimethoprim-sulfamethoxazole (SXT) is an acceptable alternative for patients intolerant of macrolides and for the rare pertussis case caused by macrolide-resistant *B. pertussis* (40). Among the fluoroquinolones, ciprofloxacin, levofloxacin, and gemifloxacin showed the highest activities against *B. pertussis* (42, 69) but are not recommended for treating children because of their potential harmful side effects. The most recent treatment and prophylaxis recommendations can be seen at http://www.cdc.gov/nip/publications/pertussis/guide.htm and http://www.cdc.gov/nip/publications/pertussis/chapter3a_update_macrolides.pdf.

Eleven instances of erythromycin resistance in *B. pertussis* have been reported from Arizona, California, Georgia, Minnesota, New York, and Utah since 1994 (2, 51, 55; K. Bromberg, personal communication), but the true prevalence of resistance in the circulating population is unknown. Susceptibility screening of *B. pertussis* isolates at several enhanced pertussis surveillance sites suggests that the prevalence of resistant strains is too low to justify routine testing of all isolates. However, resistant *B. pertussis* isolates have demonstrated wide geographic distribution and been associated with treatment failure. Consequently, surveillance for resistant organisms is recommended and isolates should be tested if associated with treatment failure (12). Resistance in *B. pertussis* is conferred by a mutation in the macrolide binding domain of the 23S rRNA.

A novel heterogeneous erythromycin resistant/susceptible phenotype has been described (100). This phenotype is distinguished by the appearance of two colony types during the resistance screening test. The first type manifests as susceptible with a zone of inhibition >40 mm after 3 to 4 days of incubation. The second colony type appears up to 8 days later within the zone of inhibition defining the susceptible type. Subculture and susceptibility testing of the resistant colonies yield uniformly resistant cultures, whereas the susceptible phenotype recapitulates the original phenotype with both the resistant and susceptible colonies.

While antibiotics are generally less active against *B. parapertussis* than against *B. pertussis* (53), *B. parapertussis* is susceptible to macrolides, fluoroquinolones, and SXT (39, 69). *B. bronchiseptica* and other *Bordetella* species generally have antimicrobial susceptibility profiles similar to those of other nonfermentative gram-negative bacilli.

Antibiotic susceptibility testing of *B. pertussis* is not standardized. Reported methods include the E test (AB Biodisk, Solna, Sweden), agar dilution, and disk diffusion using a 15-μg erythromycin disk (12, 37, 41, 51). Agar dilution has been performed with Mueller-Hinton agar supplemented with 5% horse blood (41), as well as BG agar containing 20% horse blood (37). RL agar without cephalexin is commonly used for the disk diffusion and E-test methods (37, 55). In most studies, agar dilution has served as the reference method; erythromycin MICs for 47 susceptible isolates were ≤12 μg/ml (37). It is recommended that strains determined to be nonsusceptible by disk diffusion also be tested by agar dilution (12). Studies have demonstrated good agreement among diffusion tests and agar dilution for determining erythromycin resistance in *B. pertussis* (37, 51). Less agreement was observed when testing SXT (37).

EVALUATION AND INTERPRETATION OF RESULTS

The diagnosis of pertussis can be challenging, given the frequent atypical presentation and the shortcomings of laboratory tests. Indeed, no single laboratory test can be considered a perfect diagnostic standard. While culture is virtually 100% specific, its sensitivity is often low due to poorly collected specimens, long specimen transport time, and patient factors such as duration of symptoms and prior antibiotic treatment.

Nucleic acid amplification methods such as PCR generally offer greater sensitivity than does culture. PCR has frequently been shown to provide the specific diagnosis of pertussis when culture is negative. The major shortcomings of nucleic acid amplification methods are the potential for false-positive results due to contamination, the lack of standardization among assays, and the interpretation of a positive result for *B. pertussis* DNA in the absence of a positive culture. While the combination of a positive PCR result and a negative culture result for the same patient can reflect the greater sensitivity of PCR, there are situations when a positive PCR result alone may not have clinical or public health relevance. Therefore, positive PCR results must be interpreted in conjunction with patient symptoms, treatment status, and epidemiological factors. Culture, serology, and, to a greater extent, PCR have shown that asymptomatic *B. pertussis* infections occur (35, 59). In one study, 92% of preschool-aged children with positive PCR results remained free of symptoms during a 6-week follow-up period (35). The authors attributed this to protective immunity. Comparatively, only one-third of older, school-age children remained asymptomatic throughout the follow-up period. During pertussis outbreaks, laboratories may be pressured to test specimens from asymptomatic contacts of patients. In fact, while these individuals may share a day care center or school classroom with a patient, they often do not meet the criteria for exposure to *B. pertussis.* Positive laboratory results (usually by the sensitive PCR test) for these asymptomatic persons confound treatment and outbreak response decisions. Given the general belief that a long-lasting carrier state for pertussis does not exist, PCR-positive asymptomatic persons are unlikely to contribute significantly, if at all, to the spread of pertussis.

Serology has the potential to contribute substantially to the diagnosis of pertussis, when standardized tests and interpretive criteria that reliably diagnose recent infections in vaccinated populations are available. Interpretive criteria for a single serum specimen will have more clinical utility than those that require paired sera. For the optimal use and interpretation of serology and other tests for pertussis, the laboratory must have information on the patient's symptoms, antibiotic treatment, exposure to other ill persons, vaccination status, and age and on the level of disease activity in the community.

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Francisella and *Brucella**

DAVID LINDQUIST, MAY C. CHU, AND WILL S. PROBERT

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Francisella and *Brucella*, while not closely related taxonomically, have been traditionally linked in the mind of the bacteriologist. Both are zoonotic agents capable of producing severe disease in humans. In the laboratory, they are seen as very small to tiny gram-negative coccobacilli that do not seem to grow particularly well and are not particularly active biochemically. The two genera are apt to show cross-reactivity with each other in agglutination-based serologic tests (13, 103, 178). They share a reputation for frequently causing laboratory-acquired infections (50). Finally, *Francisella tularensis*, *Brucella melitensis*, *B. suis*, and *B. abortus* are all considered to be potential bioterrorism agents (68, 138; chapter 9 of this Manual). This circumstance has arguably led to a heightened interest in these agents and has helped prompt development of tests, particularly rapid tests, for their detection, identification, and further characterization.

In the United States, *F. tularensis* and three species of *Brucella*, *B. melitensis, B. suis*, and *B. abortus,* are classified as select agents. To transfer, receive, or possess these agents, laboratories must be registered both with the Centers for Disease Control and Prevention (CDC) and with the Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture. The registration process includes a U.S. Department of Justice investigation of all personnel having access to select agents. Clinical laboratories are exempt from the registration requirement provided that within 7 calendar days of identifying one of these agents, they transfer it to a registered entity and/or destroy the agent on site. Laboratories identifying an organism as *F. tularensis* or *B. melitensis* are required to report this finding immediately to APHIS or the CDC. The identification of *B. abortus* or *B. suis* is to be reported within 7 calendar days by using the appropriate form. Report forms, contact information, laboratory registration information, and pertinent citations of the U.S. Federal Code may be found at http://www.cdc.gov/od/sap.

FRANCISELLA

Taxonomy

Tularemia, a zoonosis affecting a wide range of animals and humans, is the disease caused by infection with members of the genus *Francisella*. Tularemia was described in detail in the first part of the 20th century (67, 105). The story of its discovery and the elucidation of its pathology and epidemiology are essentially American (159). Several recent publications have reviewed the history and description of the genus *Francisella* (54, 143). The first article describing the etiologic agent of tularemia was published in 1912 by McCoy and Chapin, who isolated the agent from a ground squirrel die-off in Tulare County in California (105). Francis, for whom the genus is named, recognized and confirmed that a number of diverse and epidemiologically distinct clinical entities were really one disease, and he proposed the name "tularemia" to describe the disease in humans (67). In addition to *F. tularensis*, two other species, both first isolated in North America, are included as members of the family *Francisellaceae*. *F. novicida* was recovered in 1951 from water. *F. philomiragia* was isolated in 1969 from muskrats and the surrounding water environment (147). These two species are infrequently isolated and rarely implicated in clinical disease. The genus members have been separated into two pathogenic groups: one that causes serious illness in humans and is often lethal for animals, and one that is considered to be of lesser virulence for humans and animals (84, 89, 147). The pathogenic members of this genus exist as intracellular parasites; therefore, antibiotic therapy may have to be extended in order to eliminate the infection. All genus members are susceptible to aminoglycosides and tetracyclines. It is the widespread epizootic nature of tularemia, its potentially severe and fatal forms in humans, its reputation as a laboratory hazard, and its potential use as a biological warfare agent that have given tularemia its notoriety. *F. tularensis* has been developed as a biological weapon, and projections about its impact, if used as a weapon in an urban setting, depict a scenario devastating to public health and to the economy of health services (49).

The species of *Francisella* share some biochemical activities, a high degree of DNA relatedness (54, 63, 64, 94), and a unique cellular fatty acid (CFA) profile (83, 169). Subspecies designations continue to be in transition as new information about host relationships, geographic origin, virulence, and phenotypic, biochemical, and genetic features is revealed. Four subspecies of *F. tularensis* are listed in the most recent edition of *Bergey's Manual of Systematic Bacteriology* (147): *F. tularensis* subsp. *tularensis* (type A), *F. tularensis* subsp. *holarctica* (type B; formerly known as *F. tularensis* subsp.

*This chapter contains information presented in chapter 51 by May C. Chu and Robbin S. Weyant in the eighth edition of this Manual.

palaearctica), *F. tularensis* subsp. *mediasiatica*, and *F. tularensis* subsp. *novicida*. The last subspecies, a reclassification of *F. novicida*, does not yet have standing in taxonomy. In anticipation of its becoming validated and coming into common usage, *F. tularensis* subsp. *novicida* will be used in this chapter. The members of this genus have been associated with the natural outdoor environment, small mammals, and arthropods. *Wolbachia persica*, a bacterium isolated from *Argas persicus* ticks and two additional tick endosymbionts, from *Dermacentor andersoni* and *Ornithodoros moubata*, have 16S rRNA sequence similarity to *Francisellaceae* (64, 147).

Description of the Genus

The genus *Francisella* comprises tiny gram-negative coccobacilli that can be distinguished from other families by several features (Table 1). Members of the genus take up Gram's counterstain (safranin) poorly; they are strict aerobes, weakly catalase positive, nonmotile, and non-spore forming; and they react with a limited number of carbohydrates (Table 2). Only a few sugars (glucose, maltose, sucrose, and glycerol) are utilized by most of the members of the genus. Acid is produced without gas. Unique fatty acids are associated with the genus (Table 1) (83, 147, 169). For most species, in vitro growth is enhanced by sulfhydryl supplementation. The organisms in this genus show >98% 16S rRNA sequence similarity (147). Rare single plasmids have been associated with individual isolates of *F. tularensis* subsp. *holarctica* and *F. tularensis* subsp. *novicida* and have been found in two strains of *F. philomiragia* (M. Chu, unpublished observation).

Only a few key differences separate the two species of *Francisella* (Table 2). *F. philomiragia* is more biochemically reactive than *F. tularensis;* it differs also by its ability to ferment maltose, and it is oxidase positive using Kovács's modification. Subspecies differentiation is less distinctive; single biochemical differences in glycerol fermentation and glucose utilization are used to define the biovars. *F. tularensis* subsp. *tularensis*, designated type A, is the most virulent of the subspecies, with a 50% lethal dose of <10 cells for laboratory mice and rabbits (84, 89, 119). *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *mediasiatica* have the greatest similarity in that they utilize glycerol (2, 54), possess citrulline ureidase activity (141, 147), and along with *F. tularensis* subsp. *novicida*, share the same 16S rRNA signature nucleotide sequence (64, 141). *F. tularensis* subsp. *mediasiatica* differs by its inability to utilize glucose and its comparatively low virulence. Type B organisms (*F. tularensis* subsp. *holarctica*) may be differentiated by their inability to utilize glycerol, an absence of citrulline ureidase activity, and the presence of a unique 16S rRNA signature sequence. *F. tularensis* subsp. *holarctica* is of intermediate virulence, with a 50% lethal dose of <1,000 cells for laboratory animals (84, 89). *F. tularensis* subsp. *novicida* can be distinguished from the other subspecies by its ability to grow independently of cysteine supplementation and by its comparatively large vegetative cell size. *F. tularensis* subsp. *novicida* is considered to be of low virulence, like *F. tularensis* subsp. *mediasiatica* and *F. philomiragia*, but otherwise shares genetic characteristics with *F. tularensis* subsp. *tularensis* (147).

Epidemiology and Transmission

Francisellaceae are distributed widely within the Northern Hemisphere (Holarctic region) in a variety of natural habitats, from the Arctic Circle south to about latitude 20°N (84). Australian and Spanish isolates recovered from patients have been identified as *F. tularensis* subsp. *novicida* by using molecular techniques to augment the standard diagnostic tests (171; Chu, unpublished). *F. philomiragia* has been recovered from a patient with a history of travel to Turkey (69). Turkey has recently reported a waterborne outbreak in its western Black Sea region, an illustration of the continued appearance or reemergence of tularemia in the zone of endemicity (79).

Thus, as techniques and molecular tools are developed, it is likely that we can expect recovery of *F. tularensis*-like organisms from regions beyond the Northern Hemisphere. Tularemia is associated with a wide range of habitats and hosts, with more than 100 species of wild animals, birds, and arthropod vectors being involved (84, 90, 120). Habitats where the *Lagomorpha* (*Sylvilagus* [rabbits], *Lepus* [hares], and *Oryctolagus* [Old World hares]) and the *Rodentia* (water voles, muskrats, lemmings, voles, and beavers) thrive are important in maintaining tularemia enzootic foci (84, 90, 120, 179). Biting arthropod vectors, primarily tabanid flies (*Chrysops* and *Tabanus)*, ticks (*Dermacentor*, *Ixodes*, and *Amblyomma*), and mosquitoes (*Culicidae* in Europe and Russia), are implicated in the mechanical transmission of tularemia (53). Amebas also may carry viable *F. tularensis* cells and keep the bacteria viable in water (1). Because of the presence of *Francisella* in the natural environment, infections with *Francisella* are usually acquired in association with outdoor activities via contact with contaminated air, water, soil, vegetation, or sick or dead animals and from infective insect bites.

F. tularensis subsp. *tularensis,* the most virulent member of the genus, has been thought to occur only in North America, where it has been most closely associated with infection in lagomorphs and humans. Though *F. tularensis* subsp. *tularensis* has been recovered from mites and fleas in Europe, no human infection has been found there (159). In contrast, infection with *F. tularensis* subsp. *holarctica* is less virulent; it has a much wider distribution, in both the Old and New Worlds, and is associated with a greater variety of animals, primarily hares and rodents, and with contaminated environmental source outbreaks. *F. tularensis* subsp. *mediasiatica* is found only in circumscribed geographical regions in Kazakhstan and Turkmenistan and has been isolated from hares and ticks but not from humans (147). *F. tularensis* subsp. *novicida* has been found primarily in North America. It has also been found in Australia (171) and in Spain. Of the fewer than 20 known isolates of *F. philomiragia*, most have been from North America, with three single incidences reported from Central and Eastern Europe and Australia.

Since the 1940s, when several thousand cases annually were sometimes reported, the incidence of reported tularemia cases in the United States has steadily declined to 100 to 200 cases each year. All states except Hawaii have reported cases. From 1995 through 1999, tularemia was removed from the nationally notifiable disease list, but it was reinstated in 2000 because of concerns about its potential use as a bioterrorism agent. Despite its absence from the nationally notifiable disease list between 1995 and 1999, the number of cases reported annually to the CDC did not differ substantially from those in previous years. A total of 1,368 human cases were reported to the CDC between 1990 and 2000 (34).

The epidemiology of tularemia in the United States has not changed significantly in the past several decades. Most cases occur in south central and western states. It remains a rural disease. Most patients acquire tularemia from tick or deer fly bites, from contact with infected animals (e.g., by skinning rabbits), or as recently reported, from contact with

TABLE 1 Presumptive differentiation of *Francisella* and *Brucella* from similar gram-negative genera[a]

Test	*F. tularensis*	*Brucella* spp.	*Bartonella* spp.[b]	*Acinetobacter* spp.	*Psychrobacter phenylpyruvicus*[c]	*Oligella ureolytica*	*Bordetella bronchiseptica*	*Haemophilus* spp.[d]	*Ochrobactrum anthropi*
Oxidase	−	+	−	−	+	+	+	v	+
Urease	−	+	−	−	+	+	+	v	+
Gram stain morphology	Very tiny ccb	Tiny ccb	Thin rod	Broad ccb	Broad ccb	Small to tiny ccb	Thin rod	Small ccb	Medium rod
Specimen source	Ulcer, wound, blood, aspirates	Blood, bone marrow	Blood, bone marrow, lymph node	v	v	Urinary tract	v	Blood, cerebrospinal fluid, other	v
X and/or V factor requirement	−	−	−[e]	−	−	−	−	+	−
Cysteine enhancement	+	−	−	−	−	−	−	−	−
Motility	−	−	−	−	−	+[f]	+	−	+
Major CFA[g]	10:0; 14:0; 16:0; 3-OH-16:0; 18:1ω9c; 3-OH-18:0	16:0; 18:1ω7c; 18:0; 19:0cyc[h]	16:0; 17:0; 18:1ω7c	2-OH-12:0; 3-OH-12:0; 16:1ω7c; 16:0; 18:1ω9c	3-OH-12:0; 16:1ω7c; 16:0; 18:2; 18:1ω9c	3-OH-14:0; 16:0; 3-OH-16:0; 18:1ω7c	2-OH-12:0; 3-OH-14:0; 16:1ω7c; 16:0; 17:0cyc	14:0; 3-OH-14:0; 16:1ω7c; 16:0	18:1ω7c; 18:0; 19:0cyc; 2-OH-19:0cyc

[a] +, greater than or equal to 90% positive; −, less than or equal to 10% positive; v, variable (11 to 89% positive); ccb, coccobacilli. Data are from reference 169.
[b] Does not include *Bartonella bacilliformis*, which is the only motile species.
[c] Formerly *Moraxella phenylpyruvica*.
[d] *Haemophilus* spp. requiring X and V factors or V factor only.
[e] While not required, X factor (hemin) enhances growth for many strains.
[f] May be difficult to demonstrate.
[g] The number before the colon indicates the number of carbons; the number after the colon is the number of double bonds; ω indicates the location of the double bond counting from the hydrocarbon end of the carbon chain; OH indicates a hydroxy group at the 2- or 3-position from the carboxyl end; c indicate *cis* isomer; cyc indicates a cyclopropane ring structure. Hydroxy acids listed are at least 2% of the total CFA composition; all others are at least 10%.
[h] *B. canis* lacks 19:0cyc. CFA data for marine mammal strains are not available.

TABLE 2 *Francisella* spp. characteristics

Characteristic	*F. tularensis* subspecies				*F. philomiragia*[c]
	tularensis[a]	*holarctica*[b]	*mediasiatica*	*novicida*[c]	
Gram stain (culture)	Faintly staining, pleomorphic, single, rarely chained, gram-negative coccobacilli	As for subsp. *tularensis*	As for subsp. *tularensis*	As for subsp. *tularensis*	As for subsp. *tularensis*
Cell size (μm)	0.2 by 0.2–0.7	0.2 by 0.2–0.7	0.2 by 0.2–0.7	0.7 by 1.7	0.7 by 1.7
Growth on:					
Standard agar[d]	−	−	−	+	+
CA, 48 h	2–4-mm-diam, raised, gray, smooth, moist, butyrous, entire colonies; usually no agar discoloration	As for subsp. *tularensis*	As for subsp. *tularensis*	As for subsp. *tularensis* but 5 mm in diam	>5-mm-diam, white, smooth, mucoid, entire colonies; no agar discoloration
Cysteine heart blood agar, 48 h	2-mm-diam, pearl-white to ivory colonies with green tint and prominent opalescent sheen; smooth and butyrous; green-yellow discoloration of agar medium	As for subsp. *tularensis*	Not tested	5-mm-diam, colonies with features the same as those of type A and type B	Colonies are >5 mm in diam, creamy white-gray, mucoid, and smooth with purple-tinted opalescent sheen
Requires cystine/cysteine	+	+	+	−	−
Catalase	Weakly +	Weakly +	Weakly +	Weakly +	Weakly +
Oxidase (Kovács)	−	−	−	−	+
Acid from:					
Glucose[e]	+	+	−	+	+
Maltose	+	+	−	V[f]	+
Sucrose	−	−	+	+	V
Glycerol	+	−	+	+	+
Relative virulence (mice)	High	Intermediate	Low	Low	Low
DNA typing					
16S rRNA probe[g]	Type A	Type B	Type A	Type A	Type A
PCR primers[h]	Species specific	Species specific	Species specific	Species specific	Does not react
VNTR and IS probe[i]	Strain specific	Strain specific	Strain specific	Strain specific	Strain specific

[a] Type A, found predominantly in North America and associated with a severe form of disease in humans and rabbits.
[b] Type B, found throughout the Northern Hemisphere and associated with a less severe form of disease in humans; associated with humans and rodents (muskrats, field mice, beaver, voles, and water rats).
[c] Associated with low virulence and infrequently isolated.
[d] Standard bacteriology media: blood, tryptic soy, and brain heart infusion.
[e] Delayed or variable reaction. *F. tularensis* subsp. *mediasiatica* does not ferment glucose.
[f] V, variable or delayed reaction.
[g] Probes that define type A and type B sequences.
[h] PCR primers: Tul-4, T-cell epitope; FopA and p43,000 antigen.
[i] VNTR, variable number of tandem repeats: allelic variation, subspecies and strain specific. IS, insertion element PCR (type B specific) or Southern hybridization pattern (subspecies and strain specific).

pets and zoo animals (6, 36, 82, 87, 106). Cultures recovered in the United States from 1996 to 2001 comprise equal proportions of type A and type B genotypes, and these overwhelmingly predominate. Isolation of *F. tularensis* subsp. *novicida* and *F. philomiragia* is rare.

There is an increasing awareness of unusual occurrences of tularemia in animals and humans. Pet owners, pet owners' family members, and animal care professionals are at risk when handling sick pets. These pets may include both domestic (dogs, cats, and hamsters) and exotic animals (14, 36, 87, 106, 161). Captive prairie dogs caught in the wild have been implicated as well as commercially sold hamsters, monkeys (in monkey colonies), and zoo animals (56, 73, 82, 97, 114, 128, 129, 167). Transport of these animals poses risks to unsuspecting shipping handlers and the public of contracting an unusual and exotic disease, as illustrated by recently reported incidents

(6, 35). Cases of tularemia associated with immunocompromised patients or patients recovering from prosthetic replacement or transplantation surgery have been reported (42, 76, 102, 112, 126, 142). Additional reports on such unusual cases continue, with the recovery of *F. tularensis* subsp. *holarctica* from a patient with metastatic cancer (81) and from a kidney transplant patient (95) and a pneumonic case of *F. tularensis* subsp. *holarctica* in southern Europe. Patients with pneumonic tularemia associated with inhalation of aerosolized infectious particles are at risk of acquiring typhoidal tularemia (61, 157). Though we have gained much knowledge about the epidemiological association of lawn mowing and brush cutting with tularemia in areas like Martha's Vineyard where the disease is endemic, gaps remain in our understanding of the transmission dynamics of tularemia (61, 74, 103, 104).

Clinical Significance

F. tularensis subsp. *tularensis* and *F. tularensis* subsp. *holarctica* are the two members of the genus *Francisella* most commonly encountered in human disease. Tularemia has been known historically by a number of synonyms, such as plague-like disease, rabbit fever, deer fly fever, market men's disease, glandular type of tick fever, conjunctivitis tularensis, Ohara's or Yao-byo disease, and water rat trappers' disease. These synonyms attest to the variety of clinical presentations, the infectious agent's ubiquitous presence in nature, and the means by which humans may acquire the infection.

The clinical spectrum depends on the mode of transmission, the virulence of the infecting strain, the degree of systemic involvement, the immune status of the host, and timely diagnosis and treatment (159). Tularemia can be misdiagnosed early in infection since its symptoms are not unique: sudden onset of chills, fever, headache, and generalized malaise characterize each onset of illness. The differential diagnosis includes a wide range of infectious diseases, such as cat-scratch fever, syphilis, mycobacterial infections, anthrax, brucellosis, legionellosis, and plague. The incubation period averages 3 to 5 days, but onset may occur as early as 2 days to as late as 14 days after infection. Without treatment, nonspecific symptoms—sweats, chills, progressive weakness, and weight loss—usually persist for several weeks to months. Before the advent of antibiotics, the overall mortality from infections with the more severe type A strains was in the range of 5 to 15%; however, fatality rates as high as 30 to 60% have been reported for untreated typhoidal and pneumonic forms of the disease. In the United States, untreated type B strain infections were rarely fatal. The fatality rate for all forms in recent years has been less than 2% (49).

Patients may present with any one or more of the classically described forms of tularemia: ulceroglandular, glandular, oculoglandular, pharyngeal, typhoidal, and pneumonic. The most common form is ulceroglandular disease (45 to 80% of the reported cases), indicating that the portal of entry is via infective insect bite or other inoculation through the skin barrier, usually associated with handling contaminated materials. Pharyngeal lymphadenopathy suggests a portal of entry via ingestion of contaminated water or food, as was the case with the recent food- and waterborne outbreak in Kosovo (134). Typhoidal tularemia is the most difficult form to recognize because there is no identified portal of entry and localization signs are absent. Any of the initial forms of tularemia may be complicated by bacteremic spread, leading to various secondary tularemic symptoms such as pneumonia, sepsis, and meningitis.

Until 1989 *F. tularensis* subsp. *novicida* was defined by a single isolate recovered from water in Utah in 1951. This subspecies was not known to cause human disease. During a subsequent study of 16 unusual human bacterial isolates, 14 were identified as *F. philomiragia* and 2 were identified as *F. tularensis* subsp. *novicida* (83). These two human isolates, two isolates from Texas (37), and the molecular typing of additional isolates from the United States, Australia (171), and more recently Spain all suggest that our knowledge about the prevalence of *F. tularensis* subsp. *novicida* remains incomplete. Fever, chills, cough, shortness of breath, and chest pain are the more common symptoms described in those with airway-acquired disease. In a case from Australia, the organism was isolated from an abscessed toe. Isolates are recovered from blood, granulomatous tissue, and wounds. Patients may also present with skin lesions and cervical lymphadenopathy. Overall, cases are milder than those of disease caused by *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica*, but occasionally some are serious enough to warrant hospitalization.

F. philomiragia is an opportunistic agent infecting patients with a previous underlying condition that renders them more susceptible. More isolates have been recovered and identified since 2000, but overall, fewer than 20 cases have been described since the discovery of this species in 1974 (168). All but one case have involved a host with an impaired physical barrier to infection (near drowning) or impaired immunologic defenses (chronic granulomatous disease or myeloproliferative disease) (146). Most patients reported are from the United States (nine different states) (146). In addition, one case from Switzerland (168), one case from Australia (171), and a fatal water exposure-related case in a patient with a history of travel to Turkey (69) have been reported. In most cases *F. philomiragia* was isolated from normally sterile sites: blood, bone marrow, cerebrospinal fluid, and pericardial fluid. The drowning and water exposure cases were associated with salt water and brackish water, in contrast to *F. tularensis* infections, which are associated with freshwater sources.

Collection, Handling, Storage, and Transport of Specimens

Working with live *F. tularensis* can be hazardous. *F. tularensis* has a long and notorious history of infecting laboratory personnel working with infectious materials. Even though the use of biological safety cabinets and prophylactic antibiotic therapy (as well as vaccination, where available) provides safeguards for laboratory workers, these precautions have not fully eliminated laboratory exposures or modified behavior in the clinical laboratory to minimize risks (95, 140, 145). Clinical specimens should be handled under biosafety level 2 (BSL-2) conditions with universal precautions, with work being done using BSL-3 practices as soon as *F. tularensis* is suspected (50). Hundreds of laboratory-acquired infections have been documented, including infections of the pioneer scientists who first investigated the natural history of tularemia; they acquired infections from handling infected animals, removing arthropods from carcasses, and working with live cultures (17, 121, 140). The greatest risk of laboratory-acquired tularemia is by aerosol inhalation while working with infected materials and cultures. The infectivity of the organisms is high; *F. tularensis* should be handled only by trained and, preferably, vaccinated personnel.

Specimens should be stored chilled in appropriate medium until processed in the laboratory. Freezing of samples, unless they are kept at −80°C, is not recommended because of lysis of live bacteria upon thawing. Though *F. tularensis* may survive for long periods in the environment,

successfully culturing it from environmental specimens is difficult. Efforts should be made to maintain the integrity of such specimens and avoid delay in transporting them to the laboratory for testing. Ectoparasites may be stored intact in 2% NaCl and transported to the laboratory for identification and then processed for evidence of *F. tularensis*. Clinical specimens should be placed in Amies agar with charcoal, a commercial transport system designed for anaerobic and aerobic pathogens (BD, Franklin, N.J.). *F. tularensis* should remain viable for 7 days at ambient room temperature when stored in Amies medium (91). Stuart medium, designed for transporting gonococcal specimens, and saline are inadequate for keeping *F. tularensis* viable during transport (91). For PCR, specimens should be collected in guanidine isothiocyanate-containing buffer, which should preserve *F. tularensis* DNA for up to 1 month (91).

Direct Examination of Specimens

The *F. tularensis* subspecies cannot be clearly differentiated from one another by microscopy. Cultures and fresh clinical specimens (ulcer and wound swabs, touch-prep fresh tissues, and aspirates) but not thick blood or environmental samples can be directly examined. Under microscopic examination of Gram-stained specimens or cultures, *Francisella* cells (single and pleomorphic) appear so tiny and stain so faintly that they can be easily missed. Thus, direct Gram staining is usually of no diagnostic value. To improve visualization, direct fluorescent-antibody (DFA) staining using a labeled hyperimmune rabbit polyclonal antibody prepared by immunizations with *F. tularensis* subsp. *tularensis* cells will presumptively identify *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica*. This DFA reagent does not react with *F. tularensis* subsp. *novicida* or *F. philomiragia* or with *Legionella* species. Immunohistochemical (IHC) staining using a monoclonal antibody directed against the lipopolysaccharide (LPS) has been used successfully to visualize *F. tularensis* in formalin-fixed tissues in both intra- and extracellular sites (78). Neither the DFA reagent nor the IHC reagent is commercially available.

The presence of *F. tularensis* in specimens taken from ulcers, wounds, tissues, and the environment (animal feces and urine, water, hay infusion, mud, and ectoparasites) may be determined by detection of the *F. tularensis* LPS antigen by using an antigen capture enzyme-linked immunoassay (cELISA) or a lateral flow assay (127, 143). DNA detection by PCR directed at unique target regions has been widely applied (65, 91, 92, 125, 148, 163). The detection limit is 10^3 bacteria/ml for the cELISA and 10^2 bacteria/ml for PCR. These are specialized tests and are not widely available, although PCR technologies have become more commonly used for screening purposes.

Isolation Procedures

F. tularensis is not readily recovered by culture and is not easily identified even when it is cultured. The pathogenic *F. tularensis* bacteria are fastidious, requiring supplementation with sulfhydryl compounds (cysteine, cystine, thiosulfate, and IsoVitaleX) to grow on artificial medium. *F. tularensis* will grow well on cysteine heart blood agar supplemented with 9% chocolatized sheep blood (CHAB), chocolate agar (CA), buffered charcoal-yeast extract agar, and Thayer-Martin agar. It will grow in thioglycolate broth. When supplemented with 1 to 2% IsoVitaleX, general bacteriological media (tryptic soy broth and Mueller-Hinton broth) will support growth. The organisms grow slowly (60-min generation time); robust growth, therefore, is obtained by prolonged incubation (48 h or longer) or by plating on highly nutritive media such as CHAB. *F. tularensis* cultures should be incubated at 35 to 37°C aerobically and observed daily for 10 to 14 days; CO_2 does not impede growth. Incorporation of antibiotics (penicillin, 100,000 U/ml; polymyxin B sulfate, 100,000 U/ml; and cycloheximide, 0.1 mg/ml) into the medium will help prevent normal flora from overwhelming the *F. tularensis* organisms (125). Freezing of specimens combined with the use of antibiotics in the medium has allowed for a higher recovery rate than ever before (125). Complex protein or nutritionally enriched specimens such as blood or tissue will provide an intrinsic source of sulfhydryl compounds that permit *F. tularensis* growth. Upon subculture, the fastidious nature of *F. tularensis* will become evident as the exogenous compounds are depleted, leading to the loss of the bacterium's viability unless the subculture is propagated on cysteine-supplemented media. If a subculture fails to grow successfully on agar, inoculation of laboratory mice with a suspension of the original specimen or inoculated medium may assist in reviving the culture.

Specimens should be taken on the basis of clinical presentation and before administration of antibiotics. Fresh clinical material that is likely to contain high concentrations of *F. tularensis* organisms such as ulcer and wound specimens and lymphoid tissue (liver, spleen, or affected lymph node tissue) is inoculated directly onto an agar plate by using a sample-laden swab or bacteriological loop. Larger inocula will be necessary for the recovery of *F. tularensis* from specimens that contain a lower concentration of the organisms: blood, aspirates of pharyngeal washes, bronchial wash fluids, pleural fluids, urine, and environmental samples (159). A total of 3 to 10 ml of whole blood should be inoculated into blood culture bottles (147, 159). Environmental specimens, impinged air samples, and collected water are concentrated through a 0.22- or a 0.45-μm-pore-size nitrocellulose filter, and the filter-trapped organisms are released by vigorous agitation of the filter in less than 5 ml of sterile buffer. The inoculum should be spread onto as many agar plates as necessary at a seeding volume of 0.5 ml/agar plate. A portion of the inoculum (0.1 ml) may be injected subcutaneously into mice for enhanced recovery. Other methods for PCR and ameba culture from water have been discussed previously (1, 65). Preserved, formalin-fixed tissues should be examined by IHC staining (78).

Identification

Early recognition that an isolate may be *F. tularensis* is important from the standpoint of laboratory safety as well as helping to establish the cause of the patient's infection. There is also the possibility that a case of tularemia may be due to an act of bioterrorism. Prompt identification of an organism as *F. tularensis* may lead to an earlier recognition of such an event (chapter 9). The isolation of a poorly staining, tiny (indeed, individual cells may be difficult to discern), gram-negative coccobacillus (Fig. 1A) that produces 1- to 2-mm-diameter gray to gray-white colonies on CA after 48 h (and scant to no growth on blood agar) should raise the suspicion of *F. tularensis*. Further work on the isolate should be done in a biological safety cabinet. If further testing according to the algorithms given in chapter 9 does not rule out *F. tularensis* and the laboratory cannot do further confirmatory tests, the isolate needs to be sent to a reference laboratory that can confirm (or rule out) its identification as *F. tularensis*. Proper biohazard shipping procedures must be followed (chapter 9). In the United States, all states have at least one reference laboratory that is part of the Laboratory Response Network (LRN). These CDC- and APHIS-registered LRN laboratories are

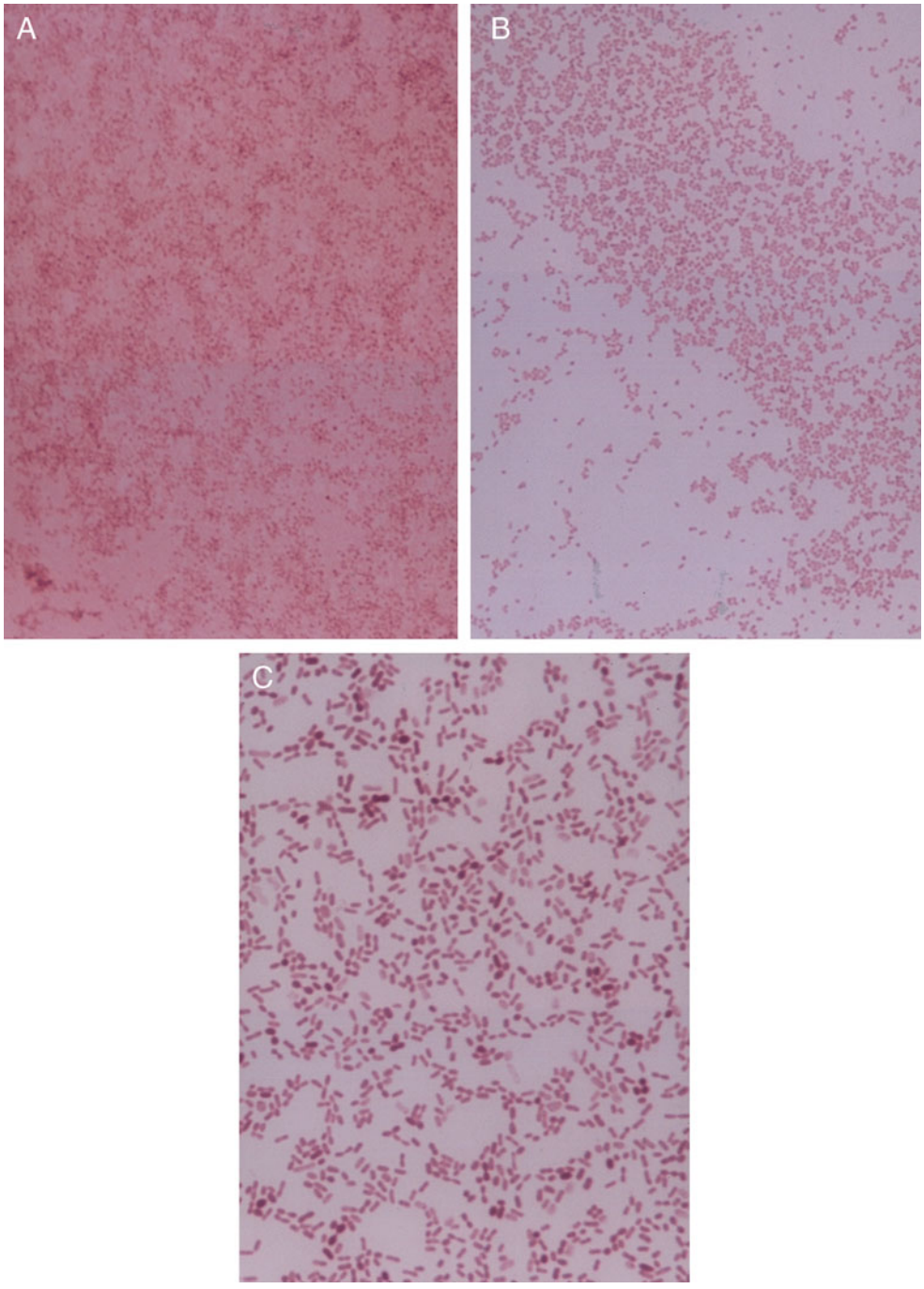


FIGURE 1 Gram stains of *F. tularensis* (A), *B. abortus* (B), and *Acinetobacter* species (C). Magnification, ×1,000.

able to confirm the identification of bacterial select agents, including *F. tularensis*. State public health laboratories can provide additional information, including location, about a state's LRN laboratories. See also http://www.bt.cdc.gov/lrn/.

A positive test result with any one of five methods—DFA staining (Fig. 2), IHC staining, the slide agglutination test, PCR, or a serology test for a single antibody titer—provides a presumptive diagnosis. Confirmation of infection would include identification of a culture as *F. tularensis* and/or a fourfold difference in titers in acute and convalescent serum samples, with one of the paired samples having a positive titer. The slide agglutination test presumptively identifies a suspect culture by mixing polyclonal rabbit anti-*F. tularensis* antibody with safranin-stained cells. Results of biochemical reactions are supportive of identification but are not reliable for confirmation since *Francisella* spp. are not very reactive. Fatty acid profiles may also be used to presumptively identify the organism as belonging to the *Francisella* genus (83, 147, 169). There is no specific bacteriophage lysis test for *F. tularensis*.

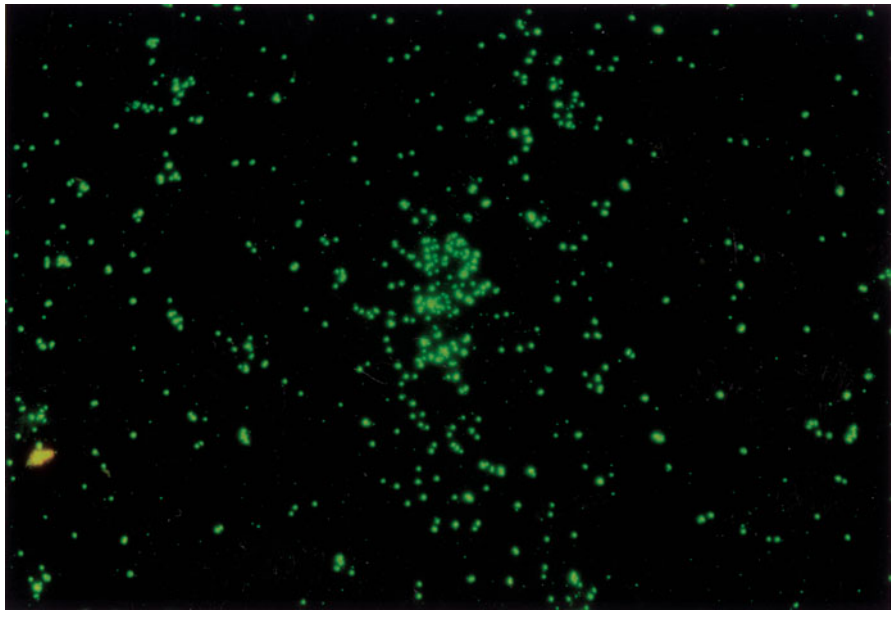


FIGURE 2 DFA staining of a culture of *F. tularensis*. Magnification, ×400.

Colonial morphology of *F. tularensis* is most distinctive when it is grown on CHAB. On CA, *F. tularensis* colonies have an entire edge; they are gray, smooth, raised, and moist, with a butyrous consistency. *F. tularensis* subsp. *novicida* grows more robustly than the other subspecies and is cysteine independent (Table 2). Colonies of *F. philomiragia* on CA are >5 mm in diameter, with an entire edge; they are white, smooth, raised, mucoid, and cysteine independent. On CHAB, *F. tularensis* exhibits a prominent and unique opalescent sheen due to its production of H_2S; this sheen is less prominent in *F. philomiragia* than in *F. tularensis* and is absent in cultures of other gram-negative organisms, such as *Yersinia*, *Brucella*, *Haemophilus,* and *Pasteurella* spp. (Fig. 3).

Typing Systems

F. tularensis biovars and *F. philomiragia* may be differentiated by protein profiling, 16S rRNA sequencing, various types of PCR approaches, and insertion element typing (60, 63, 99, 147) (Table 2). New approaches with the application of specialized PCR fragments targeting multiple-locus, variable-number tandem repeats have identified significant linkages and changes between *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* (92). Such analysis has revealed relationships between isolates demonstrated by geographical and ecological features and provides an interesting context for understanding the transmission and maintenance of *Francisella* in its environment. With the completion of the

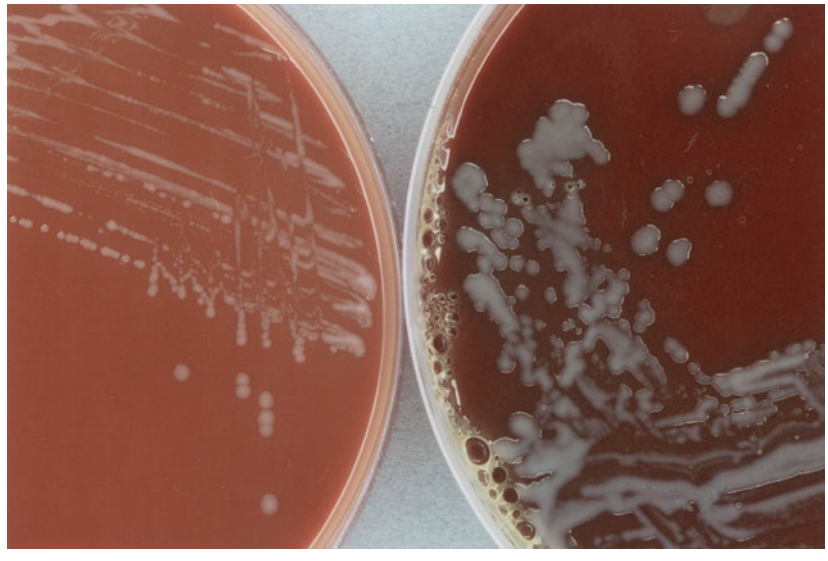


FIGURE 3 *F. tularensis* on CA (right) and cysteine heart blood agar (left) after 3 days of growth.

genomic sequencing of the prototypic type A strain (Schu 4) (98) and its comparison with the prototypic type B strain (live vaccine strain [LVS]), additional target areas may be identified for more precise typing and for study of strain pathogenesis. More recent application of technologies like DNA microarray analysis and surface-enhanced mass spectrometric methods will increase our ability to discriminate among and identify unique genomic regions for finer typing and characterization of the strains (25, 100).

Genus, species, and subspecies strains can be determined using 16S rRNA sequences, a method that has been applied in several instances to identify *Francisella* spp. successfully (69, 81, 145, 171). PCR is more sensitive than recovery by culture (83% compared to 62%) (91). Various PCR methods have been used to type isolates by genus, species, or individual isolates or small groups. Rapid PCR methods using the *Taq*man 5′ nuclease assay have a detection limit of <100 CFU under controlled laboratory conditions. These methods have been refined and applied widely in examining clinical specimens since 2003, with good correlation between culture and PCR results (125, 163).

Serologic Testing

Serology is the most common method by which *F. tularensis* infections are diagnosed (158, 159). Serum, plasma from citrated or heparinized blood, and filter paper-extracted serum proteins (172) can be used in serological assays. Antibodies may be detected as early as 1 week after onset of symptoms (about 2 weeks after infection). By 2 weeks after onset, antibodies may be detected in 89 to 95.4% of samples. Antibodies can persist for more than 10 years (17, 159, 166). Immunoglobulin M (IgM), IgA, and IgG antibodies appear simultaneously (158, 164). IgM antibodies can last for many years; thus, their presence does not indicate early or recent infection (17, 158). Agglutination testing, either by the tube agglutination (TA) or the microagglutination (MA) method, is the standard serology test for determining the presence of antibodies in tularemia (16, 27). A single specimen with a TA titer of ≥1:160 or an MA titer of ≥1:128 can be interpreted to be presumptively positive when compatible symptoms of tularemia are present and there is no history of vaccination or previous exposure. Confirmatory serodiagnosis requires a fourfold difference in titers between acute and convalescent specimens taken at least 14 days apart. One of the pair of serum specimens must show a positive titer by TA or MA. The formalin-killed whole-cell agglutination antigen (BBL Microbiology Systems, Cockeysville, Md.) may have low-level cross-reactivity primarily with *Brucella* antibodies, but the level of reactivity is typically <1:20 and will not interfere with interpretation of a positive result (6, 27, 118).

Enzyme-linked immunoassay (ELISA) formats that identify immunoglobulin class types and antigen detection have been adopted and updated for use in parts of Europe where tularemia is endemic (111, 127, 143). Although recombinant antigens have been used for ELISAs, the LPS extract remains the primary ELISA target to be used in recent test applications. Although ELISAs are more sensitive and specific than agglutination, their limitation of host specificity precludes their wide use in enzootic disease serosurveillance. Antigenic differences between the type A and type B biovars have not been demonstrated, although there is hope that such developments will be forthcoming as a result of the efforts in genomic sequencing and use of various DNA and mass spectrophotometric methods. The serology test materials thus react with anti-type A and anti-type B antibodies and usually do not react with anti-*F. tularensis* subsp. *novicida* and anti-*F. philomiragia* antibodies.

F. tularensis organisms are intracellular bacteria and are capable of eliciting both humoral and cell-mediated immunity (158). The latter response has been known to remain strong 25 years after infection (57). A skin test was previously used to measure specific reactivity by means of an intradermally injected ether extract of *F. tularensis,* but this test is no longer used. Host T cells retain proliferative responses to unique *F. tularensis* membrane proteins, with concomitant increase in interferon and interleukin-2 levels (57, 154, 155, 158). Tests for measuring the cell-mediated immune response are specialized and are not routinely used for diagnosis of tularemia. With the current emphasis on research with biodefense applications, however, new developments in this area for clinical use may be expected.

Antimicrobial Susceptibilities

Antimicrobial susceptibility testing of *F. tularensis* is not usually performed in clinical microbiology laboratories because of safety concerns in working with this organism (30, 121). Mueller-Hinton medium supplemented with 2% IsoVitaleX or CHAB must be used because of the fastidiousness of the pathogenic *F. tularensis*. This medium was recently evaluated as suitable for Clinical and Laboratory Standards Institute (formerly NCCLS) standards (26). Susceptibility of the *F. tularensis* isolate to the antibiotics recommended for treatment and prophylaxis, i.e., chloramphenicol, ciprofloxacin, gentamicin, streptomycin, and tetracycline, should be tested. No known naturally occurring strains resistant to the drugs recommended for treatment exist. Standardized methods have been published for susceptibility testing using broth, agar dilution (7), disk diffusion, and the E test (86, 93; chapter 73). No breakpoint standards have been set since there are no known naturally drug-resistant strains, except for erythromycin-resistant *F. tularensis* subsp. *holarctica* strains in Northern Europe (120).

F. tularensis infections are treatable with narrow-spectrum antibiotics, and drug resistance, with the exception of erythromycin resistance, in wild-type isolates is not known. Appropriate and early treatment, with streptomycin as the drug of choice, is effective. Streptomycin is given intramuscularly to adults in a dosage of 1 g every 12 h for 10 days (49). Gentamicin is an alternative drug that can be given intramuscularly at 3 to 5 mg/kg/day in equally divided doses at 8-h intervals for 10 days. Doxycycline or chloramphenicol may be used in combination with an aminoglycoside or used alone in less severe cases. Treatment with bacteriostatic agents, doxycycline, or chloramphenicol alone may result in treatment failures or relapses. An extended dosage schedule of 14 to 21 days may be necessary to prevent relapses. Ciprofloxacin has been used successfully to treat a limited number of patients; these clinical results, coupled with the low MIC of ciprofloxacin, suggest that this drug may become a useful addition to the armamentarium of drugs recommended for treating tularemia (156, 158). All *Francisella* isolates examined to date are β-lactamase positive, so penicillins and cephalosporins are not effective and should not be used to treat tularemia.

Prevention and Control

F. tularensis is a well-entrenched agent of enzootic disease that is unlikely to be eliminated even from limited natural foci. *F. tularensis* can survive for long periods in a cold, moist environment (15, 90, 120). Recently, extensive emphasis has

been placed on understanding the ecology and survival of and the reservoir for *F. tularensis* (1, 65). Avoidance of infective exposure is the principal means of preventing tularemia. The public should be educated about the risks for acquiring the disease in regions where tularemia is endemic and warned especially not to handle dead or sick animals, particularly rabbits, hares, and water rodents. Gloves and protective clothing should be worn when skinning or preparing such animals for food. People should be advised to cook these animals thoroughly before eating them. Repellents should be used to avoid arthropod bites, and ticks should be removed promptly. Chlorinated municipal water is safe from tularemia, but untreated water can be a ready source of infection; outbreaks have followed consumption of water from contaminated wells and streams and even consumption of freshwater crustaceans (4, 134).

Isolation of patients is not necessary, as person-to-person spread does not occur; therefore, treatment of close contacts of tularemia patients is not recommended (49). Universal precautions to avoid direct contact with contaminated secretions is advised. Laboratory personnel are at high risk of acquiring typhoidal or pulmonary tularemia via inhalation. As mentioned above, laboratory personnel should work in a class II biosafety cabinet when handling suspected tularemia specimens. All work should be performed under BSL-3 conditions once an isolate is confirmed (or even suspected) to be *F. tularensis*. People who have a recognized high-risk exposure (e.g., from a spill, aerosal spray, or puncture of skin) to *F. tularensis* should be given oral antibiotic prophylaxis. People subjected to low-risk exposure (e.g., handling a culture in the biosafety cabinet, preparing and making slides) should be placed on fever watch for 10 days and treated if they develop symptoms.

Vaccination may be advised for laboratory personnel who are working with live cultures and for others whose occupational duties put them at risk. A live attenuated vaccine has been used to immunize millions of people living in northern European countries and Russia. A variant of this vaccine, the LVS vaccine, has been used in the United States since 1959 for immunization of personnel who are at risk for laboratory-acquired infection. The vaccine is administered as a single dose of 0.06 ml by scarification. Antibodies develop within 3 weeks and may persist at reduced levels for months or years. Human volunteer studies demonstrated only partial protection against aerosol challenges after vaccination. A review of vaccination records at the U.S. Army microbiological laboratories from 1960 to 1969 revealed that there were 11 cases of laboratory-acquired tularemia (1 case per 1,000 at-risk employee-years) in vaccinated personnel (30). A recent update of this information also illustrates the protective value of vaccination (140). In the United States, vaccination was used for at-risk laboratory workers handling infective *F. tularensis* cultures. Retrospective evaluation of LVS-vaccinated people demonstrated protection against respiratory tularemia and apparent mitigation of the course of ulceroglandular disease (159). Vaccinated people maintain antibody and memory T-cell responses for at least 10 years. However, the LVS vaccine is not licensed and is not currently available in Western countries. With additional biodefense funding, several laboratories are developing vaccines using better-characterized live and killed strains and identifying and assessing the relevance of subcellular fragments for the next generation of vaccines. The challenge is that a yet-undefined mixture of T-cell-reactive bacterial proteins may be required for inducing protection (41, 122).

BRUCELLA

Taxonomy

The genus *Brucella* consists of small gram-negative coccobacilli that are pathogenic for humans and animals. In 1886 and 1887, Sir David Bruce, a British army medical officer working on the island of Malta, made the first isolation of *Brucella*, recovering it from the spleens of patients suffering from Malta fever (28). Bruce called this organism *Micrococcus melitensis*. In 1895, the Danish veterinarian Bernhard Bang isolated an organism, which he called *Bacillus abortus,* from cases of bovine abortion (8). In 1918, the American Alice Evans published her work demonstrating the close similarity of these two organisms (59). Shortly thereafter, Meyer and Shaw proposed the new genus *Brucella,* to include the agents of both Malta fever and Bang's disease as *B. melitensis* and *B. abortus,* respectively (107). In 1929, Huddleston described a third species, *B. suis,* encompassing strains originally isolated by Traum in 1914 from aborted swine (85, 160). Two additional species were identified in the 1950s: *B. ovis,* an agent of reproductive disease in sheep, and *B. neotomae,* isolated from the desert wood rat in Utah (29, 153). In 1968, Carmichael and Bruner described *B. canis,* a cause of canine abortion (31). In addition to host preference, these six species classically have been characterized phenotypically on the basis of their tolerance to dyes, requirement for CO_2, rate of urease reaction, lysis by the *Brucella* Tbilisi phage, H_2S production, agglutination by monospecific antisera, and rough-versus-smooth colony morphology (3).

Since the mid-1990s, several studies have described *Brucella* strains recovered from marine mammals. One of these studies suggested the name "*B. maris*" for these isolates (88). Others have proposed the names "*B. delphini*," "*B. pinnipediae,*" and "*B. cetaceae,*" based in part on the strains' host specificities (40, 109). None of these names have been validly published. These strains have been found to differ phenotypically from the six nomenspecies by their patterns of substrate-mediated metabolic activity (88).

Brucella, *Ochrobactrum,* and *Mycoplana* form the family *Brucellaceae. Mycoplana* species are soil organisms that have not been associated with human disease, and *Ochrobactrum anthropi* is a soil organism and an opportunistic human pathogen. The close phylogenetic relationship among the members of the family *Brucellaceae* suggests that they may have evolved from a common free-living soil organism. The *Brucellaceae* are part of the order *Rhizobiales,* which is in the class *Alphaproteobacteria* of the phylum *Proteobacteria* and the domain *Bacteria* (71). Other members of the *Rhizobiales* associated with human disease include *Bartonella* (family *Bartonellaceae*), *Afipia* (family *Bradyrhyzobiaceae*), and *Methylobacterium* and *Roseomonas* (family *Methylobacteriaceae*).

For most *Brucella* spp., the genome consists of two circular chromosomes of approximately 2.1 and 1.2 Mbp. In contrast to the larger chromosome I, the smaller chromosome II has an origin of replication and a genetic content that suggest that it was derived originally from a megaplasmid. The G+C contents of both chromosomes are 57%. The exception to the two-chromosome genome is that of *B. suis* bv. 3, which possesses a single circular chromosome of 3.1 Mbp with an overall genetic content similar to that of other *Brucella* species. Sequencing of the genomes of *B. suis*, *B. melitensis*, and *B. abortus* recently has been completed, and the sequences have been compared (48, 80, 124). Overall, the genomic content and organization are highly conserved

among the sequenced *Brucella* genomes and demonstrate similarities to the plant symbionts *Mesorhizobium loti* and *Sinorhizobium meliloti* and the plant pathogen *Rhizobium radiobacter (Agrobacterium tumefaciens)*. Chromosome I of *Brucella* contains approximately 2,100 to 2,200 open reading frames (ORFs), and chromosome II is predicted to have approximately 1,100 to 1,200 ORFs. Among the three sequenced *Brucella* genomes, the vast majority of the ORFs share greater than 99% sequence identity (80). However, through comparative genomic studies, a limited number of species-specific ORFs and variable ORFs, a significant number of which likely encode surface-exposed proteins, have been identified (80). The recent creation of an *Escherichia coli* library of 3,091 ORFs predicted from the genome of *B. melitensis* and the identification of ORFs encoding unique or highly variable proteins should greatly facilitate investigations into host tropism and *Brucella* pathogenesis, as well as the development of new diagnostic tests that target species-specific genetic sequences or polymorphic regions (52, 80).

The taxonomy of this genus is confounded by a dichotomy between the traditional (phenotypically based) and molecular definitions of its species. DNA relatedness and multilocus enzyme electrophoresis studies indicate that the six validly published nomenspecies, along with the recent marine mammal-derived strains, represent a single species (23, 70, 162). Recent molecular taxonomic studies show that although a high overall level of relatedness exists, the historically recognized species and the marine mammal strains can be differentiated by restriction polymorphisms of major outer membrane genes, insertion sequences, and whole-chromosome preparations (23, 165). This chapter follows the guidelines of the International Committee on Systematic Bacteriology subcommittee on the taxonomy of *Brucella* and retains the historical taxonomy to avoid confusion (43).

Description of the Genus

Brucellae are small, gram-negative coccoid rods, 0.5 to 0.7 μm in diameter and 0.6 to 1.5 μm in length. In Gram stains they are seen predominantly arranged singly (Fig. 1B). They are nonmotile. Their metabolism is aerobic, with oxygen or nitrate acting as the terminal electron acceptor via a cytochrome-based electron transport system. Some carbohydrates and polyhydric alcohols are oxidized; none are fermented. Nitrate is reduced, with some strains of some species producing gas. Strains are positive for catalase and, with the exception of *B. ovis*, *B. neotomae*, and some strains of *B. canis*, positive for oxidase. All species except *B. ovis* hydrolyze urea. Brucellae are negative for indole production, gelatin liquefaction, citrate utilization, and hemolysis. Growth on MacConkey agar is variable. The optimum growth temperature is 35 to 37°C. Chemo-organotrophic, most strains require complex media for growth, especially upon initial isolation. Growth may be improved by serum or blood; hemin (X factor) and NAD (V factor) are not required (3, 46).When grown on blood agar at an optimum temperature and under an optimum atmosphere, colonies are usually 0.5 to 1.0 mm in diameter, raised, and convex, with an entire edge and a smooth, shiny surface. *B. canis* and *B. ovis* characteristically produce rough colonies. Rough variants of the other species also occur (46).

Epidemiology and Transmission

Brucellae are facultative intracellular parasites, taking as their natural habitat a variety of animal species. *B. abortus* is found primarily in cattle but also occurs in camels, buffalo, yaks, and horses. Goats and sheep are the most common hosts for *B. melitensis*. This species is also found in alpacas and camels. In some parts of the world, cattle have emerged as a significant source of *B. melitensis* infections. It has been suggested that when this species occurs in cattle, smaller ruminants have provided the actual reservoir (45, 73, 178). The primary hosts for *B. suis* are swine (biovars 1, 2, and 3) and reindeer and caribou (biovar 4). In some countries, cattle have become a significant source of *B. suis* bv. 1 infection for humans. Maintenance of *B. suis* in cattle may depend on a concomitant pig reservoir (73). *B. canis* is usually isolated from kennel-bred dogs but also has been found in coyotes and foxes. Hosts for the marine mammal brucellae include seals, dolphins, and porpoises (40). Unlike the preceding taxa, *B. ovis* and *B. neotomae* are not known to cause human disease. The former has been isolated from sheep, the latter from the desert wood rat (73). Brucellae localize in their host's reticuloendothelial tissue and reproductive tract. Causing abortion, they are typically transmitted from one host animal to the next via contamination of the environment by the products of abortion (110).

Brucellosis has been described as the world's most widespread zoonosis, taking not only a serious human toll but also a severe economic toll when infecting domestic animals (73). The disease is highly endemic in the Middle East, the Mediterranean basin, Latin America, southeastern Europe, Asia, Africa, and the Caribbean (73). Though *B. abortus* has the broadest geographic distribution, *B. melitensis* causes more cases of human disease. This is attributed to bovine brucellosis's having been controlled more effectively than brucellosis in sheep and goats. In many developing countries, these small ruminants are the most important domestic animals and where they are kept, brucellosis is almost always present (45, 73). In the United States, the epidemiology of brucellosis has changed over the last 30 years. The success of programs to eradicate brucellosis in cattle, swine, sheep, and goats has decreased, though not eliminated, the risk of infection for farmers, veterinarians, and abattoir workers. Brucellosis has become primarily a foodborne disease caused by *B. melitensis* and associated with consumption of unpasteurized goat milk products from countries where caprine brucellosis is endemic (66, 132). Person-to-person transmission does not ordinarily occur but has been reported in connection with tissue transplantation and sexual intercourse (45, 175).

Brucellosis is usually transmitted to humans by direct contact through abraded skin or mucosal surfaces, consumption of contaminated food products, or inhalation. Brucellosis continues to be one of the most common laboratory-acquired infections. Practices associated with transmission in the laboratory include unprotected handling of specimens, sniffing of plates, mouth pipetting, and exposure of the eyes, nose, or mouth to infectious aerosols (50).

Clinical Significance

Of the species causing brucellosis in humans, *B. melitensis* tends to produce the most severe disease, followed by *B. suis* and *B. abortus* (110). While *B. canis* can cause clinical disease similar to that caused by the above-mentioned species, it has reduced virulence for humans (44, 175). Human brucellosis caused by marine mammal strains has been reported more recently (20, 150). The two community-acquired cases in one report presented as neurobrucellosis. The relative risk of infection with and pathogenicity of these strains for humans need further investigation.

The incubation period of brucellosis is usually 1 to 3 weeks but can be much longer. Symptoms are nonspecific.

They include fever (often undulating), malaise, night sweats, backache, muscle aches, and anorexia. The disease may affect a particular organ or organ system, causing osteomyelitis, hepatomegaly, splenomegaly, meningitis and other central nervous system symptoms, epididymo-orchitis, and endocarditis, among other complications. Although endocarditis is rare, when brucellosis is fatal, endocarditis is usually the cause. Chronic brucellosis can be defined as a disease in which compatible signs and symptoms persist for more than a year. Arguably, with brucellosis the definitions of acute and chronic are somewhat arbitrary (175). Though the fatality rate of brucellosis is now less than 2%, principally because of antibiotics, it remains a severe and debilitating disease (110).

Collection, Handling, Storage, and Transport of Specimens

Specimens for the diagnosis of brucellosis in humans include blood and bone marrow for culture and serum for serologic testing. Brucellae may also be cultured from spleen, liver, synovial fluid, or abscess specimens. For best results, multiple blood samples should be drawn during febrile episodes (110). The general collection, handling, storage, and transport guidelines for these specimens given in this Manual are applicable here (see chapters 5 and 20). Specimens for culture should be collected before the initiation of antibiotic therapy. Specimens that cannot be processed within 1 h of collection should be refrigerated. Acute-phase serum specimens should be collected as soon as possible after the onset of symptoms, and convalescent-phase specimens should be collected 14 to 21 days thereafter. Serum specimens should be shipped and stored cold or frozen. If PCR analysis is to be done, the collection method needs to adhere to the requirements of the particular assay method. Specimens from patients with suspected brucellosis should be labeled appropriately so that laboratory exposures to the disease agent can be minimized.

Direct Examination of Specimens

Direct microscopic examination of blood or bone marrow is not sufficiently sensitive to be useful in the diagnosis of brucellosis. There is no laboratory protocol for DFA staining. Direct detection methods using PCR assays have been described. When available for routine use, PCR-based tests will be particularly useful for laboratories serving areas of high endemicity (113, 131, 133). However, most PCR methods are capable of sampling only a small volume of specimen relative to that used to culture *Brucella*.

Isolation Procedures

Isolation of *Brucella* provides proof of the disease. Blood and bone marrow are the usual specimens for culture in cases of human brucellosis. Historically, bone marrow culture has been considered more sensitive, particularly in cases of chronic brucellosis and when the patient has already been treated with antibiotics (75). The greater sensitivity of modern automated blood culture systems may obviate this advantage. For example, in one study Ozturk et al., using the BACTEC 9240 system (Becton Dickinson Diagnostic Instrument Systems, Towson, Md.), cultured both blood and bone marrow specimens from 16 brucellosis patients. For 13 of the patients, *Brucella* was recovered from both specimens. For the remaining three patients, both specimens were culture negative. This study included cases in which antibiotics were given before culture but no chronic cases (123). These findings suggest that the more invasive procedure of bone marrow culture can be reserved for diagnostic problem cases in which blood cultures are negative.

Not all laboratories, particularly those in developing countries, will have an automated blood culture system. The classic biphasic blood culture technique of Casteñeda is one alternative. The culture bottle contains a broth medium with a layer of the same medium solidified with 2 to 3% agar on one side of the bottle (32, 110). Various studies have reported times to detection ranging from less than 6 days to more than 27 days by using this method (75, 137, 144).

Lysis-centrifugation involves osmotic lysis of erythrocytes followed by concentration of bacteria by centrifugation and direct plating of the concentrate onto culture media (19). This technique may be done by using the commercially available Isolator microbial tube (Wampole Laboratories, Cranbury, N.J.) or an in-house method. Lysis-centrifugation has better sensitivity and shorter time to detection than the biphasic bottle method. Since it is also technologically simple, this technique is arguably a better choice than the biphasic (Casteñeda) bottle method for laboratories that do not have an automated blood culture system (58, 101). Lysis-centrifugation also gave improved results compared to those of direct plating when synovial fluid was used for culture of *Brucella* (174). Compared with early versions of automated blood culture systems, such as BACTEC 460 and BACTEC NR, the lysis-centrifugation approach produced reduced times to detection, but in some cases the automated systems produced higher sensitivity rates (96).

Recent studies with the newer continuous-monitoring systems indicate that the BACTEC 9000 series is able to achieve times to detection that are shorter than those of lysis-centrifugation (>95% positive within 7 days of incubation) and has greater sensitivity (173). In general, modern automated blood culture systems can be depended on to detect *Brucella* within the routine incubation period used with these technologies (10, 11).

Because *Brucella* spp. have a slower growth rate than many of the bacteria encountered in the clinical laboratory, they seem more fastidious than they really are. They will grow on most of the media commonly used in this setting, including Trypticase soy agar, heart infusion agar, and brain heart infusion agar. Most strains will grow on Thayer-Martin or Martin-Lewis agar, which may be useful in recovering *Brucella* from contaminated specimens. Some strains will grow better with increased CO_2 (5 to 10%); *B. abortus* may require increased CO_2, particularly upon initial isolation.

Identification

Early recognition that an isolate may be a *Brucella* species is important for reasons of public health and laboratory safety as well as the patient's diagnosis. Brucellosis is a serious infectious disease that is frequently foodborne. Existence of a new case in the community suggests the possibility of additional cases, and *Brucella*, like *F. tularensis*, is considered a possible bioterrorism agent (chapter 9). Because of the hazard of laboratory-acquired infections, possible *Brucella* cultures should be handled using BSL-3 practices, with culture manipulations being done in a biological safety cabinet (50).

Fortunately, *Brucella* has some easily determined salient features that can lead to an early suspicion of the organism's identity. A Gram stain shows tiny, gram-negative coccobacilli. With the exception of *F. tularensis*, *Brucella* is the smallest gram-negative organism likely to be seen in the clinical laboratory. Its reputation for faint staining notwithstanding, *Brucella* may occasionally show a hint of gram positivity, especially with insufficient decolorization, leading to initial

misidentification (117). In Fig. 1 the size of *Brucella* (Fig. 1B) may be compared with that of *Acinetobacter* spp. (Fig. 1C). When streaked for isolation, discrete colonies are seldom seen in 1 day. Once evident, colonies are small (1 mm or less in diameter), translucent, soft, and easily emulsified. They are nonhemolytic on blood agar.

Brucella spp. are positive for oxidase and urease. Some of the oxidase- and urease-positive, non-glucose-fermenting, gram-negative taxa that along with *Haemophilus* spp. may be confused with *Brucella* spp. are listed in Table 1. Size difference is helpful in differentiating *Psychrobacter phenylpyruvicus (Moraxella phenylpyruvica)* from *Brucella* spp., the former being about the size of *Acinetobacter* spp. Both morphology and motility are useful in differentiating *Brucella* spp. from *Bordetella bronchiseptica* and *Ochrobactrum anthropi*. Urease-positive biotypes of *Haemophilus* spp., in addition to requiring X and V factor (or only V factor), differ from *Brucella* by not growing on sheep blood agar. *Oligella ureolytica*, also a small organism, may be more difficult to separate from *Brucella* spp. While *O. ureolytica* is described as motile, motility may be delayed or difficult to demonstrate. Most isolations of *O. ureolytica* are made from urine, an unlikely source for *Brucella*. Also, when tested in oxidation-fermentation medium, *Brucella* spp. produce acid from xylose and *O. ureolytica* does not. In all these instances, CFA analysis is another useful differential test. Laboratories using identification kits or automated identification systems need to know if the database includes *Brucella* spp. With most systems, at present, *Brucella* is not included. In the past, some of these test methodologies have been reported to misidentify *Brucella* variously as *Haemophilus influenzae* biotype IV, *Psychrobacter phenylpyruvicus*, and *Ochrobactrum anthropi* (9, 12, 55, 136).

If an identification as *Brucella* sp. cannot be ruled out (also see chapter 9) and the laboratory is unable to do further confirmatory tests, the isolate should be sent to a reference laboratory that can definitively identify and determine the species of brucellae. Use proper biohazard shipping procedures (chapter 9). In the United States, LRN laboratories are able to confirm and identify *Brucella* spp. to the species level. Again, state public health laboratories can provide additional information, including location, about a state's LRN laboratories. See also http://www.bt.cdc.gov/lrn/.

Confirmation of a culture as *Brucella* sp. can be done serologically. A suspension of the isolate equivalent to a 2 McFarland standard is made up in 1% formalin saline. The suspension is allowed to stand for 24 h, which kills the organism. The resulting product is used as an antigen in a TA test using a positive control serum such as Difco/BBL Brucella Positive Control Serum AMS (BD Diagnostics, Sparks, Md.). *B. canis* is not agglutinated by this antiserum, but this procedure will confirm as *Brucella* those species that commonly cause human brucellosis and that cause the most severe human disease, *B. melitensis*, *B. suis*, and *B. abortus*. Serologic cross-reactions have been reported with *Afipia clevelandensis*, *Vibrio cholerae*, *Yersinia enterocolitica* O:9, and *F. tularensis* (51, 178). *A. clevelandensis* is motile and its cells are larger than those of *Brucella*; *V. cholerae* and *Y. enterocolitica* are motile, glucose-fermenting, medium rods; *F. tularensis* is easily differentiated from *Brucella* by the characteristics given in Table 1.

Tests for the species-level identification of *Brucella* include sensitivity to the dyes thionin and basic fuchsin, rapidity of urea hydrolysis, H_2S production, lysis by Tbilisi (Tb) phage at the routine test dilution (RTD) and the RTD $\times 10^4$, and determination of rough versus smooth phase. Dye sensitivities are tested by swabbing a saline suspension of the test organism in a line across a plate of medium such as Trypticase soy agar or heart infusion agar containing the appropriate dye, typically at a concentration of 20 μg/ml. Known cultures of *B. melitensis*, *B. suis*, and *B. abortus* should be included as controls. The rate of urease activity can be tested conveniently by heavily inoculating a Christensen's urea agar slant with fresh growth and leaving it at room temperature for 5 min. A positive reaction will be seen with *B. suis*, *B. canis,* and some strains of *B. melitensis* within this time period. *B. abortus* and most strains of *B. melitensis* will need an additional incubation at 35 to 37°C, up to overnight, to become positive. Production of H_2S is detected using a lead acetate paper strip suspended over an inoculated slant of medium such as heart infusion agar. Read and change the strip daily for up to 6 days or until the strip blackens, indicating a positive result. Tbilisi (Tb) phage, available from the American Type Culture Collection (ATCC 23448-B1), lyses *B. abortus* when used at its RTD. It will also lyse *B. suis* when used at the RTD $\times 10^4$. The RTD is determined by making serial 10-fold dilutions of the phage and applying a 10-μl drop of each dilution to a plate inoculated for confluent growth with a suspension of a known *B. abortus* culture. The highest dilution giving confluent lysis is the RTD. The test organism is similarly inoculated onto a plate for confluent growth, and a 10-μl drop of the RTD and of the RTD $\times 10^4$ of the phage is applied. *B. canis*, a rough organism, will agglutinate in acriflavine solution; *B. melitensis*, *B. suis*, and *B. abortus*, typically smooth species, will not. For this test, suspend a small amount of growth in a drop of a freshly prepared solution of 1:1,000 neutral acriflavine in distilled water and observe for agglutination (3, 169). For additional details on these tests, see the monograph by Alton et al. (3).

Table 3 summarizes the characteristics of the four validly named species that cause human disease. The dye sensitivity and H_2S reactions noted for *B. suis* are for biovars 1 and 3 and those for *B. abortus* are for biovar 1. These are the biovars of these two species most commonly encountered in human brucellosis (44). For the reactions of the other biovars of these two species, see Alton et al. (3). With *Brucella* identification, as with any bacterial identification, it is necessary to consider the phenotypic picture as a whole before coming to a conclusion. An additional useful test for the identification of *B. canis* is CFA analysis. While *B. melitensis*, *B. suis*, and *B. abortus* all have lactobacillic acid (19:0cyc) as

TABLE 3 Differential characteristics of *Brucella* spp.[a]

Test	*B. melitensis*	*B. suis*	*B. abortus*	*B. canis*
Dye sensitivity				
Thionin	R	R	S	R
Basic fuchsin	R	V[b]	R	S
Time (min) for urea hydrolysis	>5[c]	<5	>5	<5
H_2S production	−	V[d]	+	−
Lysis by Tb[e] phage at RTD	−	−	+	−
Lysis by Tb phage at RTD $\times 10^4$	−	+	+	−
Agglutination in 1:1,000 acriflavine	−[f]	−[f]	−[f]	+

[a] Data are from references 3 and 169. R, resistant; S, sensitive; V, variable.
[b] Biovar 1, sensitive; biovar 3, resistant.
[c] Some strains are positive in <5 min.
[d] Biovar 1, positive; biovar 3, negative.
[e] Tbilisi, a *Brucella* phage originally isolated in the former USSR.
[f] Occasional strains are rough.

a major component of their CFA profile, *B. canis* lacks this compound (169). Marine mammal strains of *Brucella* are smooth, are resistant to thionin and basic fuchsin, hydrolyze urea (not rapidly), do not produce H_2S, and are variable for lysis by Tb phage (110). Strains not lysed by Tb phage will resemble *B. melitensis*.

The traditional methods of species-level identification of *Brucella* are complex and labor-intensive, involve some hazard, and in part because of the slow growth rate of *Brucella* organisms, take some days to yield a final result. Molecular methods, which involve much less manipulation of viable organisms and yield results much more rapidly, are gaining greater acceptance as a supplement or alternative to the conventional methods of identification. A number of molecular tests are now available for the identification of *Brucella* to the genus and species levels. PCR assays targeting *omp43*, *omp31*, the 16S rRNA gene, 16S to 23S spacer regions, heat shock protein genes, and the perosamine synthetase gene have been developed to detect *Brucella* spp. (18, 47, 113, 116, 130, 131, 135). 16S rRNA gene sequencing of a large panel of isolates has shown perfect sequence conservation among *Brucella* spp., limiting the usefulness of this target for species identification (72). However, resolution to species level is possible through ribotyping, amplified fragment length polymorphism analysis, amplification and DNA sequencing of *omp2* and *omp25*, and PCR assays targeting species-specific insertions of IS*711* elements (22, 38, 39, 62, 77, 115, 130, 133, 170). Several PCR assays have been formatted for use on real-time PCR instrumentation that facilitates rapid detection and reduces the risk of amplicon carryover contamination (18, 115, 130, 131, 133).

Typing Systems

B. melitensis, *B. suis*, and *B. abortus* are further divided into biovars. The three biovars of *B. melitensis* are based on agglutination with monospecific antisera for the *B. abortus* A antigen and the *B. melitensis* M antigen. *B. suis* has five biovars, defined by H_2S production, dye sensitivities, and agglutination patterns with the monospecific antisera. Differentiation among the seven biovars of *B. abortus* uses these same characteristics plus testing for CO_2-dependent growth. See the monograph by Alton et al. for details on these test procedures (3).

Molecular typing methods offer advantages over biovar determination similar to the advantages that molecular biology-based tests have over conventional tests for genus- and species-level identification of *Brucella*. In addition, molecular typing offers much greater discrimination among strains than does biovar determination. Identification beyond the species level has been demonstrated by PCR, RNA mismatch cleavage, and Fourier transform infrared spectroscopy (21, 22, 108). A PCR-based method targeting genetic segments containing variable numbers of tandem repeats has been recently developed for *Brucella* strain typing and promises to provide a useful tool for facilitating epidemiological investigations (24).

Serologic Testing

Serologic testing is a valuable adjunct to culture for the laboratory diagnosis of brucellosis. Demonstration of a specific antibody response can provide useful diagnostic information, and the characterization of the response (predominantly IgM versus IgG) assists in differentiating acute infection from chronic disease (149). The serum agglutination test (SAT) is the most widely used test in the United States (178). This test can be modified to differentiate IgG from IgM titers by pretreatment of specimens with 2-mercaptoethanol. The SAT detects antibodies against *B. abortus*, *B. suis*, and *B. melitensis* but not *B. canis*. A fourfold rise in SAT titer between the acute- and convalescent-phase serum samples is indicative of brucellosis. For single specimens, an SAT titer of $\geq$1:160 is suggestive of brucellosis. Cross-reactions may be observed with antibodies directed against *F. tularensis*, *Vibrio cholerae*, *Yersinia enterocolitica*, and *Afipia clevelandensis* (51, 178). There are few reports of using the SAT in cases of human brucellosis of marine mammal origin. Of the three reported human cases, two were positive in the SAT and one was negative (20, 150). Other serologic assays that have been described include the Rose Bengal test, the Coombs anti-human globulin test, the complement fixation test, ELISA, and the rapid dipstick test (5, 149, 152). The rapid dipstick assay, a relatively simple screening procedure for *Brucella*-specific IgM, shows promise as a field test in areas without direct access to a reference laboratory.

Antimicrobial Susceptibilities

The development of antibiotic resistance by *Brucella* has not yet been demonstrated. This has been attributed in part to the lack of plasmids (110). Additional reasons for not routinely doing in vitro susceptibility testing with this organism include safety concerns, poor correlation between high levels of in vitro activity and clinical efficacy for many agents including β-lactams and quinolones, and a general lack of well-established interpretive standards (177).

Successful treatment of brucellosis requires long-term combination antibiotic therapy. The regimen that the World Health Organization recommends is doxycycline (200 mg/day) in combination with rifampin (600 to 900 mg/day, orally) for 6 weeks (45). The combination of doxycycline and streptomycin is also effective. In children younger than 8 years, treatment combining trimethoprim-sulfamethoxazole with an aminoglycoside has been successful, without the side effects of tetracyclines in this age group (178).

The question of prophylaxis often arises after potential laboratory exposure to *Brucella*. Universally accepted practices in these cases are lacking. Some have advocated a full 6-week course of antibiotics if there has been significant exposure (151). Use of doxycycline alone for 1 week immediately following exposure (180) as well as a 3-week course of the standard combination of doxycycline and rifampin (136) has been reported.

Evaluation, Interpretation, and Reporting of Results

Isolation of *Brucella* from a patient's blood, bone marrow, or other tissue establishes a diagnosis of brucellosis. Modern blood culture methods can be quite sensitive, especially in acute infections (123, 139). An isolate presumptively identified as *Brucella* should be immediately reported to the physician as a possible *Brucella* sp., and results of confirmatory testing and identification to species level should be reported as soon as these are available. With the SAT, a fourfold or greater rise in titer is considered to indicate positivity (33). A single titer needs to be interpreted in light of epidemiologic and clinical evidence. In many settings a titer of $\geq$1:160 is considered significant in a patient with compatible symptoms (33, 176).

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*Legionella**

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53

TAXONOMY

The *Legionellaceae* are composed of a single genus, *Legionella,* and 50 validly named species (http://www.bacterio.cict.fr/l/legionella.html) (Table 1). *Legionella pneumophila, L. micdadei, L. longbeachae,* and *L. dumoffii* are the most important from a clinical standpoint, with *L. pneumophila* causing more than 90% of the cases of Legionnaires' disease (LD). The *Legionellaceae* are most closely related to the *Coxiellaceae,* and these two families constitute the proposed order "*Legionellales,*" within the class *Gammaproteobacteria* and phylum *Proteobacteria* phy. nov. (http://sn2000.taxonomy.nl/Taxonomicon/TaxonTree.aspx?id=111537). *Coxiella burnetii,* the agent of Q fever, shares many characteristics with *L. pneumophila,* including intracellular parasitism and close homologies of several virulence genes (102). Some investigators proposed the use of *Tatlockia* and *Fluoribacter* as additional genera within the *Legionellaceae* (39), but a subsequent study of 16S rRNA demonstrated that the *Legionellaceae* are monophyletic (36); use of these genus names was never widely accepted and is of historical interest only. A number of *Legionella*-like bacteria have been described to grow only within free-living amoebae and have been designated LLAP, for *Legionella*-like amoebal pathogen. One of the LLAPs, *L. lytica,* has been shown to cause human disease, and four have been assigned novel *Legionella* spp.; three of these four have been grown axenically at low temperature (1).

DESCRIPTION OF THE AGENT

The *Legionellaceae* are a diverse group of mesophilic, motile, asaccharolytic, obligately aerobic, nutritionally fastidious gram-negative rods, sharing growth dependence for L-cysteine, growth enhancement by iron, and cellular branched-chain fatty acids and ubiquinones that are unusual for gram-negative bacteria (23). *L. pneumophila* is the most extensively studied of the *Legionella* spp., with relatively little known about most of the other *Legionella* spp. Almost all of the *Legionella* spp. have been isolated from aqueous environmental sources and about one-third of the 50 validly named species have been isolated from both humans and the environment. It is assumed that the natural reservoir of all the *Legionellaceae* is our aqueous environment and that humans are an accidental host of the bacterium. Environmental *L. pneumophila* is a facultative intracellular parasite of several different free-living amoebae, such as *Acanthamoeba* and *Naegleria,* existing in microbial consortia in biofilms and free-flowing water.

L. pneumophila grows optimally at 35 to 37°C and grows less well at temperatures ranging from 20 to 42°C. Growth on solid media is enhanced by increased humidity. Incubation in 2 to 5% CO_2 can enhance the growth of some, but not most, *Legionella* spp. Bacterial phenotype, including immunogenicity, cell size, and virulence, can be altered by growth at different temperatures (23).

Amino acids, rather than carbohydrates, are used as energy sources by the *Legionellaceae* growing in vitro; this is true for intracellular bacteria as well, despite the presence of putative carbohydrate utilization genes in the *L. pneumophila* genome (23). Primary isolation of all known *Legionella* spp. requires medium supplementation with L-cysteine, as does successful propagation of all but a few species. Iron supplementation of growth media is required for optimal growth, although many *Legionella* spp. can grow, albeit poorly, in the absence of the mineral. Growth of *L. pneumophila* is enhanced by the addition of α-ketoglutarate (0.1%) to media, via an unknown, nonnutritive mechanism.

Growth of *L. pneumophila* in artificial media can be inhibited by a number of extraneous factors. These include the presence of high (100 mM, or 0.6%) NaCl concentrations, toxic peroxides, products of other bacteria and fungi, and some lipids (23). In addition, optimal growth occurs over a very narrow pH range, from 6.7 to 6.9. Solid growth media contain activated charcoal to inactivate toxic lipids and peroxides and an organic buffer (MOPS [morpholine propanesulfonic acid] or ACES [*N*-(2-acetamido)-2-aminoethanesulfonic acid]) to reduce sodium content and provide the required pH. Preparation of growth media for *Legionella* spp. can be complex and is usually best left to competent commercial sources or to specialized laboratories.

EPIDEMIOLOGY AND TRANSMISSION

LD was first recognized as a distinct entity when epidemic pneumonia with a 15% fatality rate developed during and after a convention of Pennsylvania State Legionnaires in

* This chapter contains information presented in chapter 52 by Janet E. Stout, John D. Rihs, and Victor L. Yu in the eighth edition of this Manual.

TABLE 1 Selected characteristics of *Legionella* spp.[a]

Legionella sp.[b]	Isolated from humans[c]	No. of recognized serogroups	Color under long-wave UV light	Comments
L. adelaidensis	N	1	NC	
L. anisa	Y	1	BW/YG	
L. beliardensis	N	1	YG	
L. birminghamensis	Y	1	YG	
L. bozemanae	Y	2	BW	AN, *Fluoribacter bozemanae*[d]
L. brunensis	N	1	NC	
L. busanensis	N	1	NC	
L. cherrii	N	1	BW	
L. cincinnatiensis	Y	1	YG	
L. drancourtii	N	NK	NK	Amoebic pathogen; no axenic growth
L. drozanskii	N	1	NC	Grows at 30°C but not at 37°C
L. dumoffii	Y	1	BW	AN, *F. dumoffii*[d]
L. erythra	N	2	R	
L. fairfieldensis	N	1	NC	
L. fallonii	N	1	NC	Grows at 30°C but not at 37°C
L. feeleii	Y	2	NC	
L. geestiana	N	1	NC	
L. gormanii	Y	1	BW	AN, *F. gormanii*[d]
L. gratiana	N	1	NC	
L. gresilensis	N	1	YG	
L. hackeliae	Y	2	YG	
L. israelensis	N	1	NC	
L. jamestowniensis	N	1	YG	
"L. jeonii"	N	NK	NK	Amoebic endosymbiont
L. jordanis	Y	1	YG	Partial L-cysteine dependence with serial passage
L. lansingensis	Y	1	NC	
L. londiniensis	N	1	NC	
L. longbeachae	Y	2	YG	
L. lytica	Y	NK	BW	Grows at 30°C but not at 37°C
L. maceachernii	Y	1	YG	AN, *Tatlockia maceachernii*[d]
L. micdadei	Y	1	YG	AN, *T. micdadei*[d]
L. moravica	N	1	NC	
L. nautarum	N	1	NC	
L. oakridgensis	Y	1	YG	Partial L-cysteine dependence with serial passage
L. parisiensis	Y	1	BW	
L. pneumophila	Y	16	YG	Three subspecies recognized
L. quateirensis	N	1	NC	
L. quinlivanii	N	2	YG	
L. rowbothamii	N	1	BW	
L. rubrilucens	N	1	R	
L. sainthelensi	Y	2	YG	
L. santicrucis	N	1	YG	
L. shakespearei	N	1	NC	
L. spiritensis	N	2	YG	Partial L-cysteine dependence with serial passage
L. steigerwaltii	N	1	BW	
L. taurinensis	N	1	R/yg	
L. tucsonensis	Y	1	BW	
L. wadsworthii	Y	1	YG	
L. waltersii	N	1	NC	
L. worsleiensis	N	1	NC	

[a] Abbreviations: Y, yes; N, no; NK, not known; NC, no color; BW, bright blue-white; YG, pale yellow-green; BW/YG, some strains are BW and some are YG; R, dark red; R/yg, majority of strains are red, with remainder YG.

[b] All species listed, except "*L. jeonii,*" constitute validly published names. Several other species probably exist.

[c] Severely immunosuppressed patients may acquire infections with *Legionella* spp. not previously isolated from humans.

[d] AN, alternative name; while valid, the genus names *Fluoribacter* and *Tatlockia* are not in widespread usage.

Philadelphia in July 1976 (22, 33). Joseph McDade and colleagues at the CDC determined that a novel gram-negative bacterium was the cause of the outbreak (70). Neither the disease nor the bacterium was found to be novel, with the first known epidemic of LD having occurred in 1957 (78) and isolation of the bacterium having occurred multiple times from the 1940s on.

Environmental studies found that the bacterium was widespread in natural bodies of water and occasionally in high concentration in warm waters found in plumbing systems, water heaters, warm water spas, and cooling towers. Many different *Legionella* spp. exist in nature within free-living amoebae, and as a result these otherwise fastidious bacteria can multiply within the amoebae and be protected from biocides (87). *Legionella*-infected amoebae are often found in complex consortia of microorganisms within biofilms. The bacteria are present in very low concentrations in freely flowing cold water and biocide-treated waters but can multiply in warm, and especially stagnant, water. Devices that aerosolize these contaminated waters serve to disseminate the bacteria.

The usual route of infection is thought to be by aerosol inhalation, although microaspiration of contaminated water may also be a mechanism of acquiring the disease (22). The majority of community epidemics of LD are from *Legionella*-contaminated cooling towers or other aerosol-generating devices. Contaminated potable water systems, such as water heaters and warm water in pipes, can also be a major source of disease, especially in hospitals, although they are not usually the cause of explosive outbreaks of the disease.

The bacterial inoculum required to cause human infection or disease is unknown. Animal studies performed using broth-grown bacteria show the infecting dose to be in the range of 10 to 500 CFU and the fatal dose to be about 10^4 to 10^5 CFU. Since bacterial virulence for cells or animals can be enhanced by growth in amoebae or by infection with the spore-like form, the infective doses may be even lower for naturally grown bacteria (2, 38).

Despite the ubiquity of legionella bacteria in our environment, LD is an unusual cause of pneumonia. It is estimated that 0.5 to 5% of adults requiring hospitalization for pneumonia have LD. Passive reporting indicates that the disease incidence is from 4 to 20 cases per million people per year, and a prospective study estimated that the disease incidence is about 80 cases per million people per year, or between 8,000 to 18,000 LD cases annually in the United States (69). Sporadic community-acquired LD is much more common than epidemic-associated disease, in a ratio of about 4 to 1.

The incubation period of LD is estimated to be between 2 and 14 days, with a median value of about 4 days. A study of a large outbreak extended the incubation period to as long as 19 days, with a median value of 7 days (12).

L. pneumophila causes disease by infecting human mononuclear cells, primarily alveolar macrophages. After the bacterium is inhaled into the lungs, it invades lung macrophages and multiplies in them. Detailed descriptions of pathogenesis can be found elsewhere (22, 23).

CLINICAL SIGNIFICANCE

LD is a type of bacterial pneumonia caused by *L. pneumophila* and other *Legionella* spp. The pneumonia ranges in severity from mild to fatal, with an average fatality rate of 12% (4). Major risk factors for the disease include immunosuppression of the cellular immune system, cigarette smoking, overnight travel outside the home, use of well water, chronic heart or lung disease, and chronic renal failure.

LD cannot be readily distinguished from other forms of community-acquired pneumonia by clinical, roentgenographic, or nonspecific laboratory studies (22). Several attempts at developing a clinical scoring system to distinguish LD from other pneumonias have failed.

The severity of pneumonia at presentation, underlying diseases, and promptness of specific antibiotic therapy are important prognostic factors. Promptly treated, LD can be cured in 95 to 99% of cases occurring in otherwise healthy persons. Less than one-half of patients may respond if there is a delay in therapy, immunosuppression, or respiratory failure (22). Untreated disease causes death in about 15% of previously healthy patients and up to 75% of severely immunocompromised ones.

Prospective, randomized controlled studies of adequate size have not been performed to determine the optimal therapy for LD, so great reliance is placed on experimental tissue culture and animal model studies, as well as results of nonrandomized studies (18, 22). Erythromycin, clarithromycin, azithromycin, a tetracycline, telithromycin, levofloxacin, ciprofloxacin, gatifloxacin, and moxifloxacin all appear to have roughly equivalent efficacy for nonimmunocompromised outpatients with mild LD (22). The quinolone antimicrobials, especially levofloxacin, and azithromycin are the drugs of choice for severe disease and for immunocompromised patients (22, 74).

Pontiac fever is an acute influenza-like illness that has been associated with exposure to *Legionella*-containing environmental aerosols (32, 42). The etiology and pathogenesis of this disease are unknown, but it appears as if the disease is caused by inhalation of bacterial toxins, such as endotoxin, or perhaps an acute allergic reaction to a bacterium. Since multiple microorganisms and endotoxin have been found in aerosols causing Pontiac fever, it is unclear if *Legionella* spp. play any role at all in disease causation. Pontiac fever is self-limited, with no reported deaths, little to no need for hospitalization, and no need for antibiotic therapy.

COLLECTION, STORAGE, AND TRANSPORT

Expectorated sputum and other lower respiratory specimens are the most common sources of *Legionella* spp. Other, less common sources include pleural fluid and blood. Rare sources have included pericardial fluid, kidney, liver, spleen, myocardium, respiratory sinuses, skin and soft tissues, infected wounds, peritoneal fluid, bone marrow, and intestine. Culture of available sputum, bronchoscopy specimens, lung biopsy specimens, and pleural fluid should be routine for laboratory diagnosis of LD. Lung biopsy specimens have the highest yield but may be negative. Culture of expectorated sputum or other lower respiratory tract secretions, second in yield to lung biopsy, should always be performed for optimal detection of legionella infection. Pleural fluid has low yield but should be cultured if it is available. Routine culture of other specimens for *Legionella* spp. is not indicated unless there is a high clinical suspicion of the disease affecting these sites.

Sputum microscopic scoring criteria cannot be used to determine which sputum specimens should be cultured for legionella bacteria because of limited purulence and scanty secretions in patients with LD. Up to 80% of specimens culture positive for *Legionella* spp. may be rejected when using the criterion of the presence of sputum purulence for processing specimens (31, 54).

Urine for antigen detection should be collected in a sterile container (16). Boric acid preserves the antigen, but commercial urine transport systems containing boric acid

have not been studied for antigen preservation and freedom from interactions. The urine can be transported to the laboratory at room temperature if no more than a several hour delay is anticipated. Longer transport times require specimen refrigeration; urine specimens should not be frozen, as this may reduce test sensitivity and specificity.

Blood for serum antibody testing is collected in standard tubes and transported at room temperature(19). Test performance is not adversely affected by storage of the clotted unseparated blood at room temperature for several days. Long-term storage is at –20°C in aliquots to allow parallel testing without freeze-thawing, which can lower antibody levels.

Legionella is hardy and generally survives for up to a week in clinical specimens. Sputum and other respiratory tract specimens, including lung biopsy specimens, should be collected in sterile containers and transported to the laboratory promptly at room temperature. Transportation and storage should be at 2 to 5°C if more than a several hour delay is anticipated before the specimen can be plated. Very long-term storage is best at –70°C, although this can reduce bacterial concentration to below the level of detection in cases where the starting concentration is low or the specimen is primarily aqueous. Repeated freeze-thawing is harmful to the bacteria. Some tissues, especially spleen, contain growth-preventing substances and must be plated promptly; even overnight storage at 5°C dramatically reduces culture yield; however, this is not true of lung specimens.

DIRECT SPECIMEN EXAMINATION

Microscopy

L. pneumophila found in lung and sputum is a small coccobacillus to short rod, 3 to 5 μm in length (Fig. 1). This morphology is very different from that observed for the bacterium taken from a culture plate, which is usually a long, filamentous bacillus, 10 to 25 μm in length. *L. pneumophila* is difficult to detect by Gram staining of sputum or lung biopsy specimens. Use of 0.1% basic fuchsin, rather than safranin, greatly enhances the staining of the bacterium from culture plates, but even with use of this stain it is often very difficult to visualize the bacterium in sputum and tissues. Less than 0.1% of *L. pneumophila* present in lung tissue or sputum can be visualized by Gram staining using basic fuchsin. The small size of intracellular *L. pneumophila* bacteria, the form present in human tissues, makes visualization difficult with Gram stain, as does stain uptake by the surrounding proteinaceous material found in sputum and tissue.

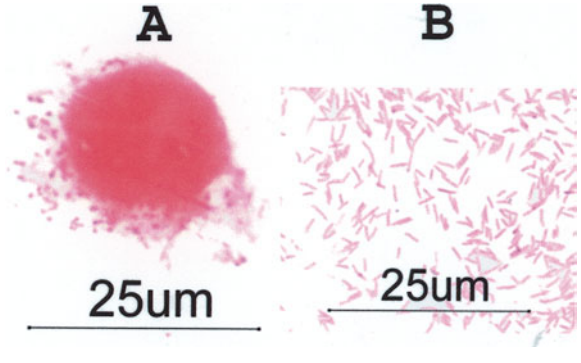


FIGURE 1 Photomicrographs of *L. pneumophila*. (A) Gimenez stain of intracellular bacteria in lung infection. (B) Gram stain using basic fuchsin counterstain of colony taken from BCYEα plate. Note the dramatic size and shape differences between the intracellular and extracellular bacteria.

Use of the Gimenez stain dramatically increases the number of *L. pneumophila* organisms detected in sputum and lung tissue, 100- to 1,000-fold over that visualized with Gram stain using basic fuchsin counterstain. Even so, this stain is very insensitive for the detection of the bacterium in sputum when compared to immunofluorescent microscopy (27). Sensitivity of the Gimenez stain for the detection of *L. pneumophila* in lung tissue has not been studied, but it appears to be high in fulminant cases. Unfortunately, the Gimenez stain is difficult to prepare, is not commercially available, requires filtration before each use, and can be unstable. Finally, the Gimenez stain cannot distinguish between *L. pneumophila* and other bacteria present in sputum and tissues. The stain detects the bacterium in Formalin-fixed, but not embedded, tissues (43).

Enhancement of bacterial staining by silver precipitate stains, such as the Warthin-Starry stain and its modifications and the Dieterle stain (29, 101), was an early approach to the detection of *L. pneumophila* in embedded tissues as well as sputum. These silver stains are useful for detection of the bacterium in embedded tissues but have no present role in the staining of the bacterium in sputum or other nonembedded specimens. Silver stains are not highly sensitive, can produce artifacts, and require expert use and interpretation for optimal sensitivity and specificity.

Immunofluorescent microscopy is the most sensitive and specific microscopic method for the detection of *L. pneumophila* in tissues and sputum (17, 27). Optimal sensitivity and specificity require exacting staining methods and great expertise by the microscopist. Polyclonal antibodies, which are commercially available, can be used to stain the bacterium in fresh, fixed, and embedded tissues and secretions. A commercially available monoclonal antibody (Bio-Rad, Hercules, Calif.) is useful for the highly specific staining of nonfixed tissues and secretions but loses sensitivity when used to stain fixed or embedded tissues (21, 41). Even when performed expertly, immunofluorescent microscopy is insensitive for the detection of *L. pneumophila* in sputum, detecting from 25 to 75% of culture-proven cases. False-positive tests with the polyclonal antibody can result from cross-reactions with non-*Legionella* bacteria, including *Pseudomonas aeruginosa, P. fluorescens, Bacteroides fragilis, Staphylococcus aureus, Bordetella pertussis, Bacillus* spp., lactobacillus-like bacteria, and candida-like yeasts; the number and variety of these potential cross-reactions make it imperative that the microscopist be skilled in distinguishing the morphology of *L. pneumophila* from those of the cross-reacting bacteria (6). Even so, some false-positive reactions will be recorded by very experienced microscopists. The monoclonal antibody has many fewer cross-reactions but may cross-react with *S. aureus* and *Bacillus* spp. In addition, *Legionella* organisms present in buffers, wash solutions, or Formalin, from specimen containers contaminated during manufacture, and from cross-contaminating, true-positive specimens can all cause false-positive tests. This means that extreme care needs to be taken to keep all slides physically separated from one another, to filter sterilize critical buffers and other aqueous solutions, and to screen specimen containers. Most important is performance of the staining and slide reading by an expert. Performance of these tests by inexperienced technologists can lead to many more false-positive than true-positive results. More details on immunofluorescent microscopy can be found elsewhere (17).

Antigen Detection

LD due to *L. pneumophila* serogroup 1 can often be diagnosed by detection of bacterial antigenuria (16). Several immunoassays are commercially available for this purpose, the most convenient of which is a rapid single-test immunochromatographic card assay (Binax, Scarborough, Maine). Several other assays utilize a microtube-based enzyme immunoassay. Only one kit (Biotest, Denville, N.J.) is designed to detect nonserogroup 1 *L. pneumophila* and appears to be somewhat sensitive for the detection of these infections (52). The strength of all these assays is their ability to detect *L. pneumophila* serogroup 1 infections, and in particular the Pontiac/MAB2/MAB3-1 monoclonal subtype (48–50). The immunochromatographic card assay may be somewhat less sensitive than the microtube-based immunoassays, although its convenience, ease of use, and robustness make up for a slightly lesser sensitivity.

Clinical test performance for all assays is dependent on the pretest probability of *L. pneumophila* serogroup 1 and on the probability of Pontiac monoclonal subtype *L. pneumophila* serogroup 1 infection (48, 50). Infection caused by *L. pneumophila* serogroup 1 Pontiac subtype is most common in epidemic and sporadic community-acquired LD and least common in nosocomial LD, especially that involving immunocompromised patients. About 80 to 90% of community-acquired LD is due to this subtype, whereas up to 60% of nosocomial LD may be caused by other *L. pneumophila* serogroup 1 subtypes, other *L. pneumophila* serogroups, and other *Legionella* species (48, 49). The assays detect about 60 to 70% of *L. pneumophila* serogroup 1 Pontiac monoclonal subtype epidemic infections and up to 90% of sporadic pneumonia caused by this subtype. The differences in test sensitivity for the same bacterial subtype are probably due to differences in disease severity, the other major factor determining test sensitivity. Patients with severe *L. pneumophila* serogroup 1 LD are the most likely to have positive urine antigen tests, for example, those requiring intensive care nursing and ventilator assistance; test sensitivity in this population is probably in the range of 90 to 95% of those infected with the Pontiac monoclonal subtype. On the other hand, urine testing may detect only 50% of outpatients with mild epidemic disease caused by the same monoclonal subtype, perhaps 40% of hospitalized patients with other *L. pneumophila* serogroup 1 subtypes, and fewer than 5 to 40% of those with infections caused by other serogroups and species, with the higher sensitivity value for testing being obtained with the Biotest kit (49, 52). The test may be negative during the first day of illness, but those with severe disease are likely to be positive on presentation to hospital. Repeat testing 2 to 3 days after the onset of illness may detect a small number of infections in patients who had negative tests initially.

Test sensitivity can be enhanced by concentrating the specimen by use of ultrafiltration devices such as Amicon concentrators (Millipore, Billerca, Mass.). In some studies this has increased test sensitivity by about 30%, without affecting specificity (14, 44). Test sensitivity decreases when specimens are frozen for weeks to months before testing.

The urine antigen assays are very specific, in the range of 99 to 99.9%. False-positive results can be due to urine rheumatoid-like factors, freeze-thawing of urine, and excessive urinary sediment. All together, these causes of false-positive results account for no more than a few percent of all positive results. Regardless, all positive results should be confirmed after boiling urine clarified by centrifugation.

Molecular Diagnosis of LD

PCR-based detection of *L. pneumophila* genes in sputum, urine, and blood has been successfully used in reference and research laboratories. The best results show that molecular diagnosis is a more sensitive method of diagnosis than culture, although some studies showed rough equivalence (73). Test sensitivities have been estimated to be 80 to 100%, 30 to 50%, and 50 to 90% for lower respiratory secretions, serum, and urine analytes, respectively; test specificities are estimated to be >90% (73). Both conventional and real-time assays have been utilized (51, 73). Until recently, only home brew assays were available.

One commercial assay, the BD ProbeTec ET *Legionella,* based on the Becton Dickinson ProbeTec methodology, is cleared for marketing by the U.S. FDA (BD Diagnostics, Sparks, Md.). Test sensitivity for 23 culture-proven archived sputum specimens was 91% (95% confidence interval [CI], 72 to 99%), and specificity was 87% (95% CI, 75 to 94%) in one study and 100% (95% CI, 96.8 to 100%) in another study. Several sputum specimens that were apparent false positives were also positive by a second molecular method (U.S. FDA 510K k033861 clearance letter, March 2004; http://www.fda.gov/cdrh/reviews/K033861.doc).

ISOLATION PROCEDURES

Specimen Plating

Optimal yield of *Legionella* spp. from clinical specimens usually requires that specimens be diluted to reduce inhibition by tissue and serum factors as well as antibiotics; that the specimen be pretreated to reduce contaminating flora; and that a variety of selective and nonselective media be used (Table 2). Culture of *Legionella* spp. from normally sterile fluids and tissues, such as pleural fluid, aseptically obtained lung tissue, or blood, is often successful without the use of multiple selective tissues and specimen decontamination.

Dilution (1:10) in tryptic soy broth increases the culture yield of most specimen types including sputum and other liquid respiratory tract specimens, lung tissue, lymph nodes, spleen, and probably other organs such as liver and kidney. Sputum and other respiratory specimens should first be examined in a petri dish for material that appears purulent, and this material should be selected for culture. Tissues (about 1 g) are ground in a tissue grinder with a small amount (1 ml) of broth, which adequately dilutes most tissues except for spleen; this tissue requires an additional 1:10 dilution for the best recovery of bacteria. Liquid specimens are roughly diluted by adding 3 drops of vortex-mixed liquid specimens to 0.9 ml of the dilution broth. Pleural fluid, joint fluid, and blood subcultured from blood culture bottles do not require dilution before plating; in fact, pleural fluid yield may be enhanced by concentration by centrifugation.

Decontamination, by either of two methods, is required to reduce contaminating flora in most sputum specimens and other respiratory tract secretions. The first method consists of diluting (1:10) the specimen in a low pH KCl-HCl buffer (pH 2.2) and incubating the mixture at room temperature (4.0 min) before plating the suspension onto culture media. Timing is critical here, since a low yield may result if the timing is off by as little as 1 min. The culture medium is sufficiently buffered so that the acidified specimen is neutralized upon being plated. The second method, an alternative to specimen acidification, consists of heating the specimen at 50°C for 30 min. Most aseptically collected tissue specimens do not require decontamination, although occasionally lung tissues contain multiple contaminating bacteria and fungi. In this case, heat or acid treatment of tissue ground in sterile distilled water may help; sometimes dilutions of the ground tissues are also required for optimal yield, with or without pretreatment.

TABLE 2 Composition and selectivity of media used to grow *Legionella* spp. from clinical and environmental specimens[a]

Medium	Synonym	Selective agents	Main use	Selectivity[b]
BCYEα	CYE	None	Clinical, culture maintenance	None
BMPA	PAC	Cephamandole, poly B, antifungal	Clinical, environmental	Normal respiratory flora, 3+; enterics, 3+; yeasts, 3+; molds, 1+; *Legionella* spp., 1+ to 4+
PAV	VAP	Vanco, poly B, antifungal	Clinical, environmental	Normal respiratory flora, 2+; enterics, 2+; yeasts, 3+; molds, 1+; *Legionella* spp., 1+
MWY	VGP	Vanco, poly B, antifungal, glycine	Environmental	Normal respiratory flora, 2+; enterics, 2+; yeasts, 3+; molds, 1+; environmental bacteria, 2+; *Legionella* spp., 1+
CCVC		Cephalothin, poly E, vanco, cycloheximide	Environmental	Normal respiratory flora, +; enterics, 3+; yeasts, 2+; molds, 2+; *Legionella* spp., 1+ to 4+
BCYEα-L		None (made without L-cysteine)	Organism identification	*Legionella* spp., 4+ (no growth of *Legionella* spp. on this medium)

[a] Abbreviations: poly B or E, polymyxin B or E; antifungal, either anisomycin or natamycin antifungal compounds; vanco, vancomycin; normal respiratory flora, normal upper respiratory tract bacteria.
[b] Selectivity scale range, 0 to 4+: 0, does not inhibit these organisms; 1+, slight inhibition, allows about 75% growth; 2+, allows about 25 to 50% growth; 3+, allows about 10% growth; 4+, allows less than 1% growth.

Inoculation of Plates

Approximately 0.1 ml is inoculated into each plate, with the bulk of the inoculum applied to the first quadrant. There is a lack of comparative studies showing whether it is better to streak plates for isolation or to uniformly distribute the inoculum over the entire plate. The plates must be thoroughly dry before being inoculated, to aid in absorption of the relatively large volume inoculum and to retard spreading of contaminants throughout the plate.

Culture Media

Buffered charcoal yeast extract medium, usually supplemented with 0.1% α-ketoglutaric acid (BCYEα), is used for isolation and growth of *Legionella* spp. Use of BCYE without α-ketoglutarate supplementation cannot be recommended for clinical use, as this amino acid greatly enhances growth of the bacterium (25).

BCYEα can be made selective by the addition of antimicrobial agents (Table 2). A variety of different antifungal agents are used in the media. Cycloheximide is a poor choice for media used for clinical specimens as it fails to inhibit *Candida albicans*. Both anisomycin and natamycin inhibit more yeasts than does cycloheximide. This dizzying array of media (Table 2) exists because no one selective medium is best for all purposes. Optimal yield of *Legionella* spp. from clinical specimens requires the use of three different media, one nonselective plate medium (BCYEα) and two selective media (BMPA and MWY). BMPA is an excellent selective medium for the vast majority of *L. pneumophila* strains, but the cephamandole present in the medium inhibits the growth of some other *Legionella* spp. and very rare *L. pneumophila* strains. Therefore, use of the less selective medium, MWY, is required for optimal growth of some *Legionella* spp. other than *L. pneumophila*. No selective medium inhibits multiresistant gram-negative bacteria found in hospitals, reducing culture yield in nosocomial disease.

Selective and nonselective media are optimized for the isolation of *L. pneumophila,* and their performance for the isolation of other *Legionella* spp. is not accurately known. One study showed that *L. micdadei* in guinea pig spleen had enhanced recovery on BCYEα medium prepared with 1% bovine serum albumin; the growth was enhanced because of decreased growth inhibition by spleen tissue (71). Whether addition of bovine serum albumin to BCYEα medium enhances *L. micdadei* recovery from human lung or sputum is unknown and probably unlikely. A BCYEα-based selective medium containing natamycin, aztreonam, and vancomycin has been reported to be useful for the isolation of *L. longbeachae* from soil (88).

Medium shelf life is around 1 year for nonselective plates and slants. This long shelf life requires thick plates (25-ml pour), complete drying of plates before storage at 2 to 4°C in sealed plastic bags, and protection from light. Selective media lose selectivity after about 3 months' storage time, but depending on the antibiotic used, they may last considerably longer.

Quality control (QC) testing of media is required before they are put into use. Current Clinical and Laboratory Standards Institute (CLSI) (NCCLS) standards are inadequate for proper QC testing of these media. About 1% of commercial media fail our laboratory QC testing. The CLSI QC testing protocol utilizes a heavy inoculum of medium-adapted *Legionella* strains and a growth/no-growth test. Minor variations in the manufacture of media, such as the addition of excess salt, overlong autoclaving, and degradation of buffers, can all seriously affect the ability of the medium to support wild strain growth, but not necessarily that of medium-adapted strains. The optimal method for medium QC testing is the inoculation of the test media with several hundred non-artificial medium-passed *L. pneumophila* bacteria (obtained from infected guinea pig lung) and quantification of the bacterial colonies after 3 to 4 days of incubation (20). In the absence of the availability of lung-passaged *L. pneumophila* bacteria, low-passage clinical strains should be used, taking care to plate only several hundred bacteria per plate. QC testing of selective media for the ability to suppress non-*Legionella* bacteria can be done by inoculation of the plate with relatively antibiotic-susceptible strains of *Escherichia coli* and *Staphylococcus aureus*, such as ATCC 25922 and 25923; the growth should be markedly suppressed.

Medium Incubation

Inoculated media are incubated at 35 to 37°C in humidified air. Regardless of the humidification method, care must be taken to keep the incubators or jars very clean and to regularly sterilize the containers or incubators. A small amount of CO_2 supplementation (2 to 5%) may enhance the growth of some of the more fastidious *Legionella* spp., such as *L. sainthelensi* and *L. oakridgenensis*. This low level of CO_2 supplementation will not harm the growth of *L. pneumophila,* but CO_2 levels higher than 5% may inhibit growth. Since the more capnophilic species are very rare human isolates, many laboratories do not use CO_2 incubation of media for *Legionella* spp.

Plate Inspection

Legionella colonies begin to appear on culture plates on day 3 of incubation. It is very unusual for the bacterial colonies to appear on plates after five incubation days. Some very rarely isolated *Legionella* spp. may require up to 14 days of incubation before growth appears; this is an extremely rare event. Regardless, it is reasonable to inspect culture plates on days 1 to 5 and then again at day 14.

The late appearance of *Legionella* spp. on culture plates can be used to great advantage if a careful record is kept of the colonies present on days 1 and 2 postincubation. New colonies appearing after day 2 should be suspected of being *Legionella* spp. Very rarely *Legionella* spp. may grow from heavily infected lung (usually from the autopsy of a fatal untreated case) on day 2, so some latitude in growth rate assumptions needs to be applied in the case of autopsy lung cultures. *Legionella* spp. never grow from clinical specimens on day 1 postincubation, a critical point in the distinction of *Pseudomonas aeruginosa* colonies from those of *Legionella* spp., as very early colonies of the latter superficially resemble those of the former.

Proper observation of culture plates requires the use of a dissecting microscope illuminated with direct light aimed at the plate surface at approximately a 30° angle. Failure to use a dissecting microscope or use of improper lighting will result in missed positive cultures, especially when there is mixed bacterial growth on the plates. In addition, very young *Legionella* colonies are very small and difficult to see with the naked eye. Therefore, use of a dissecting microscope can speed up the time to colony detection by as much as 1 day. *Legionella* growth occurs almost exclusively in the first streak quadrant, and sometimes at the edge of the plate.

The size and morphology of *Legionella* colonies change with time. Very young colonies (day 3) are flat, entire, and 0.5 to 1 mm in diameter, and when observed using a dissecting microscope and incident visible light they usually have a speckled blue, blue-green, or red color. Within 6 to 24 h of additional incubation, these colonies become smooth, convex, iridescent, and entire, measure about 1 to 3 mm in diameter, and look opal-like when observed with a dissecting microscope (Fig. 2). A thick string may form when a loop is inserted in the colony and then removed from the colony. In contrast to several mimics, the edges of the colonies are of the same consistency as the central portion and are not watery and clear. In another 1 to 2 days the colonies may increase in size up to 5 to 7 mm, become umbonate, sometimes with tuberculated or inhomogeneous texture, and develop spready edges; their iridescent nature may be lost at this stage. It is these late-stage colonies that are most difficult to distinguish from non-*Legionella* bacteria, making daily plate observation crucial for accurate detection. Very rarely some *Legionella* colonies do not change morphology with prolonged incubation.

Biosafety level 2 precautions should be used for manipulation of *Legionella* spp. cultures. It is safe to inspect culture plates and pick and subculture colonies on the open bench in a properly ventilated laboratory. Making an organism emulsion on microscope slides for the purposes of Gram staining can be safely carried out on the open bench. However, vortexing suspensions, sonication, tissue grinding, primary plating, and manipulations that may result in generation of a high-concentration aerosol should be performed in a biological safety cabinet. No well-documented cases of laboratory-acquired LD have been reported.

Initial Workup of Suspect Colonies and Look-Alike Bacteria

Colonies suspected of being *Legionella* spp. should first be subjected to Gram staining to ascertain that the bacteria are small or sometimes filamentous gram-negative rods. A small amount of the colony should be emulsified in sterile water or saline on a glass slide. It is important to completely suspend

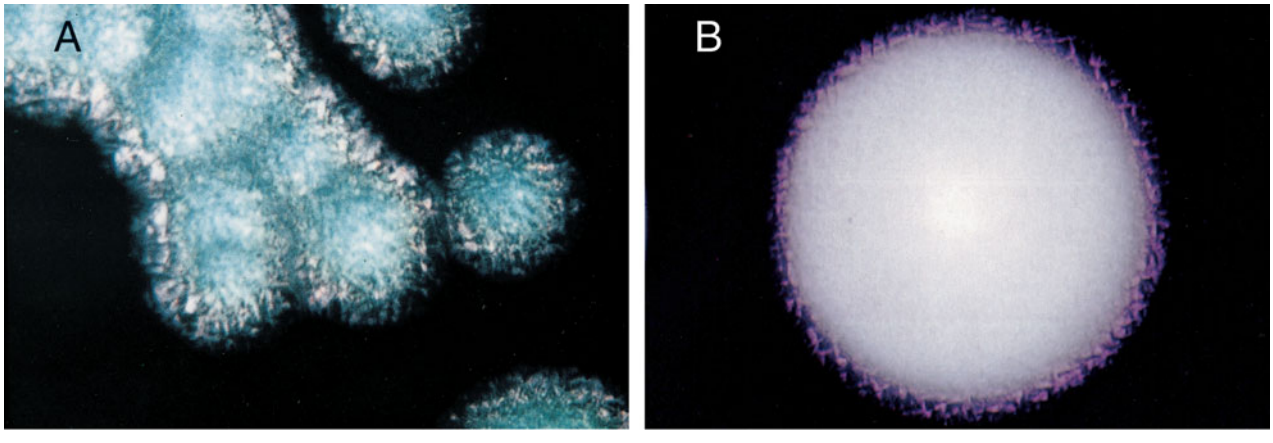


FIGURE 2 Photographs of *L. pneumophila* colonies growing on BCYEα agar. Note the internal speckling and different colors that may be seen, sometimes in the same culture. (Reproduced from Fig. 2, panels A and B, p. 814 of the 8th edition of this Manual.)

the bacteria in the liquid, as nondispersed clumps may stain as gram-positive rods. It is also crucial to use 0.1% basic fuchsin counterstain, because safranin stains these bacteria very poorly. Depending on the colony age and on the strain and species, *Legionella* spp. taken from plates vary in size from short rods measuring 0.5 by 5 μm to very long, filamentous bacteria measuring 1 by 25 μm.

Gram-negative bacteria should then be plated to two different media, BCYEα and either tryptic soy sheep blood agar (BAP) or BCYEα made without L-cysteine (BCYEα-L), in approximately equal amounts in a small (1-cm^2) area; eight or more isolates can be plated to each plate if needed (Fig. 3). Rather large amounts of the picked colony should be inoculated into these media to enable growth after 16 to 18 h of incubation, as the small inocula normally used for other bacteria may otherwise take several days to produce visible colonies on plates. If only a small single colony is available, then it can be emulsified in a small amount (~0.5 ml) of sterile distilled water (not saline) and used for staining, plate inoculation, and seroidentification. *Legionella* spp. should grow in 16 to 36 h on BCYEα medium, but not on BAP or BCYEα-L medium; this takes advantage of the L-cysteine growth dependence of *Legionella* spp. Sometimes *Legionella* spp. will grow poorly on BAP or BCYEα-L media but at most will yield only about 10% of the amount of growth on BCYEα. Nutrient carryover from the primary isolation plate is the explanation for this light growth; this can be proven by making a subculture of growth on BCYEα or BAP on a second plate of the same medium. Rare *Legionella* spp. partially lose growth dependence for L-cysteine on serial passage, but still grow more poorly on BCYEα-L than on BCYEα; these species include *L. spiritensis, L. oakridgensis,* and *L. jordanis,* none of which have been reported to cause more than two cases of LD each. BAP performs almost as well as does BCYEα-L for determining L-cysteine dependence for clinical isolates and may be less expensive, depending on the number of isolates tested per plate. If a blood-containing agar medium is used as the screening plate rather than BCYEα-L for L-cysteine dependence, great care must be taken that the medium base is not too rich. For example, brucella blood agar will support *L. pneumophila* growth almost as well as BCYEα medium, and other blood-containing media have been described to do the same (13). The plates are incubated overnight at 35 to 37°C or until there is visible growth on the BCYEα plate. Compare the relative amounts of growth on each plate to determine if there is L-cysteine dependence. Most *Legionella* spp. produce a very characteristic dank odor when growing in pure growth, which is easily identifiable to the trained nose.

Common mimics of *Legionella* colonies on BCYEα plates include *Eikenella corrodens, P. aeruginosa, Flavobacterium* spp., and some *Bacillus* spp. All of these bacteria grow equally well on BCYEα and BCYEα-L media, but when young they often grow as speckled colonies on BCYEα plates. *Francisella tularensis* can grow well on BCYEα agar but has no resemblance to *Legionella* colonies. However, *F. tularensis* is the only gram-negative bacterium other than *Legionella* spp. that exhibits L-cysteine growth dependence. The colony morphology of *F. tularensis* is not speckled; rather, it is opaque and homogeneous. Adding to the confusion is that some serotyping reagents for *Legionella* spp. may cross-react with *F. tularensis*. There is one case report of the misidentification of *F. tularensis* as *L. pneumophila* (99). Of note, some *Bacillus* sp. mimics can stain as gram-negative rods, have unapparent sporulation, and not grow on BAP (but will grow on BCYEα-L) (91). With prolonged incubation, colonies of both *E. corrodens* and *Flavobacterium* spp. change color and no longer resemble *Legionella* spp.; *E. corrodens* colonies become a light to dark green color, and *Flavobacterium* spp. turn a bright yellow color. Very young *P. aeruginosa* and *Bacillus* spp. colonies resemble the speckled flat to slightly convex young *Legionella* colonies but with prolonged incubation change their morphology, making them easily recognizable as non-*Legionella* colonies. *Bordetella pertussis* colonies may appear late on BCYEα plates, and although this bacterium neither is cysteine dependent nor possesses colony morphology similar to that of *Legionella* spp., *B. pertussis* has been reported to be misidentified as *Legionella* spp., abetted by serological cross-reactivity (76). Because many different bacteria may cross-react with serological reagents used for typing and identifying *Legionella* spp., it is crucial to become familiar with the morphology and growth characteristics of this genus; relying exclusively on serotyping to identify *Legionella* spp. could result in mistaken identification.

Microbiologists should know that some pathogenic fungi and higher bacteria grow well on BCYEα medium, presenting both potential biohazards and the opportunity to diagnose unsuspected infections. *Coccidioides* spp. often grow within a day or two on this medium and can rapidly form arthroconidia, and as such they present a biohazard. *Blastomyces dermatitidis* also grows well and converts to the mold phase within a few days. It is likely that other pathogenic fungi grow equally well on this very rich medium. *Nocardia* spp. and rapidly growing mycobacteria often grow quite well on this medium, making BCYEα medium and its selective variants plating media of choice for the laboratory diagnosis of nocardiosis (57, 98). Nocardia bacteremia can sometimes be diagnosed by subculture of blood culture bottles on BCYEα.

IDENTIFICATION

Basic Identification

Once L-cysteine dependence has been confirmed, further identification of *Legionella* spp. in a clinical laboratory relies almost exclusively on serotyping the bacteria, using either immunofluorescence or agglutination methods (17, 80). Prior to attempting identification by serotyping, the plate should be illuminated with long-wave UV light in a darkroom; some *Legionella* spp. other than *L. pneumophila* fluoresce a brilliant blue-white color, and some a brilliant red; such bacteria are best identified by a reference laboratory and are not *L. pneumophila*. *L. pneumophila* fluoresces a very pale yellow-green color, usually with diffusion of the fluorescent pigment into the culture medium; this is not specific for this species, and sometimes young cultures are completely nonfluorescent. An excellent and specific FDA-cleared fluorescein isothiocyanate-labeled monoclonal antibody to all serogroups of *L. pneumophila* is available (Monofluo, Bio-Rad). Also, an excellent non-FDA-cleared fluorescein isothiocyanate-labeled *L. pneumophila* serogroup 1 (Philadelphia 1 strain)-specific polyclonal antiserum is available (m-TECH, Atlanta, Ga. [http://www.4m-tech.com/]). Serotyping can also be performed using non-FDA-cleared latex agglutination antisera, available in a variety of formats (Oxoid, Basingstoke, United Kingdom; Denka Seiken, Tokyo, Japan); few data on performance of these reagents are available.

Since more than 90% of *Legionella* isolates are *L. pneumophila* serogroup 1, a reagent- and time-saving technique is to first test with *L. pneumophila* serogroup 1 antibody; strains negative with this antibody can then be tested with the species-specific monoclonal antibody. Extraordinarily rarely, some *L. pneumophila* serogroup 1 strains do not react with Philadelphia 1 strain antibodies, making it possible that a

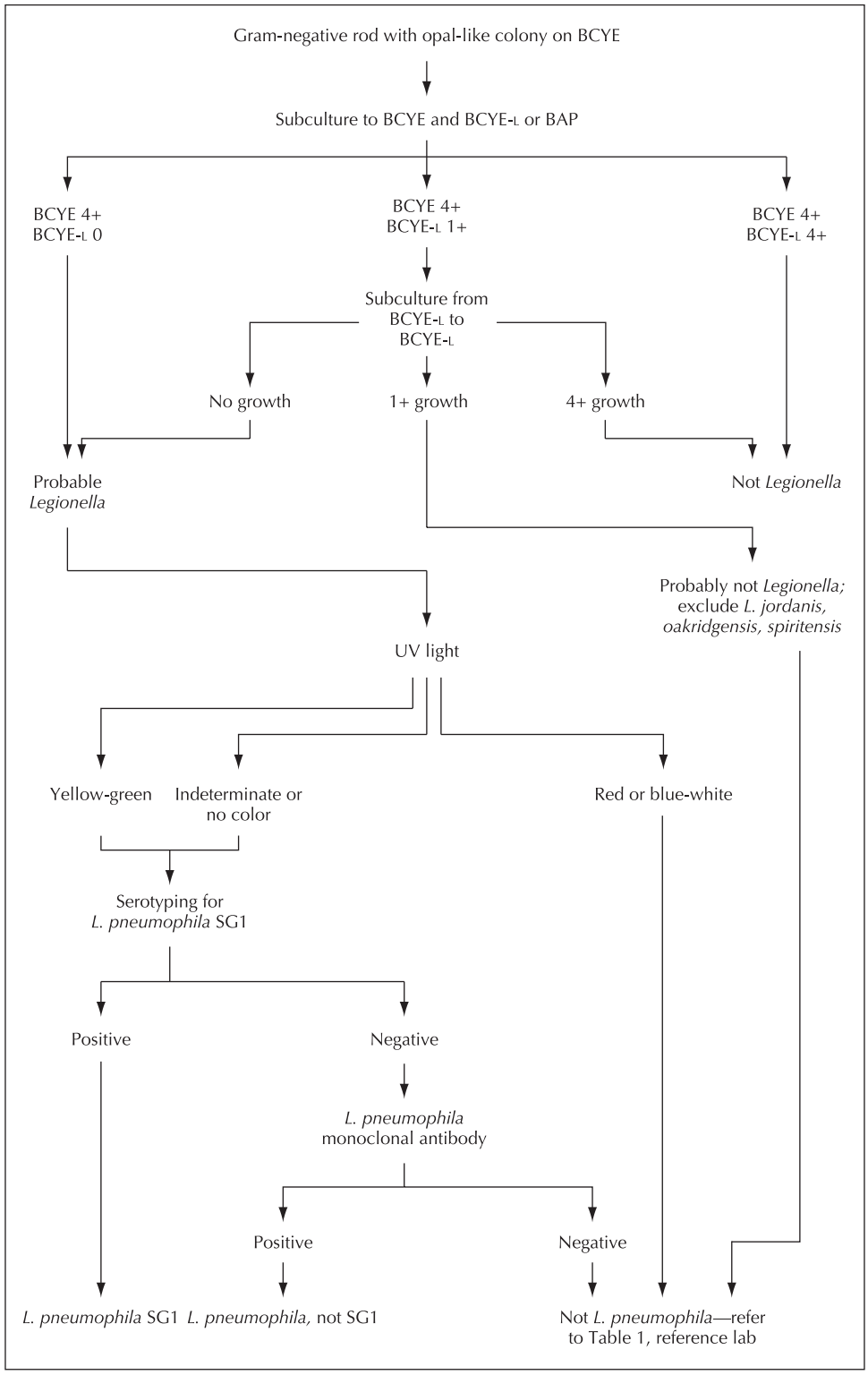


FIGURE 3 Flow scheme for basic identification of *Legionella* spp. grown from a BCYEα plate. Abbreviations: BCYE, BCYEα; BCYE-L, BCYEα made without L-cysteine; BAP, tryptic soy blood agar; UV light, colony fluorescence and color when illuminated with long-wave (360-nm) UV light. Numbers refer to the amount of growth: 4+, good growth; 1+, poor growth; 0, no growth.

serogroup 1 strain could be missed with this reagent; additional serogroup 1 antibodies are available (m-TECH). Testing has to be conducted properly for valid results, with the commonest errors being false-negative results because of a prozone phenomenon and false-positive results from contaminated buffers, wash solutions, or cross-contamination from controls (17). Strains that fail to react with *L. pneumophila* antibodies are best identified by reference or public health laboratories that have other typing sera and identification methods in use.

Gram-negative rods isolated from clinical specimens that are morphologically consistent with *Legionella* spp. and are L-cysteine dependent for growth can be reported as presumptive *Legionella* spp. in the absence of reactivity with typing sera. Gram-negative rods characteristic of *Legionella* spp. that react with *L. pneumophila* monoclonal antibody or *L. pneumophila* serogroup 1 polyvalent antibody can be presumptively identified as *L. pneumophila* or *L. pneumophila* serogroup 1, and the identification can be finalized the next day once L-cysteine growth dependence is confirmed. If the isolate is nonreactive with *L. pneumophila* species-specific antibody, or if it is *L. pneumophila* but not serogroup 1, then the isolate should be further typed by a reference laboratory. All clinical isolates should be frozen at –70°C in TSB in 10% glycerol and subcultured on a BCYEα slant for possible analysis by public health authorities.

Advanced Identification

Accurate identification of *Legionella* spp. other than *L. pneumophila* and *L. pneumophila* serogroup 1 can be quite difficult because of serologic cross-reactivities between species and serogroups, biochemical inertness, and phenotypic identity of different species. Identification to the species or serogroup level for these bacteria is usually not of major clinical significance but may have significant public health and scientific importance. Infections caused by *Legionella* spp. other than *L. pneumophila* or *L. longbeachae* almost always occur in immunocompromised patients, so identification of these other *Legionella* spp. could very rarely be a clue to occult immunosuppression (72). LD caused by *Legionella* spp. other than *L. pneumophila* may respond more poorly to erythromycin therapy (30), but whether knowing the identification of the *Legionella* spp. causing infection would influence patient outcome is debatable, especially since erythromycin is no longer the treatment of choice for severe LD (22). Investigation of LD outbreaks, and sometimes of single cases of the disease, requires knowledge of *Legionella* species identity and subtype; this identification, if needed, can be performed by a public health or reference laboratory as long as the isolates are frozen. The techniques used by reference laboratories include serotyping using collections of antisera, biochemical characterization, and sequence-based identification.

Serotyping of *Legionella* spp. is carried out using polyclonal antisera produced by the U.S. CDC, other public health agencies, and commercial laboratories (m-TECH, Denka Seiken, Oxoid, and others). Either immunofluorescence or agglutination reactions are used, with no clear evidence of superiority of one technique over the other (95). Some producers make polyvalent serum pools that react with a large number of species or serogroups, which can be useful to reduce the number of monovalent antisera used. The specificity of the polyvalent antisera has not been studied in great detail, although at least one product reacted with a number of non-*Legionella* bacteria (24). This makes it important that monovalent typing be carried out and that the bacteria meet minimal phenotypic criteria for *Legionella* spp. Unfortunately, cross-reactions between different species and serogroups occur even when monovalent antisera are used (1, 81, 84, 89, 93, 100). Use of cross-adsorbed polyvalent antisera has been described as a research tool to avoid this problem of cross-reactions, but these reagents are not available outside some research laboratories (5, 92, 94). In addition to intragenus cross-reactions, a large number of cross-reactions of monovalent polyclonal antibodies to non-*Legionella* bacteria have been reported, including *P. aeruginosa, Flavobacterium* spp., *Bacteroides fragilis, Capnocytophaga ochracea, B. pertussis, Bordetella bronchiseptica,* and possibly *Burkholderia pseudomallei* (6, 10, 11, 26, 40, 55, 58). Antibody to *L. pneumophila* serogroup 1 is quite specific, but otherwise enough cross-reactions exist to make serological identification only presumptive. Antisera to newer *Legionella* spp. are often unavailable.

Monoclonal typing antibodies reduce the number of cross-reactions with gram-negative bacteria but are commercially available only for the identification of *L. pneumophila* (9, 21, 47, 90). Nevertheless, great care must be used by experienced microbiologists, as cross-reactions with some non-*Legionella* bacteria have been reported with the *L. pneumophila*-specific monoclonal antibody, including *S. aureus,* yeasts, and *Bacillus* spp.

Legionella spp. are relatively inert biochemically and cannot be identified by using conventional tube or commercial panel biochemical tests. The few biochemical characteristics described for *Legionella* spp. that can be determined in most laboratories, such as oxidase, catalase, and β-lactamase tests, can be nonspecific and if performed improperly falsely negative. Use of a research biochemical panel to facilitate the identification of *Legionella* spp. has been described (97).

Determination of cellular fatty acid and isoprenoid quinone composition by gas-liquid chromatography and high-pressure liquid chromatography, respectively, can be successfully used to identify many *Legionella* spp. (61). These techniques require the use of expensive equipment, are highly complex, and have been supplanted by molecular methods. The cellular compositions of several newer *Legionella* spp. have not been studied using these techniques and/or are not in commercial databases, making identification of these newer species by these methods difficult.

The "gold standard" for the identification of new *Legionella* spp. has been DNA-DNA hybridization analysis (7). This method is tedious, expensive, and labor-intensive and requires special expertise as well as a large collection of reference DNA standards. As such, DNA-DNA hybridization analysis is not used to identify already known species.

Molecular identification of *Legionella* spp. is now replacing other identification techniques in research and specialty laboratories for several reasons. These include the labor cost and time required to serotype a strain, serological cross-reactions, limited availability of antibodies to newer strains, the lack of specific and easy biochemical characterization methods, and the increasing availability of inexpensive DNA sequencing. Molecular identification methods take advantage of the specific 16S rRNA or *mip* gene sequences of the different *Legionella* spp. (36, 64, 83, 86). A *mip* database, procedure instructions including primer sequences, *mip* sequence alignment software, and other genomic software tools are all freely available online (http://www.hpa.org.uk/srmd/bioinformatics/ewgli/legionellamips.htm). Sequence analysis of 16S rRNA has been used to identify several new *Legionella* spp. (35, 62, 65, 79). In addition, sequence of the *rpoB* gene has been shown to distinguish between species as well as, or better than, 16S rRNA or *mip* gene sequencing (59). Intergenic 16S-23S ribosomal spacer PCR analysis has been successful for species identification and does not require

DNA sequencing (85). The relative performances of *mip* and 16S rRNA sequencing are not accurately known. In addition, limited numbers of reference strains, especially of some unusual species, have been sequenced using either method, leaving open the possibility of incorrect classifications. A commercial plate DNA hybridization assay has been reported to correctly identify 23 different *Legionella* spp. (28) and is distributed in Japan (Kobayashi Pharmaceutical, Osaka, Japan).

Whether molecular methods can substitute for serologic ones is difficult to know because of several reports of genotypic discordances for identical serogroups and vice versa (8, 45, 66). Sequencing of the *L. pneumophila dnaJ* gene appeared to be able to distinguish between some, but not all, *L. pneumophila* serogroups (64); different serogroups that had very similar genotypes had been shown by other methods to have discordant genotypes and serotypes. More extensive testing of this method needs to be performed before it is put into routine use. Note that several molecular methods are useful for establishing strain clonality in the context of epidemiologic investigations.

TYPING SYSTEMS

Typing of *Legionella* spp. is important for public health investigations to help link culture-positive environmental sites with clinical isolates during an epidemic of the disease. Typing cannot be used by itself to determine the environmental source of an outbreak and must be accompanied by an epidemiologic investigation. Otherwise, incorrect conclusions may be made about epidemic sources (46, 60). This problem is due to clonal distributions of environmental and clinical *Legionella* spp. (3, 53, 63, 82) and to the poor specificity of some typing techniques (15, 82).

L. pneumophila serogroup 1 can be divided into at least 17 or 10 different groups by using cross-absorbed polyclonal or monoclonal antibodies, respectively (56, 96). Neither the cross-absorbed polyclonal typing sera nor all of the original monoclonal antibodies are available. Lück and colleagues made alternative monoclonal antibodies that can be used in a typing scheme (67). Monoclonal antibody typing plays a major role in subtyping *L. pneumophila* serogroup 1 isolates and, when used with molecular methods, can increase typing specificity (34, 37, 82). Used by itself, monoclonal antibody typing may not be specific enough to distinguish between closely related strains.

Sequence-based typing appears to be the most specific and precise molecular subtyping system for both *L. pneumophila* and *L. pneumophila* serogroup 1. Performed according to a standardized protocol, the method provides uniform results between laboratories, with excellent discrimination between strains (37). This method uses determination of portions of sequences of six different *L. pneumophila* genes, *flaA, pilE, asd, mip, mompS,* and *proA*. An online database, an allele subtype assignment tool, and standardized procedures are available on the Internet (http://www.hpa.org.uk/srmd/bioinformatics/ewgli/ewglisbt.htm). Amplified fragment length polymorphism analysis is an alternative molecular typing system with lower between-laboratory precision than sequence-based typing, but with high specificity (34). An amplified fragment length polymorphism analysis online database and allele assignment tool is available (http://www.hpa.org.uk/srmd/bioinformatics/ewgli/ewgliaflp.htm). Pulsed-field gel electrophoresis, plasmid typing, polyacrylamide gel protein electrophoresis, gel analysis of genomic DNA restriction fragments, and several other methods have all been successfully used to subtype *L. pneumophila,* and in some cases *Legionella* spp., but appear to be too nonspecific for epidemiologic investigative purposes.

SEROLOGIC TESTS

LD can be diagnosed by demonstration of an increase in antibodies to killed bacterial cells (19). Enzyme immunoassay, microagglutination, and indirect immunofluorescent assay methods can all be used to detect antibody changes, with indirect immunofluorescent assay considered the gold standard. While most patients develop both immunoglobulin G (IgG) and IgM responses, some develop IgM-only, IgG-only, or IgA-only responses, making it necessary to test for total immunoglobulin response and not just IgG. In addition, IgM antibodies may persist for as long as a year after infection, making IgM presence a poor marker of acute disease (75). About 75% of patients with culture-proven nosocomial *L. pneumophila* serogroup 1 LD develop seroconversion to the bacterium, whereas the test seems to have higher sensitivity in LD epidemics. Test specificity depends on several technical factors, including the method of antigen preparation, the numbers of different bacteria used in antigen preparation, the method of diluting serum, and several other factors. Seroconversion requires weeks to months after infection, with only about a 50% seroconversion rate after 2 weeks; for optimal test sensitivity, acute-phase serum should be frozen while convalescent-phase sera are collected at 2, 4, 6, 9, and 12 weeks postinfection. Sera tested 2 weeks after disease onset have only about a 37% (50% of 75%) chance of detecting seroconversion. Parallel testing of sera is required for the best specificity. Some laboratories test acute-phase sera only for evidence of early antibody production; this approach can be nonspecific and insensitive. The most specific testing is for seroconversion to *L. pneumophila* serogroup 1 only, and the least specific is the use of polyvalent antigen preparations, with approximate test specificities of 99% and 90 to 95%, respectively. Polyvalent antigen preparations are best used for screening purposes only, with follow-up monovalent testing. Almost all commercially available tests use only polyvalent antigen preparations, which can result in nonspecific results. There is also some concern that screening enzyme-linked immunosorbent assays may be insensitive, possibly making such testing both insensitive and nonspecific (68). Serologic diagnosis is best used for epidemiologic studies because of the retrospective nature of serologic diagnosis and limitations on test specificity and sensitivity.

Antimicrobial Susceptibilities and Susceptibility Testing

The antimicrobial susceptibility of *L. pneumophila* grown in broth or on agar can give results having no clinical correlate. This is because of the intracellular location of the bacterium in human infection, to which not all antimicrobial agents gain access and retain activity (18). In addition, the complex broth and agar media used to grow *L. pneumophila* inactivate many drugs. There is no indication for performing antimicrobial susceptibility testing against *Legionella* spp. except in a research setting, when correlative studies of intracellular and experimental animal model infection can be performed. The microbiologist must not assume that a particular drug will be effective for the treatment of LD simply because the drug is active against *L. pneumophila* in vitro, nor should there be an assumption that drugs having low MICs against the bacterium in vitro will be more clinically effective than will drugs with higher MICs for the organism.

Antimicrobial agents having good intracellular activity against *L. pneumophila* include most macrolide, tetracycline,

ketolide, and quinolone antimicrobial agents (18, 22). No beta-lactam agent or aminoglycoside has acceptable intracellular activity against the bacterium. It is unknown if the intracellular activity of antimicrobial agents against *L. pneumophila* can be extrapolated to all other *Legionella* spp. and to treatment of infections caused by *Legionella* spp. other than *L. pneumophila*. Some of these other *Legionella* spp. may reside in subcellular compartments different from those where *L. pneumophila* resides and thus may respond differently to antimicrobial agents (77). However, macrolide and quinolone antimicrobials appear to be effective for the treatment of LD caused by *L. micdadei, L. longbeachae, L. bozemanae,* and *L. dumoffii* (72).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Multiple laboratory methods have to be used for optimal laboratory diagnosis of LD. Culture of *Legionella* bacteria from sputum, lung, or other respiratory sites is the most specific (100%) method for diagnosis of the disease, very sensitive (~80 to 90%) in severe untreated disease and insensitive (~20%) in cases of mild disease. Culture may be the only diagnostic test giving positive results, especially when *Legionella* bacteria other than *L. pneumophila* are causing the infection. In addition, a culture isolate can be used to sort out the source of an epidemic, unlike any of the other diagnostic tests. Because of the technical difficulty of culture diagnosis, its expense, and its low sensitivity for nonsevere disease, several alternative diagnostic methods have been developed. The antigenuria assay is more sensitive than culture for the detection of community-acquired disease, and especially epidemic disease. Nevertheless, the antigenuria test is only about 60% sensitive in the best of circumstances, performs poorly for detection of nosocomial infection, and detects almost exclusively *L. pneumophila* serogroup 1. Antibody detection complements other laboratory diagnostic methods but is retrospective because it requires seroconversion for greater test specificity and sensitivity. In addition, antibody results obtained using only polyvalent antigens must be viewed with circumspection. Commercial molecular amplification tests are just being marketed and have yet to be thoroughly evaluated; prior to adoption, more-extensive studies are required. The performance of all laboratory diagnostic tests for non-*L. pneumophila* serogroup 1 LD is unknown but presumably is not as good as it is for the diagnosis of *L. pneumophila* serogroup 1 disease.

Because of the lack of any one laboratory test that is highly sensitive and specific for the diagnosis of LD caused by *L. pneumophila* serogroup 1, let alone the disease caused by other serogroups and species, multiple test types should be performed if laboratory diagnosis is crucial for clinical or public health reasons. In particular, culture needs to be performed whenever possible to enhance diagnostic yield, for public health reasons, and because it may be the only specific method capable of diagnosing LD caused by *Legionella* species other than *L. pneumophila*.

A major hindrance to the evaluation of all laboratory diagnostic methods is the lack of a good gold standard for LD diagnosis. Culture diagnosis, while very specific, is known to be imperfect and of limited sensitivity, especially for epidemic LD. Relative performance of the diagnostic tests is often compared to culture diagnosis, which tends to overestimate test sensitivity and underestimate specificity.

Positive cultures for all *Legionella* spp. are virtually diagnostic of LD, providing there are supportive clinical findings such as pneumonia. In contrast, single serum specimens showing elevated antibodies to *Legionella* spp. or to *L. pneumophila* are often not the result of LD. Only rises in antibody titers to *L. pneumophila* serogroup 1, evaluated by a test not usually commercially available, are specific enough for diagnosis, but even then use of appropriate techniques is required for optimal specificity. Detection of *L. pneumophila* serogroup 1 antigenuria is almost as specific as a positive culture, once heat-labile factors capable of causing false-positive tests are excluded. Positive test results must be reported promptly to the patient's clinician as well as to public health authorities.

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*Bartonella**

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TAXONOMY

The genus *Bartonella* is named for A. L. Barton, who first described the intraerythrocytic bacterium *Bartonella bacilliformis* in 1909. Until recently, this genus consisted of only one species, *B. bacilliformis*. The *Bartonella* genus now combines all the species of the three genera *Bartonella, Rochalimaea,* and *Grahamella* and is the only genus of the family *Bartonellaceae,* which has been removed from the order *Rickettsiales* (6, 10). *Bartonella* species are members of the alpha-2 subgroup of the class alpha-proteobacteria within the order *Rhizobiales*. The list of newly recognized species and subspecies has been increasing in the last few years (2, 9). These small gram-negative rods are erythrocyte-adherent bacilli. Based on 16S rRNA gene similarity, *Bartonella* is closely related to *Brucella* and *Agrobacterium*.

DESCRIPTION OF THE GENUS

The present family consists of 19 species and 3 subspecies, of which 10 are human pathogens (Table 1). *B. bacilliformis,* the type species of the genus, is the etiologic agent of Carrion's disease and causes either an acute bacteremic infection called Oroya fever, characterized by sepsis and hemolysis, or a chronic form called verruga peruana, characterized by cutaneous nodular vascular eruptions. *B. quintana,* the agent of trench fever, has also been found to be one of the agents of bacillary angiomatosis (BA), a vascular proliferative lesion observed in immunocompromised individuals, mainly those with AIDS (48). *B. quintana* has also been associated with endocarditis and bacteremia in homeless people (12). BA can also be caused by *B. henselae*. *B. henselae* causes cat scratch disease (CSD) in immunocompetent individuals. Similarly, *B. henselae* has been associated with several cases of endocarditis. The fourth human pathogen, *B. elizabethae,* was isolated from an immunocompetent individual suffering from endocarditis. Since then, *Bartonella vinsonii* subsp. *berkhoffii, B. vinsonii* subsp. *arupensis* (24), and *B. koehlerae* have also been associated with human cases of endocarditis (7), and "*B. washoensis*" (proposed species) has been associated with a human case of myocarditis. *B. grahamii* has been associated with a human case of neuroretinitis. *B. vinsonii* subsp. *arupensis* was also isolated from the blood of a rancher with fever and mild neurological symptoms (7). Finally, *B. clarridgeiae* is suspected to be a minor agent of CSD, based on serological evidence only. Most of the *Bartonella* species that are pathogenic to humans *(B. vinsonii* subsp. *berkhoffii, B. clarridgeiae, B. henselae, B. elizabethae,* and "*B. washoensis*") have been associated with various clinical entities, including endocarditis, in domestic dogs (7, 18).

Several other *Bartonella* species, such as *B. vinsonii* subsp. *vinsonii, B. doshiae, B. taylorii, B. peromysci, B. birtlesii, B. tribocorum, B. alsatica, B. talpae, B. bovis, B. chomelii, B. schoenbuchensis,* and *B. capreoli,* have only been isolated from the blood of various animal species, including various wild rodents, squirrels, rabbits, felids, canids, and ruminants (cattle, deer, and elk). At present, these species are not known to induce any specific disease in the infected animal.

Bartonella spp. are fastidious, aerobic, short, pleomorphic, gram-negative coccobacillary or bacillary rods (0.2 to 0.6 μm by 0.5 to 1.0 μm). Because of the slow growth of these bacteria, standard biochemical methods for identification may not be applicable. Bartonellae are oxidase and catalase negative and do not produce acid from carbohydrates. All members of the genus take from 5 to 15 days and up to 45 days in the presence of 5% CO_2 on primary culture to form visible colonies on enriched blood-containing media, as they are highly hemin dependent. The optimal growth temperature varies from 25 to 30°C for *B. bacilliformis* to 35 to 37°C for the other *Bartonella* species, such as *B. henselae, B. koehlerae,* and *B. elizabethae*. On primary isolation, some *Bartonella* species, such as *B. henselae, B. clarridgeiae, B. vinsonii,* and *B. elizabethae,* have colonies with a white, rough, dry, raised appearance and pit the medium. They are hard to break up or transfer. Other bartonellae such as *B. quintana* have colonies that are usually smaller, gray, translucent, and somewhat gummy or slightly mucoid. *B. bacilliformis, B. clarridgeiae, B. capreoli,* and *B. schoenbuchensis* are the only members of the genus that are motile by means of unipolar flagella.

NATURAL HABITATS

Most *Bartonella* species are vector-borne organisms. Some *Bartonella* species are very limited geographically, such as *B. bacilliformis,* which is found only in the Andes mountain region of South America, associated with the limited distribution of its vector, the sand fly *Lutzomyia verrucarum*. Others,

* This chapter contains information presented in chapter 53 by David F. Welch and Leonard N. Slater in the eighth edition of this Manual.

TABLE 1 *Bartonella* species or subspecies presently described, their main reservoirs, and confirmed or possible vectors[a]

Bartonella sp.	Main reservoir	Vector or potential vector
B. bacilliformis	Humans	Pheblotomines (sand flies) *(L. verrucarum)*
B. quintana	Humans	Human body lice *(Pediculus humanus corporis)*
B. henselae	Cats *(Felis catus)*	Fleas (*C. felis*), ticks?
B. clarridgeiae	Cats *(F. catus)*	Fleas (*C. felis*)
B. koehlerae	Cats *(F. catus)*	Fleas (*C. felis*)
B. vinsonii subsp. *vinsonii*	Meadow voles *(Microtus pennsylvanicus)*	Ear mites *(Trombicula microti)*?
B. vinsonii subsp. *arupensis*	White-footed mice *(Peromyscus leucopus)*	Fleas? Ticks?
B. vinsonii subsp. *berkhoffii*	Coyotes (*C. latrans*), dogs *(Canis familiaris)*	Ticks?
B. talpae	Moles *(Talpa europaea)*	?
B. peromysci	Field mice (*Peromyscus* spp.)	Fleas?
B. birtlesii	Wood mice (*Apodemus* spp.)	Fleas?
B. grahamii	Bank voles (*Clethrionomys glareolus*)	Fleas (*Ctenophthalmus nobilis*) (8)
B. taylorii	Wood mice (*Apodemus* spp.)	Fleas (*C. nobilis*)
B. doshiae	Field voles *(Microtus agrestis)*	Fleas?
B. elizabethae	Rats *(Rattus norvegicus)*	Fleas
B. tribocorum	Rats (*R. norvegicus*)	Fleas?
B. alsatica	Rabbits *(Oryctolagus cuniculus)*	Fleas? Ticks?
"*B. washoensis*"	California ground squirrels *(Spermophilus beecheyi)*	Fleas? Ticks?
B. bovis ("*B.weissii*")	Domestic cattle *(Bos taurus)*	Biting flies? Ticks?
B. chomelii	Domestic cattle *(B. taurus)*	Biting flies? Ticks?
B. capreoli	Roe deer *(Capreolus capreolus)*	Biting flies? Ticks?
B. schoenbuchensis	Roe deer *(C. capreolus)*	Biting flies, Ticks?

[a] As reported in reference 81.

such as *B. quintana, B. henselae,* and *B. elizabethae,* appear to have a worldwide distribution. *B. quintana* outbreaks had been mainly reported in Europe at the beginning of the 20th century causing hundreds of thousands of human cases of trench fever during World War I (WWI) and WWII. These outbreaks were associated with poor sanitation and personal hygiene and have now reemerged in homeless populations worldwide, associated with infestation by the human body louse, *Pediculus humanus,* possibly by inoculation of arthropod excreta through broken skin (41, 42). Humans are the only known reservoirs and victims of these two *Bartonella* species. In cases of BA caused by *B. quintana,* a history of body louse exposure is usually reported. Most cases of BA caused by *B. henselae* and almost all cases of CSD are associated with cat scratches or exposure.

The domestic cat is a healthy carrier of *B. henselae, B. clarridgeiae,* and *B. koehlerae* and constitutes the main reservoir of these organisms. *B. henselae* infection is transmitted from cat to cat mainly by the cat flea, *Ctenocephalides felis* (19), but the role of the cat flea seems limited in human infection. More likely, human infection results from the inoculation of infective flea feces at the time of the scratch (7). Some data have suggested a possible role for ticks in some human *B. henselae* infections (7). Stray cats, cats living outdoors, and young cats are more likely to be bacteremic. The prevalence of infection is usually highest in warm and humid climates where cat fleas are abundant. At least two genotypes of *B. henselae* have been identified, with type I (Houston) more common in cats in the Far East (Japan and the Philippines) and type II (Marseille) predominant in Western Europe, North America, and Australia (7). Coinfection of cats with different *Bartonella* species or genotypes has been reported (30). Additionally, *B. quintana, B. koehlerae,* and *B. clarridgeiae* have been detected by molecular methods in cat fleas, suggesting their possible role as vectors for these organisms (83). Domestic and wild canids represent the main reservoir of *B. vinsonii* subsp. *berkhoffii,* with high antibody prevalence in dogs from tropical countries (7), as well as high bacteremia prevalence in coyotes *(Canis latrans)* in California (16). Ticks may be involved in the transmission of *B. vinsonii* subsp. *berkhoffii* to dogs (9). Similarly, *Bartonella* DNA has been identified in questing adult *Ixodes pacificus* ticks from California, including several species pathogenic for humans (15), and *B. henselae* DNA was detected recently in *Ixodes ricinus* ticks collected from humans in Italy (92).

A wide range of rodent species has been reported to be infected with different *Bartonella* species (Table 1). Finally, several *Bartonella* species have been isolated from domestic and wild ruminants (Table 1), and biting flies could be an important vector of infection among these animals (7).

CLINICAL SIGNIFICANCE

Human Pathogens

Oroya Fever and Verruga Peruana (Carrion's Disease): *B. bacilliformis*

The disease caused by *B. bacilliformis,* especially its chronic form known as verruga peruana, has been recognized since pre-Columbian times in populations of the Andes. However,

the suspected link between the acute form (Oroya fever) and the chronic form was confirmed in 1885 when Carrion, a medical student, died of Oroya fever after inoculating himself with material from a verruga. The acute form of the disease, usually seen in people who are not natives of the zone of endemicity, is an acute, progressive, severe, and febrile anemia with extravascular hemolysis associated with the presence of *B. bacilliformis* in the erythrocytes. Mortality was reported to be up to 40% of the cases prior to the antibiotic era. Bacteremia is common in the general population in zones of endemicity and nonendemicity (14, 50). The second stage of infection leads in weeks to months to the eruption of nodular angioproliferative cutaneous lesions named verruga peruana; mucosal and internal lesions can occur (80). The lesions can persist for several months, but the prognosis is good at this stage.

Trench Fever, BA, Endocarditis, and Prolonged Fever or Bacteremia Caused by *B. quintana*

Trench or quintan fever is a recurrent fever with three to five, sometimes more, febrile episodes lasting 4 to 5 days each occurring after 15 to 25 days of incubation. Severe headaches and shin pain are common symptoms associated with malaise, anorexia, abdominal pain, restlessness, and insomnia. Mild forms as well as asymptomatic carriage have also been reported (12, 40). Several cases of endocarditis have been associated with *B. quintana* infection (55). In human immunodeficiency virus (HIV)-infected persons, bacteremia caused by *B. quintana* develops insidiously, involving recurrent fever, headaches, and hepatomegaly. *B. quintana* and *B. henselae* are the two *Bartonella* species involved in the etiology of BA. BA, also called epithelioid angiomatosis, is a vascular proliferative disease of the skin characterized by multiple, blood-filled, partially endothelial cell-lined cystic structures (96). It is usually characterized by violaceous or colorless papular and nodular skin lesions that clinically may suggest Kaposi's sarcoma but histologically resemble epithelioid hemangiomas. When visceral parenchymal organs are involved, the condition is referred to as bacillary peliosis hepatis, splenic peliosis, or systemic BA. Fever, weight loss, malaise, and enlargement of affected organs may develop in people with disseminated BA. Subcutaneous and lytic bone lesions are strongly associated with *B. quintana* infection (47).

Zoonotic Bartonellae

CSD

CSD is mainly caused by *B. henselae* (77, 102), even if *B. clarridgeiae* is suspected in some cases based on serological evidence (49, 63). There are now strong arguments against *Afipia felis* being one of the etiologic agents of CSD (54). In CSD, 1 to 3 weeks elapse between the scratch or bite of a cat and the appearance of clinical signs. In 50% of the cases, a small skin lesion, often resembling an insect bite, appears at the inoculation site, usually the hand or forearm, and evolves from a papule to a vesicle and partially healed ulcers. These lesions resolve within a few days to a few weeks. Lymphadenitis develops approximately 3 weeks after exposure and is generally unilateral. It commonly appears in the epitrochlear, axillary, or cervical lymph nodes. Swelling of the lymph node is usually painful and persists for several weeks to several months. In 25% of the cases, suppuration occurs. The large majority of the patients show signs of systemic infection: fever, chills, malaise, anorexia, and headache. In general, the disease is benign and heals spontaneously without sequelae. Atypical manifestations of CSD occur in 5 to 10% of the cases. The most common of these is Parinaud's oculoglandular syndrome (periauricular lymphadenopathy and palpebral conjunctivitis), but also meningitis, encephalitis, osteolytic lesions, and thrombocytopenic purpura may occur. Encephalopathy is one of the most serious complications of CSD and usually occurs 2 to 6 weeks after the onset of lymphadenopathy. However, it usually resolves with complete recovery and few or no sequelae. It is estimated that 22,000 human cases of CSD occur yearly in the United States. From 55 to 80% of CSD patients are <20 years old. There is a seasonal pattern, with most cases seen in autumn and winter.

New clinical presentations associated with *B. henselae* infection have been reported for immunocompetent persons, including neuroretinitis or bacteremia as a cause of chronic fatigue syndrome, and a case of aggressive *B. henselae* endocarditis in a cat owner. *B. henselae* was also determined as a frequent cause of prolonged fever and fever of unknown origin in children. Rheumatic manifestations of *Bartonella* infection have been described for children, including a case of myositis and a case of arthritis and skin nodules. Arthritis has been described in a very limited number of cases. Other rheumatic manifestations related to *Bartonella* infection in humans include erythema nodosum, leukocytoclastic vasculitis, and fever of unknown origin with myalgia and arthralgia (7).

For BA in immunocompromised persons, the symptoms are very different from those of CSD. BA patients with *B. henselae* infection are epidemiologically linked to cat and flea exposure (47). Cutaneous lesions are common with *B. henselae* infection, and peliosis hepatis is associated exclusively with *B. henselae* infection. Fever, weight loss, malaise, and enlargement of affected organs may develop in people with disseminated BA. Endocarditis has also been reported for patients with BA. *B. henselae* and *B. quintana* have been implicated in a few cases of HIV-associated brain lesions, meningoencephalitis and encephalopathy, dementia, and neuropsychological decline (94, 97).

Zoonotic *Bartonella* Species Associated with Endocarditis or Myocarditis

Several zoonotic *Bartonella* spp. have been recognized as causative agents of blood culture-negative endocarditis or myocarditis in humans, including *B. henselae, B. koehlerae, B. elizabethae, B. vinsonii* subsp. *berkhoffii, B. vinsonii* subsp. *arupensis* (24), and "*B. washoensis*" (7). *Bartonella* spp. account for 3 to 4% of all human cases of endocarditis in France, a percentage similar to that of endocarditis cases caused by *Coxiella burnetii,* the agent of Q fever (35, 75).

Zoonotic *Bartonella* Species Associated with Ocular Lesions, Fever, and Neurological Symptoms

Some rodent-borne *Bartonella* species are also associated with cases of neuroretinitis *(B. elizabethae* and *B. grahamii)* or fever with bacteremia and neurological symptoms *(B. vinsonii* subsp. *arupensis)* (46, 98).

Other *Bartonella* Species or Subspecies

The clinical impact on animals or humans of many *Bartonella* species is still unknown. A broad array of manifestations, including endocarditis, has been identified for dogs (9). *B. henselae, B. clarridgeiae,* "*B. washoensis,*" and *B. elizabethae* have been recognized as pathogenic for dogs (18). No specific pathology has been associated yet with *Bartonella* species infecting domestic and wild ruminants or with many rodent-borne *Bartonella* spp.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Most specimens used for *Bartonella* isolation are either blood or tissue. Approaches typically used for recovery of other pathogens from such sites are suitable, although the fastidious nature of these organisms requires that precautions be taken to minimize the interval from collection to processing. *Bartonella* spp. are difficult to isolate from blood of immunocompetent individuals, as opposed to the relative ease of isolation from blood of immunocompromised patients. Blood samples can be collected either in Isolator blood lysis tubes (Wampole, Cranbury, N.J.), in sodium citrate tubes, or in plastic EDTA tubes, which is now the most common method used for blood collection in animals. If storage of specimens prior to culture is necessary, samples should be kept frozen (at least −20°C). It was shown that blood collected from *B. henselae*-infected cats into both EDTA and Isolator blood lysis tubes yielded good recovery and no loss of sensitivity for EDTA tubes kept at −65°C for 26 days (11). *Bartonella* spp. are susceptible to a broad spectrum of antimicrobial agents; therefore, specimens should be collected prior to antimicrobial therapy.

Collection of tissue from enlarged lymph nodes, cutaneous lesions, or various organs can be performed and samples cultured after homogenization or processed for DNA extraction and PCR. Fresh tissues are preferred for PCR amplification, but paraffin-embedded tissue may be used (23). Fine-needle aspiration has also been successful for detection of *Bartonella* and is less invasive than biopsy (3).

For animals, bartonellae are isolated from blood collected in plastic EDTA tubes; these tubes prevent breakage during shipment and during storage at low temperature, which facilitates lysis of the red blood cells. Biopsies of visceral or cutaneous lesions can be performed, especially in dogs, as described for humans. Collection of aqueous humor in cat or dog retinitis cases for detection of *Bartonella* bacteria or antibodies has been suggested (51, 70).

DIRECT EXAMINATION

Microscopy

Detection of *Bartonella* in erythrocytes from blood smears is rarely performed, with the exception of stained blood films for patients with Oroya fever, as the number of bacteria present in each erythrocyte is quite low (82), reaching a high of eight bacteria in a *B. birtlesii* model in rats (93). Microscopic detection of *Bartonella* organisms in fixed tissue sections includes Warthin-Starry silver staining, which is not highly specific for *Bartonella* organisms and requires careful attention to the details of the procedure (Fig. 1). Detection of bacilli by Warthin-Starry staining is more likely to occur during the early stage of CSD lymphadenopathy, but not during the granulomatous stage of inflammation. In patients with BA, there is usually a larger number of bacilli that are identifiable by Warthin-Starry silver staining.

Antigen Detection

Immunocytochemical labeling is a specific technique but is not widely available (76). Direct immunofluorescence of blood smears allowed rapid diagnosis of *B. quintana* in a patient with acute trench fever (27) and in bacteremic homeless patients (82). In the United States and Europe, *B. henselae* and *B. quintana* immunofluorescence assay (IFA) slides are available through companies such as Focus Diagnostics, Inc. (Cypress, Calif.).

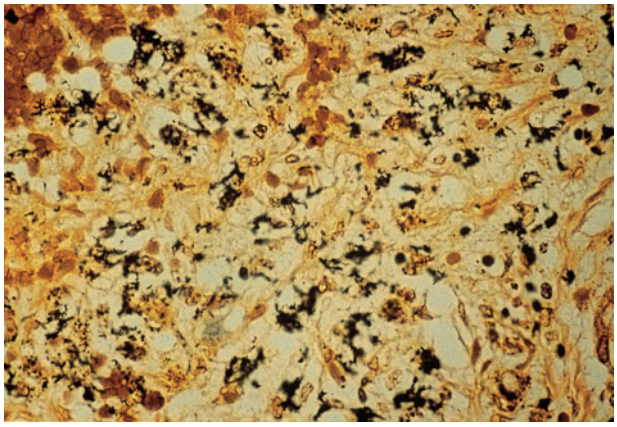


FIGURE 1 Warthin-Starry silver stain of a liver tissue section containing clusters of *B. henselae*. Magnification, ×530.

Nucleic Acid Detection Techniques

Direct detection of *Bartonella* spp. is more often performed by amplification of DNA from tissue, pus, or skin lesions. Molecular genetic methods such as restriction fragment length polymorphism (RFLP) of various genes (see recommended gene targets in Table 2) and, more recently, analysis based on PCR of random, repetitive extragenic palindromic sequences have been used to distinguish strains and species of *Bartonella.* RFLP or sequence analysis of 16S rRNA or citrate synthase genes after PCR amplification, both directly from specimens and from pure cultures, has been largely used for detecting and characterizing *Bartonella*. Identification has also been performed with the amplification of the intergenic spacer region between the 16S and 23S rRNA genes (89) or with protein-coding genes. A new tool has been proposed for the diagnosis of *Bartonella* endocarditis: a real-time nested PCR assay performed on a LightCycler apparatus using serum (23).

ISOLATION PROCEDURES (CULTURE)

Blood

Bartonellae can be recovered from the blood of bacteremic patients, especially persons infected with *B. quintana, B. bacilliformis,* and, less frequently, *B. henselae*. In immunocompromised patients, the level of bacteremia is often higher than in immunocompetent people, and most bacteremic patients have fever, chills, and weight loss (48). However, cultures from patients with infective endocarditis caused by *Bartonella* spp. are rarely positive. *Bartonella* species are optimally isolated from blood with the use of Isolator lysis centrifugation tubes or EDTA tubes and plating onto fresh chocolate or heart infusion agar containing 5% fresh rabbit blood in the absence of antibiotics. Commercial sheep or horse blood agar plates have also been used. *B. koehlerae* does not grow well on heart infusion agar and requires fresh chocolate plates (21). Plates inoculated with blood should be incubated at 35°C for at least 4 weeks in 5% CO_2 and at high humidity. Colonies usually appear after 5 to 15 days (48). Considering that the length of time required for detection of *Bartonella* spp. allows other slowly growing pathogens, including *Mycobacterium tuberculosis,* to grow on the same plates, appropriate safety precautions should be taken with positive cultures. Vials used for blood culture (broth-based or biphasic culture systems) can also be used for isolation of *Bartonella,*

TABLE 2 Sequences used as PCR primers or probes to amplify or identify *Bartonella*

Bacterium	Gene[a]	Direction[b]	Primer	Sequence	Reference(s)
B. clarridgeiae	*rib*C	F	PBC5	TACATAACGAGCCAATT	4
	*rib*C	R	PBC15	TAGCTTTAGAACAATATGGT	4
B. henselae	*rib*C	F	PBH-L1	GATATCGGTTGTGTTGAAGA	4
	*rib*C	R	PBH-R1	AATAAAAGGTATAAAACGCT	4
B. bacilliformis	*rib*C	F	PBH-L1	GATATCGGTTGTGTTGAAGA	4
	*rib*C	R	PBB-R1	AAAGGCGCTAACTGTTC	4
B. quintana	*rib*C	F	PBH-L1	GATATCGGTTGTGTTGAAGA	4
	*rib*C	R	PBQ-R1	AAAGGGCGTGAATTTTG	4
All bartonellae	*rib*C	F	PBH3	CCAAGTGCTACATAACCATC	4
	*rib*C	R	PBH4	CGGGTTGTTATTGCTCTTAC	4
All bartonellae (except *B. bacilliformis*)	*rrs-rrl* igs	F	16SF	AGAGGCAGGCAACCACGGTA	33, 90
	rrs-rrl igs	R	23S1	GCCAAGGCATCCACC	33, 90
All bartonellae	*rrs-rrl* igs	F	QHVE1	TTCAGATGATGATCCCAA	33, 90
	rrs-rrl igs	R	QHVE2	TTGGGATCATCATCTGAA	33, 90
	rrs-rrl igs	F	QHVE3	GATATATTCAGACATGTT	33, 90
	rrs-rrl igs	R	QHVE4	AACATGTCTGAATATATC	33, 90
B. bacilliformis	*rrs-rrl* igs	F	BABF	CTGGATCACCTCCTTTCTAA	33, 90
	rrs-rrl igs	R	BABR	ATGCCCTTAAGACACTTGAT	33, 90
B. quintana	*rrs-rrl* igs	F	BQF	CTCCACCATTTTAGGTCATC	33, 90
	rrs-rrl igs	R	BQR	GGTTTTGAGAATTCCCTTGC	33, 90
All bartonellae	*rrs-rrl* igs	F	16s1	(C/T)CTTCGTTTCTCTTTCTTCA	43
	rrs-rrl igs	R	16s2	GGATAAACCGGAAAACCTTC	43
	rrs-rrl igs	F	16s3	(C/T)CTTCGTTTCTCTTTCTTCA	43
	rrs-rrl igs	R	16s4	AACCAACTGAGCTACAAGCC	43
	rrs-rrl igs	F	16s5	CTCTTTCTTCAGATGATGATCC	43
	rrs-rrl igs	R	16s6	AACCAACTGAGCTACAAGCCCT	43
	*rib*C	F	BARTON-1F	TAACCGATATTGGTTGTGTTGAAG	45
	*rib*C	R	BARTON-2R	TAAAGCTAGAAAGTCTGGCAACATAACG	45
	rrs	S	Probe	ATTTGGTTGGGCACTCTAGGGG	46
	rrs		Rp2a	ACGGCTACCTTGTTAGGACTT	52
	rrs	F	357f	TACGGGAGGCAGCAG	52
	rrs	R	357ra	CTGCTGCCTCCCGTA	52
	rrs	F	536F	CAGCAGCCGCGGTAATAC	52
	rrs	R	536R	GTATTACCGCGGCTGCTG	52
	rrs	F	800F	ATTAGATACCCTGGTAG	52
	rrs	R	800R	CTACCAGGGTATCTAAT	52
	rrs	F	1050F	TGTCGTCAGCTCGTG	52
	rrs	R	1050R	CACGAGCTGACGACA	52
	35-kDa antigen gene	F	35KD1fa	GTCGCTAAAGGCTGATGA	52
	35-kDa antigen gene	R	35KD2ra	GACTGATATCGTGCGTGTG	52
	35-kDa antigen gene	F	35KDs1f	GGTACGACGACAGTAATTGTT	52
	35-kDa antigen gene	R	35KDs2r	GATTTAAGAGATACCAACCA	52
	pap	F	PAP1fa	CTTTAATGACGACTTCTGTT	52
	pap	R	PAP4ra	CCGAAATCTGAGTAACGGTA	52
	pap	R	PAP2r	CCCTAAATGTTTCAAGTTCA	52
	pap	F	PAP3f	GCTGACAGAGAAGACGCAA	52
	groEL	F	BbHs233.p	CGTGAAGTTGCCTCAAAAACC	64
	groEL	R	BbHs1630.n	AATCCATTCCGCCCATTC	64
	rpoB	F	QVE1	TTCAGATGATGATCCCAAGC	79
	rpoB	R	QVE3	AACATGTCTGAATATATCTTC	79
			Bfp1	ATTAATCTGCAYCGGCCAGA	105
			Bfp2	ACVGADACACGAATAACACC	105
			Bfs3	TTACAAAAATCYGTTGATAC	105
			Bfs4	GTATCAACRGATTTTTGTAA	105
	ftsZ	F	BaftsZF	GCTAATCGTATTCGCGAAGAA	105
	ftsZ	R	BaftsZR	GCTGGTATTTCCAAYTGATCT	105
B. henselae, *B. clarridgeiae*	*ftsZ*		Bh ftsZ 1393.n	GCGAACTACGGCTTACTTGC	105
	ftsZ		Bh ftsZ 1247.p	CGGTTGGAGAGCAGTTTCGTC	105

(Continued on next page)

TABLE 2 *(Continued)*

Bacterium	Gene[a]	Direction[b]	Primer	Sequence	Reference(s)
B. quintana	*ftsZ*	F	Bq ftsZseqF	GCACATATTCTTGATGAGAT	105
	ftsZ	R	Bq ftsZseqR	CCCCTATCATCTCATCAAG	105
B. bacilliformis	*ftsZ*	F	Bb ftsZseqF	GCGCATGTTCTTAGTGAAAT	105
	ftsZ	R	Bb ftsZseqR	CCTGTATACGTGATGCATTT	105
All bartonellae	*ftsZ*		FTS1p	GCCTTCTCATCCTCAACTT	105
	ftsZ		FTS2p	CAGCCTCTTCACGATGTG	105
	pap31		PAPn1	TTCTAGGAGTTGAAACCGAT	104
	pap31		PAPn2	GAAACACCACCAGCAACATA	104
	pap31		PAPns2	GCACCAGACCATTTTTCCTT	104
	pap31		PAPns1	CAGAGAAGACGCAAAAACCT	104
	groEL		HSPps1	CAGAAGTTGAAGTGAAAGAAAA	104
	groEL		HSPps2	GCNGCTTCTTCACCNGCATT	104
	groEL		HSPps4	GCTGGNGGTGTTGCNGTTA	104
	groEL		HSPps3	GCTGTNGAAGANGGNATTGT	104
	gltA		BhCS.781p	GGGGACCAGCTCATGGTGG	73
	gltA		BhCS.1137n	AATGCAAAAAGAACAGTAAACA	73
	gltA		CS.877p	GGGGGCCTGCTCACCGCGG	44
	gltA		CS.1258n	ATTGCAAAAAGTACAGTGAACA	44
	gltA		1240F	GATYCTTTCCGCCTTATG	74
	gltA		1497R	GAAATCCTAGAGCTTTTAATG	74
	rrs-rrl igs		321s	AGATGATGATCCCAAGCCTTCTGG	59
	rrs-rrl igs		983As	TGTTCTYACAACAATGATGAT	59
	rpoB		1400F	CGCATTGGCTTACTTCGTATG	79
	rpoB		2300R	GTAGACTGATTAGAACGCTG	79

[a] *rrs-rrl* igs, 16S rRNA-23S rRNA gene intergenic spacer region; *rrs*, 16S rRNA gene.
[b] F, forward; R, reverse.

such as BACTEC Peds Plus vials (Becton Dickinson, Sparks, Md.) (32) inoculated with about ≤5 ml and incubated, with recovery subsequently achieved by subculture onto solid media (42). Acridine orange staining and blind subculture from negative bottles before discarding them at 7 days may increase the likelihood of identifying bartonellae (1). An assay was also developed in which samples of heparinized blood are sedimented and the plasma is collected for inoculation into shell vials (42). Culture is then performed by the centrifugation-shell vial technique using ECV304 human endothelial cell monolayers (48).

In broth, *Bartonella* spp. usually do not produce turbidity or convert enough oxidizable substrate to CO_2 for CO_2 detection-based blood culture systems to indicate growth. A novel, chemically modified, insect-based liquid culture medium has been developed and supports the growth of at least seven *Bartonella* species (60). This medium also supports cocultures consisting of different *Bartonella* species, and it facilitates primary isolation of *B. henselae* from blood and aqueous fluids of naturally infected cats. This liquid growth medium may provide an advantage over conventional direct blood agar plating for the diagnosis of bartonellosis (60).

Tissue

Recovery of *Bartonella* spp. from cutaneous lesions, liver, spleen, or lymph node is not easy but is possible, especially after homogenization in inoculation medium and plating directly onto solid agar. Cocultivation with an endothelial cell line or by the shell vial method is more laborious but can yield bacterial growth on some occasions where nothing else works (48). Such methods have both been applied successfully for isolation of *Bartonella* in endocarditis cases (55). Some liquid media have been developed to allow primary isolation of *B. henselae* from blood and tissue (17, 101) but have not been used widely.

IDENTIFICATION

Bartonella spp.

Colonies of *B. henselae* can be of two morphological types, present in the same culture: (i) irregular, raised, whitish, rough (cauliflower-like), and dry in appearance and (ii) smaller, circular, tan, and moist in appearance, tending to pit and adhere to the agar (Fig. 2) (99). *B. quintana* colonies are usually smooth, flat, and shiny and do not pit the agar (48). *B. clarridgeiae* produces small white, raised, indurated, and cohesive colonies (57). They also can appear to spread on the agar during primary isolation from cats. Most *Bartonella* spp. usually appear as uniformly smooth after repeated subcultures. The bacilli are small (2 by 0.5 μm) and stain best with Gimenez stain (48). With Gram staining, *Bartonella* spp. are only weakly counterstained with safranin O. *Bartonella* spp. appear after Gram staining as small gram-negative slightly curved rods resembling *Campylobacter, Helicobacter,* or *Haemophilus* (99). Clumping of cells is quite common. The morphology of the colonies which appear usually after a lengthy incubation (more than 7 days) and negative catalase and oxidase reactions are often sufficient for a presumptive identification of *Bartonella*. After several subcultures, *Bartonella* colonies appear in less than a week. Furthermore, during primary isolation of *B. henselae* from cat blood, colonies appear most often in less than a week, whereas it usually takes 10 to 14 days to observe the first colonies for *B. clarridgeiae* or *B. koehlerae*.

B. bacilliformis, B. clarridgeiae, B. capreoli, and *B. schoenbuchensis* are the only members of the genus that are motile by means of unipolar flagella. *B. quintana* and *B. henselae* as well as several other *Bartonella* species have a twitching motility associated with fimbriae or pili. These pili are associated with cytoadherence and may mediate specific interaction with host erythrocytes and endothelial cells (62).

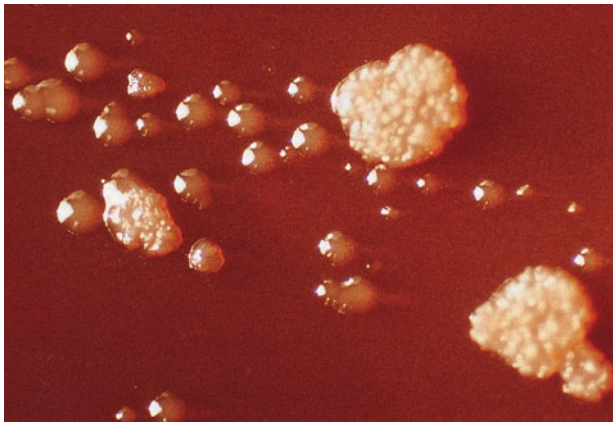


FIGURE 2 *B. henselae* after 7 days of incubation on chocolate agar, showing heterogeneity of colonies. Magnification, ×21.

Measurements of preformed enzymes and standard testing have revealed differences between species. Fatty acid methyl esters are prepared from cells harvested after 7 days of incubation on agar plates under conditions usually used for *Bartonella* isolation (see above). Bartonellae have a unique and characteristic whole-cell fatty acid composition (99). They have gas-liquid chromatography profiles consisting mainly of $C_{18:0}$, $C_{18:1}$, and $C_{16:0}$ acids. *B. elizabethae* contains a greater amount of $C_{17:0}$ than the other species.

Most species are biochemically inert except for the production of peptidases. None of the various commercially available identification systems contain *Bartonella* spp. in their databases. However, the MicroScan Rapid Anaerobe Panel (Baxter Diagnostics, Deerfield, Ill.), the RapID ANA II, and Rapid ID 32 A have been used as aids for identification. The MicroScan Rapid Anaerobe Panel has been reported to provide species identification (code 10077640 for *B. henselae,* code 10073640 for *B. quintana,* and code 10077240 for *B. bacilliformis*) (99). Overall, these identification kits are of limited use for proper diagnosis of *Bartonella* infection.

Identification of *Bartonella* isolates is largely based on molecular techniques, many allowing determination of the species or even some genotypes within a given species. Methods include Southern blotting, gel and capillary electrophoresis, PCR, DNA hybridization, RFLP, and sequence analysis and immunofluorescence detection (1, 84). The most widely targeted genes are those coding for the citrate synthase (*gltA*), a heat shock protein (*groEL*), riboflavin synthase (*ribC*), a cell division protein (*ftsZ*), and a 17-kDa antigen (23) (Table 2). The 16S rRNA gene (*rrs*) was first used by Relman et al. in 1990 for identification of the microbial etiology in a case of BA (78). However, this gene is not sensitive enough to properly discriminate *Bartonella* at the species level (56). Sequences obtained from the *gltA* and *rpoB* (RNA polymerase beta-subunit) genes are congruent with DNA-DNA hybridization for *Bartonella* species identification (56). The intergenic spacer region between the 16S and 23S rRNA genes has also been useful for *Bartonella* species identification (43, 59, 61). A genomic fingerprinting technique using infrequent restriction site PCR was also proposed to identify pathogenic *Bartonella* species (31). Use of monoclonal antibodies has been proposed for rapid identification of *B. quintana* (58).

Typing Systems

Multilocus sequence typing (MLST) has been used to examine the distribution of polymorphisms within different genes of *B. henselae* isolates (36). MLST distinguished several sequence types that resolved into three distinct lineages, suggesting a clonal population structure for the species. Similarly, multispacer typing was applied for the sequence-based typing of *B. quintana* (25).

SEROLOGICAL TESTS

Serological testing has become the cornerstone of clinical diagnosis, as it is widely used for diagnosis of *Bartonella* infections, especially cases of CSD, because culture and isolation are quite time-consuming and difficult and may yield results only after several weeks. However, cross-reactivity among *Bartonella* species is common, and it may occur with some other pathogenic bacteria such as *C. burnetii* and *Chlamydophila* spp. (53, 65). Antibody response in humans and animals is detected mainly with the IFA or different enzyme immunoassays (EIAs) (68). Western blotting tests have also been developed but are not used as widely (34, 91). In the United States, serological testing for CSD is performed by the Centers for Disease Control and Prevention (CDC) in Atlanta, Ga., by various state public health laboratories, and by commercial laboratories. Commercial slides (such as those from Focus Diagnostics, Inc.) for *B. henselae* and *B. quintana* IFA testing are available and widely used in both the United States and Europe.

B. bacilliformis

IFA and EIAs for the diagnosis of *B. bacilliformis* infection and for use in serological surveys have both been used for many years. A new IFA based on *B. bacilliformis*-infected Vero cells has been developed and shown to be very specific (92% based on 101 healthy controls) and identified 81% (86 of 106) of those with laboratory-diagnosed acute bartonellosis (13). Some other assays have been developed but showed some cross-reactivity with *Brucella* and *Chlamydophila psittaci,* suggesting that test optimization is still required (1).

B. quintana, B. henselae, and *B. clarridgeiae*

Human infection with *B. henselae* or *B. quintana* is evaluated by the presence of immunoglobulin M (IgM) or IgG directed against these bacteria, with cross-reactivity in up to 95% of samples (91). Antigen adsorption can be used to reduce cross-reactivity and to determine to which antigen the antibodies are truly directed (1). For animals, detection of antibodies is usually directed toward IgG. The first serological test for CSD, developed at the CDC, was an IFA based on *B. henselae* bacilli that were cocultivated with Vero cells to inhibit autoagglutination (77). This test was found to have good sensitivity (84 to 95%) and specificity (94 to 98%) (20, 77, 102). Lower sensitivity and specificity were reported from Europe (22, 103). However, commercial slides from at least one specific manufacturer (Focus Diagnostics) have been shown to be more sensitive for detection of cases of CSD, especially in Europe (68, 103). IFA is the most frequently used serological test, and it seems to currently be the most reliable test and the method of choice for the diagnosis of CSD (91). Dilution titers of 256 or higher are usually indicative of ongoing infection; lower titers may indicate an early or late clinical phase of the disease or prior exposure to the bacteria. In cases of endocarditis caused by either *B. henselae* or *B. quintana,* high IgG antibody titers (≥800) are usually detected (28). A titer ≥800 for IgG antibodies to either *B. henselae* or *B. quintana* has a positive predictive value of 0.810 for the detection of chronic *Bartonella*

infections in the general population and 0.955 for the detection of *Bartonella* infections among patients with endocarditis.

IFA is time-consuming, requires appropriate equipment and expertise, and is subject to interobserver variation due to the difficulty of reading the test (1). Within the last 10 years, different EIAs have been developed; they are simpler to perform, use readily available equipment, and can be automated (1). However, at present, EIAs have not been widely used and the sensitivity of the tests (17 to 35%) was lower than for one IFA (5). Giladi et al. (29) developed an EIA that used *N*-lauroylsarcosine-insoluble outer membrane antigens from agar-grown *B. henselae* for the detection of anti-*B. henselae* IgG and IgM antibodies in patients with CSD. It was found that when a positive IgG or IgM test result or both are accepted as diagnostic, the sensitivity of the EIA is 85%. Applying stringent criteria, this test is highly specific (98 and 99% for IgG and IgM, respectively), as determined by testing healthy control subjects and patients with lymphadenopathy due to defined non-CSD causes (69). EIA seems more sensitive than IFA for detection of IgM (1).

ANTIMICROBIAL SUSCEPTIBILITY

Antimicrobial susceptibility testing can be performed by incorporation of antibiotics into either blood or chocolate agar or into various media supplemented with blood using a microdilution technique (88, 95). The E test (AB Biodisk, Solna, Sweden) can also be used to determine antibiotic susceptibility (100). Results of susceptibility testing of *Bartonella* spp. are summarized in Table 3. Evaluation of susceptibility to

TABLE 3 MICs for *Bartonella* strains[a]

Antibiotic group and drug name	MIC (μg/ml)				
	B. henselae	*B. quintana*	*B. bacilliformis*	*B. vinsonii*	*B. elizabethae*
Aminoglycosides					
Amikacin	2–4	4–8	2–8	4	1
Gentamicin	0.12–0.25	0.12–2	1–2	0.5	0.12
Streptomycin	ND	ND	4	ND	ND
Tobramycin	0.5–1	0.5–4	2–4	2	0.25
Cephalosporins					
Cefotaxime	0.12–0.25	0.12–0.25	0.03–0.12	0.12	0.06
Cefotetan	0.25–0.5	0.12–0.5	2	1	1
Ceftazidime	0.25–0.5	0.25–0.5	0.12–0.25	0.25	0.5
Ceftriaxone	0.12–0.25	0.06–0.25	0.003–0.006	0.06	0.12
Cephalothin	8–16	8–16	4–8	16	8
Macrolides					
Azithromycin	0.006–0.015	0.006–0.03	0.015	0.015	0.006
Clarithromycin	0.006–0.03	0.006–0.03	0.015–0.03	0.03	0.015
Erythromycin	0.06–0.25	0.06–0.12	0.06	0.25	0.12
Roxithromycin	0.015–0.03	0.015–0.06	0.03	0.12	0.06
Telithromycin	0.003	0.006	0.015	ND	ND
Penicillins					
Amoxicillin	0.6–0.12	0.03–0.06	0.03–0.06	0.06	0.03
Oxacillin	1–2	1–4	0.25–0.5	1	4
Penicillin G	0.03–0.06	0.03	0.015–0.03	0.03	0.015
Ticarcillin	0.25	0.06–0.25	0.06–0.12	0.25	0.12
Quinolones					
Ciprofloxacin	0.25–1	0.5–2	0.25–0.5	1	0.5
Pefloxacin	4–8	2–8	1–2	4	2
Sparfloxacin	0.06	0.06–0.12	0.25	0.06	0.06
Tetracyclines					
Doxycycline	0.12	0.06–0.25	0.03–0.06	0.25	0.06
Miscellaneous					
Clindamycin	2–4	4–16	32–64	8	8
Colistin	4–16	4–16	16	8	4
Fosfomycin	16–32	32–64	8–16	16	16
Imipenem	0.5	0.25–1	0.5–1	2	0.25
Rifampin	0.03–0.06	0.06–0.25	0.003	0.12	0.03
TMP-SXT	1/5	0.25/1.25–1/5	0.4/2–0.8/4	1/5	0.5/2.5
Vancomycin	2–8	8–16	4–8	8	8

[a] Determined by agar dilution with Columbia agar supplemented with 5% horse blood. Table adapted from reference 81. Abbreviations: TMP-SXT, trimethoprim-sulfamethoxazole; ND, not done.

antibiotics has been performed either in cells or without cells, i.e., in axenic media. These various methods of culture could be used for determination of the bacteriostatic activity of antibiotics with similar results. Determination of the antibiotic susceptibility in axenic medium has been carried out either in solid media enriched with 5 to 10% sheep or horse blood or in liquid media (66, 95). It should be noted that the conditions required to grow *Bartonella* during susceptibility testing do not meet standardized criteria established for the Clinical and Laboratory Standards Institute. Bacteria of the genus *Bartonella* are susceptible to many antibiotics when grown axenically, including β-lactams, aminoglycosides, chloramphenicol, tetracyclines, macrolide compounds (including telithromycin), rifampin, fluoroquinolones, and trimethoprim-sulfamethoxazole (66, 67, 72, 86).

In vitro antibiotic susceptibilities also have been examined for *Bartonella* species cocultivated with eukaryotic cells. As with agar-based susceptibilities, these studies demonstrated that *Bartonella* spp. are susceptible to many antibiotics in vitro (37). However, all of these antibiotics (39) had only bacteriostatic activity (38, 39). Only aminoglycosides are bactericidal in vitro against *Bartonella* species grown in liquid medium (88), in endothelial cells (71), or in an erythrocyte coculture model (87).

Choice and Use of Antibiotics In Vivo

CSD typically does not respond to antibiotic therapy. Most investigators have observed no benefit with antibiotic treatment, whereas anecdotal reports have indicated that ciprofloxacin, rifampin, and trimethoprim-sulfamethoxazole may be effective (26, 75, 81).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Diagnosis of *Bartonella* infection in humans, especially for typical forms of CSD, is mainly based on serological data, which is the most cost-effective approach. As reported by Bergmans et al. (5) and Sander et al. (91), the sensitivities of different IFAs range from 14 to 100%, depending on the antigen used, the cutoff chosen, and the test procedures. For cases of endocarditis, the best approach is serological testing and performing PCR on cardiac valves. A recent procedure using a single-step serological assay against *C. burnetii* and *Bartonella* species found a sensitivity of 100%, and a positive predictive value of 98% for the diagnosis of blood culture-negative endocarditis (85). New methods of culture in liquid media are being developed, as direct isolation from blood or tissues is often negative despite detection of *Bartonella* DNA by PCR tests (60).

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Peptostreptococcus, Finegoldia, Anaerococcus, Peptoniphilus, Veillonella, and Other Anaerobic Cocci*

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TAXONOMY

Gram-positive anaerobic cocci are better known to most bacteriologists as peptococci or peptostreptococci. *Peptococcus* is only remotely related to other species of gram-positive anaerobic cocci and is rarely cultured from human clinical specimens; its main interest lies in its taxonomic significance. *Peptococcus niger* is now the sole remaining representative of this genus. Until recently, most clinical isolates of gram-positive anaerobic cocci were identified to the species level in the genus *Peptostreptococcus*, but this genus is currently under revision. *Peptostreptococcus* was described as a genus in 1936 and was considered the anaerobic equivalent of *Streptococcus*. The genus comprised 16 recognized species, with a G+C range from 27 to 37 mol%, except for *Peptostreptococcus productus* (44 to 45 mol%). Two proposals would restrict the genus *Peptostreptococcus* to *P. anaerobius* (46) and place *Peptostreptococcus magnus* and *Peptostreptococcus micros* in two new genera, *Finegoldia* and *Micromonas*, respectively (45). However, the name *Micromonas* is illegitimate because of the precedence of the microalga *Micromonas*. The name *Peptostreptococcus micros* must continue to be used because it is the single legitimate, validly published name. More recently, Ezaki et al. (19) proposed three new genera: *Anaerococcus,* which includes the saccharolytic, butyrate-producing species *(A. hydrogenalis, A. lactolyticus, A. octavius, A. prevotii, A. tetradius,* and *A. vaginalis); Peptoniphilus,* which contains the nonsaccharolytic, butyrate-producing species *(P. asaccharolyticus, P. harei, P. lacrimalis, P. indolicus,* and *P. ivorii);* and *Gallicola* (which contains a single species, *G. barnesae*). Table 1 shows the changes in classification of gram-positive anaerobic coccal species from human clinical specimens.

The taxonomy of the other validly published gram-positive anaerobic cocci from human clinical specimens such as *Streptococcus parvulus, P. productus,* and *Peptostreptococcus saccharolyticus* is also under revision. *S. parvulus* has been transferred to the genus *Atopobium* as *Atopobium parvulum* (13) (see chapter 56). *P. productus* was reclassified in the genus *Ruminococcus* (20); however, it has been recommended that *P. productus* be retained in its present taxonomic position until there is a definitive revision of the classification of ruminococci. *Peptococcus saccharolyticus* has been transferred to the genus *Staphylococcus* based on the analysis of nucleic acid relatedness data and cell wall peptidoglycan structure.

The gram-negative anaerobic cocci have been classified in a single family, the *Veillonellaceae*. *Veillonella, Acidaminococcus,* and *Megasphaera* are genera in this family, and *Anaeroglobus geminatus* gen. nov., sp. nov., was recently described (11). The genus *Veillonella* is currently subdivided into eight species (31). Among them, four have been isolated from human clinical specimens frequently. They include *V. parvula*, *V. atypica*, *V. dispar*, and the new species *V. montpelliérensis*. The other four species, *V. caviae, V. criceti, V. ratti,* and *V. rodentium*, were found only in animals, except for one *V. ratti* isolate which was recovered from human semen. Phylogenetic analysis showed that the family *Veillonellaceae* did not represent a true clade and could not be considered a relevant taxon. This family has been suppressed from the classification proposed in the latest edition of *Bergey's Manual of Systematic Bacteriology* (24). This classification grouped all genera belonging to the *Sporomusa* subbranch in a single large family named "*Acidaminococcaceae*." The phylogenetic analysis of the *Sporomusa* subbranch is in accordance with the proposed new classification mentioned above.

DESCRIPTION OF THE GROUP

The organisms included in this chapter are obligately anaerobic non-spore-forming, sometimes elongated cocci. The genera *Peptostreptococcus, Anaerococcus, Gallicola, Finegoldia, Peptoniphilus,* and *Peptococcus* are gram-positive, coccobacillary or occasionally coccoid cells. In Gram-stained preparations of pure cultures, cells vary in size from 0.3 to 2.0 μm and can be arranged in pairs, short chains, tetrads, small clusters, or irregular masses; most species are present as either chains or clumps. The ability to utilize carbohydrates varies greatly; some genera are asaccharolytic, but a few are strongly saccharolytic. For most species, the products of protein digestion appear to be the principal energy source. The genus *Staphylococcus* contains two species, *S. saccharolyticus* and *Staphylococcus aureus* subsp. *anaerobius*, that initially grow under anaerobic conditions and become aerotolerant on subcultures (see chapter 28). Strictly anaerobic *Staphylococcus epidermidis* was reported to be occasionally isolated from clinical specimens (53). The genera *Veillonella, Acidaminococcus*, *Megasphaera,* and *Anaeroglobus*

*This chapter contains information presented in chapter 55 by Bernard J., Moncla and Sharon L. Hillier and chapter 56 by Hannele R. Jousimies-Somer, Paula H. Summanen, Hannah Wexler, Sydney M. Finegold, Saheer E. Gharbia, and Haroun N. Shah in the eighth edition of this Manual.

TABLE 1 Changes in classification of gram-positive anaerobic coccal species from human clinical specimens

Current classification	Previous classification
Peptococcus niger	*Peptococcus niger*
Peptostreptococcus anaerobius	*Peptostreptococcus anaerobius*
Peptostreptococcus micros[a]	*Peptostreptococcus micros*
Peptoniphilus asaccharolyticus	*Peptostreptococcus asaccharolyticus*
Peptoniphilus indolicus	*Peptostreptococcus indolicus*
Peptoniphilus harei	*Peptostreptococcus harei*
Peptoniphilus ivorii	*Peptostreptococcus ivorii*
Peptoniphilus lacrimalis	*Peptostreptococcus lacrimalis*
Anaerococcus prevotii	*Peptostreptococcus prevotii*
Anaerococcus tetradius	*Peptostreptococcus tetradius*
Anaerococcus octavius	*Peptostreptococcus octavius*
Anaerococcus hydrogenalis	*Peptostreptococcus hydrogenalis*
Anaerococcus lactolyticus	*Peptostreptococcus lactolyticus*
Anaerococcus vaginalis	*Peptostreptococcus vaginalis*
Finegoldia magna	*Peptostreptococcus magnus*
Gallicola barnesae	*Peptostreptococcus barnesae*
Slackia heliotrinireducens corrig[b]	*Peptostreptococcus heliotrinreducens*
Atopobium parvulum	*Streptococcus parvulus*
Ruminococcus productus[c]	*Peptostreptococcus productus*
Staphylococcus saccharolyticus	*Peptococcus saccharolyticus*

[a] The name *Micromonas micros* was proposed by Murdoch and Shah (45), but it is illegitimate because it is placed in an illegitimate genus. So, the name *Peptostreptococcus micros* must be used because it is the single legitimate, validly published name.

[b] The specific epithet *heliotrinreducens* [*sic*] has been corrected in the new combination *Slackia heliotrinireducens*. However, the name "*Peptostreptococcus heliotrinireducens*" corrig. was never formally proposed before 14 December 2000. Consequently, in the "List of Prokaryotic Names with Standing in Nomenclature" (http://www.bacterio.cict.fr/index.html), the epithet *heliotrinreducens* [*sic*] has been maintained for *Peptostreptococcus heliotrinreducens*.

[c] *Ruminococcus productus* was proposed recently; however, Willems and Collins have recommended that *P. productus* be retained in its present taxonomic position (67).

are gram-negative cocci. Cells vary in size from 0.3 to 2.5 μm. They characteristically occur in pairs, but single cells, masses, or chains may also occur.

EPIDEMIOLOGY AND TRANSMISSION

Gram-positive anaerobic cocci are part of the normal microbiota of the mouth, upper respiratory and gastrointestinal tracts, female genitourinary system, and skin (42). Gram-positive anaerobic cocci constitute 1 to 15% of the normal oral microbiota (60); *P. micros* is usually considered to be the predominant species of gram-positive anaerobic cocci in the oral microbiota, and *P. anaerobius* and *Finegoldia magna* are also present in the oral cavity. The gastrointestinal tract hosts a wide variety of gram-positive anaerobic cocci, including most recognized species of *Peptostreptococcus*. *P. productus* is one of the most common organisms in the gastrointestinal microbiota; *F. magna* and *A. prevotii* are also common. Several other gram-positive anaerobic cocci are found less often. Large numbers of gram-positive anaerobic cocci can be found in the female genitourinary tract (15). *A. tetradius, A. lactolyticus,* and *A. vaginalis* were first described from vaginal discharges (21, 35); *P. anaerobius, P. asaccharolyticus, P. hydrogenalis, F. magna, P. micros, A. prevotii,* and *Peptococcus niger* have also been isolated from that site (35). The skin microbiota contains gram-positive anaerobic cocci; *F. magna* is the species identified most frequently, followed by *P. asaccharolyticus*.

Gram-negative anaerobic cocci form part of the oral, genitourinary, respiratory, and intestinal flora of humans (30). *Veillonella* species are part of the normal mouth, upper respiratory tract, gastrointestinal tract, and vaginal flora; *Acidaminococcus* and *Megasphaera* are part of the intestinal flora.

CLINICAL SIGNIFICANCE

Estimation of the clinical significance of anaerobic cocci isolated from clinical specimens is often difficult, partly due to their recovery from poorly obtained specimens. Anaerobic gram-positive cocci are opportunistic pathogens, comprising approximately one-quarter of all isolates from anaerobic infections (42). They may be present in a great variety of infections involving all areas of the human body, ranging in severity from mild skin abscesses to more serious and life-threatening infections such as brain abscess, bacteremia, necrotizing pneumonia, and septic abortion. Brain abscess is one of the more serious infections involving anaerobic cocci (10, 33, 36). Anaerobic cocci are involved in as many as 40% of pleuropulmonary infections such as lung abscess, necrotizing pneumonia, aspiration pneumonia, and empyema (37, 64). Anaerobic cocci often are isolated with other organisms in skin and soft tissue infections, including progressive bacterial synergistic gangrene, necrotizing fasciitis, and crepitant cellulitis (4, 44, 58). Other infections in which anaerobic cocci have been recognized as significant pathogens are infections of the female genital tract and intra-abdominal infections (8, 18, 56).

Although most infections involving gram-positive anaerobic cocci are polymicrobial (22), there are many instances of their isolation in pure culture (42); most relate to *F. magna*, but there are also reports of *P. anaerobius*, *P. asaccharolyticus*, *P. indolicus*, *P. micros*, *A. vaginalis,* and *P. harei* in pure culture. *F. magna* is the most pathogenic and one of the most frequently isolated gram-positive anaerobic coccal species found in human clinical specimens. It has been isolated from a wide variety of infections at various body sites in pure culture. These include cases of endocarditis (63), meningitis (10), and pneumonia (47), some of which have been fatal. *F. magna* is most commonly associated with infection of skin and soft tissue and with bone and joint infections, but it has also been isolated from cases of septic arthritis (23), prosthetic implant infections (14), breast abscess (17), diabetic foot infection (29), and upper respiratory tract infection such as sinusitis and otitis media. *P. anaerobius* is involved in polymicrobial infections, including abscesses of the brain, ear, jaw, and pleural cavity; pelvic and urogenital regions; the external genitalia; the abdominal region; and the nasal septum (7, 9, 28). The isolation of *P. anaerobius* from a patient with endocarditis has been reported (39). *P. anaerobius* has been associated with gingivitis (40) and periodontitis (65) and is one of the species found most frequently in the root canals of teeth with periapical abscess (59) and has been isolated from a peritonsillar abscess (12). *P. micros* is increasingly recognized as an important oral pathogen. Although it is considered a natural commensal of the oral cavity, elevated counts of this organism are associated with periodontal destruction (16, 50). It is also commonly isolated from other oral infections such as endodontic lesions and peritonsillar infections (38). *P. micros* is not restricted to the oral cavity; it is often isolated in mixed anaerobic infections from different body sites, including brain abscess, otitis

media, sinus infection, human bite wounds, pleural empyema, intra-abdominal infection, anorectal abscess, septicemia, gynecologic infection, vertebral osteomyelitis, and prosthetic joint infection.

Anaerobic gram-negative cocci account for a small percentage of the anaerobic cocci isolated from human specimens (3). *Veillonella* sp. strains are frequently isolated from clinical specimens in aerobic-anaerobic polymicrobial cultures. Rarely, *Veillonella* species have been the only etiologic agents identified in serious infections such as meningitis, osteomyelitis, prosthetic joint infection, pleuropulmonary infection, bacteremia, and endocarditis.

The variety of infections in which anaerobic cocci are involved has been thoroughly reviewed and summarized (42). Although the spectrum of infections has remained relatively unchanged since the extensive review by Murdoch in 1998 (42), the prevalence of these organisms as pathogens is clearly increasing.

COLLECTION, TRANSPORT, AND STORAGE OF CLINICAL SPECIMENS

Most gram-positive anaerobic cocci isolated from human clinical material are not extremely oxygen sensitive. Specimens suspected of harboring anaerobic cocci should be collected, transported, and stored by methods outlined elsewhere (see chapters 5 and 20).

ISOLATION PROCEDURES

Routinely used anaerobic plate media, such as brucella, Columbia, or Schaedler agar base supplemented with 5% sheep blood, vitamin K_1, and hemin, will support the growth of these microorganisms. However, the CDC (Centers for Disease Control and Prevention) agar base gives better recovery of gram-positive anaerobic cocci than brucella agar or other medium formulations. The usual procedures for anaerobes should be followed. Many of these organisms require high moisture content for optimal growth, so fresh medium should be used. Laboratories unable to prepare their own media may wish to consider the use of commercially prepared, prereduced, anaerobically sterilized blood agar (Anaerobe Systems, Morgan Hill, Calif.). These media have an extended shelf life, of up to 6 months, and yield results comparable to or better than those obtained with fresh media.

Gram-positive anaerobic cocci are heterogeneous; a single medium is unlikely to support the growth of all representatives and be reasonably selective. Wren (68) showed that nalidixic acid-Tween blood agar gave better isolation than neomycin blood agar, but recommended that a combination of different media be used to maximize recovery rates. The disadvantage of neomycin blood agar may be the particular inhibitory nature of neomycin to gram-positive anaerobic cocci. Petts et al. (49) reported that oxolinic acid was superior to nalidixic acid for suppression of staphylococci but permitted the growth of non-spore-forming anaerobes, including gram-positive anaerobic cocci. Turng et al. (61) described *P. micros* medium, a selective and differential medium for *P. micros* which contains colistin-nalidixic acid agar (Difco, BD Diagnostics, Sparks, Md.), a selective base for gram-positive cocci that is supplemented with glutathione and lead acetate. Strains of *P. micros* can use the reduced form of glutathione to form hydrogen sulfide, which reacts with lead acetate to form a black precipitate under the colony. Tween 80 supplementation of media may improve growth of some gram-positive anaerobic cocci.

TABLE 2 Differential characteristics of *Peptostreptococcus*, *Peptococcus*, *Peptoniphilus*, *Finegoldia*, and *Anaerococcus*[a]

Species	GLC	Inhibition by SPS	Production of:				Glucose	Production of saccharolytic and proteolytic enzymes								Gram stain and colonial morphology
			Indole	Urease	ALP	ADH		α-Gal	β-Gal	α-Glu	β-Gur	ArgA	ProA	PheA	LeuA	PyrA	
P. asaccharolyticus	A, b	–	d	–	–	–	–	–	–	–	–	+	–	–	d	–	Cells are uniform, in clumps, retaining Gram stain poorly, resembling strains of *Neisseria*. Glistening, low convex, usually whitish to lemon-yellow, musty-odor colonies.
P. indolicus	A, b	–	+	–	+	–	–	–	–	–	–	+	–	+	+	–	Similar to *P. asaccharolyticus*
P. harei	A, b	–	d	–	–	–	–	–	–	–	–	+	–	–	–/w	–	Cells variable in size and shape. Flat, translucent colonies.
P. lacrimalis	A, b	–	–	–	–	–	–	–	–	–	–	+	–	+	+	–	Cells are in short chains or clumps. Pink-white colonies.
"*trisimilis*" group[b]	A, b	–	+	–	d	–	+	–	d	–	–	–	–	–	–	+	Cells are in clumps and tetrads. Gray with white centers, entire and circular colonies.
A. hydrogenalis	B, a	–	+	d	–/w	–	+	–	–	d	–	–	–	–	–	–	Cells variable in size, arranged in clumps, tetrads, and short chains. Gray-white convex colonies, unpleasant odor.

A. prevotii	B, a	−	−	+	−	−	−	+	−	+	+	+	−	d	−	+	Cells variable in size, in clumps or tetrads. Matte gray, low convex colonies.
A. tetradius	B, a	−	−	+	−	−	+	−	−	+	+	+	−	w	+	−/w	Cells variable in size, in clumps and tetrads. Matte gray, low convex colonies.
A. lactolyticus	B, a	−	−	+	+	−	+	−	+	−	−	+	−	−	−	+	Cells are in short chains or clumps. Pink-white colonies.
A. vaginalis	B, a	−	d	−	d	+	+	−	−	−	−	+	−	−	+	−	Cells variable in size, in clumps or tetrads. Gray-white, low convex colonies.
"β-GAL" group[b]	B, a	−	−	−	w	d	+	−	w/+	−/w	−	+	−	−	+	w	Cells variable in size, arranged in clumps. Gray-white, low convex colonies.
A. octavius	B, a, c	−	−	−	−	−	+	−	−	−	−	−	+	−	−	w	Cells are arranged in clumps. Yellowish white, glistening, circular, raised, entire colonies.
P. ivorii	IV	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	Cells variable in size, arranged in clumps. Yellowish white, low convex colonies.
P. anaerobius	A, IC	+	−	−	−	−	+	−	−	+	−	−	+	−	−	−	Cells are highly pleomorphic, in chains. Colonies are gray with slightly raised off-white centers and a distinctive, sweet odor.
F. magna	A	−	−	−	d	d	−/w	−	−	−	−	+	−	−	+	+	Cells are in pairs, tetrads, or clusters and >0.6 μm in diam. Small colonies, convex and whitish, while others are flatter and translucent.
P. micros	A	−	−	−	+	−	−	−	−	−	−	+	+	+	+	+	Cells in pairs, chains, and clusters. Small colonies; typically white, glistening, and domed and often surrounded by a distinctive yellow-brown halo of discolored agar up to 2 mm wide.
P. productus	A	−	−	−	−	−	+	+	−	+	−	−	−	−	−	−	Cells are ovoid, in pairs or chains; glistening gray colonies.

[a] Data mainly from Murdoch (42) and our unpublished data. Abbreviations and symbols: A, acetate; B, butyrate; IV, isovalerate; IC, isocaproate; C, *n*-caproate; ALP, alkaline phosphatase; ADH, arginine dihydrolase; α-Gal, α-galactosidase; β-Gal, β-galactosidase; α-Glu, α-glucosidase; β-Gur, β-glucuronidase; ArgA, arginine arylamidase; ProA, proline arylamidase; PheA, phenylalanine arylamidase; LeuA, leucine arylamidase; PyrA, pyroglutamyl arylamidase; −, >90% negative; w, weakly positive; +, >90% positive; d, different reactions; w, weakly positive.

[b] Described by Murdoch and Mitchelmore (43).

A recent study (27) tested different media for recovery of *Veillonella* spp. from saliva samples and concluded that a selective medium for *Veillonella* with vancomycin and laked blood gave the greatest recovery of *Veillonella*. This medium can also be used for presumptive identification of *Veillonella* since the colonies produce a red fluorescence.

IDENTIFICATION

Phenotypic Tests

Some gram-positive anaerobic cocci, particularly strains of *P. asaccharolyticus*, decolorize readily by Gram stain and can be confused with gram-negative anaerobes such as *Veillonella*. Gram-positive anaerobic cocci can be distinguished from gram-negative anaerobic cocci by special-potency disks (vancomycin, 5 μg; kanamycin, 1,000 μg; and colistin, 10 μg). The cell morphology of older cultures of gram-positive anaerobic cocci can be very irregular, with many coccobacillary and rod-like forms. It is also important to distinguish gram-positive anaerobic cocci from microaerobic organisms, such as strains of *Streptococcus* species. A simple and reliable test is to apply a 5-μg metronidazole disk to the edge of the inoculum; gram-positive anaerobic cocci show a zone of inhibition of 15 mm or greater, whereas microaerobic strains show no zones of inhibition after incubation for 48 h (43).

P. anaerobius is the only gram-positive anaerobic coccus that gives a zone of inhibition of ≥12 mm around a sodium polyanetholsulfonate (SPS) disk. *P. micros* also exhibits a zone of inhibition with SPS; however, the zone is usually <12 mm. Most *P. anaerobius* strains form distinctive colonies on enriched blood agar; they are 1 mm in diameter after 24 h and gray, with slightly raised off-white centers and a distinctively sweet odor (43). *P. micros* and *F. magna* can be readily distinguished by a combination of colonial morphology and proteolytic enzyme profiles, supported by Gram-stained cell morphology to assess the cell size. An anaerobic coccus with a milky halo around the colonies and small cells (<0.6 μm) can be presumptively identified as *P. micros* (43, 62). *F. magna* cells are larger than those of most peptostreptococci. Published data (42) also indicate that they can be differentiated by enzymatic tests for proteolytic activity such as proline arylamidase, phenylalanine arylamidase, and tyrosine arylamidase.

P. asaccharolyticus is another gram-positive anaerobic coccal species frequently isolated from human clinical specimens, but strains that were identified phenotypically are genetically diverse. To complicate matters further, the type strain of *P. asaccharolyticus* is highly atypical in its whole-cell composition and some biochemical properties. A recent study of ours (unpublished data) indicated that all strains (a total of 33) that were identified as *P. asaccharolyticus* phenotypically shared only a low sequence similarity (<90%) with the corresponding sequence of the type strain of *P. asaccharolyticus*. They all had almost identical 16S rRNA sequences and shared a high sequence similarity (>99%) with another *P. asaccharolyticus* strain, ATCC 29743. The cellular morphology of this group of bacteria is characteristic; the cell size is more uniform than is observed with most species of gram-positive anaerobic cocci, and cells occur in clumps, retaining the Gram stain poorly and often resembling strains of *Neisseria*. The colony morphology is also distinctive; after 5 days of incubation on enriched blood agar, colonies are 2 to 3 mm in diameter, glistening, low convex, and usually whitish to lemon-yellow, and they often have a characteristic musty odor. They produce an enzyme profile very similar to that of *P. harei* strains but can easily be differentiated by their clearly different cell and colonial morphology. Cells of *P. harei* vary considerably in size (diameter, 0.5 to 1.5 μm) and shape (circular, oval, or elliptical). Colonies of 5-day cultures on enriched blood agar are approximately 1 mm in diameter, nonhemolytic, flat, and translucent. *P. indolicus* is phenotypically similar to *P. asaccharolyticus* and can be difficult to identify, but it is rarely isolated from human clinical specimens. *P. indolicus* can be tentatively distinguished from *P. asaccharolyticus* by its ability to produce alkaline phosphatase.

A. prevotii and *A. tetradius* were reported as common species of gram-positive anaerobic cocci in human clinical material in early surveys. However, nucleic acid studies indicate that they are very heterogeneous. Again, our study based on 16S rDNA sequencing indicated that a large percentage of organisms identified as *A. prevotii* or *A. tetradius* are strains of *A. vaginalis*. It is likely that strictly defined strains of *A. prevotii* or *A. tetradius* are only occasionally recovered from most clinical specimens. The activity of pyroglutamic acid arylamidase might be useful for differentiation of *A. prevotii* and *A. tetradius;* however, distinctions cannot be generalized because insufficient numbers of strains of each species have been reliably identified. *A. prevotii* and *A. tetradius* can be distinguished from other recognized species of gram-positive anaerobic cocci by production of α-glucosidase and β-glucuronidase. Strains of other saccharolytic gram-positive anaerobic cocci such as *A. vaginalis* and *A. lactolyticus* can be differentiated by their enzyme profiles.

Table 2 summarizes the differential characteristics of gram-positive anaerobic coccal species. Based on published data and our unpublished data, we developed a flowchart for rapid identification of gram-positive anaerobic cocci (Fig. 1). The identification is based on phenotypic tests that can be performed in many diagnostic laboratories. Most of the information presented here relates to the phenotypic characteristics of strains isolated from humans.

Several systems, such as Rapid ID 32A (bioMérieux, Marcy L'Etoile, France) and RapID ANA II (Remel, Inc., Lenexa, Kans.), are available commercially for the rapid identification of anaerobes. Evaluation of these biochemical kits indicates that they may be valuable for characterizing anaerobic cocci (42). However, these systems are designed to identify a wide range of anaerobes, and they contain many tests of little relevance for identification of anaerobic cocci. Furthermore, databases accompanying the kits are often incomplete or inaccurate, especially for newly described species. Our most recent comparison of the Rapid ID 32A kit with 16S rRNA gene sequencing for identification of gram-positive anaerobic cocci showed that the Rapid ID 32A system is good for accurate identification of *P. micros*, *P. anaerobius*, *F. magna,* and *P. asaccharolyticus*, but not the others.

Gram-positive anaerobic cocci are separated into five groups based on fatty acid end products of metabolism analyzed by gas-liquid chromatography (GLC) (Table 2): (i) an acetate group containing *F. magna, P. micros,* and *P. productus* that only produces acetic acid; (ii) a butyrate-acetate group that produces butyric acid as its major terminal volatile fatty acid (VFA) and acetic acid as a second lesser acid (this group contains all of the species in the genus *Anaerococcus*); (iii) an acetate-butyrate group that produces acetic acid as its major terminal VFA and butyric acid as the second acid (this group contains all of the species in the genus *Peptoniphilus* except for *P. ivorii*); (iv) a caproate group whose members produce large quantities of longer-chain VFAs (the most important species in this group is *P. anaerobius*, the only

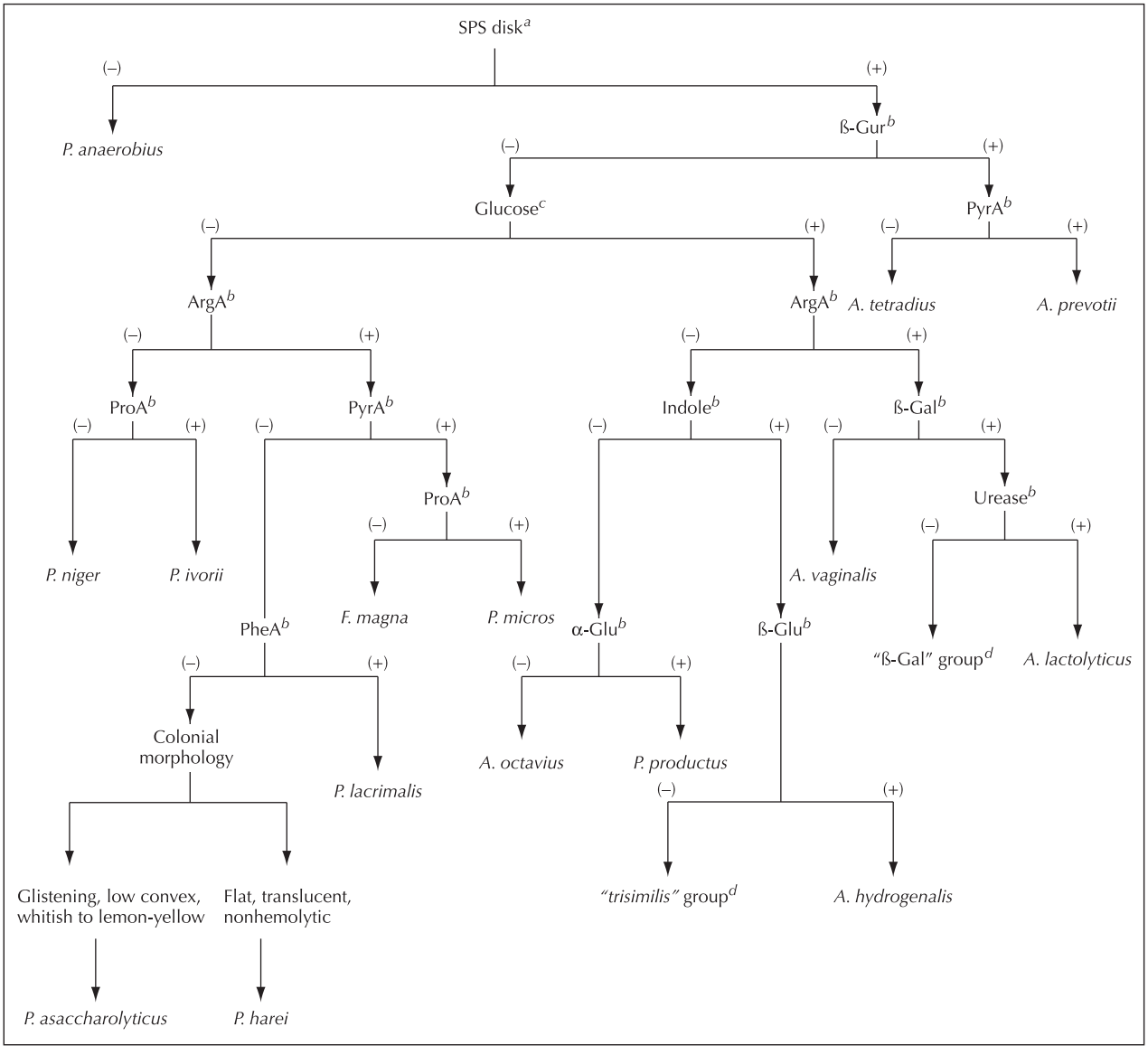


FIGURE 1 Flowchart with key characteristics for identification and differentiation of gram-positive anaerobic cocci. *a,* SPS testing was done using the SPS disk from Anaerobe Systems. All gram-positive anaerobic cocci are resistant to SPS except for *P. anaerobius,* which gives a zone of inhibition of ≥12 mm around an SPS disk. *P. micros* also exhibits a zone of inhibition with SPS; however, the zone is usually <12 mm. +, no zone or the zone of inhibition is <12 mm; −, zone of inhibition is ≥12 mm. *b,* All the enzymatic tests were done using the Rapid ID 32A system (API bioMérieux) according to the manufacturer's instructions. β-Gal, β-galactosidase; α-Glu, α-glucosidase; β-Glu, β-glucosidase; β-Gur, β-glucuronidase; ArgA, arginine arylamidase; ProA, proline arylamidase; PheA, phenylalanine arylamidase; PyrA, pyroglutamyl arylamidase. *c,* Glucose fermentation tests were performed using prereduced, anaerobically sterilized peptone-yeast-glucose (PYG) broth tube (Anaerobe Systems). A pH of ≤5.5 in the PYG tubes was interpreted as a positive result, and a pH of ≥5.9 was interpreted as a negative result. *d,* Described by Murdoch and Mitchelmore (43).

species of gram-positive anaerobic cocci to produce a major terminal peak of isocaproic acid); and (v) an isovaleric acid group that contains *P. ivorii* (this organism is the only species of gram-positive anaerobic cocci that produces a major terminal peak of isovaleric acid). GLC is also useful for identifying the rarely isolated *Peptococcus niger* and *P. octavius*, which produce *n*-caproic acid.

Veillonella, Acidaminococcus, and *Megasphaera* comprise the genera of anaerobic gram-negative cocci. Table 3 contains a key for distinguishing these three genera.

Molecular Methods

Molecular methods, such as nucleic acid probe hybridization, PCR amplification, and 16S rRNA gene sequencing,

TABLE 3 Characteristics of gram-negative cocci[a]

Organism(s)	Nitrate reduction	Catalase	Glucose fermentation[b]	Fatty acids from PYG
Veillonella spp.	+	V	−	A, p
Acidaminococcus fermentans	−	−	−	A, B
Megasphaera elsdenii	−	−	+	A, ib, b, iv, v, C

[a] A, acetate; B and b, butyrate; C, *n*-caproate; ib, isobutyrate; iv, isovalerate; v, valeric acid; V, variable; p, propionate; +, positive reaction; −, negative reaction.
[b] Glucose fermentation tests were performed using prereduced, anaerobically sterilized (PRAS) peptone-yeast-glucose (PYG) broth tube (Anaerobe Systems). The PRAS tubes were inoculated from an actively growing broth culture (without carbohydrate). A pH of ≤5.5 in the PRAS tubes was interpreted as a positive result, a pH of 5.6 to 5.8 was interpreted as weakly positive, and a pH of ≥5.9 was interpreted as negative for fermentation.

are not yet standardized or available commercially for the direct demonstration of medically important gram-positive anaerobic cocci from clinical specimens. However, several studies have used molecular techniques to identify and detect gram-positive anaerobic cocci. DNA probes targeting the 16S rRNA gene have been used to detect *P. anaerobius* and *P. micros*, and PCR assays specific for detection of *F. magna*, *P. anaerobius*, and *P. micros* directly from clinical specimens have been developed (51, 52, 55). More recently, real-time PCR has been applied for quantitative detection of *P. micros* in clinical samples (2, 5). Our group evaluated the utility of 16S rRNA gene sequencing as a means of identifying clinically important gram-positive anaerobic cocci (57). Our study indicated that problems exist in the public database. For example, among the 13 type strains of gram-positive anaerobic coccal species that we tested, only 4 gave a "perfect" match with their corresponding sequences in GenBank, whereas the other 9 had lower sequence similarities (<98%) (Table 4). Based on the 16S rRNA sequences we deposited in GenBank, a multiplex PCR scheme was developed for rapid identification of clinically significant gram-positive anaerobic coccal species. Molecular methods undoubtedly will offer a potential option for accurate identification and rapid diagnosis in laboratories supplied with the appropriate equipment and competence.

ANTIMICROBIAL SUSCEPTIBILITIES

New information regarding the antimicrobial susceptibilities of gram-positive anaerobic cocci is sparse compared with the information available for other anaerobic species. Current information is summarized in Table 5. Many reports fail to give results for individual species, opting instead to combine data for the group.

Penicillins are considered to be effective first-line therapy for gram-positive anaerobic cocci. Most evidence suggests that *P. asaccharolyticus*, *F. magna*, and *P. micros* are usually susceptible to penicillins, although Wren (69) reported resistance among isolates of *F. magna* and *P. micros* (16 and 8%, respectively) to penicillin. Cephalosporins are usually, but not always, effective. Carbapenems are extremely active. Several authorities maintain that most gram-positive anaerobic cocci are susceptible to metronidazole, but resistance has frequently been reported. Strains that are microaerobic are much more likely to be resistant to metronidazole. Susceptibilities to clindamycin vary widely, and local geographic variation should be taken into account. A French multicenter study (41) reported clindamycin resistance (28%) among *Peptostreptococcus* spp., and an American study by Sanchez et al. (54) noted >10% resistance of *F. magna* to clindamycin. Wren (69) reported that 9% of *F. magna* isolates were resistant to clindamycin in London, England. Erythromycin, clarithromycin, and azithromycin have similar efficacies and are probably not active enough to be recommended. Several studies (25, 48, 66) indicated that narrow-spectrum quinolones such as ciprofloxacin have only moderate activity, but more recently developed agents such as trovafloxacin and clinafloxacin are extremely active (48). Goldstein et al. (26) reported that gemifloxacin is the most active fluoroquinolone tested for peptostreptococci. In two studies (1, 34), ciprofloxacin and ceftazidime showed much lower activities against gram-positive anaerobic cocci than the other antimicrobial agents tested. In another recent study, Koeth et al. (32) reported that the levofloxacin and clindamycin susceptibility rates of *F. magna* were 72.4 and 84.7%, respectively. It is highly desirable that future investigations present data on different species separately. A study by Brazier et al. (6) presented data on different gram-positive anaerobic coccal species separately. They found that the highest percentage of overall resistance detected among gram-positive anaerobic cocci was 41.6% resistance to tetracycline, followed by 27.4% resistance to erythromycin. Among gram-positive anaerobic cocci as a group, 7.1% of isolates were resistant to penicillin and clindamycin, and 3.5% of isolates were resistant to amoxicillin-clavulanate. There was no resistance among gram-positive anaerobic

TABLE 4 List of type strains and the corresponding accession numbers of 16S rRNA gene sequences in GenBank

Species	Strain	Accession no.
Anaerococcus hydrogenalis	ATCC 49630	D14140
Anaerococcus lactolyticus	ATCC 51172	AF542233
Anaerococcus octavius	CCUG 38493	Y07841
Anaerococcus prevotii	ATCC 9321	AF542232
Anaerococcus tetradius	ATCC 35098	AF542234
Anaerococcus vaginalis	ATCC 51170	AF542229
Finegoldia magna	ATCC 15794	AF542227
Peptoniphilus asaccharolyticus	ATCC 14963	AF542228
Peptoniphilus harei	ATCC BAA-601	Y07839
Peptoniphilus indolicus	ATCC 29427	AY153431
Peptoniphilus ivorii	ATCC BAA-602	Y07840
Peptoniphilus lacrimalis	ATCC 51171	AF542230
Peptostreptococcus anaerobius	ATCC 27337	L04168
Peptostreptococcus micros	ATCC 33270	AF542231
Veillonella atypica	ATCC 17744	AF439641
Veillonella dispar	ATCC 17748	AY995770
Veillonella montpellierensis	ADV 281.99	AF473836
Veillonella parvula	ATCC 10790	AY995767
Acidaminococcus fermentans	ATCC 25085	X65935
Megasphaera elsdenii	ATCC 25940	U95027

TABLE 5 Antimicrobial susceptibilities of gram-positive anaerobic cocci[a]

Species (no. of strains)	Range of MICs (μg/ml)[b]									
	Pen (1)	Amp/Sul[c] (8)	Amox/Clav[c] (8)	Pip/Tazo[c] (64)	Cefox (32)	Imi (8)	Clinda (4)	Metro (16)	Cipro (2)	Trova (4)
A. lactolyticus (2)	≤0.5	≤0.25	0.25		0.5	0.12	≤0.12	1		
A. lactolyticus-I (5)	≤0.5–2[d]	≤0.25–0.5	0.12–0.25	0.5	0.5–2	0.12–0.5	0.25–64[h]	1	0.5–1	
A. lactolyticus-II (3)	≤0.5–1	≤0.25			0.5–1	0.12–0.5	≤0.12	4		
A. prevotii (3)	2–16	1–4	2	2	1–4	0.5–1	32–128	1–2	16	
A. tetradius (1)	≤0.5	≤0.25			0.5	≤0.062	0.25	2		
A. vaginalis (16)	≤0.5	0.12–0.25	0.12–0.25	0.062–0.12	≤0.12–0.25	≤0.062–0.12	0.062–0.5	0.25–4	4–8	
F. magna (12)	0.12–1	≤0.25–0.5	0.12–0.25		0.5–2	0.062–0.5	0.062–2	0.25–2	0.12–2	0.062–0.12
P. asaccharolyticus (10)	≤0.5	0.12–0.25	0.12–0.25	0.062	≤0.12	≤0.062	≤0.12	0.5–4		
P. harei (2)	≤0.5–1	≤0.25			0.25	≤0.062	0.5	2		
P. anaerobius (16)	0.12–32[e]	≤0.25–16[g]	0.12–32		0.5–32	≤0.062–2	≤0.062–0.5	0.12–8	1	0.12
P. micros (15)	0.062–64[f]	0.062–0.5	0.062	0.062	0.5–1	0.062–0.12	0.062–1	0.25–1	1–16	0.062

[a] Data were obtained from the Wadsworth Anaerobic Bacteriology Laboratory. Strains were tested by Clinical and Laboratory Standards Institute (NCCLS) agar dilution procedures.

[b] MIC breakpoints are indicated in parentheses next to each antimicrobial agent. Abbreviations: Pen, penicillin; Amp/Sul, ampicillin-sulbactam; Amox/Clav, amoxicillin-clavulanic acid; Pip/Tazo, piperacillin-tazobactam; Cefox, cefoxitin; Imi, imipenem; Clinda, clindamycin; Metro, metronidazole; Cipro, ciprofloxacin; Trova, trovafloxacin.

[c] Results for these three drug combinations given for amoxicillin and piperacillin.

[d] One of 5 strains showed resistance.

[e] Three of 16 strains showed resistance.

[f] Two of 15 strains showed resistance.

[g] Three of 16 strains showed resistance.

[h] One of 5 strains showed resistance.

cocci to piperacillin-tazobactam, chloramphenicol, cefoxitin, imipenem, or metronidazole. Data from our laboratory are presented in Table 5.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

The initial report should provide Gram stain results and bacterial and human cell morphologies. The relative quantities of different organisms seen in the smear give a good overall impression of specimen quality, the nature of the polymicrobial infection, and the relative importance of each organism. In general, bacterial isolates that are predominant, virulent, and resistant to antimicrobial agents should be given the greatest attention. Bacteria present in pure culture or in large numbers are probably of major importance, as are organisms recovered in multiple cultures and isolated from normally sterile sites. Furthermore, Gram stain results can guide the laboratory in choosing media for optimal recovery of the predicted organisms.

The significance of finding anaerobic gram-positive or gram-negative cocci in clinical specimens depends on the specimen and the likelihood that it was contaminated by the microbiota of the skin or mucous membranes. Hence, interpretation of culture results is dependent on the nature and quality of the specimen submitted to the laboratory.

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Propionibacterium, Lactobacillus, Actinomyces, and Other Non-Spore-Forming Anaerobic Gram-Positive Rods*

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TAXONOMY AND DESCRIPTION OF AGENTS

The anaerobic gram-positive non-spore-forming rods are widely distributed among two gram-positive phyla: *Actinobacteria* and *Firmicutes* (Table 1). Several new genera have been described, and numerous taxonomic revisions within the genera covered in this chapter have been performed since the 8th edition of this Manual (Table 2). Although it may be confusing for those in clinical microbiology laboratories, the clarification of taxonomic relationships allows definitive associations between taxa and disease to be uncovered and greater precision in the laboratory identification of pathogens. In addition, molecular ecological analysis of clinical specimens has revealed numerous as-yet-uncultivable bacterial taxa belonging to this group (15, 35, 70, 117, 118, 126).

Phylum *Actinobacteria*

The family *Actinomycetaceae* currently includes five genera: *Actinomyces, Actinobaculum, Arcanobacterium, Varibaculum,* and *Mobiluncus*. It has long been known that species assigned to the genus *Actinomyces* constitute a heterogeneous group. New genera have been proposed to include both newly described species related to, and species formerly included in, *Actinomyces*. For example, *A. bernardiae* and *A. pyogenes* have been moved to the genus *Arcanobacterium* (116) (see chapter 34). *Actinomyces* and related genera contain anaerobic and aerotolerant, non-acid-fast, gram-positive organisms with variable morphology, ranging from characteristic branching rods to coccobacilli. *Actinomyces* contains a rapidly increasing number of species (Table 2) that usually produce succinic acid from glucose. *Actinobaculum* currently includes *A. schaalii* and *A. urinale* from humans and the type species *A. suis* from pigs (63, 97), but the proposal of more new species in the genus is warranted (35, 58). *Varibaculum* (67) is a monophyletic genus related to *Mobiluncus* but distinct on the basis of 16S rRNA gene sequence identity. *Varibaculum cambriensis* is related to *Actinomyces neuii* but would appear to deserve consideration as a separate genus. *Mobiluncus* contains two species, *M. curtisii* and *M. mulieris,* which are strictly anaerobic, curved bacilli with variable Gram reactions and corkscrew motility. They resemble *Actinomyces* in that succinic acid is the major metabolic end product from glucose. It has been shown recently that *Falcivibrio vaginalis* and *Falcivibrio grandis* are later synonyms of *M. curtisii* and *M. mulieris,* respectively (76).

Propionibacterium species are anaerobic and aerotolerant, pleomorphic, gram-positive rods that produce propionic acid from glucose. Four *Propionibacterium* species have been isolated from human clinical infections: *P. acnes, P. avidum, P. granulosum,* and *P. propionicum*. The last species (formerly *Arachnia propionica*) exhibits some characteristics similar to *Actinomyces* species. Two related taxa, formerly members of *Propionibacterium, Propionimicrobium lymphophilum* (146) and *Propioniferax innocua* (172), can also be encountered in clinical material.

Members of the genus *Bifidobacterium* and the closely related genera *Parascardovia* and *Scardovia* are strictly anaerobic or occasionally microaerobic, gram-positive, pleomorphic rods, appearing as uniform to branched or club-shaped. Typically, bifidobacteria produce fructose-6-phosphate phosphoketolase as well as acetic and lactic acids as major metabolic end products. Bifidobacteria are aciduric and are nutritionally fastidious. There are currently 29 *Bifidobacterium* species. Of these, 10 species *(B. bifidum, B. breve, B. gallicum, B. longum, B. adolescentis, B. catenulatum, B. dentium, B. angulatum, B. pseudocatenulatum,* and *B. scardovii)* have been isolated from the human gut and oral cavity. *B. longum* now includes the former species *B. infantis* and *B. suis* (129). Two species isolated from dental plaque, *B. inopinatum* and *B. denticolens* (27), were later shown to represent novel genera on the basis of HSP60 gene sequence relatedness and were transferred to *Scardovia* and *Parascardovia,* respectively (81).

Numerous taxa have been misassigned to *Lactobacillus* in the past, including the so-called "anaerobic lactobacilli," which are now recognized to constitute two new genera, *Atopobium* and *Olsenella,* within the family *Coriobacteriaceae* (26, 30). *Atopobium* species produce lactic acid as the major glucose metabolic end product. *A. minutum* and *A. rimae* were formerly *Lactobacillus* species, and *A. parvulum* and *A. fossor* formerly belonged to the genera *Streptococcus* and *Eubacterium,* respectively (26, 87). *A. vaginae* is a novel species, originally isolated from the healthy vagina (124). The genus *Olsenella* is closely related to *Atopobium* (30) and currently includes two species: *O. uli* (formerly *Lactobacillus uli*) and *O. profusa,* both isolated from the human oral cavity.

Most *Eubacterium* species belong to the *Firmicutes,* but until recently, the genus included a number of species now

* This chapter contains information presented in chapter 55 by Bernard J. Moncla and Sharon L. Hillier in the eighth edition of this Manual.

TABLE 1 Some features of non-spore-forming anaerobic gram-positive genera

Phylum and genus	G+C mol%	Cell characteristics	Aerotolerance[a]	Gram reaction[b]	Major end product(s)[c]	Other features
Actinobacteria						
Actinobaculum	50–57	Straight/slightly curved, branching; singly/in clusters	+/−	(+)	A	Saccharolytic
Actinomyces	55–68	Variable, often branching; singly/in pairs	+/−	+	S, L	Saccharolytic
Atopobium	35–46	Short, elliptical; singly/in pairs/short chains	−/+	+	L	Saccharolytic
Bifidobacterium	57–64	Variable	−/+	+	A, L	Aciduric
Collinsella	60–61	Short; in chains	−	+	A, F, L	Saccharolytic, H_2 production
Cryptobacterium	50–51	Short	−	(+)	None	Asaccharolytic
Eggerthella	62	Coccobacilli/short rods; in pairs/short chains	−	+	(A, L, S)	Asaccharolytic
Mobiluncus	49–52	Curved with tapered ends; singly/in pairs; motile	−	v	S, L, A	Saccharolytic
Olsenella	63–64	Short, elliptical; singly/in pairs/short chains	−	+	L, A	Saccharolytic
Parascardovia	54–56	Small, slender, variable	−	+	A, L	Saccharolytic
Propionibacterium	59–67	Variable	−/+	+	P	Saccharolytic
Propionimicrobium	53–54	Variable; often diphtheroid/club-shaped	−	+	P, A, S	Saccharolytic
Scardovia	44–46	Small, coccoid, variable	−	+	A, L	Saccharolytic
Slackia	60–64	Cocci/coccobacilli/short rods; singly/in clumps	−	(+)	(A)	Asaccharolytic
Varibaculum	52	Short, straight/curved, diphtheroid	−/+	+	L, S	Saccharolytic
Firmicutes						
Anaerofustis	70	Thin rods	−	+	A, B	Saccharolytic
Anaerostipes	46	Thin rods; in short chains	−	(+)	A, B, L	Saccharolytic
Anaerotruncus	54	Thin rods	−	+	A, B	Saccharolytic
Bryantella	50	Short; in pairs/short chains	−	+	A (S, L)	Formate required
Bulleidia	38	Short, straight/slightly curved; singly/in pairs	−	+	A, L	Saccharolytic
Catenibacterium	36–38	Short; in long tangled chains	−	+	A, B, L	Saccharolytic
Dorea	40–46	Short/long; in pairs/in chains	−	+	A, F	Saccharolytic, H_2 production
Eubacterium	30–57	Variable	−	v	B, A, L (F)	Saccharolytic
Faecalibacterium	47–57	Pleomorphic rods	−	−	B, F, L	Saccharolytic
Filifactor	34	Short, regular	−	−	B	Asaccharolytic
Holdemania	38	Short; in pairs/short chains	−	(+)	A, L	Saccharolytic
Lactobacillus	35–53	Short/long, slender; in chains	−/+	+	L	Aciduric
Mogibacterium	41–50	Short; singly/in clumps	−	(+)	PAA	Asaccharolytic
Oribacterium	42	Elongated, ovoid; singly/in pairs; highly motile	−	−	A, L	Saccharolytic
Pseudoramibacter	61	Pleomorphic; in pairs	−	+	A, B, C, F	Saccharolytic
Roseburia	29–42	Thin, pleomorphic rods	−	v	B, L	Saccharolytic, H_2 production
Shuttleworthia	50–51	Short/slightly curved; singly/in pairs/short chains	−	+	B, A (L)	Saccharolytic
Solobacterium	37–39	Short, straight/slightly curved; singly/in pairs	−	+	A, L	Saccharolytic
Turicibacter	37	Irregular, long; in long chains	−	+	L	Saccharolytic

[a] +/− or −/+, variable aerotolerance within the genus; −, the genus contains only strictly anaerobic species.
[b] +, positive; −, negative; v, variable; (+), decolorization in old culture.
[c] A, acetic acid; B, butyric acid; C, caproic acid; F, formic acid; L, lactic acid; P, propionic acid; S, succinic acid; PAA, phenylacetic acid; parentheses, strain variation.

shown to belong to the *Actinobacteria,* such as the former *E. lentum,* now renamed *Eggerthella lenta* (162); *E. aerofaciens*, now *Collinsella aerofaciens* (88); and *E. exiguum,* now *Slackia exigua* (162). *E. lenta* and *C. aerofaciens* are numerically important bacteria in the human intestinal tract (88, 162), whereas *S. exigua* is strongly associated with oral infections (162). Recently, two new *Eggerthella* species, *E. hongkongensis* and *E. sinensis,* have been proposed (96). A novel species found in oral infections, *Cryptobacterium curtum* (108), is related to this group.

Phylum *Firmicutes*

Lactobacillus, a large and heterogeneous genus, contains microaerobic, catalase-negative, non-spore-forming gram-positive rods, which produce lactic acid as their single or major metabolic end product from glucose fermentation. Members of *Lactobacillus* sensu stricto constitute a branch of the *Firmicutes* subdivided into seven groups: *L. buchneri, L. casei, L. delbrueckii, L. plantarum, L. reuteri, L. sakei,* and *L. salivarius* (69). *Catenibacterium mitsuokai* (84), isolated from human feces, forms a cluster with *L. vitulinus* and *L. catenaformis,* two misclassified *Lactobacillus* species.

Organisms assigned to *Eubacterium* are defined by default; they do not produce propionic acid as a major acid product, lactic acid as the sole major acid product, succinic and lactic acids with small amounts of acetic or formic acids, or acetic and lactic (acetic > lactic) acids, with or without formic acid, as the sole major acid products (103). It has

TABLE 2 Recently classified or reclassified genera and species (from 2002 onwards) of non-spore-forming anaerobic gram-positive rods isolated from humans

Phylum and genus	New genus	Species	Previous nomenclature	Reference
Actinobacteria				
Actinobaculum		*A. massiliae*	New sp.	58
		A. urinale	New sp.	63
Actinomyces		*A. cardiffensis*	New sp.	62
		A. dentalis	New sp.	66
		A. hongkongensis	New sp.	169
		A. nasicola	New sp.	65
		A. oricola	New sp.	64
Bifidobacterium		*B. longum*	*B. infantis, B. longum, B. suis*	129
		B. scardovii	New sp.	77
Eggerthella		*E. hongkongensis*	New sp.	96
		E. sinensis	New sp.	96
Parascardovia	Yes	*P. denticolens*	*Bifidobacterium denticolens*	81
Propionimicrobium	Yes	*P. lymphophilum*	*Propionibacterium lymphophilum*	146
Scardovia	Yes	*S. inopinata*	*Bifidobacterium inopinatum*	81
Varibaculum	Yes	*V. cambriensis*	New sp.	67
Firmicutes				
Anaerofustis	Yes	*A. stercorihominis*	New sp.	48
Anaerostipes	Yes	*A. caccae*	New sp.	140
Anaerotruncus	Yes	*A. colihominis*	New sp.	99
Bryantella	Yes	*B. formatexigens*	New sp.	168
Dorea	Yes	*D. formicigenerans*	*Eubacterium formicigenerans*	152
		D. longicatena	New sp.	152
Faecalibacterium	Yes	*F. prausnitzii*	*Fusobacterium prausnitzii*	37
Lactobacillus		*L. antri*	New sp.	127
		L. gastricus	New sp.	127
		L. kalixensis	New sp.	127
		L. ultunensis	New sp.	127
Mogibacterium		*M. diversum*	New sp.	109
		M. neglectum	New sp.	109
Oribacterium	Yes	*O. sinus*	New sp.	17
Roseburia		*R. intestinalis*	New sp.	36
Shuttleworthia	Yes	*S. satelles*	New sp.	32
Turicibacter	Yes	*T. sanguinis*	New sp.	6

been proposed that *Eubacterium* sensu stricto should be restricted to *E. limosum, E. callanderi,* and *E. barkeri* (165). The remaining *Eubacterium* species would therefore require reclassification. Clinically important *Eubacterium* species are widely distributed among the *Firmicutes*. *E. alactolyticum*, a saccharolytic species found in the oral cavity of humans, and a close phylogenetic relative of the strictly defined genus *Eubacterium,* has been moved to a new genus as *Pseudoramibacter alactolyticus* (165). *E. biforme, E. cylindroids,* and *E. dolichum* fall within the family *Erysipelotrichaceae* with the recently described saccharolytic species, *Bulleidia extructa*, isolated from the human mouth (34), and *Holdemania filiformis* (166) and *Solobacterium moorei* (86), isolated from human feces. *E. brachy, E. infirmum, E. minutum, E. nodatum, E. saphenum,* and *E. sulci* (formerly *Fusobacterium sulci*), together with *Mogibacterium*, a genus of five species indistinguishable by phenotypic tests (109, 110), are a group of asaccharolytic taxa isolated from the human mouth. On the basis of the 16S rRNA phylogeny and their phenotypic characteristics, novel genera should be created for the majority of these species, with the exception of the pairs of species *E. minutum* and *E. nodatum* and *E. sulci* and *E. infirmum*, which are closely related. Many *Eubacterium* species are closely related to clostridia. Spore formation has long been the primary criterion for assignation of gram-positive anaerobic rods to *Clostridium,* but phylogenetic analysis indicates that the taxonomic importance of this characteristic may have been overemphasized. Numerous phylogenetic clusters contain both sporing and nonsporing representatives. *E. budayi, E. moniliforme,* and *E. nitritogenes* are found in clostridial Cluster 1 (25), while *E. siraeum* belongs to a clostridial group that includes *Clostridium leptum* and the recently described *Anaerotruncus colihominis* (99). *E. tenue* and *E. yurii* belong to the clostridial Cluster XI described by Collins et al. (25). *E. tenue* is related to *Clostridium ghonii* and *Clostridium sordellii;* further work is required to determine whether these species constitute a novel genus. *E. yurii* is related to the genus *Filifactor,* which includes *F. alocis* (formerly *Fusobacterium alocis*) isolated from oral infections in humans (80). *E. contortum, E. eligens, E. hadrum, E. hallii, E. ramulus, E. rectale, E. saburreum,* and *E. ventriosum* belong to the family *Lachnospiraceae*. *E. rectale* and *E. ramulus* are related to *Roseburia intestinalis* (36). This family also includes the recently described formate-requiring species *Bryantella formatexigens* (168), isolated from human feces without any disease association so far, and *Oribacterium sinus* (17), a highly motile species isolated from pus of a human sinus. *E. eligens* and *Lachnospira pectinoschiza* are close phylogenetic neighbors and are both motile rods whose growth in broth culture is stimulated by the presence of

fermentable carbohydrate. *E. eligens* should be transferred to the genus *Lachnospira*. *Anaerostipes caccae* (140) forms a loose group with *E. hallii* and *Coprococcus eutactus*, all common species in human feces.

Turicibacter sanguinis (6) is a recently described gram-positive anaerobic rod from human blood, belonging to the phylum *Firmicutes* but which, on the basis of 16S rRNA sequence comparisons, appears to represent a novel lineage within this phylum. Further work is required to clarify the taxonomic position of this genus. Similarly, *Anaerofustis stercorihominis* (48), isolated from human feces, is another deep-branching gram-positive anaerobic rod within *Firmicutes*.

EPIDEMIOLOGY AND TRANSMISSION

Many of the organisms described in this chapter are integral components of the commensal microbiota associated with the mucocutaneous surfaces of the human and animal digestive tract, being found in the mouth, small and large intestines, urogenital tract, and skin (1, 2, 117, 118, 127, 160). Microbial colonization of an individual occurs in a successive manner during the first weeks and months of life. *Actinomyces* are among the first colonizers in the mouth (134), whereas bifidobacteria and *Collinsella* have been suggested to play a significant role in the development of the gut microbiota (70). *Eubacterium rectale, Faecalibacterium prausnitzii,* and lactobacilli are also among the dominant members of the intestinal microbiota (1, 37, 149). In contrast to previous culture-based reports indicating *L. acidophilus* to be the predominant *Lactobacillus* species in the female genital tract, molecular techniques have revealed significant numbers of *L. crispatus, L. gasseri, L. iners, L. jensenii,* and *L. vaginalis* (2, 145, 160, 161, 164). These species, except for *L. iners,* are responsible for hydrogen peroxide (H_2O_2) production, which may help to control the overgrowth of bacterial vaginosis-associated organisms (2).

CLINICAL SIGNIFICANCE

Non-spore-forming anaerobic gram-positive rods seldom cause infections alone but are typically found in polymicrobial infections associated with mucosal surfaces (Table 3). Many anaerobes involved in infections of the head and neck originate from the oral cavity, and most vaginal and bladder pathogens are of fecal origin. In intra-abdominal infections due to organ perforation, the predominant recoveries reflect the microbiota at the site of the leakage (163). For surgical patients, anaerobes remain a significant cause of morbidity and mortality (39). Anaerobic bacteria can occasionally spread to adjacent tissues and even the bloodstream with serious consequences. In anaerobic bacteremias, the gastrointestinal tract is the most common source, followed by abscesses, gynecologic infections, and wound infections (131). Anaerobic blood culture methods tend to be targeted at *Clostridium* species and *Bacteroides fragilis,* which are aerotolerant and relatively fast growing and do not have complex nutritional requirements.

Actinomyces and Related Bacteria

Actinomyces and related bacteria have been isolated with increased frequency, as contaminants or etiologic agents, from clinical specimens representing a wide range of infections (Table 3). *Actinomyces* and related species occur typically in polymicrobial infections together with anaerobic and/or capnophilic, gram-negative species (23, 102, 128, 136). These include several types of oral infection, such as dental caries, endodontic infections, odontogenic abscesses, and dental implant-associated infections (4, 8, 24, 64, 66, 133, 136, 142). Traditionally, *A. israelii* has been thought to be the etiologic agent of classical actinomycosis but *A. gerencseriae,* which is very similar to *A. israelii, A. graevenitzii,* and *A. meyeri,* has also been isolated from actinomycotic lesions (23, 102, 136, 154). Several recognized *Actinomyces* species, especially *A. israelii* and *A. turicensis* but also *A. naeslundii, A. odontolyticus, A. gerencseriae, A. cardiffensis,* and a novel, closely related species, *V. cambriensis,* have been isolated from intrauterine device-associated infections in the female genital tract (50, 62, 67, 68). In addition, *A. naeslundii* and *A. israelii* have been isolated from infectious hip prostheses (148, 171). *A. turicensis,* in particular, seems to dominate in both female and male genital and urinary tract infections (128). *A. funkei, A. naeslundii, A. odontolyticus,* and *A. turicensis* as well as two *Actinobaculum* species, *A. schaalii* and *A. urinale,* have been isolated from blood (46, 68, 97, 98, 115, 122). Taken together, *A. turicensis, A. radingae, A. israelii,* and *A. naeslundii* seem to be the most frequent *Actinomyces* findings in clinical specimens, present in polymicrobial infections or, occasionally, alone (23, 51, 68, 128).

Propionibacterium

Propionibacteria can be found in various systemic or disseminated opportunistic infections (Table 3), such as endocarditis, central nervous system infections, osteomyelitis, osteitis, and arthritis (11, 29, 43, 51, 79), and in about 20% of infected dog and cat bite wounds (151). When *P. acnes* is isolated from blood, it is mainly seen as a causative agent of clinically insignificant bacteremia (11, 95, 131) or as a contaminant from the skin. Typically, *P. acnes* is isolated from sebaceous follicles and lesions of acne vulgaris (51). However, the pathogenic potential of *P. acnes* should not be underestimated when there are predisposing factors present, such as a foreign body, surgery or trauma, diabetes, or immunosuppression (11, 59, 79, 157). *P. acnes* has been connected with some, often severe, late postsurgical infections after implantation of a foreign body, e.g., intraocular lenses, prosthetic heart valves, ventriculoperitoneal shunts, and posterior implants for scoliosis patients (11, 22, 60, 79, 95, 131, 157). It can be a causal agent in infective endocarditis with a predilection for prosthetic valves and foreign bodies (59, 157). The suggested association of *P. acnes* with SAPHO (synovitis, acne, pustulosis, hyperostosis, and osteomyelitis) syndrome with arthritis is based on its repeated growth in pure cultures from bone specimens (91). *P. acnes* and *P. granulosum* have been recently linked as possible etiologic agents to sarcoidosis (42), in which host factors play an important role. In contrast to *P. acnes* and other propionibacteria, the spectrum of infections with involvement of *P. propionicum* is somewhat similar to that of *Actinomyces;* for example, *P. propionicum* has been isolated from actinomycosis-like eye infections (9) and endodontic infections (18, 142).

Lactobacillus

Despite the reputation of lactobacilli as beneficial organisms, they can be involved in serious infections (Table 3), especially in immunocompromised individuals (10, 12, 16, 45, 89, 111, 130). The most common *Lactobacillus* species isolated from various human infections are *L. rhamnosus, L. casei, L. fermentum, L. gasseri, L. plantarum, L. acidophilus,* and *L. ultunensis* (15, 16, 130). The most frequent infections caused by lactobacilli are bacteremia and endocarditis, which carry a relatively high mortality rate (16). Detection of lactobacilli, alone or with other microorganisms, in blood cultures of patients with underlying diseases may be clinically

TABLE 3 Genera of non-spore-forming anaerobic gram-positive rods detected in human infections

Site/disease association	Genera isolated
Brain and/or central nervous system	*Actinomyces, Eubacterium, Propionibacterium, Pseudoramibacter, Varibaculum*
Eye infections	*Actinomyces, Propionibacterium, Varibaculum*
Mouth	
Abscesses	*Actinomyces, Atopobium, Eubacterium, Filifactor, Mogibacterium, Olsenella, Pseudoramibacter, Slackia, Varibaculum*
Dental caries	*Actinomyces, Bifidobacterium, Lactobacillus, Olsenella, Propionibacterium*
Endodontic infection	*Actinomyces, Atopobium, Bifidobacterium, Eubacterium, Filifactor, Mogibacterium, Lactobacillus, Olsenella, Propionibacterium, Pseudoramibacter, Slackia*
Periodontal diseases	*Cryptobacterium, Eubacterium, Filifactor, Mogibacterium, Olsenella, Pseudoramibacter, Slackia*
Respiratory tract infections	*Actinomyces, Eubacterium, Lactobacillus, Oribacterium*
Abdomen/intestine	
Abscesses	*Actinomyces, Eggerthella, Eubacterium, Lactobacillus*
Appendicitis	*Actinomyces, Eggerthella*
Cholecystitis	*Actinomyces, Lactobacillus*
Peritonitis	*Eggerthella, Lactobacillus*
Genital tract	
Abscesses	*Actinomyces, Atopobium, Eubacterium*
Bacterial vaginosis	*Atopobium, Mobiluncus*
Intrauterine device infections	*Actinomyces, Eubacterium, Varibaculum*
Pelvic inflammatory disease	*Actinomyces, Atopobium, Eubacterium, Lactobacillus*
Preterm labor/delivery	*Mobiluncus*
Urinary tract infections	*Actinobaculum, Actinomyces*
Skin and/or soft tissue	
Abscesses	*Actinobaculum, Actinomyces*
Acne vulgaris	*Propionibacterium*
Cellulitis	*Actinomyces, Pseudoramibacter*
Necrotizing soft tissue infections	*Actinomyces, Eubacterium, Mogibacterium*
Lymphadenitis	*Propionibacterium, Propionimicrobium*
Bone and joint infections	*Actinomyces, Propionibacterium*
Wounds	
Bite wound infections (animal)	*Filifactor, Propionibacterium*
Bite wound infections (human)	*Actinomyces, Collinsella, Eggerthella, Eubacterium, Mogibacterium, Lactobacillus, Propionibacterium*
Postoperative wound infections	*Bifidobacterium, Propionibacterium, Pseudoramibacter*
Diabetic foot infections	*Actinomyces, Propionibacterium*
Cardiovascular sites	
Bacteremia	*Actinobaculum, Actinomyces, Atopobium, Eggerthella, Eubacterium, Lactobacillus, Olsenella, Propionibacterium*
Endocarditis	*Actinomyces, Lactobacillus, Propionibacterium*
Infected atheroma	*Actinomyces*
Foreign body infections	*Actinomyces, Mogibacterium, Propionibacterium*

significant. *L. rhamnosus* was the most frequent species in *Lactobacillus* bacteremia (16, 45, 95, 130). *L. rhamnosus* GG, a common probiotic strain, has been occasionally detected in clinical specimens (93, 101, 120, 130). In two pediatric patients with sepsis, the *Lactobacillus* strain from blood samples was indistinguishable from the probiotic strain ingested (93), suggesting that further work needs to be done. Vancomycin-resistant lactobacilli have been connected to dialysis-related peritonitis after extended use of glycopeptides (89, 111). In contrast to other *Lactobacillus* species, *L. iners* has been associated with an intermediate state of bacterial vaginosis (161). In the oral cavity, lactobacilli form a considerable part of the species present in advanced caries lesions (19), with *L. gasseri* and *L. ultunensis* numerically dominant (15). Interestingly, a dental procedure or condition can be a potential predisposing factor for *Lactobacillus* endocarditis (16).

Eubacterium and Related Bacteria

Low G+C organisms representing the former genus *Eubacterium* are often detected in various oral infections (Table 3). Careful isolation of tiny-colony-forming anaerobes from periodontal pockets in adult patients with advanced periodontitis showed that former *Eubacterium* strains (mostly asaccharolytic) dominated (155), thus suggesting their role in the etiology of chronic periodontitis. Downes et al. (33) characterized *Eubacterium*-like strains from oral infections and found *Mogibacterium timidum* to be among the most frequently detected species. *Mogibacterium vescum, Bulleidia extructa, Filifactor alocis,* and *Pseudoramibacter alactolyticus*

were also found among the isolates from severe (some of them requiring treatment in intensive care units) odontogenic infections. Less frequently isolated were *E. sulci*, *E. saburreum*, and *E. yurii* (33). Many species found in odontogenic infections are also common in endodontic infections (71, 107, 109, 141, 143). *F. alocis, E. nodatum, E. saphenum,* and *M. timidum* have been associated with periodontal diseases (5, 92, 117). Due to their presence in the oral cavity, various *Eubacterium* and related species are among the anaerobic findings in human bite wound infections (150). *Filifactor villosus,* a species of animal origin, has been isolated from infected cat bite wounds in humans (151). *E. nodatum* has been found in infections of the female genital tract (73). *E. tenue* and *E. callanderi,* an environmental anaerobe, have been detected in clinically significant bacteremia (95, 153).

Eggerthella and Related Bacteria

Of the four high G+C content genera previously classified as *Eubacterium, Eggerthella* has been shown to possess particular pathogenic potential (Table 3). *E. lenta* has been isolated from a wide range of clinical specimens, mainly from intra-abdominal sites (10, 12, 104, 121). *E. lenta* and two recently described *Eggerthella* species, *E. hongkongensis* and *E. sinensis,* have been found in blood in association with clinically significant infections of relatively high mortality (95, 96). *Cryptobacterium curtum* and *Slackia exigua* have been associated with chronic periodontitis (5, 92), and the latter has also been associated with endodontic infections (71).

Atopobium

The genus *Atopobium* can be isolated from various infections (Table 3) and includes several clinically relevant species. Although *A. vaginae* was first isolated from the healthy vagina (124), it has been increasingly reported to be involved in infections of the genital tract, especially bacterial vaginosis (14, 47, 52, 161). *A. minutum* has been isolated from various infections of the lower part of the body, and *A. parvulum* has been isolated from respiratory specimens (113). In the oral cavity, *A. parvulum* and *A. rimae* have been detected in periodontal pockets (113, 117); however, in another study, these species have been shown to be more prevalent in healthy individuals than in periodontitis patients (92). Among *Eubacterium*-like isolates from severe odontogenic infections, *A. rimae* proved to be the most frequent finding (33). An *Atopobium* species of animal origin, *A. fossor,* has been isolated as one of the most frequent species from horses with pneumonia and lung abscesses (119).

Olsenella

Olsenella species show disease associations similar to those observed for lactobacilli in the oral cavity, *O. profusa* being detected in advanced dental caries lesions (106), and *O. uli,* in particular, in endodontic infections (18, 107, 123). Both species can also be found in subgingival sites of periodontitis patients (30, 113). In addition, *O. uli* has been reported as one of the causative organisms in clinically significant bacteremia (95).

Bifidobacterium and Related Bacteria

Bifidobacteria, one of the dominant bacterial groups in the human intestinal tract (70), are generally considered nonpathogenic but appear in some infectious lesions (Table 3). Dental caries is the most common clinical entity where *Bifidobacterium,* mainly *B. dentium,* and related species *P. denticolens* and *S. inopinata* may have a pathogenic role (4, 19, 27, 81). Some *Bifidobacterium* species, such as *B. adolescentis* and *B. dentium*, are occasionally isolated from other infections, mainly in immunocompromised individuals (10, 12). In addition, a novel *Bifidobacterium* species, *B. scardovii,* has been isolated from human clinical samples, including blood, urine, and the hip (77).

Mobiluncus

The presence of vibrio-like *Mobiluncus* species in smears of vaginal fluid has been widely used as one of the indicators of bacterial vaginosis, the most common infection in the female genital tract (112). Of the two *Mobiluncus* species, *M. curtisii* seldom occurs in the vaginas of healthy women but, instead, is highly associated with bacterial vaginosis and its treatment failure due to the persistence of the organism (138). The disturbed composition of the vaginal microbiota can be a risk for adverse pregnancy outcome when ascending to the upper genital tract (75). In addition to bacterial vaginosis, *M. curtisii* has been occasionally isolated from endometrial smears and pus specimens of the female genital tract (3) and some extragenital infections (40, 57).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Careful specimen collection, avoidance of contamination with the commensal microbiota where necessary, and anaerobic transport techniques are essential for the successful recovery of clinically significant anaerobic bacteria (see chapters 5 and 20). Specimens suitable for the isolation of non-spore-forming, gram-positive anaerobic rods present as organisms of etiologic importance include aseptically collected peripheral blood, tissue biopsy specimens, aspirates (e.g., cerebrospinal fluid, joint fluids, and pus), root canal exudates, and subgingival plaque. Mucosal or cutaneous swabs are not recommended (53). A comprehensive description of different specimen collection and transport methods for anaerobic bacteriology can be found elsewhere (83).

DIRECT EXAMINATION

Direct examination is of unequivocal value for the diagnosis of actinomycosis. The presence of "sulfur granules" in pus, which when crushed, Gram stained, and viewed under the microscope reveal a mass of gram-positive branching filaments, is characteristic of this disease. Similarly, in cervical smears of women with an intrauterine contraceptive device, the presence of branching gram-positive organisms suggests an infection with *Actinomyces* (50, 128). Gram stains of vaginal smears have been considered more useful than culture for laboratory confirmation of bacterial vaginosis, and the diagnostic criteria for this common infection have been based on the standardized Nugent scoring system (112). The system relies on Gram stain characteristics of vaginal smears, recognizing individual morphotypes or their combination. Although intercenter reliability for gram-positive cocci was poor, the study documented moderate agreement for large gram-positive rods (lactobacilli) and small gram-variable and/or gram-negative rods (*Gardnerella vaginalis* and *Bacteroides/Prevotella),* and good agreement for curved gram-variable rods *(Mobiluncus)* (112). A simplified assessment of Gram-stained smears, taking lactobacillary and mixed bacterial morphotypes into account, has been proposed (78). It has been noted that the image area observed in microscopes requires standardization, in particular, when interpreting the intermediate state of bacterial vaginosis (94). Subsequently,

a strong association between *A. vaginae* and an abnormal vaginal state has been established (47, 161). This gram-positive, variably shaped, often coccoid species may confuse scoring systems relying on the Gram stain.

In cases where there is no typical microbiota associated with a particular infection, it can be difficult to make a preliminary decision regarding appropriate empiric antimicrobial treatment on the basis of the Gram stain. For example, branching/pleomorphic rods can be tentatively identified as facultatively anaerobic *Actinomyces* or strictly anaerobic *E. nodatum* (73), and coccoid cells of *A. radicidentis* may suggest a genus other than *Actinomyces* (24), while easily decolorizing species (e.g., *Eubacterium*-like species) can yield a false gram-negative reaction (33). The misinterpretation can lead to antimicrobial coverage targeted against facultative organisms instead of anaerobes and/or gram-negative bacteria.

CULTURE AND ISOLATION

Specimens should be processed without delay using appropriate culture media, including standard anaerobic blood agar enriched with hemin and vitamin K_1 and a variety of selective media based on the expected microbiota at the collection site, or in the case of bite wounds, on the oral microbiota of the attacker (human or animal). Fresh or prereduced culture media, including phenyl ethyl alcohol blood agar and/or colistin nalidixic acid blood agar, can be useful for enhanced recovery rates of gram-positive organisms (20). Many asaccharolytic species exhibit enhanced growth on solid media augmented with 0.5% arginine (156). In general, members of the aciduric genera *Bifidobacterium* and *Lactobacillus* can be selectively cultured using agar media with an acidic pH, such as Rogosa or deMan Rogosa Sharpe (MRS) agar. However, some nutritionally fastidious *Lactobacillus* strains fail to grow on these agar media. Munson et al. (106) observed that lactobacilli grew as well on nonselective media with neutral pH and suggested that acidic-pH medium is not required for their detection. Notably, *L. iners*, one of the predominant lactobacilli in the vagina, can grow only on blood agar and not on typical solid media used for *Lactobacillus* (44).

Similar species among facultatively anaerobic *Actinomyces, A. gerencseriae* and *A. israelii,* can be isolated preferably from aerobically *(A. gerencseriae)* or anaerobically *(A. israelii)* incubated plates (8). On the other hand, to maintain bacterial viability, processing the samples and performing all the steps throughout the protocol in an anaerobic chamber favor the growth of some fastidious anaerobes, e.g., *Eubacterium*-like species (31). An anaerobic atmosphere-generating system has been shown to be superior to an anaerobic chamber in culturing *Eubacterium*-like strains from stock (28). When anaerobic jars are used for incubation, anaerobic growth should not be exposed to oxygen by opening the jar before 48 h of incubation in order to facilitate the detection of fastidious organisms (20). The availability of an anaerobic chamber may enable examination of the culture whenever necessary. For reliable detection of slow-growing organisms, the incubation time should be sufficient; for instance, an extended incubation period may be needed for some clinically relevant *Eubacterium*-like species (33, 73, 108–110, 153). In heart tissue specimens from endocarditis patients, grinding the tissue can improve the detection of anaerobic bacteria (59, 79). A lytic anaerobic medium can increase the recovery rate of anaerobes and facultative bacteria in automated blood culture systems (125).

IDENTIFICATION PROCEDURES

Traditionally, the identification of bacterial isolates in clinical microbiology laboratories is performed by phenotypic tests. For organisms inert in most conventional biochemical tests or with unusual biochemical profiles, as is the case for many *Eubacterium* and related species, or in cases where fastidious organisms require specific nutrients or temperatures, identification strategies based on phenotypic characteristics can be challenging.

Presumptive Identification

The initial differentiation is based on aerotolerance (growth in air or in air plus 5% CO_2), colonial morphology, pigmentation, fluorescence under long-wave UV illumination, and presence of hemolysis. The colonial morphology can provide clues regarding the organism involved; for instance, an easily recognizable "molar tooth" appearance is typical for *A. israelii* but, notably, also for *E. nodatum* (73). Sometimes a species has various colonial morphologies, as is the case for *Shuttleworthia satelles,* with three separate colony types; the last two types (satellite and opaque) appear after incubation for 5 days (32). Other rapidly recognizable features are fluorescence and/or pigment production: *E. lenta* shows orange to red fluorescence under UV light (104), and pigmentation of colonies is typical for *A. odontolyticus,* but *A. graevenitzii* and some other *Actinomyces* species can also produce pigment (68, 135).

Gram stain morphology can be helpful; it can show whether organisms are gram-positive anaerobic rods, which can be very short (e.g., *C. curtum* and *E. lenta*), long (e.g., many *Lactobacillus* species), pleomorphic (e.g., *Bifidobacterium*), branching (e.g., many *Actinomyces* spp.), or curved and motile (e.g., *Mobiluncus* spp.); sometimes a specific cell morphology can be seen, such as "flying birds" (e.g., *P. alactolyticus*). The morphology may vary when cells are grown on different culture media. The two *Mobiluncus* species can be tentatively separated based on the length of the curved, motile cells: in contrast to the short, gram-variable cells of *M. curtisii, M. mulieris* reveals clearly longer cells, which often appear as gram negative (139). Although *Actinomyces* organisms have been traditionally described as branching rods, many new species within the genus are nonbranching, and some have very short or even coccoid cells (24, 158). Staining of cells can vary with different culture conditions. Certain gram-positive anaerobes, e.g., *F. alocis* and *M. mulieris*, routinely stain gram negative, whereas older cultures (>3 days) of *Actinobaculum* and some *Eubacterium* species and related genera are gram variable (97, 108, 110). Decolorization of gram-positive organisms may be due to exposure to oxygen or to damage from fixatives and reagents causing a breakdown of the physical integrity of the cell wall; therefore, anaerobic working conditions or, if not available, limited exposure time to oxygen between incubation and staining improves the reliability of the Gram stain for anaerobic bacteria (82). Routine screening of special-potency antibiotic susceptibility disk patterns is valuable to confirm the accuracy of the Gram stain reaction (83): gram-positive species are generally resistant to colistin (10 μg) and susceptible to vancomycin (5 μg) and often to kanamycin (1 mg). However, the intrinsic resistance of some *Lactobacillus* species/strains, e.g., *L. rhamnosus,* to glycopeptides should be considered (16, 45, 89, 111), in addition to intrinsic resistance of *Bifidobacterium* to aminoglycosides (105). *Holdemania filiformis* has been reported to be resistant to vancomycin (34). For rapid confirmation of the Gram reaction, a simple test based on dissolution of gram-negative cell wall and cytoplasmic membrane with a solution of 3% potassium hydroxide

(61) can be used: when suspended in the solution, gram-negative cells display increased viscosity and stringing within 30 s, whereas the absence of stringing, i.e., a negative reaction, suggests that the isolate is gram positive.

Additional rapid tests for initial grouping of non-spore-forming gram-positive anaerobes include testing for production of catalase (H_2O_2 at a concentration of 15%) and indole, nitrate reduction, and motility (20). If presumptive identification to the genus level has been made correctly, this may give valuable information to clinicians in deciding the initial treatment. However, differentiating members of the "normal flora" of human skin and mucous membranes from pathogenic non-spore-forming gram-positive rods can be difficult. Identification of nonsporing gram-positive rods to the species level should be performed whenever they are present in pure cultures in clinical specimens or as the predominant organism from normally sterile sites; otherwise, the potential pathogenicity of these less often suspected species may remain undetected.

Biochemical Testing

For a more advanced phenotypic classification of anaerobic organisms and distinguishing individual species, sugar fermentation reactions, preferably using prereduced, anaerobically sterilized carbohydrates, and enzyme profiles with individual diagnostic tablets, fluorogenic substrate tests, or preformed enzyme kits must be determined. Insufficient growth or poor reproducibility of reactions can cause difficulties in interpretation of results obtained with biochemical tests; therefore, young cultures and heavy inoculum should be used (135). A well-designed selection of key tests provides a tentative identification of various isolates to the species level prior to confirming their identifications by more definitive methods. Table 4 presents some biochemical characteristics of *Actinomyces* and related organisms. Since the description of many novel species, such as *A. dentalis, A. hongkongensis, A. nasicola, A. oricola, A. urinale,* and "*A. massiliae,*" is based on a single strain (58, 63–66, 169), discrepancies in test reactions may appear. Table 5 presents simple enzymatic reactions useful in distinguishing propionibacteria encountered in human infections, and Table 6 shows tests for *Atopobium* and *Olsenella* species. Although the cultivation and identification of *Eubacterium*-like species are very laborious, not only because of their oxygen sensitivity and slow growth but also due to their nonreactivity in conventional biochemical testing, some simple reactions may be helpful for grouping these organisms (Table 7).

In culture-based identification of anaerobic non-spore-forming gram-positive rods, the determination of major volatile fatty acid end products of glucose metabolism, as detected by gas chromatography, is a prerequisite, even for the genus level (Table 1). Typically, *Actinomyces* strains produce succinic and lactic acids as their major metabolic end products but *A. dentalis* seems to lack this characteristic succinic acid (66). An *Actinomyces*-like *Propionibacterium* species, *P. propionicum,* is easily separated from *Actinomyces* based on its production of propionic acid (51). For microaerobic *Lactobacillus* spp., defining characteristics are their ability to grow in acid media and ferment carbohydrates to produce lactic acid as the major end product with or without small amounts of acetate, whereas in the case of *Bifidobacterium* spp. the production of acetic acid dominates that of lactic acid. Downes et al. (33) successfully used the determination of metabolic end products by gas chromatography together with sugar fermentation by prereduced, anaerobically sterilized carbohydrates and enzyme profiles generated by a commercial identification kit (API Rapid ID 32A; bioMérieux, Marcy-l'Etoile, France) for identification of *Eubacterium*-like isolates to the genus and species level; for instance, the lack of enzyme activity and formation of caproic acid or phenylacetic acid distinguish *P. alactolyticus* or *Mogibacterium* spp., respectively, from other related taxa. However, phenotypic criteria are particularly unreliable for identification of many *Actinomyces* species (68) and members of the *L. acidophilus* complex and related species (159). In addition, gas chromatographic analysis of cellular fatty acids and examination of protein patterns by polyacrylamide gel electrophoresis have been used taxonomically to distinguish among strains within a species and among organisms within a genus or family. However, the availability of gas chromatography methods is often restricted to reference laboratories. The precise identification to the species level of heterogeneous groups within non-spore-forming gram-positive bacteria can be difficult, even when using the determination of volatile fatty acids by gas chromatography and biochemical profile analyses because biochemical tests do not accurately reflect genotypic groups (33, 45, 68, 145).

Preformed enzyme and carbohydrate fermentation profiles can be obtained using commercially available identification test kits, such as the API (bioMérieux), RapID (Remel, Lenexa, Kans.), and BBL Crystal (Becton Dickinson Diagnostic Systems, Sparks, Md.) systems, according to manufacturers' instructions. Although this approach is often hindered by similarities in fermentation profiles of separate species within a genus, kits serve as a widely used adjunct to anaerobe diagnostics in most hospital laboratories (53), since they are easy to use and much faster than conventional anaerobic procedures. The main problems with these kits are their incomplete or inaccurate databases (7, 132). The databases of the API Coryne (bioMérieux) and RapID CB Plus (Remel) tests, designed for coryneform bacteria, currently include some aerotolerant *Actinomyces* and *Propionibacterium* species as well. Notably, different test kits and individual tests differ in their substrate specificities so that an indicated reaction can vary depending on the system used (23, 132, 135). For example, one key reaction for *A. naeslundii,* urease production, can remain negative with one test, and thus the strain can be misidentified as *A. israelii,* while another test yields a correctly positive reaction (132, 171). Isolates of a novel *Atopobium* species, *A. vaginae,* have been misidentified as *Gemella morbillorum* by the API Rapid ID 32A (bioMérieux) and RapID ANA II (Remel) test kits (47, 52). In contrast, a clinically relevant *Bifidobacterium* species, *B. scardovii,* was readily separated from other bifidobacteria by using the Rapid ID 32A kit (bioMérieux) (77). The carbohydrate fermentation test kit API 50 CH (bioMérieux), which is specifically designed for lactobacilli, can be valuable in identification to the genus level but fail at the species level (7). Despite the lack of reliability of species level identifications, commercial test kits can be useful for the detection of positive reactions and identification of many organisms from clinical sources to the genus level. Clinical microbiologists should be aware of the possibility of erroneous identification and adjust the interpretation of their results accordingly, in conjunction with cellular and colonial morphology and other information available. Indeed, practical, discriminatory, and cost-effective methods are needed for identification of fastidious gram-positive bacteria. Molecular identification offers an opportunity for the description of new bacterial species encountered in clinical microbiology laboratories, and commercial 16S rRNA gene sequence-based identification kits are coming into the market (170).

TABLE 4 Biochemical characteristics of *Actinomyces* and related bacteria encountered in human infections[a]

Genus and species	Aerotolerance	Pigment	Production of:		Nitrate reduction	Esculin hydrolysis	Production of:			CAMP test	Fermentation of:				
			Catalase	Urease			α-Glu	β-NAG	β-Gal		Mal	Man	Raf	Suc	Tre
Actinobaculum															
A. massiliae	+	−	−	−	−	−	+	−	−	−	+	−	+	ND	+
A. schaalii	+	−	−	−	−	−	+	−	−	Weak	+	−	−	v	v
A. urinale	(+)	−	−	+	−	−	−	−	−	−	v	−	−	v	−
Actinomyces															
A. cardiffensis	(+)	−	−	−	v	−	+	−	−	−	v	−	v	v	−
A. dentalis	−	−	−	−	−	+	+	−	+	−	+	−	+	+	+
A. europaeus	+	−	−	−	v	v	+	−	+	−	+	−	−	v	v
A. funkei	+	−	−	−	+	−	+	v	v	v	v	−	−	+	−
A. georgiae	+	−	−	−	v	+	+	−	+	−	+	−	−	+	+
A. gerencseriae	+	−	−	−	v	+	+	−	+	−	+	v	v	+	v
A. graevenitzii	+	+	−	−	v	−	v	+	+	−	+	−	v	+	v
A. hongkongensis	−	−	−	−	−	−	−	−	−	−	ND	ND	−	ND	−
A. israelii	(+)	−	−	−	+	+	+	−	+	−	+	−	+	+	+
A. meyeri	−	−	−	−	v	−	+	+	+	v	+	−	−	+	−
A. naeslundii	+	−	−	+	+	v	v	−	+	−	+	−	+	+	+
A. nasicola	(+)	−	−	−	−	−	+	+	+	−	−	−	−	−	−
A. neuii															
subsp. *anitratus*	+	−	+	−	−	−	v	−	+	+	+	+	v	+	v
subsp. *neuii*	+	−	+	−	+	−	+	−	+	+	+	+	v	+	v
A. odontolyticus	+	+	−	−	+	v	−	−	+	−	+	−	−	+	−
A. oricola	+	−	−	−	−	+	+	−	v	−	−	−	+	+	+
A. radicidentis	+	+	+	v	v	+	+	−	+	−	+	+	+	+	+
A. radingae	+	−	−	−	v	+	+	+	+	v	+	−	v	+	v
A. turicensis	+	−	−	−	−	−	+	−	−	−	+	−	v	+	v
A. urogenitalis	+	+	−	−	+	+	+	+	+	−	+	−	−	+	+
A. viscosus	+	−	+	v	+	v	v	−	v	−	+	v	+	+	v
Varibaculum															
V. cambriensis	(+)	−	−	−	+	−	+	−	v	−	+	v	−	+	−

[a] Biochemical data from references 51, 58, 62–67, 135, 158, and 169. Symbols and abbreviations: +, positive; −, negative; (+), better growth in anaerobic condition; v, variable; ND, no data; α-Glu, α-glucosidase; β-NAG, β-*N*-acetyl-glucosaminidase; β-Gal, β-galactosidase; Mal, maltose; Man, mannitol; Raf, raffinose; Suc, sucrose; Tre, trehalose.

TABLE 5 Biochemical characteristics of propionibacteria encountered in human infections[a]

Genus and species	Aerotolerance	Catalase	Indole	Nitrate reduction	Esculin hydrolysis
Propionibacterium					
P. acnes	+	+	+	+	−
P. avidum	+	+	−	−	+
P. granulosum	+	+	−	−	−
P. propionicum	−	−	−	+	−
Propionimicrobium					
P. lymphophilum	−	v	−	v	ND

[a] Biochemical data compiled from references 51 and 146. Symbols and abbreviations: +, positive; −, negative; v, variable; ND, no data.

Identification by DNA Sequence Analysis

As mentioned above, the use of conventional and biochemical tests for the identification of this group carries a significant risk of misidentification. Far more precise identifications can be obtained by 16S rRNA gene sequence analysis (90). A major advantage is that methods do not need to be adapted for different groups of organisms; thus, there are no special considerations needed when analyzing gram-positive nonspore-forming anaerobes.

A major impetus to the study of unculturable bacteria has been the development of 16S rRNA/PCR amplification/cloning/sequencing methodology (167). The phylum *Firmicutes,* in particular, has been found to harbor a number of lineages without culturable representatives. For example, branches within the families *Eubacteriaceae* and *Lachnospiraceae* have been found in advanced carious lesions, endodontic infections, and subgingival plaque in periodontitis (19, 107, 117). Similarly, the colonic microbiota includes novel branches within the *Clostridiaceae* and *Erysipelotrichaceae* (149). Culture-independent analysis of the microbiota in bacterial vaginosis identified a novel taxon related to *A. vaginae* (161), and novel taxa belonging to both the *Actinobacteria* and *Firmicutes* were found in a corneal ulcer (137).

SEROLOGICAL TESTS

Serological tests are of little diagnostic value for this group of organisms, which are found almost exclusively in polymicrobial infections. Furthermore, infections are frequently opportunistic in nature, with the causative organism being a member of the commensal microbiota, rendering serological tests difficult to interpret.

ANTIMICROBIAL SUSCEPTIBILITIES

In the clinical setting, empirical information is used for the initial diagnosis of infection and choice of antimicrobial therapy, while awaiting culture and susceptibility test results. Published data regarding antimicrobial susceptibilities of nonsporing gram-positive anaerobes may be difficult to interpret. Changes in the bacterial taxonomy, e.g., among the former *Eubacterium* genus, and more precise classification of tested isolates may result in antimicrobial resistance patterns different from those given in previously published surveys (144). In general, penicillin and other β-lactams exhibit activity against gram-positive bacteria together with parenteral carbapenems, such as imipenem, ertapenem, and meropenem (13, 41, 54, 55, 74, 100, 105, 144). Metronidazole has been considered a drug of choice for treatment of anaerobic infections; however, resistant strains are common among non-spore-forming gram-positive genera with facultative/microaerobic strains, i.e., *Propionibacterium*, *Actinomyces*, *Bifidobacterium*, and *Lactobacillus*, but also among the strictly anaerobic genera *Atopobium* and *Mobiluncus* (3, 47). In addition, some strains of *Eggerthella* and *Eubacterium* resistant to metronidazole have been reported (21, 74, 100). Failures or relapses are common in the treatment of bacterial vaginosis, but whether metronidazole-resistant *A. vaginae* or *M. curtisii* (3, 47, 52) could play a role is not known. Occasional strains among various genera of non-spore-forming gram-positive anaerobic rods show

TABLE 6 Enzyme reactions useful in distinguishing species within the genera *Atopobium* and *Olsenella*[a]

Genus and species	Aerotolerance	Production of:		Nitrate reduction	Hydrolysis of:		Acid phos	β-Gal
		Catalase	Indole		Esculin	Arginine		
Atopobium								
A. minutum	−	−	−	−	ND	+	−	−
A. parvulum	−	−	−	−	v	−	+	+
A. rimae	−	−	−	−	v	v	+	−
A. vaginae	(−)[b]	−	−	−	−	+	+	−
Olsenella								
O. profusa	−	−	−	−	+	−	ND	ND
O. uli	−	−	−	−	+	+	ND	ND

[a] Biochemical data compiled from references 26, 30, 33, 113, and 124. Symbols and abbreviations: +, positive; −, negative; v, variable; ND, no data; Acid phos, acid phosphatase; β-Gal, β-galactosidase.

[b] In the original description, *A. vaginae* is facultatively anaerobic.

TABLE 7 Biochemical characteristics of some human *Eubacterium*-like organisms[a]

Phylum/genus and species	Glucose fermentation	Production of:		Nitrate reduction	Hydrolysis of:		Production of:		
		Catalase	Indole		Esculin	Arginine	β-Gal	β-Glu	β-NAG
Actinobacteria									
Collinsella aerofaciens	+	−	−	−	v	v	v	v	−
Collinsella intestinalis	+	ND	ND	ND	ND	ND	−	−	+
Collinsella stercoris	+	ND	ND	ND	ND	ND	+	Weak	+
Cryptobacterium curtum	−	−	−	−	−	+	ND	ND	ND
Eggerthella hongkongensis	−	+	−	−	ND	+	−	+	−
Eggerthella lenta	−	+	−	+	−	+	−	−	−
Eggerthella sinensis	−	+	−	−	ND	+	−	−	−
Slackia exigua	−	−	−	−	−	+	ND	ND	ND
Firmicutes									
Bulleidia extructa	+	−	−	−	−	+	ND	ND	ND
Catenibacterium mitsuokai	+	−	ND	−	−	ND	ND	ND	ND
Eubacterium brachy	−	−	−	−	−	−	ND	ND	ND
Eubacterium limosum	+	−	−	−	+	v	ND	ND	ND
Eubacterium minutum	−	−	−	−	−	−	ND	ND	ND
Eubacterium nodatum	−	−	−	−	−	+	ND	ND	ND
Eubacterium rectale	+	−	−	−	+	−	ND	ND	ND
Eubacterium saburreum	+	−	+	−	+	+	ND	ND	ND
Eubacterium saphenum	−	−	−	−	−	−	ND	ND	ND
Eubacterium sulci	−	−	−	−	−	−	ND	ND	ND
Eubacterium tenue	Weak	−	+	−	−	ND	ND	ND	ND
Eubacterium yurii	Weak	−	+	−	−	−	ND	ND	ND
Filifactor alocis	−	−	−	−	−	+	ND	ND	ND
Holdemania filiformis	+	−	−	−	+	−	ND	ND	ND
Mogibacterium spp.	−	−	−	−	−	−	ND	ND	ND
Pseudoramibacter alactolyticus	+	−	−	−	−	−	ND	ND	ND
Shuttleworthia satelles	+	−	+	−	+	−	ND	ND	ND
Solobacterium moorei	+	−	−	−	+	+	ND	ND	ND

[a] Biochemical data compiled from references 32, 33, 73, 83–85, 96, 108, 109, 165, and 166. Symbols and abbreviations: +, positive reaction; −, negative reaction; v, variable reaction; ND, no data; β-Gal, β-galactosidase; β-Glu, β-glucosidase; β-NAG, β-*N*-acetyl-glucosaminidase.

resistance to clindamycin (13, 21, 38, 56, 72, 114, 144). For example, of the 304 *P. acnes* isolates collected from various human infections in 13 European countries, 15% of isolates demonstrated resistance to clindamycin (114). Isolates were also resistant to erythromycin (17%) and to tetracycline (3%), whereas all isolates were susceptible to penicillin, vancomycin, and linezolid. Although vancomycin and teicoplanin are considered active against most gram-positive bacteria, species-related resistance to glycopeptides is frequent among species of the genus *Lactobacillus*. Less than one-quarter of the isolates from 80 cases of *Lactobacillus* infections were reported as susceptible to vancomycin (16). Notably, the vancomycin-resistant *L. rhamnosus* is the most common *Lactobacillus* species in clinical specimens (45, 130). In contrast to vancomycin and teicoplanin, ramoplanin, a novel glycolipodepsipeptide, showed good activity against lactobacilli (21, 49). A novel glycopeptide, telavancin, proved to be more active than vancomycin against lactobacilli, except for *L. casei*, and demonstrated very good activity against the tested strains of *Propionibacterium*, *Actinomyces*, *Eggerthella*, and *Eubacterium* (55). Oxazolidinones represent a new class of synthetic antimicrobial agents, having relatively good in vitro activities against gram-positive cocci but also against anaerobes, and ranbezolid may have lower MICs than linezolid (13, 41). Also telithromycin, a novel ketolide, and streptogramin antimicrobial agents, such as pristinamycin and quinupristin-dalfopristin, have considerable activities against non-spore-forming gram-positive rods (13, 56, 105). Fluoroquinolones have a broad spectrum of antibacterial activity and good absorption from the gastrointestinal tract. Novel quinolones, such as garenoxacin, gatifloxacin, and moxifloxacin, exhibit better antianaerobic activity than the older quinolone compounds levofloxacin and ciprofloxacin (13, 38, 72, 100), suggesting their potential in treating mixed organism infections.

The potential presence of anaerobic gram-positive non-spore-forming rods in infections associated with biofilms and increased antimicrobial resistance of biofilms (147) needs to be taken into account. Due to the involvement of biofilms, mechanical intervention (drainage of abscesses, decompression of infected spaces, debridement of necrotic tissue, and removal of foreign bodies) can be critical in the management of these infections. In addition, appropriate coverage for both facultative and anaerobic bacteria is often necessary.

INTERPRETATION AND REPORTING OF RESULTS

Once clinically relevant isolates are recovered, their accurate classification is needed to estimate the seriousness of the infection and to select the appropriate antimicrobial therapy. Conventional identification protocols for anaerobic non-spore-forming gram-positive bacteria to the species level are laborious and time-consuming and involve substantial

subjective judgment that can result in inconclusive identification. In reference anaerobic laboratories, the use of gas chromatography and/or sequencing of the 16S rRNA gene provides useful tools for organism identification.

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*Clostridium**

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TAXONOMY

The genus *Clostridium* comprises obligately anaerobic (or occasionally aerotolerant) rods that usually stain gram positive in young cultures. Clostridia, mainly from our indigenous microbiota, are involved in a variety of infections that are commonly associated with considerable morbidity and mortality (44, 86). Virulence factors (particularly toxins), inoculum size, and other properties play important roles in diseases involving *Clostridium* species. Impaired host defenses also enhance susceptibility to clostridial infections. Currently, 202 clostridial species are validly published (http://www.dsmz.de/bactnom/bactname.htm); however, the number of clinically significant clostridia from human infections is limited (Table 1).

Phylogenetically, the genus *Clostridium* is extremely heterogeneous, with many species intermixed with other spore-forming and non-spore-forming genera (53, 66). Traditionally, the species have been defined based on a few morphological, ultrastructure (72), and physiological features (44, 173). During the past decade, analyses of 16S rRNA gene sequences indicated that the "clostridia" could be divided into 19 clusters and 11 groups (53). Homology group I forms the basis of the genus *Clostridium* and is analogous to group I proposed in 1975 (96, 173). The type species, *Clostridium butyricum* (44), and most of the clinically important *Clostridium* species cluster within homology group I. The heterogeneous non-group I clostridia require reclassification (53, 173), but a difficulty in resolving the heterogeneity within the genus is that 16S rRNA gene sequences may not be adequate alone in distinguishing genera, and it is necessary to find genetic and phenotypic characters that enable rapid discrimination among genera within this group (173; P. Lawson, personal communication).

Since the last edition of this Manual, some new pathogenic species have been designated within the clinically significant *Clostridium* species. Two new species clustering within the *C. coccoides* rRNA group, *C. hathewayi* (174) and *C. bolteae* (169), were described from human feces. Phenotypically, *C. clostridioforme* is a relatively heterogeneous anaerobe. Sequencing analyses of 16S rRNA genes from 107 strains that were previously identified phenotypically as *C. clostridioforme* in various clinical laboratories revealed that "*C. clostridioforme*" in fact represents three distinct species: *C. bolteae, C. clostridioforme,* and *C. hathewayi* (67). *C. bartlettii* is another new *Clostridium* species described from human feces (168); the clinical significance of this organism remains unknown. *C. neonatale* was proposed as a novel species recovered from bacteremia in patients with necrotizing enterocolitis (NEC) (9); however, this species is not yet validly published.

Anaerotruncus colihominis is a new genus and species within the *C. leptum* rRNA cluster of organisms originally described from human feces (120), and subsequently found in bacteremia (118). *A. colihominis* was originally described as a non-spore-forming organism; however, further studies have revealed that this organism does produce spores under some conditions (118) and should therefore be considered in *Clostridium* identification schemes.

DESCRIPTION OF THE GENUS

The description of the genus *Clostridium* is outlined in *Bergey's Manual of Systematic Bacteriology* (44) and *The Prokaryotes* (89). Recent analyses of rRNA genes have greatly expanded the clostridia to include 5 new genera and 11 new species combinations (53). The clostridia, as currently named, belong to the phylum *Firmicutes* and comprise a heterogeneous (paraphyletic) group consisting of at least 12 lineages. The clostridia have a wide range of G+C contents, from 22 to 55 mol%, while the toxigenic species have a much narrower range of G+C contents, 24 to 29 mol% (44, 86, 89, 173). Common morphological and phenotypic properties that have traditionally been used to define the genus include (i) the formation of endospores, (ii) an anaerobic energy metabolism, (iii) the inability to reduce sulfate to sulfide, and (iv) a gram-positive cell wall structure (44, 89).

Vegetative cells of *Clostridium* species are rod shaped, commonly pleomorphic, and are often arranged in pairs or short chains; the cells have rounded or sometimes pointed ends (44, 86, 90). Rods may join to form tight coils or spiral configurations in certain species, such as *C. cocleatum* and *C. spiroforme*. Clostridia usually stain gram positive at least in early stages of growth, although some species, such as *C. clostridioforme, C. hathewayi, C. innocuum,* and *C. ramosum,* almost always appear gram negative. Several species (e.g., *C. tetani*) appear gram negative by the time spores have formed. The cells form oval or spherical endospores (44, 86, 89). The spores are often wider than the vegetative organisms, imparting characteristic

* This chapter contains information presented in chapter 54 by Stephen D. Allen, Christopher L. Emery, and David M. Lyerly in the eighth edition of this Manual.

TABLE 1 Characteristics of *Clostridium* species of clinical significance[a]

Species	Gelatin hydrolysis	Lecithinase	Lipase	Indole	Esculin hydrolysis	Nitrate	Fermentation of:		
							Milk digestion	Glucose	Arabinose
Saccharolytic, proteolytic									
C. bifermentans[b]	+	+	–	+	+⁻	–	+	+	–
C. botulinum[c]									
Types A, B, F	+	–	+	–	+	–	+	+⁻	–
Types B, E, F[d]	+	–	+	–	–	–	–	+	w⁻
Types C, D	+	–⁺	+	–⁺	–	–	+	+	–
C. cadaveris	+	–	–	+	–	–	+	+	–
C. difficile[e]	+	–	–	–	+	–	–	+ʷ	–
C. novyi A	+	+	+	–	–	–	–	+	–
C. perfringens	+	+	–	–	v	v	+	+	–
C. putrificum	+	–	–	–	–⁺	–	+	+ʷ	–
C. septicum[f]	+	–	–	–	+	v	+	+	–
C. sordellii[b]	+	+	–	+	–⁺	–	+	+	–
C. sporogenes[f]	+	–	+	–	+	–	+	+	–
Saccharolytic, nonproteolytic									
C. baratii	–	+	–	–	+	+⁻	–	+	–
C. bolteae[g]	–	–	–	–	–⁺	–	–	+	+
C. butyricum	–	–	–	–	+	–	–	+	+⁻
C. carnis[h]	–	–	–	–	+	–	–	+	–
C. clostridioforme[g]	–	–	–	–	+	–	–	+	+
C. glycolicum	–	–	–	–	–⁺	–	–	+	–
C. hathewayi[g]	–	–	–	–	+ʷ	–	–	+	v
C. indolis	–	–	–	+	+	+⁻	–	+	w⁻
C. innocuum[e]	–	–	–	–	+	–	–	+	–⁺
C. paraputrificum	–ʷ	–	–	–	+	–⁺	–	+	–
C. ramosum	–	–	–	–	+	–	–	+	–
C. sphenoides	–	–	–	+	+	+⁻	–	+	–ʷ
C. symbiosum	–ʷ	–	–	–	–	–	–	+ʷ	v
C. tertium[h]	–	–	–	–	+	+⁻	–	+	–
Asaccharolytic									
C. argentinense	+	–	–	–	–	–	+	–	–
C. hastiforme	+	–	–	–	–	–⁺	–⁺	–	–
C. histolyticum[h]	+	–	–	–	–	–	+	–	–
C. limosum	+	+	–	–	–	–	+	–	–
C. orbiscindens[i]	–ʷ	–	–	v	–	–	–	–	–
C. subterminale	+	–⁺	–	–	–⁺	–	+	–	–
C. tetani[f]	+	–	–ʷ	v	–	–	+	–	–

[a] +, positive reaction; –, negative reaction; v, variable reaction; w, weakly positive reaction; ST, subterminal; T, terminal. Superscript indicates rare variability. Capital letters indicate major metabolic products from peptone-yeast-glucose (PYG), lowercase letters indicate minor products, and parentheses indicate a variable reaction for fatty acids as follows: A, acetic; P, propionic; IB, isobutyric; B, butyric; IV, isovaleric; V, valeric; IC, isocaproic; L, lactic; S, succinic; and PA, phenylacetic.

[b] *C. bifermentans* is urease negative, and *C. sordellii* is urease positive. *C. bifermentans* usually forms chalk-white colonies on egg yolk agar.

[c] Toxin neutralization test required for identification. Send suspected isolates or *C. botulinum*-containing material to the appropriate local or state public health agency.

Fermentation of:											Spore location	Metabolic end products from PYG
Cellobiose	Fructose	**Lactose**	Maltose	Mannitol	Mannose	Melibiose	Ribose	Salicin	**Sucrose**	Xylose		
–	–^{w}	–	w^{-}	–	–^{w}	–	–	–	–	–	ST	A (iv, ic, p, ib, b, l, s)
–	–^{w}	–	–^{w}	–	–	–	–	–	–	–	ST	A, B, IV, ib (ic, v, p)
–	+^{w}	–	+$^{-}$	–^{w}	+^{w}	–^{w}	v	–	+^{w}	–	ST	B, A (1)
–	v	–	v	–	v	–^{w}	v	–	–	–	T	B, P, A (v, l, s)
–	v	–	–	–	–^{w}	–	–	–	–	–	T	B, A
v	+^{w}	–	–	w^{+}	v	–	–^{w}	–^{w}	–	–^{w}	ST^{T}	B, A, ic, iv, ib (v, l)
–	–^{w}	–	v	–	–	–^{w}	v	–^{w}	–	–	ST	A, B, P
–$^{+}$	+	+	+	–	+	v	v	–$^{+}$	+	–	ST	A, B, L (p, s)
–	–^{w}	–	–^{w}	–	–	–	–	–	–	–	TST	A, B, ib, iv (p, ic, v, l, s)
+^{w}	+	+	+	–	+	–	v	v	–	–	ST	B, A (p, l)
–	v	–	w	–	–^{w}	–	–^{w}	–	–	–	ST	A (IC, p, ib, iv, l)
–^{w}	–	–	–^{w}	–	–	–	–	–	–	–	ST	A, B, iv, ib (p, ic, v, l, s)
+	+	w^{+}	w^{+}	–	+	–^{w}	w^{-}	+$^{-}$	+	–	ST	B, A, L (p, s)
–$^{+}$	+	–	+	–	+		–	–$^{+}$	+	+	ST	A (l)
+	+	+	+	–^{w}	+	+^{w}	+^{w}	+^{w}	+	+	ST	B, A (l, s)
w^{+}	v	v	w^{+}	–	w^{+}	–	–	w	w^{+}	–	ST	B, A, L (s)
+	+^{w}	+	+	–	+	v	v	+	+^{w}	+^{w}	ST	A (l)
–	+^{w}	–	v	–	–	–	–	–	–	+$^{-}$	ST	A, IV, IB (p, l, s)
+	+	+	+	–	+	+	+	+	+	+	ST	A (l)
+^{w}	w^{+}	w^{+}	w^{+}	–^{w}	–^{w}	–^{w}	–^{w}	w^{-}	v	v	T	A
+	+	–^{w}	–	+^{w}	+	–	v	+^{w}	+^{w}	–^{w}	T	B, L, a (s)
+	+^{w}	+	+	–	+	–	w^{-}	+	+	–	TST	B, A, L (s)
+	+	+	+	+$^{-}$	+	+$^{-}$	v	+	+	–^{w}	T	A, l (s)
+^{w}	+^{w}	w^{+}	+^{w}	w^{+}	+^{w}	v	–^{w}	v	w^{-}	v	ST^{T}	A (l, s)
–	+	–$^{+}$	–	–	v	–	–	–	–	–	ST	A, B, L
+^{w}	+	+	+	w^{+}	+^{w}	+^{w}	+^{w}	+^{w}	+	v	T	A, B, L
–	–	–	–	–	–	–	–	–	–	–	ST	A, b, ib, iv (l)
–	–	–	–	–	–	–	–	–	–	–	T	A, B, iv, ib(p, ic)
–	–	–	–	–	–	–	–	–	–	–	ST	A (l, s)
–	–	–	–	–	–	–	–	–	–	–	ST	A (l, s)
–	–	–	–	–	–	–	–^{w}	–	–	–	ST	a, B (l)
–	–	–	–	–	–	–	–	–	–	–	ST	A, B, IV, ib (p, ic, l, s)
–	–	–	–	–	–	–	–	–	–	–	T	A, B, p (l, s)

[d] Nonproteolytic.
[e] L-Proline-aminopeptidase differentiates: C. *difficile* is positive, and C. *innocuum* is negative.
[f] Swarming.
[g] Cigar shaped; C. *bolteae* is lactose and β-NAG negative, C. *clostridioforme* is lactose positive and β-NAG negative, and C. *hathewayi* is lactose and β-NAG positive.
[h] C. *tertium*, C. *carnis*, and most C. *histolyticum* isolates grow aerobically.
[i] Described from a case of bacteremia; frequently isolated from stool specimens.

spindle shapes of clostridia (47, 99). Most strains are motile by means of peritrichous flagella. Nonmotile species from clinical specimens include *C. perfringens, C. ramosum,* and *C. innocuum*.

Clostridium species are metabolically diverse. As currently designated (53, 89), most species are chemoorganotrophic; some species may be chemoautotrophic and chemolithotrophic. They can be saccharolytic, proteolytic, neither, or both; they do not carry out dissimilatory sulfate reduction. They usually produce mixtures of organic acids and alcohols from carbohydrates, proteins and peptides, or purines and pyrimidines (86, 89).

Most species are obligately anaerobic, although the tolerance to oxygen varies widely; some species (e.g., *C. tertium*) will grow but not sporulate in the presence of air, and a few aerotolerant species, such as *C. carnis, C. histolyticum,* and occasional strains of *C. perfringens,* give scant growth on solid media incubated under 5 to 10% CO_2. The distinction between aerotolerant clostridia and certain *Bacillus* species may be made by virtue of the fact that clostridia usually form spores only under anaerobic conditions, they grow better anaerobically than in air, and clostridia usually do not produce catalase. Although *Clostridium* species are usually catalase and superoxide dismutase negative, trace amounts of these enzyme activities may be detected in some strains. In addition, clostridia lack a cytochrome system and are thus oxidase negative. Clostridia often occur in nature and in infections as consortia of mixed species, wherein aerobic and facultative organisms utilize oxygen, provide nutrients or other factors, and create an environment favorable for clostridial growth. Since clostridia often are present as mixed cultures, it is important to carefully ascertain purity by plating for single colonies and by microscopic analysis.

Clostridia produce a diversity of biologically active proteins, including toxins, many of which are important in human disease. The clostridia produce more kinds of protein toxins than any other bacterial genus, and more than 25 toxins lethal to mice have been identified (14, 23, 85). At least 15 species of cluster I *Clostridium* produce protein toxins, and new toxins and virulence proteins have been discovered through traditional isolation techniques and genomic analyses (38, 159). These proteins include neurotoxins, enterotoxins, cytotoxins, collagenases, permeases, necrotizing toxins, lipases, lecithinases, hemolysins, proteinases, hyaluronidases, DNases, ADP-ribosyltransferases, neuraminidases, and some others that are simply known as lethal toxins (85). Certain clostridial toxins have extraordinary potency, and botulinum and tetanus neurotoxins are the most poisonous toxins known, with lethal doses of 0.2 to 10 ng per kg for various animals, including humans (23, 75, 85, 154). The virulence traits of clostridia that cause disease in hosts are incompletely understood. Recently, genomic sequences of pathogenic clostridia have become available (38, 159), which should facilitate a comprehensive approach for understanding virulence factors involved in clostridial pathogenesis.

EPIDEMIOLOGY AND TRANSMISSION

Clostridium species are widespread in the environment partly due to their ability to form resistant endospores. They are commonly found in soil, feces, sewage, and marine sediments (44, 86, 163). The common presence of *C. perfringens* in soil is seldom of clinical significance (165). Infection of war wounds, for example, typically is due to the subject's indigenous microbiota and the lack of sufficient hygiene. On the other hand, the incidences of tetanus and food-borne botulism are commonly related to the incidence of their spores in soil, water, and many foods (86, 164). Outbreaks of hospital-acquired *C. difficile* enteric infections are often traceable to environmental sources and other typical background factors for nosocomial infection (142). Many clostridia are present in large numbers as part of the indigenous microbiota of the intestinal tract of humans and animals (44, 86) and may be found in the female genital tract and oral microbiota as well.

CLINICAL SIGNIFICANCE

Although the exogenous clostridial infections or intoxications such as tetanus, food-borne botulism, and hospital-acquired *C. difficile* colitis are serious, endogenous infections by clostridia that are a part of the host's indigenous microbiota are commonly encountered. Nearly any type of infection in the body may involve clostridia, often in association with non-spore-forming anaerobes and facultative or aerobic organisms. These infections tend to be more severe than comparable infections without clostridia as part of the infecting microbiota. Head and neck infections, brain abscess, sinusitis, otitis, aspiration pneumonia, lung abscess, pleural empyema, cholecystitis, other intra-abdominal infections, gynecologic and obstetric infections, and soft tissue and bone infections all may involve clostridia (79). Common predisposing factors are surgical procedures, trauma, vascular stasis, obstruction, malignancy with or without chemotherapy and immunosuppressive agents, diabetes mellitus, prior aerobic infection, and use of antimicrobial agents with poor activity against clostridia (e.g., trimethoprim-sulfamethoxazole) (64, 83).

Clostridial Bacteremia

C. septicum is isolated only rarely from the feces of healthy individuals but is found in anaerobic blood cultures in bacteremia associated with underlying disease processes, often neoplastic disease, particularly in the ileocecal region. As many as 70 to 85% of patients whose blood cultures are positive for this organism have some underlying malignancy (29, 103). *C. septicum* has been found in the lumen of 10 to 68% of normal appendices (27, 72). Another clinically important association has been observed between *C. septicum* bacteremia, neutropenia of any origin, and neutropenic enterocolitis involving the terminal ileum or cecum (102, 113). Patients with diabetes mellitus, severe atherosclerotic cardiovascular disease, or anaerobic myonecrosis (gas gangrene) may also develop *C. septicum* bacteremia (77, 109). The clinical importance of recognizing *C. septicum* bacteremia and starting appropriate treatment immediately cannot be overemphasized. Patients with this condition are usually gravely ill and may have metastatic spread of myonecrosis to distant anatomic sites. Mortality rates are very high. *C. septicum* has also been recovered from bacteremia in cirrhotic patients, as have *C. perfringens, C. bifermentans,* and other clostridia (49). Some of these patients demonstrated septic shock.

Another clostridial species of importance in patients with serious underlying disease such as malignancy is *C. tertium*. This organism, as well as *C. septicum* and *C. perfringens,* may be seen in bacteremia in such patients, with or without neutropenic enterocolitis (122). *C. tertium* may present special problems in terms of both identification and treatment. This organism may appear gram negative, is aerotolerant, and is resistant to metronidazole, clindamycin, and cephalosporins. *C. tertium* has also been found in bacteremia associated with acute pancreatitis (157).

An important study by Woo et al. (188) involved sequencing of genes encoding 16S rRNA of non-*C. perfringens* clostridia in a study of anaerobic bacteremia. *C. perfringens* accounted for 79% of clinically relevant clostridial bacteremias; 11 other species accounted for 17 other blood cultures positive for clostridia other than *C. perfringens*. There were four bacteremias due to *C. paraputrificum,* three due to *C. ramosum,* two due to *C. tertium,* and one each due to *C. baratii, C. difficile, C. disporicum, C. indolis, C. innocuum, C. orbiscindens, C. septicum*, and *C. sporosphaeroides*. The mortality rate of clinically relevant clostridial bacteremia was 29%. A study of risk factors for mortality in patients with anaerobic bacteremia (187) found, in a multivariate analysis, that the only significant factors were liver disease and patient age. In this study, there were 25 patients with clostridial bacteremia; the crude mortality rate in this group was 35%. The *C. clostridioforme* group (including *C. clostridioforme, C. hathewayi,* and *C. bolteae*) has been reported from a number of cases of bacteremia (67, 189).

Enteric Infections

Food Poisoning

C. perfringens is one of the most common bacterial causes of food-borne illness in the United States and Canada (32, 145). Almost all U.S. outbreaks and cases of *C. perfringens* food-borne gastroenteritis appear to be due to type A strains (32, 162). In *C. perfringens* type A food-borne disease, the food vehicle is typically an improperly cooked meat or meat product, such as gravy, that has cooled slowly after cooking or may have been inadequately reheated. Spores surviving the initial cooking germinate, and vegetative cells proliferate during slow cooling or insufficient reheating. Illness results from the ingestion of food containing about 10^8 or more viable vegetative cells, which sporulate in the alkaline environment of the small intestine, producing an enterotoxin (*C. perfringens* enterotoxin [CPE]) in the process. *C. perfringens* type A food-borne illness occurs within 7 to 30 h of ingestion of the suspect food and is generally mild and self-limiting (162). In the very young, the elderly, and the immunocompromised, symptoms are more severe, occasionally resulting in death (33). Enterotoxin-producing *C. perfringens* has been implicated as an etiologic agent of persistent diarrhea in elderly patients in nursing homes and tertiary-care institutions (30) and has been considered to play a role in antibiotic-associated diarrhea (AAD) without pseudomembranous colitis.

C. perfringens strains associated with food poisoning contain the enterotoxin gene *(cpe)* on the chromosome and produce the *C. perfringens* enterotoxin (CPE), which generally acts by forming pores in membranes of host cells (162). *C. perfringens* strains isolated from non-food-borne diseases, such as AAD and sporadic diarrhea, carry *cpe* on a plasmid (41, 68). The plasmid possessing *cpe* may be transmissible, since cases of food-associated diarrhea as well as AAD may be associated with a relatively small number of CPE-positive cells (68, 171, 184). These results indicate that *C. perfringens* strains bear a plasmid-borne enterotoxin gene that should not be disregarded as causative agents of food poisoning.

In addition to CPE, various strains of *C. perfringens* collectively produce about 15 protein toxins, several of which are involved in diseases of animals and livestock (85, 162). Epsilon-toxin is a 33-kDa protein produced by *C. perfringens* types B and D strains and is among the most lethal of clostridial toxins, next to botulinum and tetanus neurotoxins, and epsilon-toxin is considered a potential bioterrorism agent (23, 162). In animals, it causes edema and hemorrhage in the brain, heart, spinal cord, and kidneys (162).

Enteritis Necroticans (Pig-bel and Darmbrand), Necrotizing Enteritis, and NEC

Enteritis necroticans is caused by alpha-toxin- and beta-toxin-producing strains of *C. perfringens* type C, and beta-toxin is probably mainly responsible for the pathogenesis (162, 170). The gene encoding beta-toxin is located on a plasmid in *C. perfringens* (68). Enteritis necroticans is a life-threatening infection with ischemic necrosis of the jejunum (10). It was recognized in Papua New Guinea during the 1960s as the most frequent cause of death in children; it has been associated with pig feasts and occurs both sporadically and in outbreaks (143). Immunization against the beta-toxin resulted in a decreased incidence of the disease in New Guinea (119). Enteritis necroticans has also been recognized in the United States, the United Kingdom, Germany, and other developed nations, especially involving adults who are malnourished or who have chronic illnesses (e.g., diabetes or alcoholic liver disease) (60, 78, 81, 148) and also in a child with neutropenia (124). Enteritis necroticans may be underreported (128). It should be noted that NEC, a disease resembling enteritis necroticans but associated with *C. perfringens* type A, has been found rarely in North America in previously healthy adults (167).

NEC is a serious gastrointestinal disease that affects low-birth-weight (premature) infants who are hospitalized in neonatal intensive care units (110). Kliegman et al. (105) noted that the etiology and pathogenesis of this condition remain speculative in spite of more than 1,000 publications on NEC over the last 4 decades. Pathological similarities between NEC and enteritis necroticans include the patterns of bowel necrosis and inflammation that occur in these conditions (104, 105). Both diseases may manifest intestinal gas cysts (pneumatosis cystoides intestinalis). The sources of the gas, which contains hydrogen, methane, and carbon dioxide, are probably the fermentative activities of intestinal bacteria, including clostridia in particular (104). Epidemiological data support an important role for *C. perfringens* or other gas-producing microorganisms (e.g., *C. neonatale,* certain other clostridia, or *Klebsiella* spp.) in the pathogenesis of NEC (9, 104, 110).

CDAD and Colitis

C. difficile, the major cause of antibiotic-associated pseudomembranous colitis, is also the most frequently identified cause of hospital-acquired diarrhea (97). It has been estimated that *C. difficile* is responsible for more than 250,000 cases of diarrheal disease per year in the United States (186), with a cost exceeding $1 billion (114). *C. difficile* has been isolated from various natural habitats and the feces of domestic animals and humans (129). *C. difficile* is present asymptomatically as part of the bowel microbiota in up to half of all healthy neonates during the first year of life; the carriage rate decreases to the adult rate of 3% or less by the age of 2. Hospitalized patients frequently become colonized with this organism (73). McFarland et al. (136) reported that 21% of 399 patients with negative cultures on admission to a hospital with a high prevalence of *C. difficile*-associated disease (CDAD) acquired *C. difficile* during hospitalization. Of these patients, 63% remained asymptomatic, while 37% developed diarrhea. Antimicrobial agents of all classes and several anticancer chemotherapeutic agents have been implicated in the development of CDAD or pseudomembranous colitis (97). As noted above, certain *C. difficile* strains contain mobilizable transposable elements with antibiotic resistance to chloramphenicol, tetracycline, or erythromycin (4, 172). Metronidazole and vancomycin

have been active against all strains tested, but there have been a number of failures with metronidazole therapy recently (20, 146). Higher likelihood of treatment failure was noted in patients who remain on the predisposing antibiotics while undergoing treatment for CDAD (138).

In CDAD, the primary initiating event involves the disruption of the commensal intestinal microbiota during treatment with antibiotics or antineoplastic agents (73, 97, 129). As the levels of antibiotics drop below inhibitory concentrations, nosocomial pathogens such as *C. difficile* are able to grow. Toxigenic as well as nontoxigenic isolates are capable of forming spores and existing in the hospital environment. As a result, either type can infect the colon because of changes in populations of the intestinal microbiota. Only the toxigenic isolates are associated with disease, and nontoxigenic isolates may be protective by competitive exclusion (31). Toxigenic strains produce and release toxins TcdA and/or TcdB. TcdA and TcdB each have a molecular mass of approximately 270 kDa, similar to the TcsH hemorrhagic and TcsL lethal toxins from *C. sordellii* and alpha-toxin from *C. novyi* (179). The cytotoxic actions of these toxins and the associated inflammatory response result in the histopathology of CDAD.

In the early 1990s, an isolate of *C. difficile* that produced only toxin TcdB was described, and detailed studies suggested that the isolate contained a truncated *tcdA* gene (140, 179, 186). Furthermore, bioassays indicated that toxin B from this particular isolate was more toxic than toxin B from typical A^+/B^+ isolates. Multiple reports of atypical $TcdA^-/TcdB^+$ isolates and their association with clinical disease were documented (6, 8, 98, 130). Such isolates may be missed in laboratories that base their diagnosis solely on the use of toxin A-specific tests. In $TcdA^-/TcdB^+$ isolates, a large portion of the *tcdA* gene encoding the repeating region (i.e., the binding portion of the toxin) is deleted (100, 140). In addition to the deletion in TcdA, the active site of the TcdB gene is modified in $TcdA^-/TcdB^+$ isolates, resulting in a TcdB protein that more closely resembles the lethal toxin of *C. sordellii* (129, 140, 179). A limited number of cases of pseudomembranous colitis are caused by $TcdA^-/TcdB^+$ organisms (6, 8, 98, 130). Strains of *C. difficile* may only produce TcdA (97, 130, 179). Recently, *C. difficile* isolates that produce the highly potent iota-toxin, which is related to the iota-toxin of *C. perfringens* and *C. spiroforme,* have been noted (179). Virulent clones of *C. difficile* strains, characterized as toxinotype III, North American PFGE type 1, and PCR ribotype 027 (NAP1/027), have been linked to increased mortality in North America and Europe (135, 147, 181). The severity of CDAD caused by NAP1/027 may be due to hyperproduction of toxins A and B. Dissemination of this strain has led to important changes in the epidemiology of CDAD (181).

C. difficile is responsible for 20% or fewer of cases of AAD (24, 186). Reports suggest that *C. perfringens* is a less frequently identified cause of AAD (30). Enterotoxin-producing *C. perfringens* type A has been isolated from AAD patients who are negative for *C. difficile* and who have no other apparent cause of the disease. Coinfection with *C. difficile* and enterotoxigenic *C. perfringens* type A has been reported for AAD patients (43). The incidence of *C. perfringens*-associated AAD has been estimated at 5 to 20%, but additional epidemiological studies are needed to accurately determine the role of this organism in AAD (43).

C. difficile is an important cause of enteric infection in the elderly and in nursing home residents (160). *C. difficile* may be a problem even when antibiotics are not used, in patients with bowel stasis and those who have had bowel surgery. The clinical picture of *C. difficile* infection ranges from mild diarrhea to pseudomembranous colitis with or without toxic megacolon. In the most severe form, the disease may lead to bowel perforation and death.

Skin and Soft Tissue Infections

Soft tissue infections with clostridia or other organisms are sometimes not regarded as serious. However, it should be appreciated that necrotizing soft tissue infections (such as necrotizing fasciitis and myonecrosis) have a poor prognosis and require early surgical intervention as well as antimicrobial therapy. An interesting study published recently (15) reviewed 166 patients with necrotizing soft tissue infection of various types and with differing etiologies. The overall mortality was 16.9% in this series, and loss of limbs occurred in 26%. Clostridial infection was consistently associated with poor outcome and was an independent predictor for both mortality and limb loss; it was strongly associated with intravenous drug use and high levels of leukocytosis.

Clostridial Myonecrosis

C. perfringens is the clostridial species most commonly isolated from human clinical specimens, excluding feces (78, 79). One of the serious and unique infections in which this organism plays a major role is myonecrosis (gas gangrene) that involves a breakdown of muscle tissue related to the action of potent exotoxins, particularly alpha-toxin (a phospholipase C) and theta-toxin (a thiol-activated cytolysin) (39, 40). Alpha-toxin produces the opaque zone of lecithin hydrolysis products that surrounds colonies of *C. perfringens* growing on egg yolk agar plates. It gives rise to an outer zone of partial hemolysis that surrounds a smaller zone of complete hemolysis produced by theta-toxin (a heat-labile, oxygen-labile hemolysin easily detected around colonies of *C. perfringens* growing on sheep blood agar).

Clostridial myonecrosis is rapidly progressive and has a high mortality rate. Associated systemic manifestations may include shock, renal failure, and sometimes bacteremia with intravascular hemolysis (78, 79). In addition to *C. perfringens,* gas gangrene may involve *C. septicum, C. novyi, C. histolyticum, C. bifermentans, C. sordellii,* and other clostridia. Gas gangrene of the uterus, now rare, occurred most frequently as a consequence of illegal or self-induced abortions but has also followed spontaneous abortion, vaginal delivery, and cesarean section (78, 79, 128). Crepitant cellulitis, also called anaerobic cellulitis, is seen principally in diabetic patients and characteristically involves subcutaneous tissues or retroperitoneal tissues and can progress to fulminant systemic disease (78, 79, 128). The muscle and fascia are not involved. When clostridia are involved in this process, the disease may be more severe than when only non-spore-forming anaerobes and other bacteria are responsible.

Other Skin and Soft Tissue Infections

In addition to the well-known exogenous clostridial infections, emerging clostridial diseases have been recognized during the past decade. An emerging infection with a high mortality rate is soft tissue infection and toxic shock among injecting drug users. These individuals manifest hypotension, marked edema, and high leukocyte counts. The principal pathogens have been found to be *C. novyi* type A and *C. perfringens* (21, 36, 144). *C. novyi* types B, C, and D are unlikely to be isolated from human clinical material. Cases of *C. histolyticum* infection with cellulitis, abscess formation, or endocarditis have also been found (21). An outbreak of necrotizing fasciitis due to *C. sordellii* has been reported for black-tar heroin users (101).

C. sordellii is responsible for a rare, rapidly fatal postpartum endometritis in young, previously healthy women with a unique clinical picture of little or no fever or purulent discharge, shock, extensive edema and fluid accumulation in body cavities, and a markedly elevated white blood cell count (137, 151). *C. sordellii* has also been implicated in toxic shock-like syndrome following spontaneous childbirth or abortion; these women had refractory hypotension, multiple effusions, peripheral edema, hemoconcentration, and profound leukocytosis with lack of fever (161, 185). Also, *C. sordellii* was accountable for endophthalmitis after suture removal from a corneal transplant (192).

Botulism

C. botulinum is the cause of the rare but occasionally fatal illness known as botulism. Spores of *C. botulinum* are widely distributed in soil and aquatic habitats (94, 164). *C. botulinum,* along with unique strains of *C. butyricum, C. baratii,* and *C. argentinense,* can produce botulinum neurotoxin (BoNT), the most lethal poison known (46, 71, 75, 85, 154). The intravenous lethal dose for BoNT has been estimated as 0.1 to 0.5 ng per kg of body weight, and BoNT is among the most potent protein toxins by oral ingestion, with an estimated oral lethal dose of 0.2 to 1 μg per kg (17, 75, 154). There are seven antigenic serotypes of BoNT (A through G), which serve as useful clinical and epidemiological markers (84, 94, 115). Toxin serotypes A, B, and E of *C. botulinum* are the principal causes of botulism in humans (52, 84, 94). Neurotoxigenic strains of *C. butyricum* (63, 71, 86) and *C. baratii* (86) that produce type E and F neurotoxins, respectively, have been implicated mainly in infant botulism. Type E botulinal toxin-producing *C. butyricum* strains were confirmed by sequencing of the16S rRNA gene (48, 71), leading to the conclusion that neurotoxigenic *C. butyricum* must be regarded as an emergent food-borne pathogen. *C. argentinense,* which produces type G neurotoxin (84), has been isolated from soil in Argentina. Its reported isolation from autopsy materials from five individuals who died suddenly has not been substantiated, and *C. argentinense* has not been clearly implicated in botulism (86). *C. botulinum* types C and D are associated primarily with botulism in birds and mammals (94, 163, 164). Strains of *C. botulinum* that produce more than one serotype of BoNT, generally with one serotype formed in much higher levels, have been isolated from the environment and human and animal botulism cases (71, 84, 93). The BoNTs are coexpressed with nontoxic proteins of toxin gene clusters (34), and evidence suggests that the complexes are much more stable in the gastrointestinal tract (153). The genes for BoNT complex formation are associated with unstable genetic elements in certain serotypes, enabling toxin gene transfer to nonpathogenic clostridia (57, 92, 191).

There are four naturally occurring types of botulism: (i) classical food-borne botulism, an intoxication caused by the ingestion of preformed botulinal toxin in contaminated food; (ii) wound botulism, which results from elaboration of botulinal toxin in vivo after growth of *C. botulinum* in an infected wound; (iii) infant botulism, in which botulinal toxin is elaborated in vivo in the gastrointestinal tract of an infant colonized with *C. botulinum;* and (iv) botulism due to intestinal colonization in children and adults (16, 46, 71, 84, 106, 164). Intestinal colonization in adults has been associated with surgery and administration of antibiotics (80, 84). Recently, *C. botulinum* has been isolated from patients colonized with *C. difficile* (63), with viral infections (62), or with Crohn's disease (80).

Regardless of the category of botulism, the toxin enters the bloodstream at a peripheral site (e.g., gut, wound, or lung) and is transferred to the neuromuscular junctions of motor neurons, where it binds irreversibly to the presynaptic membranes. The site of action of all serotypes of BoNT is the presynaptic terminal of motor neurons (50, 117, 155). Elucidation of the three-dimensional structures of botulinum and tetanus toxins and their constituent domains has provided considerable insights into their mechanisms of action (116, 117, 155, 176). BoNT penetrates the plasma membrane by receptor-mediated endocytosis, and the light chain of 50 kDa (the catalytic domain) is internalized into the nerve cell through a protein channel (117, 155). Once internalized, BoNT specifically cleaves SNARE proteins involved in vesicle trafficking of neurotransmitters to the membrane (155). Exocytosis of acetylcholine is prevented at the nerve terminal to the neuromuscular junction, with consequent blockage of innervation of muscle activity (155). The clinical hallmark of botulism is an acute flaccid paralysis, which begins with bilateral cranial nerve impairment involving muscles of the eyes, face, head, and pharynx and then descends symmetrically to involve muscles of the thorax and extremities. At physiological levels, botulinum toxin, unlike tetanus neurotoxin (TeNT), probably does not enter the central nervous system (CNS). In naturally occurring food-borne botulism, gastrointestinal symptoms (e.g., abdominal cramps, nausea, vomiting, or diarrhea [more often constipation or obstipation]) may precede the neurologic signs of descending flaccid paralysis. Death may result from respiratory failure caused by paralysis of the tongue or muscles of the pharynx leading to occlusion of the upper airway or from paralysis of the diaphragm and intercostal muscles (17, 50). Generally, the patient's hearing remains normal, consciousness is not lost, and the victim is cognizant of the progression of the disease. Certain episodes of botulism have been reported that affect autonomic and sensory functions. A patient's awareness of loss of muscle activity can lead to considerable emotional distress, such as anxiety and depression (50).

Wound Botulism in Intravenous Drug Users

Wound botulism is another exogenous clostridial intoxication that may be found among injecting drug users (37, 133). Although wound botulism is rare in non-drug users, cases occasionally occur, such as a fatal case that occurred in a young girl who had a tooth extracted (182). An association between botulism and subcutaneous injection of Mexican black-tar heroin was reported in the United States and in the United Kingdom (37, 133). A study found 33 clinically diagnosed cases of wound botulism in the United Kingdom and Ireland between 2000 and 2002 (37). All individuals affected had injected black-tar heroin into muscle or by "skin popping." The clinical diagnosis was confirmed by laboratory tests in 20 of these cases; 18 cases were caused by type A toxin and 2 by type B toxin. Users of drugs other than heroin are also at risk of illness caused by clostridia; injection or snorting cocaine has resulted in cases of botulism (112).

Infant Botulism

In the United States, infant botulism is the most frequently recognized form of botulism. Although infant botulism has been documented in at least 15 other countries (16, 70, 71), it is comparatively rare outside the United States, perhaps due to failure of recognition and diagnosis (16). Of the nearly 2,000 cases reported in the United States from the discovery of infant botulism in 1976 (16, 149), approximately 45% of cases were in California, where an intensive epidemiological and microbiological program has been used for recognition and treatment of infant botulism (16).

Across the United States, the geographic distribution of toxin types in infant botulism cases has roughly paralleled the spore distribution of C. *botulinum* toxin types in soil sampled from different locations (16), although certain geographic locations have comparatively high incidences. Type A has been the most frequent BoNT type in cases of infant botulism in states west of the Mississippi River, whereas type B cases have predominated east of the Mississippi River (16, 164). Three known cases have been caused by a strain(s) of C. *botulinum* that produced toxins requiring both type B and F antitoxins for neutralization (84). Type E infant botulism, caused by neurotoxigenic strains of C. *butyricum*, was initially confirmed in two infants from Italy (71, 86), and later in additional patients. Type F infant botulism has been caused by neurotoxigenic C. *baratii* (71, 86).

Although most infants that contract botulism are 3 weeks to 6 months old, the age range is a few hours following birth to 363 days (16). Botulinum spores have not been detected in any food or liquid ingested by these infants other than honey (16). To date, the only clearly defined risk factors have been exposure to soil, dust, and honey (16, 70). It is recommended that honey not be fed to infants less than 1 year of age. Another possible risk factor, although not proven, is that spores could be transmitted in corn syrup, and possibly infant foods within the household environment (95). Whatever the sources, the ingested spores of C. *botulinum* germinate within the intestinal tract, and the vegetative cells multiply and produce the neurotoxin, which is then absorbed into the bloodstream (16, 84). Decreased frequency of bowel movements, a sign of decreased intestinal motility, may be an additional risk factor for infant botulism. The first sign of illness is usually constipation, which is often overlooked. Infants who are ultimately hospitalized develop lethargy and mild weakness, with feeding difficulties, pooled oral secretions, and an altered cry (16). They eventually lose head control and may go on to develop ophthalmoplegia, ptosis, flaccid facial expression, dysphagia, other signs of cranial nerve deficits, and generalized muscular weakness. Respiratory insufficiency and inability to swallow necessitating intubation and intragastric feeding also may occur. There is likely a spectrum of clinical features in infant botulism, ranging from mild illness not requiring hospitalization to severe botulism requiring intensive care. It has been postulated that fulminant, rapid-onset botulism can cause sudden death, which may account for a small percentage of cases of sudden infant death syndrome (17, 25). Human immune globulin that neutralizes BoNT (BabyBIG) is now approved by the FDA for use in therapy of infant botulism; this product has moderate titers of neutralizing antibodies against types A and B toxin, but its efficacy against serotypes E and F is unknown (18, 70).

Aerosolized Botulinum Toxin and Potential Use as a Bioterrorism Agent

Inhalational botulism, which results from aerosolization and inhalation of botulinum toxin, has been considered a fifth category of botulism (17, 150). Botulism could also result from covert contamination of foods (17, 183). These two routes of botulism have been considered most likely to occur in a bioterrorism incident (17, 42) (see chapter 9). Inhalational botulism has been demonstrated experimentally in monkeys (17, 150), accidentally in three veterinary personnel in Germany who were exposed to reaerosolized BoNT from rabbits and guinea pigs with aerosolized BoNT on their fur (17), and three researchers who were exposed to an aerosol during BoNT manipulations (91). Terrorists have attempted to use aerosolized botulinum toxin as a bioweapon in Japan but were not successful. After Operation Desert Storm ended in 1991, Iraq admitted to having produced 19,000 liters of concentrated botulinum toxin and to having loaded more than half of it into specially designed military weapons (e.g., missiles). According to Arnon et al. (17), 19,000 liters of concentrated botulinum toxin is more than enough to kill the entire human population by inhalation. Although inhalational botulism is possible, the toxin is unstable in aerosols and the more likely route of intentional intoxication is by food contamination and oral ingestion.

Tetanus

Tetanus, caused by C. *tetani*, is often associated with puncture wounds that do not appear to be serious. The organism and its spores can be isolated from a variety of sources, including soil and the intestinal contents of numerous animal species. A potent neurotoxin (TeNT), often referred to as tetanospasmin, is elaborated at the site of trauma and rapidly binds to neural tissue, provoking a characteristic paralysis and tonic spasms (28, 65). Tetanus is largely a disease of nonimmunized people, particularly in poor regions of the world and primarily affecting infants, since an effective toxoid for immunization has been used for many years.

Tetanus is an intoxication analogous to botulism except that it occurs solely through wound infection and production of tetanospasmin (TeNT). TeNT is synthesized as a single, inactive polypeptide chain (150 kDa), which is cleaved by an intrinsic protease to produce an active dichain form, consisting of a heavy chain (100 kDa) and a light chain (50 kDa) linked by a disulfide bond (155). The heavy chain is the part of the molecule that binds to neuronal cells, and the three-dimensional structure of this region has been elucidated (155). The light chain, a zinc endopeptidase, enters the cell cytoplasm and traverses from the nerve terminal to the nerve cell body by retrograde axonal transport (28, 155), eventually reaching neurons in the spinal cord and brain stem, where it affects glycinergic and GABA (γ-amino-*n*-butyric acid)-ergic neurotransmission (28, 155). Inhibitory impulses to CNS neurons are blocked, while uninhibited firing of motor nerve transmission continues, resulting in prolonged muscle spasms of both flexor and extensor muscles that can persist for weeks. The mechanism by which exocytosis of neurotransmitter release is inhibited is analogous to that of BoNT; in fact, TeNT cleaves the SNARE protein (VAMP) at the same peptide bond as BoNT B (117). In contrast to the pathophysiology of botulism, TeNT is retrogradely transported in neurons to the CNS and its site of action (28, 155).

In the United States, tetanus is reported most frequently in California, Michigan, Texas, and Florida and areas of the rural South (65). While most cases involve older, nonimmunized persons, injection of drugs (i.e., skin popping) has recently become an important risk factor in younger patients (22). It would be an unusual request for the clinical laboratory to isolate C. *tetani* from a wound, since tetanus usually presents few diagnostic problems for the clinician. The worldwide incidence of tetanus has been estimated to be as many as 500,000 cases per year (28, 65). Neonatal tetanus is endemic in developing countries despite the fact that it could be prevented by immunization of the mother and acquisition of passive antibodies by the fetus.

ADDITIONAL CLOSTRIDIAL SPECIES OF INTEREST

C. *innocuum* is an important organism in infections of immunocompromised hosts. C. *innocuum* has also been

recovered from patients with recurrent CDAD (2). It is often resistant to multiple drugs used to treat anaerobic infections (2). *C. ramosum* is the second most common *Clostridium* species (after *C. perfringens*) isolated from clinical specimens, including blood cultures and intra-abdominal and soft tissue infections. This species may be resistant to clindamycin and multiple cephalosporins. As noted earlier, *C. tertium* is often isolated from blood cultures from immunocompromised patients and has been reported as a cause of neutropenic enterocolitis and meningitis (51, 111, 122, 175). *C. hathewayi* and *C. bolteae* have been described from various human infections (67, 189), including fatal sepsis (127). Phenotypically similar *C. clostridioforme* is one of the clostridia most commonly isolated from human infections and appears to be associated with more serious or invasive human infections than *C. hathewayi* or *C. bolteae*.

The emergence of 16S rRNA gene sequencing technology has provided a means for identification of strains that may previously have been misidentified or classified as *Clostridium* without species identification. Examples are bacteremia caused by *C. hathewayi* (189), *C. intestinale* (59), and *C. symbiosum* (58); a fatal sepsis due to *C. fallax* in a previously healthy 16-year-old (87); and abscesses yielding *C. celerecrescens* (76). Microarray analysis of DNA from fecal samples has also been useful in determination of predominant species in the large bowel (180). It is likely that in this era of molecular identification techniques, a more accurate picture of clostridial infections will emerge.

COLLECTION, TRANSPORT, AND STORAGE OF CLINICAL SPECIMENS

As with other anaerobic infections, the proper selection, collection, and transport of clinical specimens are extremely important for the laboratory diagnosis of clostridial infections. For recommended collection and transport procedures in general, refer to chapters 5 and 20. Multiple tissue specimens should be sampled from the active sites of infection when gas gangrene is suspected, because clostridia are often not distributed uniformly in pathologic lesions. In addition to aspirates and tissues, selected clostridial illnesses require special specimens.

Confirmation of *C. perfringens* Food-Borne Illness

A freshly passed fecal specimen is the preferred specimen for *C. perfringens* culture and toxin assay, and appropriate specimens include the suspect food. Swab specimens are inadequate for the toxin assay because the sample volume is insufficient. These specimens should be placed into sterile unbreakable containers. All specimens should be stored at 4°C and shipped on cold packs as soon as possible. For optimal recovery, stool specimens should be processed within 24 h of collection.

Diagnosis of Enteritis Necroticans (*C. perfringens* Type C)

If enteritis necroticans is suspected, the appropriate specimens include three blood cultures from three different venipuncture sites, stool (at least 25 g, or 25 ml if liquid), and bowel luminal contents or tissue from the involved bowel (e.g., surgical specimen or autopsy material). Specimens should be transported in tightly sealed leakproof containers for the following: direct Gram stain, culture, isolation, identification, and typing of *C. perfringens*. PCR assays for genotyping *C. perfringens* are being used in certain research or referral laboratories to aid in diagnosis (170). Accordingly, DNA can be extracted for this purpose from formalin-fixed intestinal tissue or culture and amplified by PCR using primers specific for the *cpa* and *cpb* genes of *C. perfringens* type C.

C. difficile Culture and Toxin Assay

A single, freshly passed fecal specimen (ideally 10 to 20 ml of watery stool; minimum of 5.0 ml or 5 g) is the preferred specimen for *C. difficile* culture and toxin assay. To lessen the chance of obtaining positive culture results from patients merely colonized with the organism, only liquid or unformed stool specimens should be processed. Swab specimens are inadequate for the toxin assay because the sample volume is insufficient. Other appropriate specimens include bowel luminal contents and surgical or autopsy samples of the large bowel. Specimens should be transported in tightly sealed, leakproof plastic or glass containers. For optimal recovery, stool specimens should be cultured within 2 h of collection; although spores will survive in refrigerated stool for several days, there will probably be a large decrease in the number of viable vegetative cells of *C. difficile* in refrigerated specimens. Stools should be placed in an anaerobic environment (anaerobic transport vial or bag) if culture must be performed after storage. Adequate recovery of *C. difficile* may be expected from stools stored at 4°C for up to 2 days. Specimens for toxin assay may be stored at 4°C for up to 3 days and should be frozen at −70°C if performance of the assay is delayed. Freezing at −20°C results in a dramatic loss of cytotoxin activity. *C. difficile* toxin is unstable and will degrade at room temperature within 2 h after collection.

Suspected Neutropenic Enterocolitis Involving *C. septicum*

The specimens of choice for suspected neutropenic enterocolitis involving *C. septicum* are three blood cultures collected from three different venipuncture sites, stool (at least 25 g, or 25 ml if liquid), and luminal contents or tissue from the involved ileocecal area collected at surgery or autopsy and transported in tightly sealed leakproof containers. In addition, a biopsy sample of muscle (or an aspirate of fluid from the involved area, taken with a needle and syringe) should be collected if the patient is also suspected of having myonecrosis or another form of progressive infection.

C. botulinum Culture and Toxin Assay

Most hospital laboratories are not properly equipped to process specimens from patients suspected of having botulism. Before collecting any specimens, the medical care providers who suspect a diagnosis of botulism in a patient should immediately call their state health department's emergency 24-h telephone number or the CDC in Atlanta, Ga. (770-488-7100, 24-h/7-day emergency service), so that appropriate action can be taken to establish the diagnosis, initiate treatment, and investigate the case. Acceptable specimens include feces, enema fluid, gastric aspirates or vomitus, tissue or exudates, and postmortem specimens. These specimens should be placed into sterile unbreakable containers. Serum specimens (preferably >10 ml) should be collected as soon as possible after the onset of symptoms. Clinical swabs should be collected in an anaerobic transport medium; environmental swabs (from which spores may be isolated) may be sent in plastic containers without any medium. Food specimens should be left in their original containers if possible, or placed in sterile unbreakable containers. All specimens should be stored at 4°C and shipped on cold packs as soon as possible. Further information can be found at the CDC

botulism website (http://www.bt.cdc.gov/agent/botulism). Laboratories should have all of the pertinent information and contact numbers handy. During investigations of possible bioterrorism, sera, gastric aspirates, feces, and environmental or nasal swabs could be useful for detecting aerosolized botulinum toxin that may have been inhaled (11, 190). All specimens should be refrigerated until they can be transported to the laboratory for testing.

Certain clostridial toxins, particularly BoNT and TeNT and iota-toxin, are extremely toxic molecules and are considered very potent poisons. The CDC recommends biosafety level 3 primary containment and personnel precautions for facilities producing BoNTs for study. Personnel who work in the laboratory may be immunized with a pentavalent (A to E) toxoid available from the CDC. A biosafety manual should be posted in the laboratory and should contain the proper emergency phone numbers and procedures for emergency response. Regulations governing personnel safety for research with select agents are outlined in the *Code of Federal Regulations* and the manual *Biosafety in Microbiological and Biomedical Laboratories* (CDC/NIH; available on the Internet at http://www.cdc.gov/od/ohs/biosfty/ bmbl4/bmbl4toc.htm).

DIRECT EXAMINATION

Gas gangrene and necrotizing fasciitis represent extremely urgent situations requiring rapid clinical diagnoses. The direct examination of a Gram-stained smear of the wound may be useful for supporting the diagnosis of gas gangrene (11). Characteristic findings in *C. perfringens* infections include the absence of leukocytic infiltration and the presence of clostridia in smears prepared from central areas of the lesion. Special note should be made of gram-positive rods, with or without spores, because sporulation in tissue is not common for the two species most frequently encountered in wound and abscess materials, *C. perfringens* and *C. ramosum*. *C. perfringens* usually appears as large, relatively short, fat, gram-positive rods with blunt ends and often in short chains in tissue smears; the cells of *C. ramosum* are more slender and longer (Fig. 4). *C. perfringens* may or may not be encapsulated in smears from wounds; capsules usually are present in smears of endometrial specimens from postabortion *C. perfringens* infections. Spore stains offer no advantage over Gram stains for demonstration of spores, but examination with a phase-contrast or dark-field microscope may be helpful if the spores are close to maturity. If spores are present, shapes (spherical or oval) and positions (terminal, subterminal, or central) in the cells should be noted.

Direct Toxin Detection

Bioassays for BoNT and TeNT are currently the most important laboratory tests for diagnosis of botulism and tetanus (46, 56, 84). The definitive diagnosis of botulism is the detection of BoNT (not the organism) (84). Currently, the only reliable assay for BoNT is the mouse bioassay together with neutralization of mouse toxicity with type-specific antitoxins (84). Detection of neurotoxins is usually performed on fecal specimens, blood (serum), suspect foods in food-borne botulism cases, and culture fluid following enrichment by growth of the organism (46, 84). Enzyme-linked immunosorbent assays (ELISAs) have also been used to detect BoNT (56, 158), and cell culture systems (82) and biosensor platforms (55, 158) are in development for BoNT detection (158). Real-time PCR assays for detection of *C. botulinum* BoNT gene fragments specific to BoNT A, B, and E have been developed as alternatives to the mouse bioassay; this approach was found to demonstrate a sensitivity and specificity similar to those of conventional approaches (5).

Antigen Tests for Diagnosis of *C. difficile* Disease

Controversy exists about which of the multiple methods for the detection of *C. difficile* and its toxins is optimal. Toxin detection and neutralization by a cell culture cytotoxin assay are often considered the "gold standard" when new detection methods are evaluated. A confirmed diagnosis of *C. difficile*-associated enteric disease on the basis of both clinical and laboratory criteria represents the ultimate gold standard. Cytotoxicity assay for toxin B is considered the gold standard due to its high sensitivity (94 to 100%) and high specificity (99%) (35, 54). However, the assay is costly and technically demanding and has a slow turnaround time. Therefore, it is not widely used in clinical laboratories. Cytotoxin B neutralization assays are commercially available (Table 2).

The commercially available enzyme immunoassays (EIAs) (Table 2) generally show lower sensitivities and specificities (45 to 95% and 75 to 100%, respectively) than tissue culture assay (35, 134, 178). In general, the relative sensitivities and specificities for the various tests vary considerably, depending upon the laboratory and the method to which the test is compared. Although the sensitivity is lower, the rapid turnaround time of the EIAs and their ease of use explain the preference of many laboratories for an EIA procedure (186). EIAs that detect both toxins A and B are preferable since some strains of *C. difficile* produce only one toxin or the other (186).

Most of the EIAs for detection of toxin A and/or toxin B in stool are microwell format assays, but several membrane-based EIAs are also available (Table 2). The membrane-based EIAs offer an even faster turnaround time than many of the microwell formats, although that of the microwell EIAs from Meridian Bioscience (Cincinnati, Ohio) and TechLab (Blacksburg, Va.) can be shortened considerably by utilizing a shaking incubation step. The microwell formats offer increased sensitivity and specificity over membrane-based EIAs (61). However, one study found the Triage *C. difficile* Micro Panel (Biosite Diagnostics, San Diego, Calif.) and two new chromatographic immunoassays (Clearview *C. difficile* A [Inverness Medical Professional Diagnostics, Princeton, N.J.] and ColorPac Toxin A [Becton Dickinson, Sparks, Md.]) to be more sensitive than conventional EIAs (178). Similarly, the ImmunoCard Toxins A&B (Meridian Bioscience) was found to provide a high specificity and sensitivity and to be suitable for rapid screening for CDAD (177).

The common antigen glutamate dehydrogenase (GDH) does not differentiate between toxigenic and nontoxigenic strains and cannot establish a diagnosis for CDAD because colonization with *C. difficile* is frequent in hospitalized patients. However, testing for GDH has proven useful as a screening test due to its high negative predictive value. A preferred laboratory approach for the diagnosis of CDAD is to test the stool specimens for GDH and, if positive, to test the specimens by EIA for toxin detection (134, 166). The GDH-positive but toxin-negative specimens should be further tested with a cell culture-based cytotoxin assay. The Triage *C. difficile* Micro Panel provides simultaneous detection of toxin A and GDH. Several studies have found the Triage to provide a suitable, rapid alternative for the diagnosis of CDAD (121, 134, 166, 178). Laboratory results obtained with the commercial kits must be correlated and interpreted within the context of the patient's clinical presentation.

Currently PCR is used mostly as a research tool in laboratories for detecting *C. difficile* toxin genes directly in fecal

specimens. Real-time PCR has been successfully used for quantitative detection of *C. difficile* and its toxins in fecal samples (26, 177). Also, a multiplex PCR toxin approach has been designed for simultaneous identification and toxigenic type characterization of *C. difficile* isolates (123).

Tests for Diagnosis of CPE

Testing of food sources of *C. perfringens* illness is a public health function (see chapter 12). Several methods have been described for the detection of CPE in feces, including cell culture assays, ELISA, and reversed-phase latex agglutination (RPLA) (139). The cell culture assay using Vero cells is not as sensitive or reproducible as other methods (19, 69). The RPLA kit (PET-RPLA; Oxoid, Hampshire, United Kingdom, and Remel Inc., Lenexa, Kans.) was reproducible and reasonably sensitive; however, nonspecific interference by fecal matter has been reported (139). Similarly, the background bacterial DNA in stool has been reported to interfere with PCR amplification of the enterotoxin gene (139). While an in-house ELISA system developed by the Food Safety Microbiology Laboratory of the Public Health Laboratory Service has been reported to be the most sensitive assay and considered the gold standard, the TechLab *C. perfringens* enterotoxin ELISA system provided a specific, reliable, and practical tool for detecting CPE in fecal samples (19, 69).

ISOLATION PROCEDURES

A summary of useful procedures for culture and isolation of clostridia is provided below. Clostridia usually produce good growth on commercially available CDC anaerobe blood agar and phenylethyl alcohol blood agar (PEA) after 1 to 2 days of incubation. Brucella agar with 5% sheep blood, Columbia agar, or brain heart infusion agar supplemented with yeast extract, vitamin K, and hemin may also be used as the nonselective blood agar medium. Colony characteristics vary on different media. A few species, such as *C. perfringens,* form colonies after overnight incubation or in as little as 6 h. When clostridia are suspected in wound or abscess specimens (e.g., gas gangrene), egg yolk agar (modified McClung-Toabe formula; see chapter 21) may also be inoculated.

After incubation, the blood agar and PEA cultures should be examined under a dissecting microscope, with attention to the hemolysis pattern, colony structure, and any evidence of swarming or motile colonies. Egg yolk agar should be examined for evidence of lecithinase (Fig. 1) or lipase production. Lecithinase activity is indicated by the development of an insoluble, opaque, whitish precipitate within the agar. An iridescent sheen or oil-on-water appearance (pearly layer) indicates lipase activity. Proteolysis, the third reaction that can be seen on egg yolk agar, is indicated by a zone of translucent clearing in the medium around the colonies. In addition to the modified McClung-Toabe egg yolk agar formulation, the same reactions can be visualized on the hemin-supplemented egg yolk agar formulation recommended by Jousimies-Somer et al. (99) or on Lombard-Dowell egg yolk agar (187a).

Isolation of additional strains in the presence of swarming *Proteus* species or *C. septicum* may require short incubation times (18 to 24 h), subculture onto PEA, or use of anaerobe blood agar with 4% agar ("stiff blood agar"). When isolated colonies can be picked, they should be subcultured to chopped-meat medium, and the culture should be incubated overnight and used for the inoculation of differential media. In addition, prereduced, anaerobically sterilized (PRAS) peptone-yeast-glucose media may be inoculated for gas-liquid chromatography if the laboratory has that capability.

Spore Selection Techniques

Heat (80°C) or ethanol treatment procedures can aid in detecting spores (99, 108). Ethanol may be more effective than heat if the specimen contains relatively heat-sensitive clostridia (e.g., *C. botulinum* type E and some strains of *C. perfringens* involved in food-borne outbreaks). Heat treatment may be more effective than alcohol if homogenization is incomplete and the specimen contains particulate matter that is not penetrated adequately by the alcohol. For any spore selection technique, an untreated control subculture should be prepared.

Alcohol Treatment

To a 1-ml sample of a fecal suspension or homogenate of a wound or exudate, in a sterile screw-cap tube, an equal volume of absolute (or 95%) ethanol is added. The specimen is gently mixed at room temperature (22 to 25°C for 1 h). An Ames aliquot mixer (Miles Laboratories, Inc., Elkhart, Ind.) is a convenient way to provide continuous mixing. The treated material is used to inoculate chopped-meat–glucose or thioglycolate medium, anaerobe blood agar, or egg yolk agar. The culture is incubated and inspected for growth.

Heat Treatment

For heat treatment, a tube of chopped-meat–glucose or thioglycolate medium (5 ml) is preheated in an 80°C water bath for 5 min, and 1 ml of sample suspension is added. The culture is heated for 10 min, and the tube is removed and cooled in cold water. The treated sample suspension is subcultured into an unheated tube of chopped-meat–glucose or thioglycolate medium, anaerobe blood agar, or egg yolk agar. The cultures are incubated anaerobically and examined for growth.

Isolation of *C. difficile*

Culture alone (without subsequent testing of *C. difficile* isolates for toxin production) results in lower specificity and misdiagnosis of CDAD, especially when *C. difficile* carriage is prevalent. Testing isolates with a toxin assay by cell culture or EIA provides the organism for epidemiological studies and for antimicrobial susceptibility testing, if required. To perform a culture for *C. difficile,* a stool specimen should be inoculated directly onto cycloserine-cefoxitin-fructose agar (CCFA) (see chapter 21). The culture should be incubated anaerobically at 35 to 37°C for 18 to 24 h before observation. Following incubation, the plates should be examined by using a dissecting microscope. Colonies of *C. difficile* are yellowish to white, circular to irregular, and flat, with a rhizoid or erose edge and a ground-glass appearance (Fig. 2). The colonies have a distinctive odor like *para*-cresol (or horse manure). In addition, *C. difficile* colonies on CCFA fluoresce chartreuse under UV light (99).

In addition to the use of CCFA medium, *C. difficile* from fecal samples can be isolated by using the alcohol or heat shock spore selection technique. After 18 to 24 h of incubation on blood-containing agar, colonies from treated samples are nonhemolytic, 2 to 4 mm in diameter, creamy yellow to gray-white, and irregular. The colonies have a coarsely mottled to mosaic internal structure and a matte or dull surface, and they are seen to be slightly raised when viewed under a dissecting microscope. The odor is distinctive. Gram staining of *C. difficile* reveals gram-positive to gram-variable rods that are thin, with parallel sides, and 0.5 μm wide by 3 to 5 μm long. If spores are present, they are subterminal. Presumptive

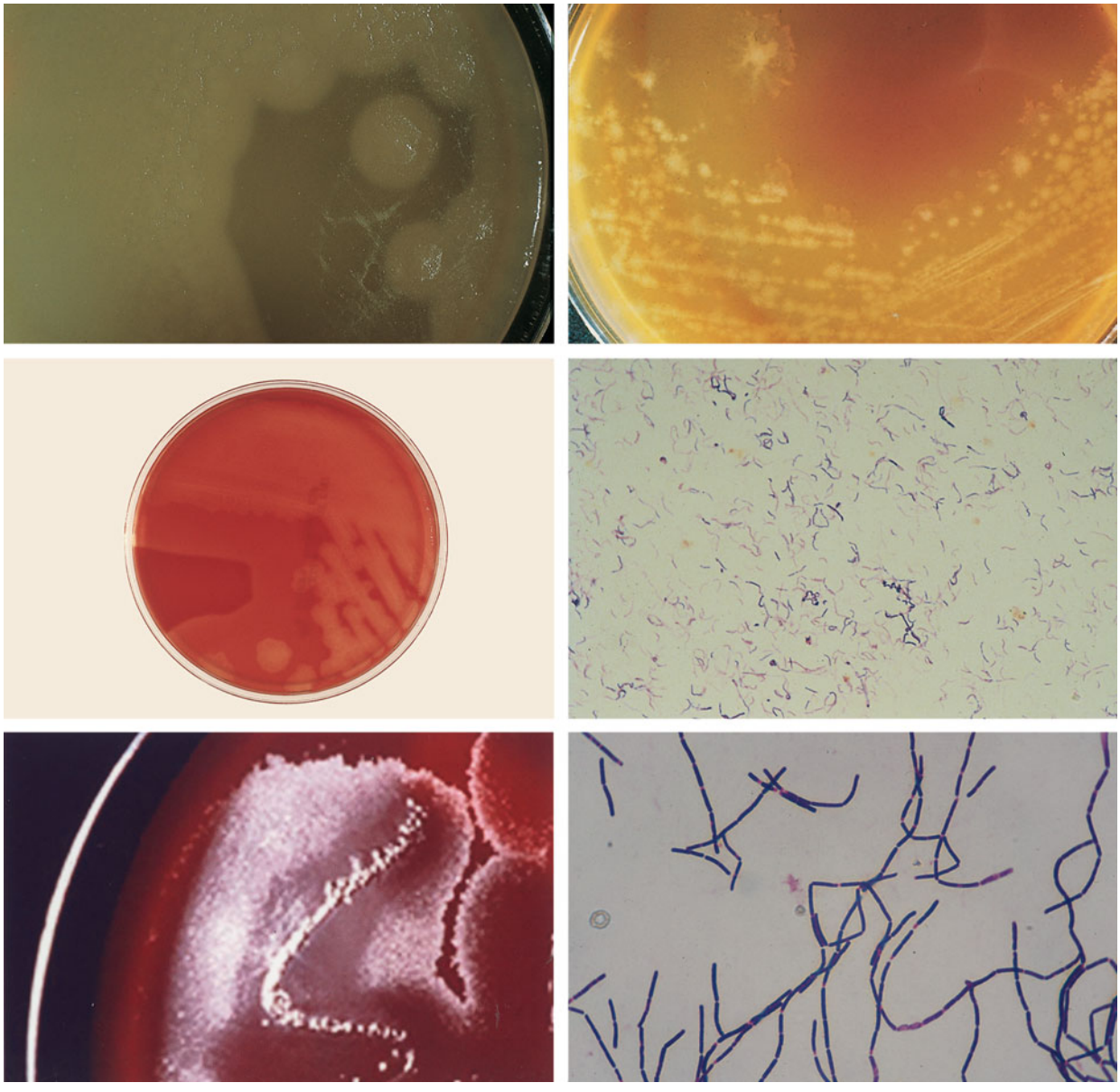


FIGURE 1 (top row, left) Lecithinase reaction on egg yolk agar. Note opacity of agar surrounding colonies due to precipitation of complex fats. (Reprinted with permission from reference 99.)

FIGURE 2 (top row, right) *C. difficile* on CCFA. Note the yellow, ground-glass appearance of colonies. (Reprinted with permission from reference 99.)

FIGURE 3 (middle row, left) *C. perfringens* on blood agar showing double zone of beta-hemolysis. (Reprinted with permission from reference 99.)

FIGURE 4 (middle row, right) Gram stain of *C. ramosum*. Note thin, gram-variable bacilli with distinct spores. (Reprinted with permission from reference 99.)

FIGURE 5 (bottom row, left) *Clostridium septicum* on blood agar after 12 h of incubation. Note spreading colonies. (Courtesy of E. J. Baron.)

FIGURE 6 (bottom row, right) Gram stain of *C. septicum*. Note long, filamentous bacilli with rare spores. (Courtesy of E. J. Baron.)

TABLE 2 Commonly used tests for the diagnosis of *C. difficile* disease

Entity detected	Method	Principal advantages	Principal limitations	Available tests and sources
Organism	Toxigenic culture	Sensitive, specific	Efficiency varies from lab to lab; must add method for toxin detection	CCFA, available from various manufacturers; prereduced media preferred (Anaerobe Systems)
GDH	Latex agglutination	Rapid, simple	Low sensitivity; does not distinguish between toxigenic and nontoxigenic *C. difficile*	CDT (BD) Meritec *C. difficile* (Meridian Bioscience)
	Membrane EIA	Rapid, simple	Higher sensitivity; does not distinguish between toxigenic and nontoxigenic *C. difficile*	Triage Micro *C. difficile* panel (Biosite Diagnostics Inc.) Immunocard *C. difficile* (Meridian Bioscience) *C. diff* Chek (Inverness Medical Professional Diagnostics and TechLab, Inc.)
Toxin B	Tissue culture	Sensitive, specific	Requires 24–48 h to complete; toxin B can be inactivated, resulting in false-negative results	*C. difficile* Toxin/Antitoxin (TechLab) *C. difficile* Tox B Test (Inverness Medical Professional Diagnostics and TechLab)
Toxin A	EIA	Rapid, simple	Sensitivity and specificity vary considerably Does not detect A^-/B^+ isolates	Triage *C. difficile* panel (Biosite Diagnostics) Premier *C. difficile* Toxin A (Meridian Bioscience) ProSpecT-II *C. difficile* Toxin A microplate (Remel Inc.) *C. difficile* Tox-A Test (TechLab) Culturette Toxin CD Test (BD) VIDAS-CDA (bioMerieux)
	Membrane EIA	Rapid, simple	Sensitivity and specificity vary considerably Does not detect A^-/B^+ isolates	Triage *C. difficile* panel (Biosite Diagnostics) ImmunoCard Stat! Toxin A (Meridian Bioscience) *C. difficile* Toxin A (Oxoid and Remel) Clearview *C. difficile* Toxin A (Inverness Medical Professional Diagnostics and Hardy Diagnostics, Santa Maria, Calif.)
	Optical immunoassay	Rapid, simple	Less sensitive than EIA	ColorPAC Toxin A Test Kit (BD) BioStar OIA CdTOX A (Inverness Medical Professional Diagnostics)
Toxins A and B	EIA	Rapid, simple	Sensitivity and specificity vary considerably Detects A^-/B^+ isolates	Premier Toxins A and B (Meridian Bioscience) ProSpecT *C. difficile* Toxin A/B microplate (Remel Inc.) *C. difficile* TOX A/B II (Inverness Medical Professional Diagnostics and TechLab)
	Membrane EIA	Rapid, simple	Sensitivity and specificity vary considerably Detects A^-/B^+ isolates	ImmunoCard Toxins A and B (ICTAB; Meridian) Xpect *C. difficile* Toxin A/B (Remel and Oxoid) Tox A/B Quik Chek (Inverness Medical Professional Diagnostics)

identification of *C. difficile* can be made by demonstrating typical colonies, Gram stain morphology, and characteristic odor. Definitive identification depends on demonstration of the unique pattern of short-chain fatty acid metabolic products by gas-liquid chromatography (GLC) and by biochemical characterization of isolates (12, 74, 90) (see Table 1).

IDENTIFICATION

Preliminary Identification of *Clostridium* Species

Clostridia are typically gram-positive rods by microscopic morphology. Some clostridia appear gram negative, especially *C. ramosum, C. innocuum,* and the *C. clostridioforme* group, but the special-potency antibiotic disk pattern will verify the presence of gram-positive organisms. Second, it may be difficult to detect spores, so an ethanol or heat spore treatment may be necessary, and phase-contrast or dark-field microscopy may be helpful. Third, the colonial morphology of pure cultures may be variable, so the culture may appear to be mixed. Subcultures of single, well-isolated colonies yield the same variable morphologies. Examination of colonies by stereomicroscopy is helpful for noting colonial characteristics. Fourth, the aerotolerant clostridia may be confused with *Bacillus* or *Lactobacillus* spp. *Clostridium* species sporulate anaerobically only, grow much better anaerobically (larger colonies), and are almost always catalase negative. *Bacillus* spp. sporulate aerobically only, usually grow better aerobically, and are usually catalase positive. Aerobically grown *C. tertium* shares similar colonial and cellular morphologies with *Lactobacillus* spp. Certain clostridia can be identified with relative ease by Gram stain and colony morphology, indole reaction, hemolysis on blood agar, and tests decribed below (Table 3).

Special-Potency Disks

The isolate should be subcultured on blood agar with special-potency disks containing vancomycin (5 μg), kanamycin (1 mg), or colistin (10 μg). The plates should be incubated anaerobically for 48 to 72 h at 35 to 37°C. Clostridia are colistin resistant and vancomycin susceptible (Table 3), except for occasional *C. innocuum* isolates, which may be only moderately susceptible to vancomycin (7).

Lecithinase and Lipase

The isolate should be subcultured on egg yolk agar and incubated anaerobically for 48 to 72 h at 35 to 37°C. Lecithinase activity is demonstrated by a white, opaque, diffuse zone around the colonies and extending into the medium (Fig. 1). Lipase activity is indicated by an iridescent sheen on the surface of bacterial growth and on the agar surface around the colonies.

Spore Test

Media for the demonstration of spores include chopped-meat agar slant or broth and thioglycolate medium. The culture should be incubated anaerobically at 5 to 7°C below the optimum temperature (30°C) for growth and sporulation of clostridia, except for *C. perfringens* (should be induced at 37°C). Actively growing cultures may stand at room temperature for several days to 1 week, and ethanol or heat spore treatments can be performed as described above.

Definitive Identification of *Clostridium* Species

The traditional method for the phenotypic characterization and identification of clostridia is the use of PRAS media for the determination of fermentation profiles and other characteristics combined with GLC analysis of metabolic end products (99). However, only a few laboratories have PRAS media or GLC available. Table 1 lists characteristics that are useful for definitive identification of clinically relevant clostridia. The key reactions (bold in Table 1) require minimal PRAS media and can be used in conjunction with commercial identification kits or individual preformed-enzyme tests such as Wee Tabs (Key Scientific, Round Rock, Tex.) or Rosco Diagnostic Tablets (Rosco, Taasrup, Denmark). Gelatin and esculin hydrolysis, carbohydrate fermentation reactions, and metabolic end product analysis (Table 1) are based on results obtained with PRAS media (Anaerobe Systems, Morgan Hill, Calif.).

PRAS Biochemical Inoculation

Actively growing broth cultures (without carbohydrate) or cell pastes suspended in broth medium (e.g., peptone-yeast or thioglycolate) may be used to inoculate PRAS media. The cultures are incubated for 48 to 72 h at 35 to 37°C, and overnight incubation may be sufficient for many clostridia.

Gelatin Hydrolysis

A PRAS gelatin tube with an actively growing culture is refrigerated along with an uninoculated tube for at least 1 h. The tubes are removed to room temperature, inverted immediately, and observed for liquefaction every 5 min. In a positive reaction, the gelatin has been hydrolyzed and thus fails to solidify and will drop to the top of the inverted tube immediately. In a negative reaction, the medium fails to liquefy when it reaches room temperature (>30 min).

TABLE 3 Rapid identification of some lecithinase-positive and/or swarming *Clostridium* spp. of clinical significance[a]

Species	Lecithinase	Lipase	Indole	Swarming	Urease	Spore location	Other characteristics
C. bifermentans	+	–	+	–	–	ST	Chalk-white colonies on EYA
C. novyi A	+	+	–	+	–	ST	Robust beta-hemolysis
C. perfringens	+	–	–	–	–	ST	Double zone of beta-hemolysis Reverse CAMP positive
C. sordellii	+	–	+	–	+	ST	Large gram-positive bacilli
C. septicum	–	–	–	+	–	ST	Rare spores Spreading, adherent colonies
C. sporogenes	–	+	–	+	–$^{+}$	ST	Abundant oval spores
C. tetani	–	–^{w}	v	+	–	T	Drumstick shaped

[a] EYA, egg yolk agar; for other abbreviations and symbols, see Table 1, footnote *a*.

A weakly positive reaction yields liquid medium at the time it reaches room temperature (<30 min).

Esculin Hydrolysis

Five drops of 1% ferric ammonium citrate are added to a tube of actively growing bacteria in a PRAS esculin tube, and the tube is observed for a color change and fluorescence under UV (366 nm) light. In a positive reaction, a black or dark brown color may develop, and there is no fluorescence under UV light. In a negative reaction no color develops, and the tube fluoresces white-blue under UV light. Since many clostridia produce hydrogen sulfide (H_2S), which also reacts with the reagent to form a black complex, all tubes that darken after the addition of reagent should be confirmed under UV light.

Carbohydrate Fermentation

The pH of actively growing organisms (at least 2+ turbidity) should be measured in a PRAS carbohydrate tube. A positive reaction ("acid") yields a pH below 5.5, and a negative reaction results in a pH exceeding 5.9. "Weak acid" is indicated by a pH of 5.6 to 5.8. Details of GLC procedures used for the analyses of metabolic end products listed in Table 1 are outlined elsewhere (99).

Commercial kits, based on the detection of preformed enzymes with chromogenic or fluorogenic substrates, have been marketed for the rapid identification of anaerobes. These panels include RapID ANA II (Remel), RapID 32A (bioMerieux, Durham, N.C.), Vitek ANI Card (bioMerieux), and BBL Crystal Anaerobe Identification System (BD, Franklin Lakes, N.J.). The overall performance of these panels varies, and the panels are generally not satisfactory as the sole identification method for clostridia. In the RapID ANA II system, 74% of the 130 clostridia tested were correctly identified to the species level (132). Identification was correct for *C. perfringens* and *C. ramosum* but less likely to be correct for other commonly encountered clostridia, such as *C. difficile, C. innocuum,* and *C. clostridioforme* (132). Similarly, in another study, 100% of 20 *C. ramosum* isolates, 24% of 21 *C. innocuum* isolates, 50% of 20 *C. clostridioforme* isolates, and 91% of 11 *C. perfringens* isolates were correctly identified by RapID ANA II (7). In the same study, Rapid ID 32A identified 70% of *C. ramosum* isolates, 0% of *C. innocuum* isolates, 40% of *C. clostridioforme* isolates, and 46% of *C. perfringens* isolates correctly (7). Neither the RapID ANA II nor the Rapid ID 32A test system was found to provide a reliable method for identification of *C. botulinum* (126). The Vitek ANI Card identified only 64% of clostridia correctly; all *C. perfringens* isolates (total of 8) and 64% of *C. difficile* isolates (7 of 11 total isolates) were correctly identified to the species level (156). The BBL Crystal Anaerobe Identification System (BD) correctly identified 79 of 103 (76.7%) *Clostridium* strains, but the system failed to identify any of the 7 *C. innocuum* or 9 *C. tetani* strains tested (45).

In general, Gram reaction, cellular morphologies, colonial characteristics, and aerotolerance of isolates should be determined in conjunction with the use of commercial microsystems. Supplementation of tests in these kits with individual tablets (e.g., Wee Tabs or Rosco Tablets) can be helpful. Other useful supplemental tests for clostridia include the tests outlined above, such as lipase and lecithinase, the reduction of nitrate, gelatin and esculin hydrolysis, carbohydrate fermentation, and metabolic end product analysis using GLC.

Characteristics of Commonly Encountered Clostridia

Key characteristics that aid in the presumptive identification of the most common species are listed below. See Tables 1 and 3.

- *C. bifermentans:* colonies chalk-white on egg yolk agar, irregular, scalloped edge; many free spores, often chaining; urease negative; indole and lecithinase positive. *C. sordellii* is similar but is usually urease positive.
- *C. bolteae:* colonies usually have a slightly irregular edge; greening of agar around colonies; gram negative; tapered ends; spores are rare. Lactose negative and β-*N*-acetylglucosaminidase (β-NAG) negative.
- *C. butyricum:* very large, irregular colonies with mottled to mosaic internal structure; subterminal spores; ferments many carbohydrates.
- *C. cadaveris:* white-gray, entire or slightly irregular, raised to slightly convex; oval terminal spores; spot indole positive.
- *C. clostridioforme:* same as for *C. bolteae* but lactose positive and β-NAG negative.
- *C. difficile:* colonies creamy yellow to gray-white (Fig. 2), irregular, coarse, mottled to mosaic internal structure, matte or dull surface, horse stable odor; subterminal to free spores or spores infrequent; gelatin hydrolysis can be slow. Mannitol and proline positive; colonies fluoresce chartreuse on CCFA.
- *C. glycolicum:* colonies gray-white, entire to scalloped edge, convex; subterminal and free spores.
- *C. hathewayi:* same as for *C. bolteae* but lactose and β-NAG positive.
- *C. innocuum:* gray-white to brilliant greenish colonies, coarsely mottled to mosaic internal structure, entire edge usually; terminal spores may be difficult to find; nonmotile; mannitol positive; lactose, maltose, and proline negative.
- *C. novyi* type A: lecithinase and lipase positive, may swarm, strong beta-hemolysis.
- *C. perfringens:* double zone of beta-hemolysis around colonies (Fig. 3), boxcar-shaped rods, spores rare, lecithinase positive (Fig. 1).
- *C. ramosum:* colonies resemble *Bacteroides fragilis* but usually have a slightly irregular edge; gram-variable, palisading, slender rods; small round or oval terminal spores (Fig. 4); nonmotile; mannitol positive.
- *C. septicum:* swarms (Fig. 5), large, filamentous bacilli (Fig. 6), subterminal spores often "lemon" forms, DNase positive, sucrose negative.
- *C. sporogenes:* medusa-head colonies, possible swarming, colonies adhere firmly to agar; subterminal and many free spores; lipase positive.
- *C. symbiosum:* rods with tapered ends, football shaped, may form chains, often has spores.
- *C. tertium:* aerotolerant, terminal spores when anaerobically incubated.
- *C. tetani:* may form a thin film of growth over entire agar plate, especially on moist media; drumstick spores.

Toxin tests are necessary for the identification of a few species. *C. sporogenes* cannot be differentiated with certainty from the proteolytic group I strains of *C. botulinum* unless

toxin tests are used. A few strains of *C. botulinum* produce lecithinase as well as lipase and are difficult to distinguish from *C. novyi* type A except by toxin tests or by the use of a *C. novyi* fluorescent-antibody conjugate (165). An excellent source of diagnostic clostridial antisera is TechLab. As a supplement to the methods described, the various types of *C. botulinum* and other clostridia can be presumptively identified on the basis of differences in their cellular fatty acid profiles (12, 74, 90) and by typing methods such as pulsed-field gel electrophoresis or other molecular analyses. Finegold et al. (67) described a multiplex PCR procedure for the rapid distinction among the three species of the *C. clostridioforme* group.

ANTIMICROBIAL SUSCEPTIBILITIES

Antimicrobial susceptibility studies with strains of a number of clostridial species are summarized in Table 4. Now that more laboratories are identifying anaerobes by 16S rRNA gene sequencing, more accurate species identification will be available and more reliable susceptibility data can be generated. Drugs lacking antimicrobial activity against various clostridia include trimethoprim-sulfamethoxazole (a drug noted for promoting intestinal overgrowth of clostridia [83]), ampicillin, and clindamycin. No resistance of clostridia to ampicillin-sulbactam or piperacillin-tazobactam was noted, and antimicrobial resistance was uncommon among clostridia with respect to imipenem, metronidazole, and vancomycin. Five species (all with small numbers of strains) and *C. perfringens* showed little or no resistance to the antimicrobial agents under consideration (Table 4). Organisms with some resistance to three drugs included *C. ramosum, C. innocuum,* and *C. clostridioforme;* two strains of *C. orbiscindens* displayed resistance to ampicillin as well as linezolid.

Multiple studies have described resistance to various antibiotics among clostridia. Resistance to penicillin is especially common in *C. ramosum, C. clostridioforme,* and *C. butyricum* (64, 152); these species produce β-lactamases that are induced by β-lactam antibiotics (88). *C. tertium* has resistance features unusual among clostridia, including resistance to β-lactam antibiotics, metronidazole, and clindamycin. Resistance to clindamycin has been documented for strains of *C. perfringens* (as noted above), *C. ramosum, C. difficile, C. tertium, C. subterminale, C. butyricum, C. sporogenes,* and *C. innocuum* (187a).

Chloramphenicol, piperacillin, metronidazole, imipenem, and combinations of β-lactams with β-lactam inhibitors (e.g., ampicillin-sulbactam) were active against most clostridia (187a). The clostridia are variably resistant to cephalosporins and tetracyclines, and they are usually resistant to the aminoglycosides. Many clostridia other than *C. perfringens* (particularly *C. ramosum, C. clostridioforme,* and *C. innocuum*) are resistant to cefoxitin, cefotaxime, ceftazidime, ceftizoxime, cefoperazone, and other broad-spectrum β-lactams (7, 64, 152, 187a). Most strains of *C. innocuum* were only moderately susceptible to vancomycin (7). *C. innocuum* may be intrinsically resistant to this compound (141).

Earlier quinolones, such as ciprofloxacin, levofloxacin, and lomefloxacin, have demonstrated low or intermediate activity against anaerobes, but more recently introduced quinolones, like moxifloxacin, gatifloxacin, trovafloxacin, and garenoxacin, have good activity in vitro against most anaerobes, including clostridia (1, 125). A high frequency of moxifloxacin resistance has been described among *C. difficile*

TABLE 4 Activities of various drugs against *Clostridium* spp. (Wadsworth agar dilution procedure)[a]

Antimicrobial agent	CLSI MIC breakpoint (μg/ml)		% Susceptible[b]																	
	Susceptible	Intermediate	*C. bifermentans*	*C. bolteae*	*C. butyricum*	*C. cadaveris*	*C. clostridioforme*	*C. difficile*	*C. disporicum*	*C. glycolicum*	*C. hathewayi*	*C. innocuum*	*C. orbiscindens*	*C. paraputrificum*	*C. perfringens*	*C. ramosum*	*C. septicum*	*C. sordellii*	*C. sporogenes*	*C. subterminale*	*C. tertium*
Ampicillin[c]	0.5	1	100	67	86	100[d]	67	26	100	100		100	53	94	100	100	100[d]	100	100	100	100
Amoxicillin-clavulanate	4/2	8/4	100	66	100		75	100	100	100	100	100	100	100	100	100		100	100	100[d]	100[d]
Piperacillin-tazobactam	32/4	64/4	100[d]					100				100			100	100		100	100[d]		
Ticarcillin	32	64						100							100	100		100	100[d]		
Clindamycin	2	4	100	100	100	100[d]	90	56	100	92	93	83	100	87	100	82	100[d]	94	8	85	100[d]
Vancomycin	8	16	100	91	100	100[d]	100	100	100	100	100	98	100	100	100	100	100[d]	100	100	100	100
Imipenem	4	8	100[d]	100[d]	100[d]	100[d]		94				100		100[d]	100	100		100	100		
Linezolid	2	4	100	100	85		86	91	100	100	100	94		75	100	12		100	100	83	100[d]
Metronidazole	8	16	100	100	100	100	100	100	100	100	100	98	100	100	97	98	100[d]	95	100	100	100
Trimethoprim-sulfamethoxazole	32	64	0	67	50	0[d]	100	26	78	0		3	12	31	4	57	100[d]	0	0[d]	0	14
Trovafloxacin	2	4		0[d]	100[d]	100[d]	100[d]	86				100	100	100	8	86	100[d]	100	100		

[a] Clinical and Laboratory Standards Institute (CLSI) approved method M11-A6 (50a); data from Wadsworth Anaerobic Bacteriology Laboratory.
[b] According to the CLSI-approved breakpoints (M11-A6) (50a), the intermediate category is susceptible.
[c] Strains producing β-lactamase should be considered resistant.
[d] Five or fewer strains were tested.

isolates (3), and quinolone resistance is a problem among pathogens.

Severe *C. difficile*-associated intestinal disease is usually treated with oral vancomycin or metronidazole, but recently a number of metronidazole failures have been noted. Relapse of disease following antibiotic therapy occurs in about 30% of patients. Surgical therapy is required in the event of bowel perforation and toxic megacolon.

Patients diagnosed with food-borne or wound botulism should immediately receive trivalent (type ABE) antitoxin and intensive respiratory care (17, 46, 84). Early administration of antibodies can decrease the severity of the disease and slow its progression, resulting in a shorter hospital stay and more rapid recovery (16, 18, 84). Convalescence and recovery from botulism are generally prolonged, requiring weeks to months depending on the quantity of toxin exposure and serotype (serotype A has the longest duration, 1 to 6 months), but recovery is usually complete and patients regain normal function (16, 50, 84). Symptoms of fatigue, muscular weakness, and constipation have been reported to persist for months in certain patients (50).

Surgical measures are especially important in the treatment of gas gangrene and a number of *Clostridium* infections. The effect of clindamycin (and metronidazole) on toxin suppression by *C. perfringens* and other gram-positive organisms is an important reason to utilize such an agent in addition to penicillin or other β-lactam agents in the management of serious infections such as anaerobic myonecrosis (gas gangrene).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

The isolation of a *Clostridium* species from a clinical specimen, even a blood culture, may or may not be significant clinically, and culture results should be interpreted in relation to the patient's clinical findings. Clostridia of the patient's own intestinal microbiota may be present on the skin and may contaminate blood samples or other specimens. Bacteremia may be transient or clinically insignificant. In addition, most clostridia currently encountered in wounds, exudates, blood, and other normally sterile body fluids are opportunistic and may not cause serious or progressive disease unless conditions are suitable in the host. As discussed earlier in this chapter, one exception to this generalization is *C. septicum,* which rarely is encountered in blood cultures except from patients who have an underlying malignancy or neutropenic sepsis. *C. septicum* sepsis is an infectious disease emergency that requires prompt and clear communication between the laboratory and the clinician in order to institute early surgical measures and treatment with antimicrobial agents to improve outcomes. *C. tertium, C. perfringens,* and other clostridia, to a lesser extent, may be involved in serious infections that require emergency measures. The best approach for preventing tragic consequences that may be avoidable is good communication between microbiologists and clinicians.

The accurate and timely reporting of preliminary results (e.g., findings from direct microscopic examinations of clinical specimens), as well as early culture results (after 24 and 48 h of incubation), can be extremely valuable to the physician. For smaller laboratories without anaerobic chambers, incubation of the appropriate media in anaerobic jars provides acceptable recovery for most clinically significant anaerobes, assuming that optimal collection and transport of specimens are performed. The colony characteristics and microscopic features of some clostridia (e.g., *C. perfringens, C. sordellii,* and *C. sporogenes*) may be distinctive, so preliminary or presumptive reports may be released before aerotolerance studies are completed. Accurate, definitive identification is needed to better define the role of clostridia in disease, to aid the clinician in selecting optimal treatment, and for public health purposes (e.g., hospital-acquired *C. difficile* disease).

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Bacteroides, Porphyromonas, Prevotella, Fusobacterium, and Other Anaerobic Gram-Negative Rods*

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58

TAXONOMY

The anaerobic gram-negative bacteria are part of the normal flora of the mouth, upper respiratory tract, intestinal tract, and urogenital tract of humans and animals. Anaerobic spirochetes are covered in chapter 63, and *Campylobacter* spp. other than *Campylobacter rectus, Campylobacter curvus, Campylobacter gracilis,* and *Campylobacter showae* are discussed in chapter 59. The anaerobic gram-negative rods belong to the phyla *Bacteroidetes* and *Fusobacteria* and the families *Bacteroidaceae, Porphyromonadaceae*, *Prevotellaceae*, and *Fusobacteriaceae*. Species definition is based on biochemical characteristics, nucleic acid base composition, and homology (see chapter 19). *Veillonella* and the other anaerobic gram-negative cocci are covered in chapter 55. In the majority of clinical specimens, organisms of the genera *Bacteroides, Porphyromonas, Prevotella, Fusobacterium, Campylobacter*, *Sutterella,* and *Bilophila* are most often encountered (65).

The taxonomy of the gram-negative rods has been changing in recent years (14, 25, 64). A number of new genera and species have been named since the last edition of this book (Table 1). The methods used for taxonomic studies have been based mainly on nucleic acid analyses such as DNA-DNA hybridization and 16S rRNA and other gene sequencing (25). The latter classification approach, based on phylogenetic relatedness, does not necessarily correlate with phenotypic characteristics, such as Gram-staining properties, morphology, atmospheric growth requirements, and sporulation, concepts that were earlier considered cornerstones in the classification of anaerobes (68, 102). In fact, several species currently thought of as anaerobic gram-negative rods (*Butyrivibrio* spp., *Catonella morbi, Dialister pneumosintes, Fusobacterium* spp., *Leptotrichia* spp., *Johnsonella ignava, Tissierella* spp., *Mitsuokella multiacida, Selenomonas* spp., *Centipeda periodontii)* cluster within the *Clostridium* subphylum of the gram-positive bacteria (23, 60). Some of them are discussed in chapters 56 and 57.

The genus *Bacteroides* includes bile-resistant species that were formerly described as the "*Bacteroides fragilis*" group (including *Bacteroides eggerthii*) (78, 102, 125). However, the *B. fragilis* group organisms *Bacteroides distasonis, B. goldsteinii,* and *B. merdae* are related to *Tannerella forsythensis* (formerly *Bacteroides forsythus*) and cluster close to *Porphyromonas* on the basis of 16S rRNA sequencing (102, 103). A new genus, *Parabacteroides*, has been proposed to encompass these three species (M. Sakamoto, unpublished data). Similarly, *Bacteroides splanchnicus* falls far outside the group and probably represents a new genus (103). On the other hand, the bile-sensitive *Prevotella heparinolytica* and *Prevotella zoogleoformans* cluster among the *B. fragilis* group (102). Inclusion of these species warrants redefinition of the *B. fragilis* group. The taxonomic positions of other species still included in the genus *Bacteroides* remain uncertain, but all of these will ultimately be transferred to other genera. *Porphyromonas catoniae* is the only nonpigmented taxon in the genus *Porphyromonas* (144). Animal strains similar to *Porphyromonas gingivalis* are called *Porphyromonas gulae* (43). 16S rRNA sequencing results have identified two new closely related gram-negative species named *Alistipes finegoldii,* which is pigmented, bile resistant, and weakly saccharolytic, and *A. putredinis*, which is asaccharolytic and nonpigmented (111). The asaccharolytic, formate- and fumarate-requiring gram-negative rods *Bacteroides ureolyticus, C. gracilis* (formerly *B. gracilis*), *C. curvus, C. rectus* (formerly *Wolinella curva* and *W. recta*), and *Sutterella wadsworthensis* are actually microaerophiles. *Campylobacter hominis* is a truly anaerobic campylobacter from the human gastrointestinal tract (77). *Wolinella succinogenes,* isolated from the bovine rumen, is the only species remaining in the genus *Wolinella.* The taxonomic position of *B. ureolyticus* has remained uncertain; additional studies are required to determine whether these organisms should be transferred to the genus *Campylobacter* or if they represent a new genus (139). Sulfate-reducing *Desulfomonas pigra* was renamed *Desulfovibrio piger,* and the genus *Desulfomonas* is no longer valid (80).

DESCRIPTION OF THE GROUP

The true anaerobic gram-negative bacteria are differentiated from facultatively anaerobic bacteria by their inability to grow in the presence of oxygen and their susceptibility to metronidazole. Metronidazole resistance in these genera is rare and usually due to a laboratory error or an incorrect identification. Some of the species included here are indeed microaerophiles and are often resistant to metronidazole; the appropriate atmospheric requirements should be determined for all isolates. Cell morphology varies from short coccobacilli

*This chapter contains information presented in chapter 56 by Hannele R. Jousimies-Somer, Paula H. Summanen, Hannah Wexler, Sydney M. Finegold, Saheer E. Gharbia, and Haroun N. Shah in the eighth edition of this Manual.

TABLE 1 New anaerobic gram-negative rods named since the last edition of this Manual

New genus or species	Previous designation	Isolated from:	Reference
Alistipes putredinis	*Bacteroides putredinis*	Appendicitis specimens	111
Alistipes finegoldii	New	Appendicitis specimens	111
Bacteroides coprocola	*B. vulgatus-like*	Human feces	69
Bacteroides goldsteinii	*B. distasonis*-like	Human infections (intestinal origin)	126
Bacteroides nordii	*B. uniformis*-like	Human infections (intestinal origin)	127
Bacteroides plebeius	*B. vulgatus*-like	Human feces	69
Bacteroides salyersiae	*B. uniformis*-like	Human infections (intestinal origin)	127
Cetobacterium somerae	New	Children's feces	40
Desulfovibrio piger	*Desulfomonas pigra*	Human infections (intestinal origin)	80
Dialister micraerophilus	New	Human clinical samples	67
Dialister propionicifaciens	New	Human clinical samples	67
Faecalibacterium prausnitzii	*Fusobacterium prausnitzii*	Feces	32
Fusobacterium canifelinum	*F. nucleatum*-like	Oral cavity of cats and dogs; bite infections	24
Fusobacterium equinum	*F. necrophorum*-like	Oral cavity of horses	28
Porphyromonas gulae	*P. gingivalis*—animal strain	Gingival sulcus of animals	43
Porphyromonas somerae	*Porphyromonas levii*-like	Human skin, soft tissue, bone infections	130
Porphyromonas uenonis	*P. endodontalis*-nonoral	Human infections (nonoral)	41
Prevotella baroniae	New	Human oral cavity	30
Prevotella marshii	New	Human oral cavity	30
Prevotella multiformis	*P. denticola*-like	Human subgingival plaque	113
Prevotella multisaccharivorax	New	Human subgingival plaque	117
Prevotella salivae	*P. oris*-like	Human oral cavity	114
Prevotella shahii	*P. loeschii*-like	Human oral cavity	114
Sneathia sanguinegens	*Leptotrichia sanguinegens*	Human blood	22
Tannerella forsythensis	*Bacteroides forsythus*	Human periodontal pockets	115

to long, thin, pointed forms. The age of the culture, nutritional status, and exposure to air strongly affect morphology.

EPIDEMIOLOGY AND TRANSMISSION

Gram-negative anaerobic rods inhabit the mucosa of animals and humans. They are the predominant species in the oral cavity, the gastrointestinal tract, and the vaginal tract (in bacterial vaginosis; otherwise lactobacilli predominate). Most infections are acquired endogenously, when the integrity of the colonized mucosa or lumen is breached by trauma, disease (cancer), or iatrogenically (during surgery). Along the gastrointestinal tract, luminal populations appear to differ from adherent/mucosal strains. *B. vulgatus* and *B. thetaiotaomicron* are luminal and thus more commonly isolated from feces (100, 108). The predilection to adherence among *B. fragilis* may contribute to its predominance among the group as a pathogen, which, along with its relative aerotolerance, allows survival in hostile, oxygenated environments prior to establishment of the infection (112). Aspiration pneumonia, characterized by mixed anaerobes including gram-negative rods from the mouth, occurs during lapses of consciousness or other sedation events. Exceptions to endogenous acquisition of infection include clenched-fist wounds and animal and human bite wounds, which occur when the oral contents of an animal or person are implanted traumatically through the skin into the underlying tissue (131, 132). These infections invariably involve mixed flora, often with aerobic organisms. It is possible that *B. fragilis* is associated with diarrhea in children and that some toxigenic *B. fragilis* group may be transmitted from livestock, but that has not been proven (104).

CLINICAL SIGNIFICANCE

Gram-negative anaerobic rods predominate among anaerobes in clinical infections; they are found in more than one-half of the specimens yielding anaerobes (37, 65). Among the anaerobes encountered in clinical specimens, members of the bile-resistant *B. fragilis* group are the most commonly encountered and are more virulent and resistant to antimicrobial agents than most other anaerobes (91). *B. fragilis* and *Bacteroides thetaiotaomicron* are of the greatest clinical significance (44). Members of the *B. fragilis* group are major constituents of the normal colonic flora but are not common in the genital tract, mouth, or upper respiratory tract (108). These species are often found among the mixed microbiota of abscesses in tissue adjacent to their normal mucosal environments (intra-abdominal abscess or female genital tract abscess, for example). Some *B. fragilis* strains produce a potent zinc-dependent metalloprotease or enterotoxin with a variety of pathological effects on intestinal mucosal cells (119). The enterotoxin induces interleukin-8 production, which probably accounts for the inflammatory damage seen in *B. fragilis* diarrhea (145). *B. fragilis* also possesses lipopolysaccharides (LPS) and capsular polysaccharides of varying structure, antigenicity, and biological activity, which appear to play a role in periodontal disease and abscess formation (137, 141).

The pigmented anaerobic gram-negative rods are composed of saccharolytic and asaccharolytic species of the genera *Prevotella, Porphyromonas,* and *Alistipes finegoldii*. Several species of these genera are found in human clinical specimens. *Prevotella corporis, Prevotella denticola, Prevotella intermedia, Prevotella loescheii, Prevotella melaninogenica, Prevotella nigrescens, Prevotella pallens, Prevotella tannerae, Porphyromonas endodontalis, P. gingivalis,* and the nonpigmented species *P. catoniae* are found in the human oral cavity (116). Some are important pathogens in oral, dental, and bite infections and may produce infections of the head, neck, and lower respiratory tract. *P. gingivalis* and *Tannerella forsythensis* are the putative key pathogens (with *Treponema denticola*) in aggressive forms of adult periodontitis and, together with *P. endodontalis,*

are often involved in root canal infections and complications of these infections such as odontogenic sinusitis (56, 115). Some of the above-named pigmented organisms and *Porphyromonas asaccharolytica* and *P. uenonis* are also prevalent in the urogenital or intestinal tracts and are important in infections arising from these sources (25, 41, 111). In addition to *P. uenonis* and the pigmented bile-resistant organisms mentioned above, *P. gingivalis* has also been isolated from extraoral sources, especially from patients with appendicitis (93, 138). *Porphyromonas* spp. of animal origin have been encountered in humans with animal bite infections (19, 45, 57). *Porphyromonas somerae* has been isolated from various types of human clinical infections including pleuropulmonary infections, skin and soft tissue infections, and bacterial vaginosis (130).

The bile-sensitive, nonpigmented, saccharolytic *Prevotella* and related strains recovered from human samples are found in the same settings as the pigmented gram-negative rods (38). *Prevotella bivia* and *Prevotella disiens* are found in female patients with genital tract infections, in patients with soft tissue infections, and less frequently in oral infections (4); these strains are often resistant to the β-lactam antibiotics, including penicillin, aminopenicillins, and cephalosporins (140). *Prevotella oris* and *Prevotella buccae* are found in a variety of oral, pleuropulmonary, and other infections (21, 38, 65). Members of the *P. oralis* group are relatively infrequently encountered in routine human clinical samples but common in oral and dental infections (30, 114).

P. zoogleoformans (indole negative) is rarely isolated from human clinical specimens, whereas *P. heparinolytica* (indole positive) is often found in the oral cavity of humans and animals and in association with oral infections. *Prevotella dentalis* (formerly *Mitsuokella dentalis*) is a common isolate from infected root canals, periodontal pockets, mandibular and gum abscesses, and in sialadenitis (128). *M. multiacida,* a nonmotile gram-negative anaerobic rod, sometimes isolated from rumen and the mammalian intestinal tract, is not related to *P. dentalis* but is most closely related to *Selenomonas* species, which in turn seem to belong to cluster IX of the *Clostridium* subphylum of the gram-positive bacteria (23). The nonpigmented, saccharolytic organism *Megamonas hypermegas* has not been isolated from human clinical specimens.

The nonpigmented, asaccharolytic, or weakly fermentative species *Anaerorhabdus furcosus, Bacteroides capillosus, Bacteroides coagulans, Dialister pneumosintes, Alistipes putredinis, Desulfovibrio* spp., and *Tissierella praeacuta* inhabit the intestinal tract and have occasionally been recovered from miscellaneous infections (37, 38, 48). *Bacteroides tectus* and the phenotypically similar *Bacteroides pyogenes* are often associated with canine or feline oral flora and are prevalent in infections in humans who have sustained animal bites (1, 38, 132). *Bilophila wadsworthia,* a bile-resistant organism, is present in small numbers in the bowel flora of healthy persons, yet it is the third most common anaerobe recovered from gangrenous or perforated appendixes and is a common constituent of the microbiota in other intra-abdominal infections (5, 7, 11). It has also been isolated from various other clinical specimens including blood, brain abscess, liver abscess, pericardial fluid, joint fluid, and pleural fluid as well as from human feces and vaginal and oral secretions (6, 36). *B. wadsworthia* is easily overlooked in cultures owing to its fastidious growth.

Members of the formate-fumarate-requiring *B. ureolyticus-Campylobacter* group, including *B. ureolyticus, C. gracilis, C. curvus, C. rectus,* and *S. wadsworthensis,* have been isolated from various types of infections (122). *B. ureolyticus* has been recovered from pulmonary, head and neck, intra-abdominal, urogenital, bone, and soft tissue infections. *C. gracilis* has been recognized as an important pathogen in serious visceral or head and neck infections and, in general, in infections above the diaphragm, and *S. wadsworthensis* is an important pathogen in infections below the diaphragm, especially abdominal infections (94, 143). *Campylobacter* spp. are primarily oral isolates found in patients with oral infections and periodontitis; they occasionally enter the bloodstream in immunocompromised patients. *C. rectus* has been implicated as a putative pathogen at sites of active periodontal breakdown (58, 110, 122). Many species of oral anaerobes have recently been associated with atherosclerosis, although a causal relationship has not been proved (59, 118, 124).

Among *Fusobacterium* species, *F. nucleatum* is most commonly encountered in clinical infections (13, 65). This organism is found in the mouth and in the genital, gastrointestinal, and upper respiratory tracts. It is often involved in the same types of infections as the pigmented *Prevotella* spp. and *Porphyromonas* spp. *F. nucleatum* may be found as the sole infecting agent in pleuropulmonary infections (21). *Fusobacterium necrophorum* is a very virulent anaerobe that may cause severe infection, usually in children or young adults, originating from pharyngotonsillitis, sometimes in association with infectious mononucleosis (70). It was the most common anaerobe isolated from peritonsillar abscesses in young adults and may in fact be an etiological agent of pharyngitis as often as *Streptococcus pyogenes* (2, 10, 63). Complications include Lemierre's disease, an acute jugular vein septic thrombophlebitis often complicated by sepsis and metastatic abscesses most frequently in the lungs, pleural space, liver, and large joints (63, 70). *F. necrophorum* is now encountered in serious infections less often than in the pre-antimicrobial agent era, but this makes it more treacherous, because many clinicians may not be familiar with the syndrome (74). There are two subspecies; *F. necrophorum* subsp. *necrophorum* contains lipase-positive, hemagglutinin-producing biovar A, and *F. necrophorum* subsp. *funduliforme* contains lipase-negative, non-hemagglutinin-producing biovar B. *Fusobacterium mortiferum* and *Fusobacterium varium* are encountered mainly in patients with intra-abdominal infections. *Fusobacterium russii* and *F. canifelinum* have been implicated in animal bite infections (24, 38), and *Fusobacterium ulcerans* is found in tropical ulcer. Other fusobacteria are isolated from clinical specimens rarely, usually oropharyngeal or genital sites. The former *F. prausnitzii,* abundant in the human feces, is not a fusobacterium but, rather, is related to the *Clostridium* spp. and has been renamed *Faecalibacterium prausnitzii*. Its pathogenic potential is unknown (26, 32). Other former gram-negative anaerobes closely related to clostridia include *Butyrivibrio fibrisolvens* and *B. hungatei* (from rumen) and *Catonella morbi* and *Johnsonella ignava* (from human gingival crevice). Routine clinical laboratories are unlikely to encounter these organisms.

Capnocytophaga spp. of human origin are often isolated only in anaerobic culture, and they are common in the oral cavity. *Leptotrichia buccalis* is a common mouth organism and may be found in the vagina and intestinal tract. Isolation of *Capnocytophaga* spp. or *Leptotrichia* spp. from blood cultures is often linked to patients (often adolescents) with hematological malignancies and is a direct clue to the presence of oral mucosal lesions in the patient (3, 38, 92). *Sneathia sanguinegens* has been isolated from cultures of blood from pregnant and elderly women and neonates (53).

Selenomonas sputigena, Selenomonas artemidis, Selenomonas dianae, Selenomonas flueggei, Selenomonas infelix, and *Selenomonas noxia* are all oral organisms, as is *Centipeda*

periodontii, which closely resembles and phylogenetically clusters with *Selenomonas* in the *Clostridium* subphylum of the gram-positive bacteria (23, 133); these organisms are found in subgingival sites in patients with periodontitis (16, 75, 133).

The motile organisms *Succinivibrio dextrinosolvens, Butyrivibrio fibrisolvens,* and *Desulfovibrio* spp. including the nonmotile species *D. piger* are found as members of the normal colonic flora but may occasionally be encountered in patients with clinical infections, such as appendicitis (48). *Desulfovibrio* has been recovered in blood cultures from an immunocompromised patient, an immunocompetent patient, and from a pyogenic liver abscess in another patient (48, 80). The sites of normal carriage of *Anaerobiospirillum succiniciproducens* and *Anaerobiospirillum thomasii* in humans are unknown, but *Anaerobiospirillum* species are common in the fecal flora of cats and dogs (82). Strains of *Anaerobiospirillum* have been isolated from cultures of blood from compromised patients and from fecal specimens of patients with diarrhea (82, 83, 89, 134). In the latter case, a zoonotic role for *Anaerobiospirillum* spp. has been proposed.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

General guidelines for collection, transport, and storage of specimens are discussed in chapters 5 and 20. Sites normally harboring a rich indigenous flora, such as the intestinal tract or vagina, should not be sampled and cultured for anaerobes except under special circumstances and by using special methods (e.g., quantitative study of upper small bowel flora in patients with the blind loop syndrome). Lower respiratory tract specimens and endometrial samples are especially difficult to obtain without contaminating the sample with indigenous flora. Double-lumen-catheter bronchial brushings and bronchoalveolar lavage fluid transported immediately to the laboratory under anaerobic conditions and cultured quantitatively, as well as pleural fluid, represent good respiratory tract specimens. An endometrial suction curette (Pipelle; Unimar, Wilton, Conn.) biopsy provides a good sample from the endometrium (39). Instructions for collection of specimens from different body sites and by various methods are given in more detail elsewhere (14, 39, 65).

Ulcers should be carefully debrided, and proper tissue samples should be collected from the base or progressive edge, where bacteria actively multiply, rather than from unremoved crust or surface pus, which is often contaminated by other bacteria not reflecting the true infecting flora.

Pus, when present, is best aspirated into a syringe through a needle and injected into an anaerobic transport vial containing an oxidation-reduction indicator. Syringes used for aspiration should not be used as transporters because of the potential danger of needlestick injuries or accidental expulsion and because oxygen diffuses through plastic syringes. Pieces of infected tissue obtained by excision or biopsy are always preferable to pus.

Specimens obtained with a swab are the least desirable and should be discouraged unless special circumstances exist. Swabs are intrinsically aerobic and prone to drying if not in anaerobic transport vials, often carry a volume of sample too small to be cultured on several media or quantitatively, and leave some of the specimen in the transport medium. However, swabs are used in many institutions. One study showed that aerobic and anaerobic organisms in purulent specimens survived for up to 48 h in anaerobic swab transport systems (20). Tissue samples are best transported in anaerobic transport vials or in loosely capped containers sealed in gas-impermeable bags in which an anaerobic atmosphere was generated. For small tissue and biopsy specimens and for subgingival and root canal samples, a semisolid anaerobic transport medium in which the specimen can be submerged may be used (Dental Transport Medium; Anaerobe Systems, Morgan Hill, Calif.). The dental specimens can be collected with a curette or with the use of paper points after careful removal of supragingival plaque (27, 65). The material from curettes or the paper points are transported in a transport medium, such as VMGA III (Viability Medium, Göteborg, anaerobically prepared and sterilized), containing glass beads to facilitate dispersion of aggregates in plaque (27). In the case of failed peri-implants, the whole fixture is placed in VMGA III (27, 65).

The conditions and time of transport should not affect the viability or relative proportions of bacteria present in the specimen if appropriate transport systems are used (8). Rapid transport is important when Gram stain and culture results are needed early for guidance of therapy. Contrary to previous recommendations, specimens in glass may be transported at room temperature or refrigerated (not frozen), as the organisms, especially facultative fastidious species, seem to survive at lower temperatures in newer transport systems (31, 136). More detailed information on transport systems and anaerobic techniques can be found elsewhere (8, 14, 65).

DIRECT EXAMINATION

The gross appearance (purulence, necrotic tissue), fluorescence of the sample under long-wave (366-nm) UV light, and the odor of the specimen can give the laboratory valuable clues to the presence of anaerobes. A fetid or putrid odor due to volatile short-chain fatty acids and amines is always associated with the presence of anaerobes in the sample. Black necrotic tissue and/or red fluorescence of the sample may be indicative of the presence of pigmented gram-negative rods.

Microscopy

The Gram stain is still the fastest, simplest, and the most likely to yield significant information among rapid methods and should be prepared from all specimens accepted for anaerobic culture. The morphotypes and relative quantities of both the host and the bacterial cells present in the preparation should be reported. Furthermore, the Gram stain information also provides quality control for specimen transport and isolation efficiency (39). Thick films from exudates and bloody fluids should be spread thin as for a blood smear. Smears should be fixed in methanol for 30 s to preserve the host and bacterial cell morphologies (87). Standard Gram stain procedures and reagents are used, except that 0.1 to 0.5% basic fuchsin, which enhances the staining of gram-negative anaerobes, is substituted for safranin as the counterstain (65, 87). Dark-field and phase-contrast microscopy may be helpful in the detection of small, poorly staining organisms *(D. pneumosintes),* for the direct observation of motility (*Campylobacter* spp.), for the notation of spores (*Clostridium* spp.), and for the recognition of morphotypes not cultivable on ordinary media (spirochetes). Gram stain using Nugent criteria for interpretation of vaginal discharge is still considered the best method for diagnosis of bacterial vaginosis (76, 120). Chapter 20 outlines this procedure.

Antigen or Product Detection

Although a direct fluorescent antibody for *B. fragilis* has been reported, it is not in routine use (105). Gas-liquid chromatography (GLC) of specimens has been proposed, but

it has not gained acceptance. Newer methods for identification of volatile products in specimens, such as the electronic nose, are in developmental stages (54, 106, 135).

Molecular methods, such as nucleic acid probe hybridization, PCR amplification, and direct demonstration of nucleic acid sequences by 16S rRNA gene sequencing, are not used for routine diagnostic studies but are widely used in specialized oral and other research microbiology laboratories (16, 116). Eventually, molecular methods will become more commonplace (see chapter 16) (2).

ISOLATION PROCEDURES

The use of selective media along with nonselective media increases the yield and saves time in terms of recognition and isolation of colonies. Freshly prepared or prereduced and anaerobically sterilized media (Anaerobe Systems) should be used (88, 107). Different basal media differ in their abilities to support the growth of anaerobes; brucella base is superior to Trypticase soy (CDC base agar) and Schaedler base for isolation of gram-negative rods, but CDC base better supports the growth of anaerobic gram-positive cocci. Brain heart infusion base is superior to Trypticase soy in isolation efficiency for *Eubacterium* species but is inferior for pigmented gram-negative rods from subgingival and other samples (65). Fastidious anaerobe agar (Lab M, Bury, England) produces luxuriant growth of fusobacteria and some formate-fumarate-requiring species and can be used as a basal medium with or without selective agents. In academic centers performing large-scale anaerobic bacteriology, it would be ideal to use two different basal media to maximize isolation efficiency (39, 86) (chapter 21).

Isolation methods have been published elsewhere (15, 65, 86) (chapter 20). The minimum medium setup includes (i) a nonselective, enriched, brucella base sheep blood agar plate supplemented with vitamin K_1 and hemin (BAP); (ii) a kanamycin-vancomycin laked sheep blood agar (KVLB) for the selection of *Bacteroides* and *Prevotella* spp. (KVLB allows growth and rapid pigmentation of most *Prevotella* spp., but the concentration of vancomycin [7.5 μg/ml] inhibits most *Porphyromonas* spp.; for the isolation of *Porphyromonas* spp., KVLB medium with a reduced vancomycin concentration [2 μg/ml] may be prepared); and (iii) a *Bacteroides* bile-esculin agar plate (BBE) for specimens from areas below the diaphragm for the selection and presumptive identification of the *B. fragilis* group and *Bilophila* sp. BBE and KVLB are also available as biplates. When indicated, a phenylethyl alcohol-sheep blood agar plate, used to prevent overgrowth by aerobic gram-negative rods and swarming of some clostridia, may be inoculated. When fusobacteria are clinically suspected as the cause of infection, *Fusobacterium* neomycin-vancomycin agar or *Fusobacterium* selective agar may be used for isolation (65). A selective medium for culturing *Anaerobiospirillum* spp. from fecal specimens has been described (83). Use of a metronidazole disk on nonselective agar may help detect strict anaerobes, as resistant colonies would suggest facultative anaerobes or gram-positive anaerobes which are often resistant. After inoculation, the anaerobic plates should immediately be incubated at 36°C in an anaerobic environment, such as an anaerobic bag, jar, or chamber. Alternatively, setup and incubation may all be done in an anaerobic chamber. Plates should not be exposed to air during the first 48 h to avoid death of the more oxygen-sensitive species. In routine clinical microbiology, a total incubation period of at least 5 days for primary plates is recommended. In our experience with shorter incubation times, some anaerobic species, such as *Porphyromonas* and *Bilophila* spp., may not be detected.

Use of anaerobic blood culture media has been controversial, as strict anaerobes now comprise <5% of most clinically relevant blood culture isolates (98, 99). At the least, blood cultures from patients with abdominal or gynecological processes, peritoneal abscess, dirty wound, decubitus ulcers, osteomyelitis, oropharyngeal disease, and elderly patients should be cultured in an anaerobic bottle (9). However, since many facultative organisms may be recovered faster from the anaerobic bottle, both aerobic and anaerobic media are recommended for at least one blood culture set.

IDENTIFICATION

Colonies should be examined with a dissecting microscope to facilitate detection. Use a broken sterile wooden stick point to obtain material from each different colony type. Use this single colony sample to inoculate (i) a brucella BAP to which special-potency antibiotic disks (colistin, 10 μg; kanamycin, 1,000 μg; and vancomycin, 5 μg) are applied (a nitrate disk may also be added) (Fig. 1); (ii) a chocolate agar plate that is incubated in 5 to 10% CO_2 (can be a pie plate) for aerotolerance testing and detection of *Haemophilus* and capnophilic organisms; and finally (iii) an area on a slide for Gram staining. A blood agar pie plate is optional, but it is useful for seeing β-hemolysis of microaerophilic streptococci. A laked rabbit blood agar plate (LRBA) for the rapid demonstration of pigment production (laked rabbit blood agar is the most reliable and effective medium) (57, 65) and an egg yolk agar plate (EYA) for the demonstration of lipase, lecithinase, and proteolytic activities may also be inoculated at this point. The primary plates are reincubated along with the purity and test plates.

Preliminary Examination of Isolates

The characteristics that are noted from BAP include detailed Gram stain and colony morphology, pigment, fluorescence (long-wave UV light) (the results of the last two tests are also recorded from an LRBA), hemolysis, and greening or pitting of the agar. Furthermore, the spot indole reaction (*para*-dimethylaminocinnamaldehyde reagent), the nitrate reduction test, tests for sensitivities to special-potency antibiotic disks (Fig. 1), and catalase (15% H_2O_2) are performed. Most commonly isolated anaerobes are nonmotile, but in case of an unusual strain, motility tests from a broth culture or plate (hanging drop or wet mount) may be performed. Motile isolates are most likely to be *Campylobacter* spp. Instead of spot tests, Lombard-Dowell quadrant plates I, II, and III (Remel, Lenexa, Kans.) developed at the Centers for Disease Control and Prevention can be used to test for kanamycin resistance, indole, esculin, bile, gelatinase, lecithinase, lipase, and several carbohydrate fermentation reactions (35). Good growth is necessary for the proper interpretation of test results on this medium.

The primary plates are reinspected after 4 or more days to detect slow growers, new morphotypes, or late pigmenters. In oral microbiology laboratories, two rapid in situ tests have been used. The rapid differentiation of lactose-fermenting species from lactose-nonfermenting species is determined by applying 4-methylumbelliferyl-D-galactoside reagent (catalog no. M-1633; Sigma Chemical Co., St. Louis, Mo.) to the colonies and screening for fluorescent (lactose-positive) colonies under long-wave UV light. The carboxy-L-arginine-7-amino-4-methylcoumarin amide HCl (CAAM) test demonstrates the trypsin-like activities of suspected colonies of *P. gingivalis;* the colonies are treated with the CAAM reagent (catalog no. C-9396; Sigma) and screened for blue-white fluorescence under long-wave UV light (123).

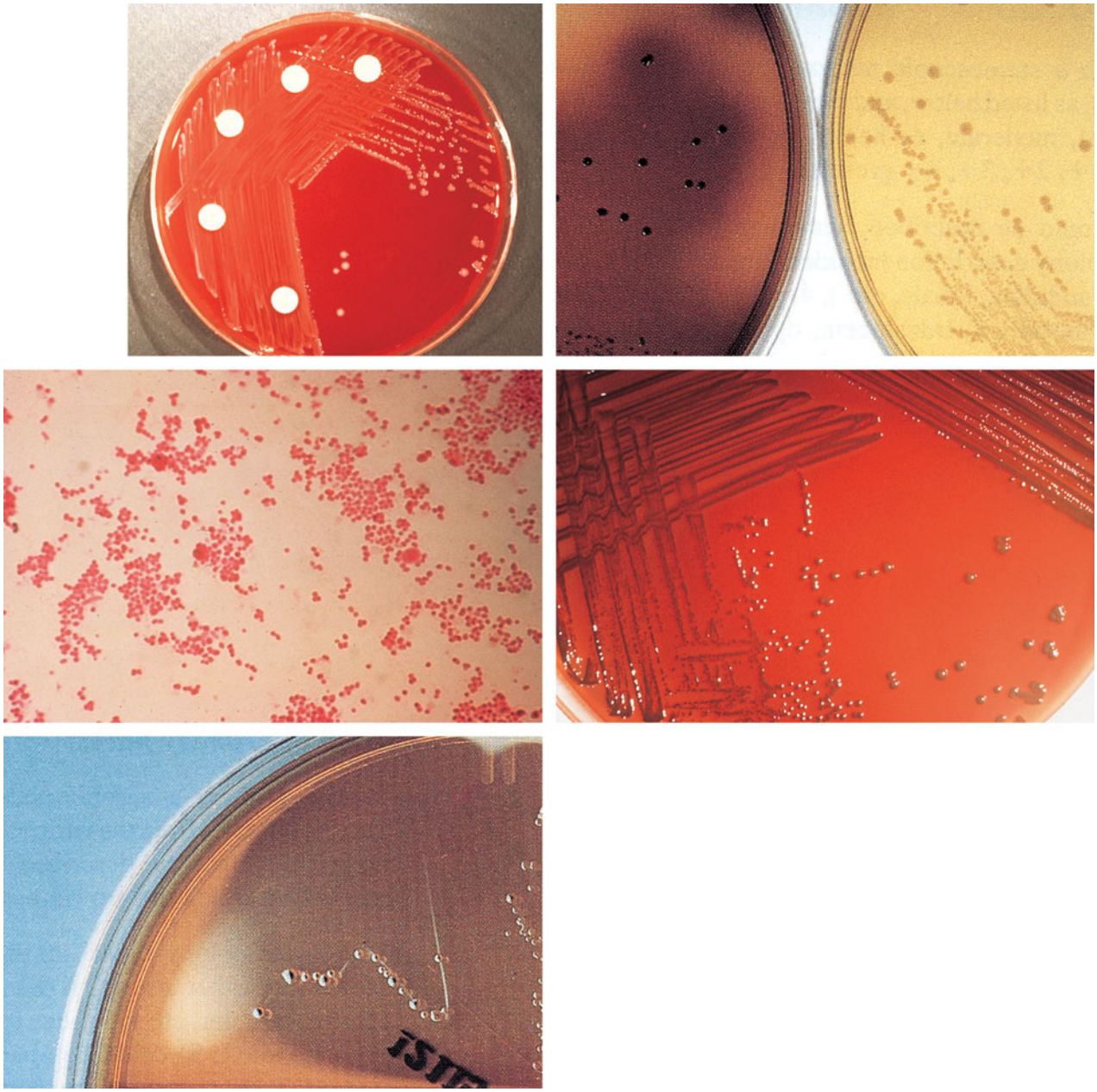


FIGURE 1 (top left) Placement of special-potency antibiotic disks. A blank disk for indole testing may be added (usually after growth has occurred).

FIGURE 2 (top right) Colonies of *Bacteroides fragilis* (left) and *Bacteroides vulgatus* (right) on BBE. Note the blackening of the agar and colonies due to esculin hydrolysis and bile precipitation.

FIGURE 3 (middle left) Coccobacillary cells of *Prevotella melaninogenica.*

FIGURE 4 (middle right) Pigmented colonies of *Porphyromonas* species.

FIGURE 5 (bottom) Colony morphology of *Bilophila wadsworthia* on BBE. Note the black centers due to H_2S production.

Presumptive Identification of Species

Most of the clinically significant gram-negative rods can be placed into broad groups with relatively few tests, and some isolates, including *Bilophila wadsworthia* and *Fusobacterium nucleatum*, can be presumptively identified with ease (Table 2) (101). The special-potency antibiotic disk pattern can be used to separate the gram-negative rods into several groups. A zone size equal to or greater than 10 mm is considered sensitive (65).

Most of the *Bacteroides* and *Prevotella* spp. are resistant to vancomycin and kanamycin and are variable in sensitivity to colistin; *Porphyromonas* spp. are generally sensitive to vancomycin and resistant to colistin. The *B. fragilis* group can be

identified presumptively by their resistance to all three special-potency antibiotic disks and by growth equal to or greater than the control growth in 20% bile, as determined by a tube test, by a bile disk test (65), or on BBE (Fig. 2). Coccobacillary organisms that fluoresce red or produce black colonies are in the pigmented *Prevotella* spp.-*Porphyromonas* spp. group (Fig. 3 and 4).

Fusobacterium spp., *B. ureolyticus, Campylobacter* spp., *Bilophila* sp., *Sutterella* sp., and *Leptotrichia* spp. are resistant to vancomycin but sensitive to both colistin and kanamycin. *B. ureolyticus, Campylobacter, Bilophila,* and *Sutterella* colonies are usually much smaller and more translucent than those of fusobacteria and typically reduce nitrate; *Leptotrichia* colonies are large and gray and have a convoluted ("brain surface") texture. Furthermore, the most commonly encountered fusobacteria, *F. nucleatum* and *F. necrophorum,* are indole positive and nitrate negative. On microscopic inspection, fusobacteria are usually larger than the other bacteria with the same identification disk profile, excluding the large tapered cells of *Leptotrichia.* An anaerobic, gram-negative, catalase-negative rod that requires formate and fumarate for growth in broth culture or that pits agar may be presumptively identified as *B. ureolyticus* or a *Campylobacter* sp.; *B. ureolyticus* is urease positive (Table 2, footnote *d*). To document the formate-fumarate requirement, inoculate one tube of peptone yeast (or thioglycolate) broth medium containing the additive (0.3%) and one tube not containing the additive (control) (Table 2, footnote *b*). Compare the two tubes for intensity of growth. A strongly catalase-positive, bile-resistant, often urease-positive gram-negative rod can be presumptively identified as *Bilophila* sp. (Fig. 5). Microaerophilic and catalase- and urease-negative but bile-resistant (by the disk test) organisms growing in *Campylobacter* atmosphere (2 to 6% oxygen) are probably *S. wadsworthensis*.

Desulfovibrio piger (nonmotile), other *Desulfovibrio* spp. (motile), and the capnophilic, often yellow-pigmented *Capnocytophaga* spp. are resistant to vancomycin and colistin but sensitive to kanamycin (142). *Desulfovibrio* cells are curved or spiral; *Capnocytophaga* cells resemble fusobacteria with tapered ends. *Selenomonas* spp. may have the same special-potency disk patterns as *Desulfovibrio* and *Capnocytophaga* spp., but a few strains are sensitive to colistin. *Selenomonas* cells are curved and motile, as are those of *C. periodontii,* a closely related species that forms swarming colonies.

Most organisms not fitting the above-described groupings are *Bacteroides* spp. or *Prevotella* spp. but occasionally are representatives of the other genera listed in Table 1.

Rapid Identification

Whenever possible, simple tests should be used for the rapid and, to the extent that they permit, definitive identification of anaerobes (65, 85, 101). These include tests for colony and microscopic morphologies; the spot indole, catalase, lipase, and lecithinase tests; the nitrate disk test; the bile disk test; growth stimulation tests; and tests for sensitivities to special-potency antibiotic disks. Furthermore, rapid tests based on the presence of preformed enzymes produce clinically meaningful results in a timely fashion (see "Other Approaches to Identification"). See Tables 2 to 7, which incorporate the results of these tests; they can be used for the rapid identification of many commonly encountered anaerobic gram-negative rods.

Identification of the most commonly encountered bile-resistant members of the *B. fragilis* group is based on the special-potency disk profile and the results of a few tests that can be performed rapidly, including the catalase, indole, esculin, and α-fucosidase tests (Table 3, footnote *b*). Based on these reactions, the members of this group can be reported as *B. fragilis* group, most closely related to *B. fragilis,* or other species in this group (Tables 2 and 3). Further tests are performed when indicated. *Porphyromonas* spp. are easily identified even to the species level with the aid of the special-potency disk profile and the results of a few tests including indole, catalase, lipase, and some rapid enzyme tests (see Tables 2 and 6) (57, 66, 85, 138). *Porphyromonas asaccharolytica* may be confused with some *Prevotella* species without the vancomycin special-potency disk results (*Prevotella* spp. are resistant). An indole- and lipase-positive coccobacillus that forms black-pigmented colonies or that fluoresces red may be identified as belonging to *Prevotella intermedia/P. nigrescens* group; *P. pallens* resembles these two species but has lighter pigment and is lipase negative (71, 72). A rapid enzyme test for α-glucosidase (with Rosco Diagnostic Tablets [Rosco Diagnostics, Taastrup, Denmark] or the WEE-Tabs system [Key Scientific Products, Round Rock, Tex.]) is helpful; *P. intermedia/P. nigrescens* and *P. pallens* are positive, and *P. asaccharolytica* is negative (57). Furthermore, *Porphyromonas asaccharolytica* is sensitive to the special-potency vancomycin disk. Any indole-negative strains must be identified further by other biochemical tests. A lipase-positive, indole-negative, pigmented gram-negative rod could be *P. loescheii*.

B. ureolyticus, C. gracilis, other "anaerobic" *Campylobacter* spp., and *Sutterella* sp. are thin gram-negative rods with rounded ends that produce the *Fusobacterium* disk pattern. The colonies are small and translucent or transparent and may produce greening of the agar. Three colony morphotypes exist: smooth and convex, pitting (Fig. 6), and spreading. All colony types can occur in the same culture. These organisms are asaccharolytic and nitrate reducing, and they require supplementation of broth media with formate and fumarate for growth (Table 2, footnote *b*). *C. rectus, C. curvus, C. concisus,* and *C. showae* are motile and oxidase positive; unlike the first three, *C. showae* is catalase positive. *C. gracilis* is nonmotile and urease negative and, thus, is differentiated from urease-positive *B. ureolyticus* (Table 2, footnotes *d* and *e*). *Bilophila* sp., which phenotypically resembles *B. ureolyticus,* is distinguished from the above-mentioned species by its resistance to bile and its strong catalase reaction. *Sutterella* sp. is also resistant to bile but is urease and catalase negative (7, 143).

F. nucleatum is a thin rod with tapering ends (Fig. 7) and is indole positive. The needle-shaped morphology is shared with the microaerophilic, indole-negative *Capnocytophaga* spp. and *Leptotrichia* spp., as discussed above. *F. nucleatum* fluoresces chartreuse under UV light and often produces greening of the agar after exposure to air due to H_2O_2 production. At least three different colony morphotypes of *F. nucleatum* exist (13, 14, 34, 68). Due to considerable phenotypic and genotypic heterogeneity and uncertainty of valid criteria for the separation of the *F. nucleatum* subspecies, it is hard to judge whether the reported colonial morphologies consistently coincide with the subspecies designations. *F. nucleatum* subsp. *nucleatum* colonies may be small, grayish white, and smooth; *F. nucleatum* subsp. *fusiforme* colonies may also be small (<0.5 to 1 mm), granular, and irregular (bread crumb shaped) (Fig. 8); and *F. nucleatum* subsp. *polymorphum* colonies often are large, speckled, smooth, translucent, and butyrous. *F. necrophorum* subsp. *necrophorum* is lipase positive and usually bile sensitive. It is a pleomorphic long rod with round ends and often has bizarre forms, especially in older

TABLE 2 Group identification of anaerobic gram-negative organisms[a]

Group or organism	Vancomycin (5 μg)	Kanamycin (1,000 μg)	Colistin (10 μg)	Growth in 20% bile	Catalase	Indole	Growth stimulated by formate-fumarate[b]
B. fragilis group	R	R	R	+	V	V	
Other *Bacteroides* spp.	R	R	V	–$^{+}$	–$^{+}$	V	
Pigmented species	V	R	V	–	–$^{+}$	V	
Porphyromonas spp.	S	R	R	–	V	+$^{-}$	
Prevotella spp.	R	R^{S}	V	–	–	V	
P. intermedia-P. nigrescens-P. pallens	R	R^{S}	S	–	–	+	
P. loescheii	R	R	V	–	–	–	
Other *Prevotella* spp.	R	R	V	–	–$^{+}$	–$^{+}$	
Campylobacter spp./*B. ureolyticus*	R	S	S	–	–$^{+}$	–	+
B. ureolyticus	R	S	S	–	–	–	+
Campylobacter spp.[g]	R	S	S	–	–	–	+
C. *gracilis*	R	S	S	–	–	–	+
Sutterella sp.	R	S	S	+	–	–	+
Bilophila sp.[h]	R	S	S	+	+	–	–
Desulfovibrio spp.[i]	R	S	R	V	V	–$^{+}$	–
Fusobacterium spp.	R	S	S	V	–	V	
F. nucleatum	R	S	S	–	–	+	
F. necrophorum	R	S	S	–$^{+}$	–	+	
F. varium/F. mortiferum	R	S	S	+	–	V	
Anaerobiospirillum spp.	R	S	S	–	–	–	
Capnocytophaga spp.	R	S	R	–	–	–	
Centipeda periodontii	R	S	R	–	–	–	
Leptotrichia/Sneathia	R	S	S	+$^{-}$	–	–	
Selenomonas spp.	R	S	R	–	–	–	
Dialister pneumosintes	R	S	R^{S}	–	–	–	
Veillonella spp. (cocci)	R	S	S	–	V	–	

[a] R, resistant; S, susceptible; R^{S}, most strains resistant, some strains susceptible; V, variable; +, positive reaction for majority of strains; −, negative reaction; +$^{-}$, most strains positive but some strains negative; −$^{+}$, most strains negative but some strains positive.

[b] Compare the growth of the organism in an unsupplemented thioglycolate broth with growth in a broth supplemented with formate and fumarate additive: dissolve 3 g of sodium formate, 3 g of fumaric acid, and 20 pellets of sodium hydroxide in 50 ml of distilled water; adjust the pH to 7; and filter sterilize. Add 0.5 ml of additive to 10 ml of culture broth (55, 65).

[c] Use the nitrate disk test (65).

[d] Make a heavy suspension of the organism in 0.5 ml of sterile urea broth (BBL; BD, Franklin Lakes, N.J.) or in sterile water and insert a urea tablet (Rosco Diagnostic Tablets, A/S Rosco, Taastrup, Denmark) or WEE-Tabs (Key Scientific Products; Round Rock, Tex.). Incubate the tubes aerobically for up to 24 h. A bright pink or red is positive; this color usually appears within 15 to 30 min.

TABLE 3 Characteristics of *B. fragilis* group and *B. splanchnicus*[a]

Species	Growth on BBE	Indole	Catalase	Esculin hydrolysis	α-Fucosidase[b]
B. caccae[d]	+	–	–$^{+}$	+	+
B. distasonis[e]	+	–	+$^{-}$	+	–
B. eggerthii	+	+	–	+	–
B. fragilis[d]	+	–	+	+	+
B. goldsteinii	+	–	V	+	–
B. merdae[e]	+	–	–$^{+}$	+	–
B. nordii	+	+		+	–
B. ovatus	+	+	+$^{-}$	+	+$^{-}$
B. salyersiae	+	+	–	+	–
B. stercoris	+	+	–$^{+}$	+$^{-}$	V
B. thetaiotaomicron	+	+	+	+	+
B. uniformis	W^{+}	+	V	+	+
B. vulgatus	+	–	–$^{+}$	–$^{+}$	+
B. splanchnicus	+	+	–	+	+

[a] +, positive reaction for the majority of strains; −, negative reaction; V, variable reaction; W, weak reaction; W^{+}, weak reaction but some strains more strongly positive; +$^{-}$, most strains positive; −$^{+}$, most strains negative, some strains positive; −^{w}, most strains negative, some strains weakly positive. For sugars: +, pH < 5.5; W, pH = 5.5 to 5.8; −, pH > 5.8.

[b] Make a heavy suspension (heavier than a McFarland no. 2 standard) of the organism in 0.25 ml of sterile saline. Add a tablet of substrate (Rosco Diagnostic Tablets or WEE-Tabs). Incubate for 4 h (or overnight). Yellow color, positive; colorless, negative.

Nitrate reduction[c]	Lipase	Pigment	Red fluorescence	Urease[d]	Motility[e]	Pitting of agar[f]	Slender cells with pointed ends
−	−		−	−			
−	−			−			
−	V	+	+⁻	−			
−	−⁺	+⁻	+⁻	−			
−	−⁺	+	+	−			
−	+⁻	+	+	−			
−		+	+	−			
−	−⁺	−	−				
+	−			V	V	V	
+	−			+	−	V	
+	−			−	+⁻	V	
+	−			−	−	V	
+	−			−	−	V	
+	−			+⁻	−	−	
V	−			−⁺	+⁻	−	
−⁺	V			−	−		V
−	−			−			+
−	+⁻			−			−
−	−			−			−
−	−			−	+		
−	−			−	−		+
+				−	+		
−	−			−	−		
−	−			−	+		
−	−			−	−		
+	−		V	−	−		

[e] Check motility with a young broth culture supplemented with formate and fumarate or prepare a suspension in broth directly from the blood agar plate.
[f] Formate and fumarate should be added to broth media for this group of organisms.
[g] *C. rectus, C. curvus,* and *C. showae.*
[h] Growth stimulated by 1% pyruvate (final concentration).
[i] Desulfoviridin positive. Swab cell paste from a pure culture on blood agar, add 1 drop of 2N NaOH to the swab, and immediately observe under long-wave (366-nm) UV light. Positive, red fluorescence; negative, no fluorescence.

cultures that have been exposed to air. It produces indole, fluoresces chartreuse, produces greening of the agar, and often demonstrates beta-hemolysis around the grayish to yellow dull, umbonate colonies (Fig. 9). Lipase-negative strains require further biochemical testing. *F. mortiferum* is indole negative and extremely pleomorphic; it has filaments containing swollen areas with large, round bodies and exhibiting irregular staining (Fig. 10). *F. necrophorum* may have a similar

Fermentation of:								Fatty acids from PYG[c]
Arabinose	Cellobiose	Rhamnose	Salicin	Sucrose	Trehalose	Xylose	Xylan	
+	+⁻	+⁻	−⁺	+	+	+	−	A, p, S, (iv)
−⁺	+	V	+	+	+	+	−	A, p, s, (pa, ib, iv, l)
+	−⁺	+⁻	−	−	−	+	+	A, p, S, (ib, iv, l)
−	+⁻	−	−	+	−	+	−	A, p, S, pa, (ib, iv, l)
−	+	+	+	+	+	+	−	A, p, S, (ib, iv)
−	V	+	+	+	+	+	−	A, p, S, (ib, iv)
−	+	+	−	+	−	+	−	
+	+	+	+	+	+	+	+	A, p, S, pa, (ib, iv, l)
+	+	+	−	+	−	+	−	
−	−⁺	+	−⁺	+	−	+	+	A, p, S, (ib, iv)
+	+⁻	+	−⁺	+	+	+	−	A, p, S, pa, (ib, iv, l)
+	+	−⁺	+⁻	+	−[w]	+	−	a, p, l, S, (ib, iv)
+	−	+	−	+	−	+	−⁺	A, p, S
+	−	−	−	−	−	−	−	A, P, S, ib, b, iv, (l)

[c] Capital letters indicate major metabolic products from peptone-yeast-glucose (PYG), lowercase letters indicate minor products, and parentheses indicate a variable reaction for the following fatty acids: A, acetic; P, propionic; IB, isobutyric; B, butyric; IV, isovaleric; V, valeric; L, lactic; S, succinic; PA, phenylacetic. Note that isoacids are primarily from carbohydrate-free media (e.g., peptone-yeast extract) in the case of saccharolytic organisms.
[d] *B. caccae* is L-arabinose positive with Rosco Diagnostic Tablets; *B. fragilis* is negative.
[e] *B. merdae* and *B. goldsteinii* are β-glucuronidase positive with Rosco Diagnostic Tablets; *B. distasonis* is negative. *B. goldsteinii* is α-glucosidase positive; *B. merdae* is negative.

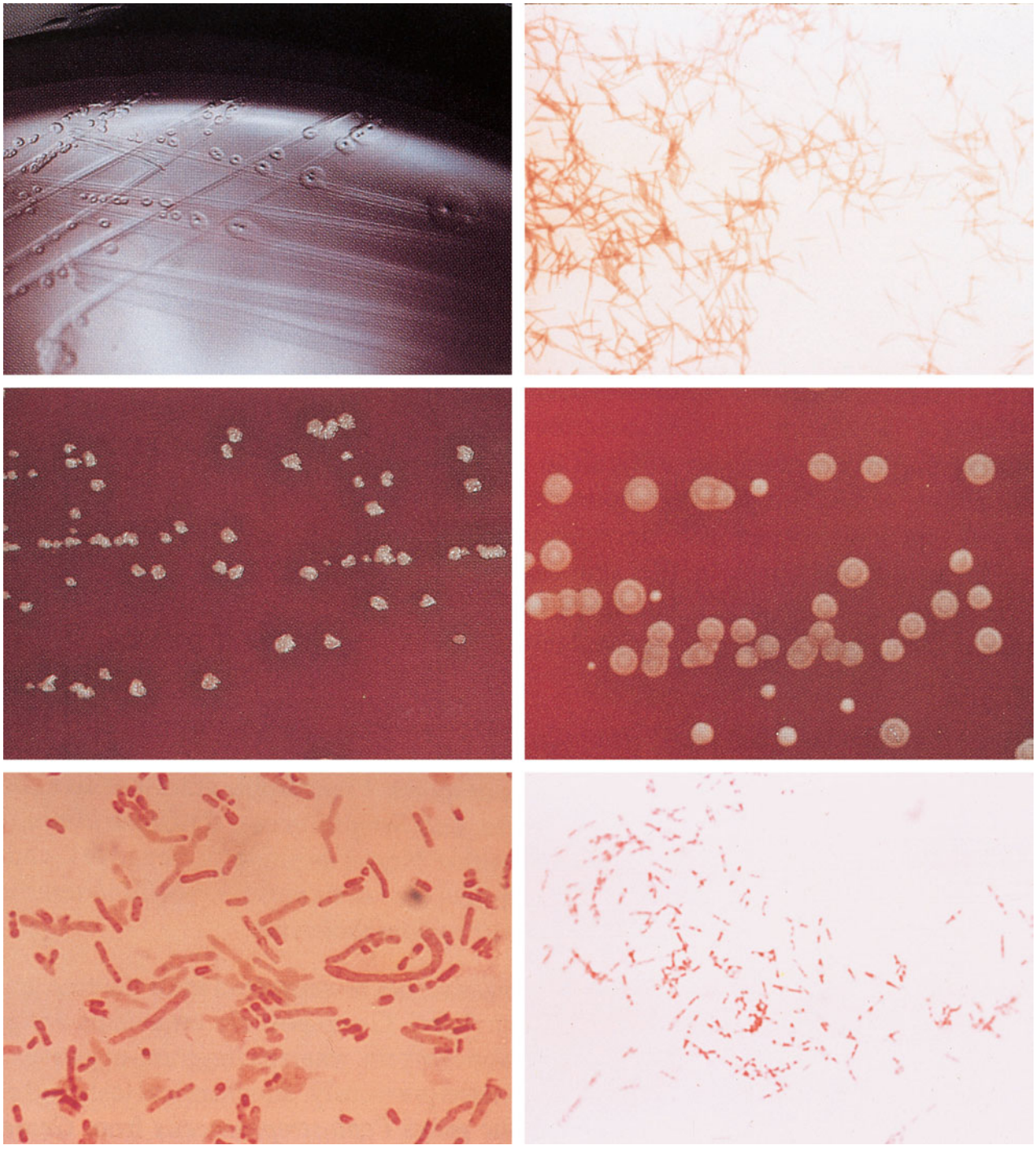


FIGURE 6 (top left) Pitting colonies of *Bacteroides ureolyticus*.

FIGURE 7 (top right) Cells of *Fusobacterium nucleatum*. Note the slender shape with pointed ends.

FIGURE 8 (middle left) Bread crumb-shaped colonies of *Fusobacterium nucleatum*. Note the greening of the agar.

FIGURE 9 (middle right) Umbonate colonies of *Fusobacterium necrophorum*.

FIGURE 10 (bottom left) Microscopic morphology of *Fusobacterium mortiferum*. There is marked pleomorphism and irregularity of staining. Note the filaments with swellings.

FIGURE 11 (bottom right) Pleomorphic, irregularly staining cells of *Bacteroides fragilis*.

morphology but usually has fewer round bodies. A bile-resistant fusobacterium isolated from BBE may be identified as *F. mortiferum* or *F. varium*; *F. mortiferum* is positive for *o*-nitrophenyl-β-D-galactopyranoside (Table 3, footnote *b*), whereas *F. varium* is negative.

Definitive Identification

A definitive identification of an anaerobic isolate should be obtained for all isolates from normally sterile body sites such as blood, spinal fluid, and organs or body cavities; when the patient is gravely ill and not responding to treatment; and when prolonged treatment is necessary. Definitive identification is also indicated in unusual case presentations and in some teaching-hospital settings, particularly if the case is to be published.

The definitive identification of some species requires certain additional biochemical tests, metabolic end product analysis, and/or cell wall fatty acid profiling by GLC (65). Even in good research or reference laboratories, a percentage of strains will not be identified definitively. If such strains are isolated from blood or closed-space infections, molecular methods such as 16S rRNA sequencing may be helpful. Motility may be helpful for characterizing curved rods that are not *Campylobacter,* as shown in Table 2. Organisms with small, translucent, spreading colonies that are not *B. ureolyticus*-like should also be checked for motility. The results in Tables 3 to 7 are based on reactions in prereduced, anaerobically sterilized (PRAS) liquid media (55, 65). A shortened and simplified scheme (spot indole, arabinose, sucrose, trehalose, rhamnose, and xylan tests) for the identification of the *B. fragilis* group with commercial PRAS biochemicals and the addition of bromthymol blue at 24 h postincubation has been described (18, 125). Other systems that depend on color changes of pH-dependent indicators for interpretation of reactions may not yield the same results as reactions in PRAS biochemicals. Gas chromatographic analysis may be performed on peptone-yeast glucose broth that shows good growth of the organism (55, 65). Each lot of uninoculated broth must be assayed in parallel with samples to determine the background amounts of acetic and succinic acids, and if chopped-meat broth is used, an uninoculated broth is assayed for lactic acid. Fermentation end products vary depending on the substrate available to the organism. Use of substrates other than those on which the results in the identification tables are based may lead to misinterpretation of the GLC pattern and misidentification of the organism. For instance, saccharolytic organisms produce larger amounts of isoacids in the absence of a fermentable carbohydrate, and a fermentable carbohydrate is required for the detection of lactic acid.

Colonies of the *B. fragilis* group on brucella BAP are 2 to 3 mm in diameter, circular, entire, convex, and gray to white. The cells may be uniform, bipolarly stained, or pleomorphic (some may contain vacuoles) (Fig. 11); this difference is medium and age dependent. The presence of ovoid cells suggests *B. ovatus.* Good growth in or stimulation by 20% bile (2% oxgall) is characteristic of the *B. fragilis* group; the exception to this rule is the poor growth of *B. uniformis* in bile. Some organisms not belonging to the *B. fragilis* group (non-*B. fragilis* group) are bile resistant. These include *B. splanchnicus, B. tectus, B. pyogenes, M. multiacida, Bilophila* sp., *Alistipes* spp., and some fusobacteria (111). Not all of these, however, grow on BBE. Morphologic characteristics, special-potency identification disks, and some biochemical reactions differentiate these species. Most of the *B. fragilis* group organisms blacken the BBE (esculin hydrolyzed), although *B. vulgatus* may not hydrolyze esculin. Table 3 is a key for the differentiation of members of the bile-resistant *B. fragilis* group.

Members of the non-*B. fragilis* group, nonpigmented anaerobic gram-negative rods, form two major subgroups: saccharolytic and asaccharolytic or weakly saccharolytic. Tables 4 and 5 list the more commonly encountered or clinically important species in these groups.

The saccharolytic organisms fall into two categories, i.e., pentose (arabinose and xylose) fermenters and pentose nonfermenters. Pentose fermentation may be difficult to demonstrate owing to suboptimal growth in the test medium; therefore, a screening test for the presence of the preformed enzyme β-xylosidase is recommended (for the procedure, see Table 3, footnote *b*). *P. oris* and *P. buccae* are pentose fermenters (Table 4). They are phenotypically very similar but can be differentiated by the α-fucosidase and *N*-acetyl-β-glucosaminidase tests (Table 3, footnote *b*) (33, 57); furthermore, *P. buccae* is usually sensitive to the special-potency colistin disk, whereas *P. oris* is not. *P. zoogleoformans* and *P. heparinolytica* may also ferment pentoses. Both produce viscous material in broth cultures, and on solid media the colonies often adhere to the agar. The positive indole reaction of *P. heparinolytica* differentiates these two species. The indole production is sometimes very difficult to demonstrate and should be tested from a pure culture on an EYA plate and/or from an old (>5-day-old) culture in chopped-meat broth because in some strains it can be a weak reaction. Other pentose fermenters include bile-resistant *M. multiacida* and *B. splanchnicus* and bile-sensitive *P. dentalis.* Unlike most *Prevotella* and *Bacteroides* species, *M. multiacida* is both nitrate and inositol positive. *P. dentalis* forms characteristic "water-drop," viscous colonies on blood agar. A positive *N*-acetyl-β-glucosaminidase reaction differentiates it from *P. buccae,* and a negative α-fucosidase reaction differentiates it from *P. oris.* The far better growth of *P. dentalis* on CDC blood agar than on brucella blood agar is an additional feature to facilitate the identification of the species.

Salicin, cellobiose, xylan, and sucrose are the key sugars in the differentiation of *P. baroniae, P. oralis, P. buccalis, P. veroralis, P. marshii, P. oulorum,* and *P. enoeca* (Table 4). In addition, *P. oulorum* produces catalase and is lipase positive (97). Certain strains of the saccharolytic pigmented *Prevotella* spp. require more than 21 days to develop pigment, and these strains (especially *P. loescheii*) closely resemble *P. veroralis.* Darker, more opaque colonies and salicin and cellobiose reactions may aid in differentiation of the strains.

P. bivia and *P. disiens* are both saccharolytic and strongly proteolytic (Table 4). Gelatin and milk are usually digested within 2 to 3 days (milk digestion may take longer). Differentiation is based on lactose fermentation; *P. bivia* is lactose positive, and *P. disiens* is lactose negative. Furthermore, *P. bivia* is both *N*-acetyl-β-glucosaminidase and α-fucosidase positive, but *P. disiens* is negative for both. Under long-wave UV light, *P. bivia* and *P. disiens* colonies may fluoresce light orange to pink (coral), and *P. disiens* also may produce a brown pigment on LRBA that makes differentiation from the phenotypically similar *P. corporis* difficult. *P. bivia* and *P. enoeca* share the same phenotypic characteristics, but *P. enoeca* is gelatin negative; it is usually isolated from oral or oral-associated sources, whereas *P. bivia* is more often found in nonoral samples.

Asaccharolytic, nonpigmented *Bacteroides* spp. and *Tissierella* spp. are infrequently isolated from clinical specimens (Table 5). *Anaerorhabdus furcosus,* another rare species, is currently considered to be within *Bacteroidaceae* but may

TABLE 4 Characteristics of nonpigmented saccharolytic *Prevotella* spp. and other genera[a]

Subgroup and species	Growth in 20% bile	Gelatin	Indole	Esculin hydrolysis	α-Fucosidase[b]	ONPG[b,c]	*N*-Acetyl-β-glucosaminidase[b]	β-Xylosidase[b]
Pentose fermenters								
M. multiacida	+	−	−	+	+	+	+	+
P. buccae	−	+	−	+	−	+	−	+
P. dentalis	−	−	−	V	−	+	+	V
P. heparinolytica[e]	−	−	+	+	+	+	+	+
P. multisaccharivorax	−	+	−	+	−	V		+
P. oris	−	V	−	+	+	+	+	+
P. zoogleoformans[e]	−	V	−	+	+	+	+	+
Not pentose fermenters								
P. buccalis	−	−	−	+	+	+	+	−
P. baroniae	−	+	−	+	+	+	+	−[f]
P. bivia	−	+	−	−	+	+	+	−
P. disiens	−	+	−	−	−	−	−	−
P. enoeca	−	+	−	V	+	+	+	−
P. marshii	−	+	−	−	+	−	−	−[f]
P. multiformis	−	+	−	−	V	+		−
P. oralis	−	+	−	+	+	+	+	−[f]
P. oulorum[g]	−	−	−	+	−	+	+	−
P. shahii	−	+	−	−	+	W	+	−[f]
P. veroralis	−	V	−	+	+	+	+	−
Other								
Anaerobiospirillum succiniciproducens[h]	−	−	−	−		+		
Capnocytophaga spp.	−		−	+−				
Leptotrichia buccalis	+−	−	−	+				
Leptotrichia sanguinegens			−	+				
Selenomonas sp.	−		−	V				

[a] See Table 3, footnote *a*.
[b] See Table 3, footnote *b*.
[c] ONPG, *o*-nitrophenyl-β-D-galactopyranoside.
[d] See Table 3, footnote *c*.

TABLE 5 Characteristics of nonpigmented weakly saccharolytic or nonsaccharolytic gram-negative rods[a]

Species	Growth in 20% bile	Glucose	Catalase	Indole	Nitrate	Motility
Alistipes putredinis	+−	−	+−	+	−	−
Anaerohabdus furcosa	+−	W	−	−	−	−
Bilophila wadsworthia[g]	+	−	+	−	+	−
Bacteroides capillosus	−+	W−[h]	−	−	−	−
B. coagulans	+	−	−	−	−	−
B. pyogenes[f]	+	W	−	−	−	−
B. tectus[f]	+	W	−	−	−	−
B. ureolyticus	−	−	−+	−	+	−
Campylobacter spp.	−	−	−	−	+	+−
C. gracilis	−	−	−	−	+	−
Desulfovibrio piger[g]	+	−	−	−	−	−
D. desulfuricans[g]	−	−	−	−	+	+
D. fairfieldensis[g]	+	−	+	−	+	+
D. vulgaris[g]	+	−	−	+	−	+
Dialister pneumosintes	−	−	−	−	−	−
Sutterella wadsworthensis	+	−	−	−	+	−
Tannerella forsythensis	−	−	−	−	−	−
Tissierella praeacuta	+	−	−	−	V	+

[a] See Table 3, footnote *a*.
[b] F/F, formate-fumarate. See Table 3, footnote *b*.
[c] See Table 2, footnote *i*.
[d] See Table 2, footnote *d*.

Fermentation of:								Fatty acids from PYG[d]
Arabinose	Cellobiose	Glucose	Lactose	Salicin	Sucrose	Xylose	Xylan	
+	+	+	+	+	+	+		A, L, S
+	+	+	+	+	+	+		A, S, (p, ib, iv)
+	+	+	+	−	W	V		A, S
+	+	+	+	+	+	+		A, p, S (iv)
V	+	+	+	V	+	+		A, S, iv
+−	+	+	+	+	+	+		A, S, (P, ib, iv)
V	+	+	+	V	+	V		A, P, S, (ib, iv)
−	+	+	+	−	+	−	−	a, iv, S
−	+	+	+	+	+	−		A, S, (l)
−	−	+	+	−	−	−		A, iv, S, (ib)
−	−	+	−	−	−	−		A, S, (p, ib, iv)
−	−	+	+	−	−	−		a, S
−	−	+	−	−	−	−		A, S, (l)
−	+	+	+	−	+	−		A, S, iv
−	+	+	+	+	+	−	−	A, S, (l)
−	−	+	+	−	+	−	−	A, S
−	−	+	+	−	+	−		A, S, (l)
−	+	+	+	−	+	−	+	a, S
−	−	+	+	−	+	−		A, S
−	−+	+	V	−	+	−		A, S
−+	+−	+	+−	+−	+	−		L, (a, s)
−	+	+	−	+	−	−		L, (a)
−	−	+−	V	−	+−	−		A, P

[e] Produces viscous sediment in broth and colonies usually adhere to agar.
[f] Positive with 4-methylumbelliferyl substrates (81).
[g] Catalase and lipase positive.
[h] Corkscrew motility.

F/F required[b]	Desulfoviridin[c]	Urease[d]	Esculin hydrolysis	Gelatin hydrolysis	Fatty acids from PYG[e]
−	−	−	−	+	a, P, ib, b, IV, S, (1), pa
−	−	+	+	−w	a, l, (s)
−	W	+−	−	−	A, (s)
−	−	−	+	−w	a, s, (p, l)
−	−	−	−	+	a, (p, 1, s)
−	−	−	+	+	a, P, ib, b, IV, S, (l)
−	−	−	+	+	A, p, iv, S, pa
+	−	+	−	−	A, S
+	−	−	−	−	a, S
+	−	−	−	−	a, S
−	+	−	−	−	A
−	+	+	−	−	A
−	+	+	−	−	A
−	+	−	−	−	A
−	−	−	−	−w	a, (l, s)
+	−	−	−	−	a, S
−	−	−	+	+	A, S, pa
−	−	−	−	+	A, p, ib, B, IV, s, (l)

[e] See Table 3, footnote c.
[f] Animal origin; also isolated from bite infections in humans. Quantitative differences between some cellular fatty acids may help in differentiation of these species (42).
[g] *Bilophila* sp. is colistin sensitive; *Desulfovibrio* spp. are colistin resistant.
[h] W−, most strains weakly positive, some strains negative.

be more related to gram-positive rods. *B. capillosus* coagulates milk and may grow better with Tween 80-supplemented media. *D. pneumosintes* is a very tiny rod best seen by darkfield examination (it almost resembles *Veillonella* spp. by Gram staining) and forms minute colonies that may require magnification to be seen. Other species of *Dialister* from clinical specimens have been described recently (67). *T. forsythensis* is a fusiform rod that exhibits a wide variety of enzyme activities including trypsin-like activity and *N*-acetyl-β-glucosaminidase, α-fucosidase, and β-glucuronidase activities. However, its growth is minimal in broth media, and it requires *N*-acetylmuramic acid; therefore, it is often seen as satellite colonies around colonies of other organisms, especially fusobacteria. *Desulfovibrio* spp. are sulfate-reducing bacteria and can be confirmed by the desulfoviridin test (Table 2, footnote *i*). The species are motile (except for the recently included *D. piger*) and produce copious amounts of H_2S (142).

The pigmented *Prevotella* spp. and *Porphyromonas* spp. (Table 6) vary greatly in the degree and rapidity of pigment production, which ranges from buff to tan to black depending primarily on the type of blood and the composition of the base medium used in the agar. A period of 2 to 21 days may be required even on LRBA to detect pigmentation in some strains. The identities of strains not showing pigmentation must be established by other biochemical tests to avoid confusion with the *P. oralis* group of organisms. The pigmented *Prevotella* and *Porphyromonas* spp. fluoresce pink, orange, or brick red under UV light. Fluorescence is best demonstrated in young cultures; in older cultures, especially on laked blood agar, the fluorescence is more or less masked depending on the intensity of pigment production (66).

The indole-positive species *P. intermedia* and *P. nigrescens* produce dark pigment; the pigment produced by *P. pallens* is lighter. The use of oligonucleotide probes or determination of arbitrarily primed PCR and enzyme electrophoretic mobility profiles are needed for species differentiation (51, 66). The unusual special-potency antibiotic disk pattern (sensitivity to vancomycin), in addition to asaccharolytic properties, separates most *Porphyromonas* spp. from the other pigment producers. *P. asaccharolytica, P. endodontalis, P. uenonis*, and *P. gingivalis* are all asaccharolytic and phenotypically very similar. The key differential tests include tests for phenylacetic acid production, trypsin-like activity, and *N*-acetyl-β-glucosaminidase and α-fucosidase activities (Table 6). Unlike most *Porphyromonas* spp., *P. catoniae, P. levii,* and *P. somerae* (frequently isolated from human clinical specimens) are indole negative and weakly saccharolytic; the latter characteristic is also shared with the animal strains of *Porphyromonas macacae.* Most of the *Porphyromonas* spp. of

TABLE 6 Characteristics of pigmented *Porphyromonas* spp. and *Prevotella* spp.[a]

Species and origin	Indole	Lipase	Catalase	Esculin hydrolysis	α-Fucosidase[b]	α-Galactosidase[b]	β-Galactosidase[b]
Porphyromonas, asaccharolytic or weakly saccharolytic							
Human origin							
P. asaccharolytica	+	−+	−	−	+	−	−
P. catoniae[d]	−	−	−	−	+	−+	+
P. uenonis	+	−	−	−	−	−	−
P. endodontalis	+	−	−	−	−	−	−
P. gingivalis[e]	+	−	−	−	−	−	−[f]
P. somerae[e]	−	−	−	−	−	−	+
Animal origin[g]							
P. canoris	+	−	+	−	−	−	+
P. cangingivalis	+	−	+	−	−	−	−
P. cansulci	+	−	+[w]	−	−	−	−
P. circumdentaria	+	−	+	−	−	−	−
P. crevioricanis	+		−	−	−		
P. gingivicanis	+		+	−	−		
P. gulae	+	−	+	−	−	−	−[f]
P. levii[e]	−	−	−	−	−	−	+
P. macacae	+	+	+	−	−	+	−[f]
Prevotella, saccharolytic							
P. corporis	−	−	−	−	−	−	−
P. denticola	−	−	−	+−	+	+	+
P. intermedia	+	+−	−	−	+	−	−
P. loescheii	−	V	−	+−	+	+	+
P. melaninogenica	−	−	−	−+	+	+	+
P. nigrescens[h]	+	+−	−	−	+	−	−
P. pallens	+	−	−	−	+	−	−
P. tannerae	−	−	−	−	+	−	+

[a] See Table 3, footnote *a*. +[w], most strains positive; some strains weakly positive.
[b] Reaction by the API ZYM system or with Rosco Diagnostic Tablets (see Table 3, footnote *b*). Reactivity in these systems is not always identical (see footnote *f*).
[c] See Table 3, footnote *c*.
[d] Nonpigmented.

animal origin are differentiated from the human strains by a positive catalase reaction; *Porphyromonas crevioricanis,* however, is catalase negative (66).

Table 7 characterizes the more commonly isolated fusobacteria. Bizarre pleomorphic rods with very large, round bodies are suggestive of *F. mortiferum* (Fig. 10). This organism may grow on BBE and turn the agar black due to H_2S production. *F. ulcerans,* isolated from tropical ulcer, closely resembles indole-negative strains of *F. varium* but is nitrate positive. *F. periodonticum,* an oral isolate, is indole positive and bile sensitive. The national anaerobic laboratory of Finland failed to demonstrate glucose, fructose, or galactose fermentation by the type strain; this bacterium is indistinguishable from *F. nucleatum* except by 16S-23S rRNA internal transcribed spacer sequencing (26). *Fusobacterium russii* is indole negative and bile sensitive (26).

Other Approaches to Identification

Several microsystems for the identification of anaerobes are currently available. The API 20A system (bioMérieux Vitek, St. Louis, Mo.) is a microtube biochemical system that uses bromcresol purple as indicator, produces results after 24 to 48 h of incubation, and has a computerized database. The API 20A is best suited for identification of saccharolytic, fast-growing organisms such as members of the *B. fragilis* group and many clostridia. Most asaccharolytic organisms cannot be identified by API 20A, and some fastidious organisms (e.g., some *Prevotella* spp.) fail to grow. Even for many saccharolytic organisms, supplemental tests are often required for definitive identification. The color reactions are not always clear-cut, as shades of brown or no color can make interpretation of the test results difficult.

The rapid (2- to 4-h) identification systems based on detection of preformed (constitutive) enzymes by use of chromogenic or fluorogenic substrates, or a combination of both, include the RapID ANA II system (Remel), Rapid ID 32A, ANI Card, and API ZYM systems (bioMérieux), the MicroScan system (Dade Behring MicroScan, Inc., Sacramento, Calif.), and the BBL Crystal Anaerobe Identification system (Becton Dickinson Diagnostic Systems, Franklin Lakes, N.J.), as mentioned in chapter 15. The overall performance of these systems has varied from moderate to good; 53 to 100% of the isolates are identified to the species level (17, 29, 61, 79, 90, 129). The Crystal Anaerobe (ANR) Identification (ID) system was reported to identify 91% of the *B. fragilis* group and all of the *Prevotella* and *Porphyromonas* strains tested to the species level; 53% of the non-*B. fragilis* group *Bacteroides* spp. were identified correctly (17). The Rapid ID 32A system was found to be 78.4 to 90.6% accurate for the *B. fragilis* group (29, 79); a supplemental test for catalase

N-Acetyl-β glucosaminidase[b]	Chymotrypsin	Trypsin	Fermentation of:				Fatty acids from PYG[c]
			Glucose	Cellobiose	Lactose	Sucrose	
−	−	−	−	−	−	−	
+	+−	−+	W	−	W	−	A, P, iv, l, S
−	−	−	−	−	−	−	A, p, ib, B, IV, s
−	−	−	−	−	−	−	A, p, ib, B, IV, s
+	+	+	−	−	−	−	A, p, ib, B, IV, s, pa
+	+	−	W	−	W	−	A, p, ib, B, IV, s
+	+	−	−	−	−	−	A, P, ib, b, IV, s
−	−	−	−	−	−	−	A, p, ib, B, IV
−	−	−	−	−	−	−	A, P, ib, B, IV, S, pa
−	−	−	−	−	−	−	A, P, ib, b, IV, s, pa
−	NA	−	−	−	−	−	A, p, ib, B, IV, s
−	NA	−	−	−	−	−	
+	+	+	−	−	−	−	A, p, ib, B, IV, s, pa
+	+	−	W	−	W	−	A, p, ib, B, IV, s
+	+	+	W	−	W	−	A, p, ib, B, IV, s, pa
−	+−	−	+	−	−	−	A, ib, iv, S, (b)
+	−	−	+	−+	+	+	A, S, (ib, iv, l)
−	−	−	+	−	−	+−	A, iv, S, (p, ib)
+	−	−	+	+	+	+	a, S, (1)
+	−	−	+	−+	+	+	A, S, (ib, iv, l)
−	−	−	+	−	−	+−	A, iv, S, (p, ib)
−	−	−	+	−	−	+	A, S, (p, ib)
+	−	−	+	−	+	V	a, iv, S, (ib)

[e] *P. gingivalis* does not show fluorescence; *P. somerae* and *P. levii*-like may show weak or no fluorescence.
[f] Negative by the API ZYM system; positive by the Rosco *o*-nitrophenyl-β-D-galactopyranoside test.
[g] May be isolated from bite infections.
[h] *P. intermedia* shares the same characteristics. Differentiation based on enzyme electrophoresis, oligonucleotide probe analysis, or arbitrarily primed PCR (93).

TABLE 7 Characteristics of more common *Fusobacterium* species from human clinical samples[a]

Species	Distinctive cellular morphology	Indole	Growth on BBE or 20% bile	Lipase	ONPG[b]	Fermentation of:				Lactate converted to propionate	Threonine converted to propionate	Fatty acids from PYG[c]
						Glucose	Fructose	Lactose	Mannose			
F. nucleatum[d]	Slender, pointed ends	+	−	−	−	$-^{w}$	$-^{w}$	−	−	−	+	A, p, B, (l, s)
F. necrophonum[e]	Large, pleomorphic	+	$-^{+}$	$+^{-}$	−	$-^{w}$	$-^{w}$	−	−	+	+	A, p, B, (l, s)
F. mortiferum	Bizarre; round bodies	−	+	−	+	$+^{w}$	$+^{w}$	+	$+^{w}$	−	+	a, p, B, (v, l, s)
F. varium	Large, rounded ends	$+^{-}$	+	$-^{+}$	−	w^{+}	w^{+}	−	$+^{w}$	−	+	a, p, B, L, (s)
F. gonidiaformans	Gonidial forms	+	−	−	−	−	−	−	−	−	+	A, p, B, (l, s)
F. naviforme	Boat shape	+	−	−	−	w^{-}	−	−	−	−	−	a, B, L, (p, s)
F. russii	Large, rounded ends	−	−	−	−	−	−	−	−	−	−	a, B, L
F. canifelinum	Slender, pointed ends	+	−	−	−	$-^{w}$	$-^{w}$	−	−	−	+	A, p, B, (l, s)
F. ulcerans[f]	Large, rounded ends	−	+	−	−	+	−	−	$+^{-}$	−	+	a, p, B, l, (s)

[a] See Table 3, footnote *a*. $+^{w}$, most strains positive; some strains weakly positive; w^{-}, most strains weakly positive, some strains negative; w^{+}, most strains weakly positive, some strains positive.
[b] ONPG, *o*-nitrophenyl-β-D-galactopyranoside. Reaction by the API ZYM system or with Rosco Diagnostic Tablets (see Table 3, footnote *b*). Reactivity in these systems is not always identical.
[c] See Table 3, footnote *c*.
[d] *F. periodonticum* shares the same characteristics with, and is very similar to, *F. nucleatum*.
[e] Lipase-positive strains, *F. necrophonum* subsp. *necrophorum;* lipase-negative strains, *F. necrophorum* subsp. *funduliforme*.
[f] Nitrate positive.

activity increased the accuracy to 94.6% (61). The Rapid ID 32A system identified 95.5% of the fusobacteria correctly (79); however, poor discrimination between *F. nucleatum* and *F. necrophorum* has been noted (29). Similarly, the Rapid ID 32A system accurately assigned *Prevotella* and *Porphyromonas* species to the genus level, but it less accurately differentiated between the species (29). The RapID ANA II system identified 62% of the gram-negative rods correctly (90). Use of the rapid identification systems is indicated when an identification is not achieved by using the tests described in "Rapid Identification" above but before more time-consuming tests, such as fermentation tests and GLC, are used. These systems are also suited for the identification of slowly growing fastidious gram-negative rods and cocci, since no growth in the test medium is required. When using rapid identification systems, all the information available on the organism should be considered and correspond to the profile number identification. The performance of all these systems is, of course, affected by the source and nature of the isolates and the extent of the system's database; the accuracy can be further increased by certain simple supplemental tests. The API ZYM system, which allows the detection of 19 preformed enzymes in 4 h, does not have a database; only data compiled from different publications are available, but it is a useful supplement for identification of clinically encountered anaerobic bacteria, especially the *Porphyromonas* spp. (Table 6) (33, 138).

Individually available tablets containing single, dual, or triple enzyme substrates (Rosco Diagnostic Tablets [Rosco]; WEE-Tabs [Key Scientific Products]) are much cheaper than commercial kits; they can be applied in a number of situations and allow flexibility in tailoring the set to best suit special needs (33, 57). The use of 4-methylumbelliferone derivatives of many substrates (Sigma) permits rapid and inexpensive spot tests based on fluorescence (84, 85, 95, 109). It should be noted, however, that reactions obtained by fluorogenic and the different chromogenic test applications may not completely agree, owing to divergent substrate concentrations, affinities, and buffering conditions in the different systems. Therefore, it is important to name the system used for identification when reporting enzyme reactions, particularly in publications.

A method for identification of anaerobes based on analysis of whole-cell fatty acids by capillary column GLC has been adopted primarily by some reference laboratories (96). An extensive database (MIDI, Inc., Newark, Del.) for anaerobes has been compiled, largely by the Virginia Polytechnic Institute Anaerobe Laboratory, and it is updated frequently. An option for creating a cumulative database is also available. Nucleic acid probes are not commercially available for the identification of clinically important anaerobes, but they are being used in some dental laboratories (16). Determination of arbitrarily primed PCR profiles may be useful in differentiation of certain species such as *P. intermedia* and *P. nigrescens* (72).

Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF-MS) may have some utility for rapid identification of anaerobic bacteria (121). Ions from colony paste activated by a pulsed laser beam travel through a tube to a detector, where they are separated by differences in mass-to-ionic charge ratios, which are used to form a pattern. In practice, minimal sample preparation from a single colony is required (121). At the generic level, representative members of *Bacteroides, Prevotella,* and *Porphyromonas* may be readily distinguished. This new tool has not yet been introduced into clinical laboratories.

Identification of isolates by 16S rRNA and other genetic sequencing is gradually being adopted by dental, reference, and clinical laboratories and is also commercially available (see chapter 16) (25, 63, 68, 72, 78, 116, 125).

TYPING SYSTEMS

Due to the endogenous nature of most infections, no generally agreed upon typing systems exist.

SEROLOGICAL TESTS

Serological procedures are not practical for the identification of anaerobic bacteria from colonies. Furthermore, no standardized tests are available for the detection of antibodies or antigens in clinical specimens that would be useful for this group of organisms.

ANTIMICROBIAL SUSCEPTIBILITIES

Susceptibility testing of anaerobes is discussed in chapter 76. The susceptibility patterns for the most commonly encountered gram-negative rods of clinical significance are noted in Table 8. Resistance is increasingly common among anaerobic gram-negative rods. This is particularly true for the members of the *B. fragilis* group, which are not uncommonly resistant to expanded- and broad-spectrum cephalosporins (including β-lactamase-resistant drugs such as cefoxitin) and clindamycin. Strains of the *B. fragilis* group with resistance to imipenem and metronidazole are rarely encountered. The originally reported resistance to several antimicrobial agents in the group containing both *C. gracilis* and *S. wadsworthensis* was found to be partly due to a technical artifact (lack of formate-fumarate additive in susceptibility testing media), highlighting the importance of good growth for these assays. The true resistance seems to be confined to *Sutterella; Sutterella* spp. are more resistant to antimicrobial agents such as metronidazole and some β-lactam drugs than *C. gracilis* (94, 143).

β-Lactamase production (nitrocefin test positivity) and penicillin resistance are present in the species of the *B. fragilis* group; thus, routine testing of known *B. fragilis* group strains is unnecessary. Approximately 50% of *Prevotella* spp. are also β-lactamase producers, and even higher proportions of β-lactamase producers have been reported among certain species when several colonies per plate were tested (62, 73). An increasing number of β-lactamase-positive *F. nucleatum* strains have been encountered. Occasional strains of *C. gracilis, B. coagulans, B. splanchnicus, Desulfovibrio* spp., *F. mortiferum, F. varium, Megamonas hypermegas, M. multiacida,* and *Porphyromonas* spp. may produce β-lactamase (52). Animal-derived *Porphyromonas* spp. are more often β-lactamase producers than those derived from humans (66). *Leptotrichia* spp. as well as *Capnocytophaga* spp. are sometimes β-lactamase producers and are resistant to aminoglycosides and vancomycin; *Leptotrichia* spp. are resistant to erythromycin as well. Erythromycin and all the newer extended-spectrum macrolides except azithromycin, commonly used for the treatment of upper respiratory tract infections, such as tonsillitis, otitis media, and maxillary sinusitis, are not active against fusobacteria.

Piperacillin-tazobactam is active against most strains of anaerobic gram-negative rods, although some members of the *B. fragilis* group are resistant to ampicillin-sulbactam and amoxicillin-clavulanate. Ertapenem, imipenem, and meropenem are also consistently active against most anaerobes (49). A new antimicrobial, tigecycline, (a glycylcycline

TABLE 8 Percent susceptibility of clinically important anaerobic gram negative rods to selected antimicrobial agents[a]

Antimicrobial agent	*B. fragilis*	*B. fragilis* group	*Prevotella*	*Porphyromonas*	*F. nucleatum*	*F. mortiferum/varium*
Carbapenems						
Ertapenem	>95	>95	>95	>95	>95	>95
Imipenem	>95	>95	>95	>95	>95	>95
Meropenem	>95	>95	>95	>95	>95	>95
β-Lactamase inhibitor combinations						
Ampicillin-sulbactam	>95	90	>95	>95	>95	>95
Amoxicillin-clavulanate	>95	90	>95	>95	>95	>95
Piperacillin-tazobactam	>95	>95	>95	>95	>95	>95
Ticarcillin-clavulanate	>95	>95	>95	>95	>95	>95
Penicillins						
Penicillin	0	0	50	95	>95	90
Ampicillin	0	0	50	95	>95	90
Cephalosporins						
Cefoxitin	85	70	90	>95	>95	>95
Ceftizoxime	85	50	90	>95	>95	>95
Chloramphenicol	>95	>95	>95	>95	>95	>95
Tetracyclines						
Tetracycline	20	10	50	90	>95	90
Doxycycline	30	20	80	90	>95	90
Tigecycline	90	90	>95	>95	>95	>95
Lincosamides						
Clindamycin	80	50	90	>95	>95	80
Fluoroquinolones						
Moxifloxacin	90	80	90	>95	>95	90

[a] Data from references 12, 46, 47, and 49 through 52.

derived from minocycline) is also very active against the full spectrum of anaerobic gram-negative rods (12). Resistance to quinolones is increasing, although moxifloxacin remains moderately active against many of these strains (46, 50, 51). Mechanisms of resistance other than β-lactamase (including high-level metalloenzyme production) include changes in penicillin-binding proteins and in outer membrane porin channels. Efflux pumps are present in many *Bacteroides* species and contribute to other mechanisms of quinolone resistance. Plasmids conferring resistance to metronidazole have been reported but have not spread resistance to this antimicrobial agent.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Because anaerobic bacteriology is time-consuming, several interim reports are desirable. The initial report can give Gram stain results. Bacterial and host cell morphologies and the relative quantities seen in the smear give a good overall impression of the specimen quality, the nature of the polymicrobial infection, and even morphologies suggestive of certain anaerobes. Furthermore, Gram stain results can guide the laboratory in choosing media for the optimal recovery of the predicted organisms. At 24 h, preliminary information on the anaerobic as well as the aerobic and facultatively anaerobic flora is available if the laboratory incubates plates in an anaerobic chamber. At 48 h, more definite information on the nonanaerobes and preliminary but clinically useful information on the anaerobes can be given (39).

Interpretation of results of a mixed culture containing multiple species is difficult. In general, bacterial isolates that are most predominant, empirically most virulent, and most resistant to antimicrobials should be given the most attention. Rough quantitation of the different isolates recovered, together with Gram stain results (provided that the specimen was properly taken and transported), is helpful. The bacteria present in pure culture or in large numbers are probably of major importance, as are organisms recovered on repeat culture and organisms isolated from normally sterile sites. The nature of the bacteria found can also give clues to their importance in the infectious process. Certain taxa, including the *B. fragilis* group, *Fusobacterium* spp., and some *Prevotella* spp., are much more important clinically in terms of the frequency of occurrence, the severity of the infection produced, and antimicrobial resistance.

Microbiologists must be willing to consult with the clinician on interpretation of the relevance of the findings. The laboratory should provide the clinician with a gradual introduction to new taxonomy by reporting both the new and old names in parallel for at least 2 years. Statements of specimen quality and possible limitations of methods used serve as the most important feedback to the clinician. Dialogue between the clinician and the microbiologist should be frequent.

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Campylobacter and *Arcobacter*

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TAXONOMY

Three closely related genera, *Campylobacter, Arcobacter,* and *Sulfurospirillum,* are included in the family *Campylobacteraceae* (95, 129). The family *Campylobacteraceae* includes 17 species in the genus *Campylobacter,* 6 species in the genus *Arcobacter,* and 6 species in the genus *Sulfurospirillum.* Since the last edition, several new species have been proposed, including *Campylobacter insulaenigrae* (32), isolated from marine mammals. Two new *Arcobacter* species have recently been described: *Arcobacter cibarius,* isolated from chickens (48), and *A. halophilus,* an obligate halophilic species (23). Some isolates previously identified as *C. gracilis* have been renamed as *Sutterella wadsworthensis* and are more closely related to *Alcaligenes* and *Bordetella* species (144).

DESCRIPTION OF AGENTS

Campylobacters are curved, S-shaped, or spiral rods that are 0.2 to 0.9 μm wide and 0.5 to 5 μm long. Certain species, such as *C. hominis,* form straight rods. *Campylobacter* species are gram-negative, non-spore-forming rods that may form spherical or coccoid bodies in old cultures or cultures exposed to air for prolonged periods. Organisms are usually motile by means of a single polar unsheathed flagellum at one or both ends but may lack flagella. Species are generally microaerobic with a respiratory type of metabolism; however, some strains grow aerobically or anaerobically. An atmosphere containing an increased hydrogen concentration is required by some species for microaerobic growth (131).

Arcobacters are gram-negative, slightly curved, curved, S-shaped, or helical non-spore-forming rods that are 0.2 to 0.9 μm wide and 1 to 3 μm long. Organisms are motile with a single polar unsheathed flagellum. Arcobacters grow at 15, 25, and 30°C but have variable growth at 37 and 42°C. Organisms are microaerobic and do not require increased hydrogen for growth. Arcobacters may grow aerobically at 30°C and anaerobically at 35 to 37°C. Most strains are nonhemolytic. *A. skirrowii* may be alpha-hemolytic (133). *A. halophilus* is an obligate halophile and grows poorly on media containing less than 2% NaCl (23).

Originally classified as free-living *Campylobacter* species, *Sulfurospirillum* spp. are slender, curved, gram-negative rods that are 0.1 to 0.5 μm wide and 1 to 3 μm long. All of the species are sulfur reducers and exhibit variable metabolic activities. *Sulfurospirillum deleyianum* is the type species of the genus. These species have no known pathogenicity for humans or animals, are environmental organisms isolated from water sediments, and will not be further discussed in this chapter (129).

EPIDEMIOLOGY AND TRANSMISSION

Campylobacter species are primarily zoonotic, with a variety of animals implicated as reservoirs for human infection (Table 1). In addition to food animals such as poultry, cattle, sheep, and pigs, *Campylobacter* species may be present in domestic pets. Humans appear to be the only recognized reservoirs for the periodontal pathogens, *C. concisus, C. rectus, C. curvus,* and *C. showae.*

Campylobacter infections are common in both the developed and the developing world. The reported incidences of culture-confirmed infections vary considerably from country to country, and as culturing practices and reporting requirements can vary, direct comparison of the reported incidences can be complex. In the United States, where reporting practices vary from state to state, the food-borne-disease active surveillance program FoodNet (http://www.cdc.gov/foodnet) provides uniform reporting from a panel of sentinel sites, giving an accurate measure of the incidence of diagnosed infections. FoodNet data suggest that numbers of campylobacteriosis cases in the United States reached a peak in the mid-1990s and showed a decline from 1996 to 1999 (110). This decline continued somewhat through 2003 and into 2004, with the incidence of diagnosed *Campylobacter* infections at 12.6 and 12.9 per 100,000 people, respectively, lower than that of salmonellosis (17). A comparison of the data from 1996 to 1998 with the 2004 data shows that the estimated incidence of infection with *Campylobacter* decreased 31%. However, *C. jejuni* subsp. *jejuni,* referred to as *C. jejuni,* continues to be the most common enteric pathogen isolated from patients in some states, according to FoodNet reports, with 1.4 million cases estimated to occur in the United States annually (110). Because of underdiagnosis and underreporting, the actual incidence in any country is substantially greater than the reported incidence. For example, in the United States, it was estimated that the true incidence was 35-fold higher than the reported incidence, or 515 per 100,000 people in 1999 (110).

TABLE 1 Reservoirs for and diseases associated with *Campylobacter* and *Arcobacter* species[a]

Species	Humans	Cattle	Sheep	Pigs	Wild birds	Poultry	Pets	Rodents	Types of infection[b]
C. jejuni subsp. *jejuni*	×	×			×	×	×		GI, B
C. jejuni subsp. *doylei*	×								GI, B
C. coli				×	×	×			GI, B
C. fetus subsp. *fetus*		×	×						GI, B
C. upsaliensis						×	×		GI, B
C. lari					×		×		GI, B
C. hyointestinalis		×		×		×	×	×	GI
C. helveticus							×		NR
C. sputorum	×[c]	×[d,e]	×[e]						GI[c]
C. concisus	×								D
C. curvus	×								D
C. rectus	×								D, GI
C. showae	×								D
C. gracilis	×								D
C. mucosalis				×					NR
A. butzleri		×		×		×			GI, B, T
A. cryaerophilus		×	×	×		×			GI
A. skirrowii		×	×	×		×			GI

[a] The information in this table is from references 66, 83, 112, and 130.
[b] GI, gastrointestinal; B, bloodstream; D, oral or dental; T, soft tissue; NR, not reported to be associated with human infections.
[c] Biovar sputorum.
[d] Biovar paraureolyticus.
[e] Biovar faecalis.

The epidemiology of campylobacteriosis does not appear to have changed over the last 5 years. *Campylobacter* infections are usually sporadic, occurring in the summer months and early fall, and usually follow ingestion of improperly handled or improperly cooked food, primarily poultry products. A large case control study concluded that numerous food sources are associated with *Campylobacter* infections but that a large proportion of infection is attributable to consumption of poultry and other meats in restaurants (33). The incidence of infection follows a bimodal age distribution, with the highest incidence in infants and young children, followed by a second peak in young adults 20 to 40 years old (110). Outbreaks usually occur in the spring and fall months, and in recent years, most outbreaks have been food (poultry and unpasteurized dairy products) or water associated. Milk-borne outbreaks, while common in the 1980s and early 1990s, are less frequently reported now; the most recent milk-borne outbreaks were reported in Wisconsin in 2001 (44) and Michigan in 2003 (http://www.cdc.gov/foodborneoutbreaks/us_outb/fbo2003/summary03.htm).

The incidence of *Campylobacter* infection in developing countries such as Mexico and Thailand is much higher than that in the United States. In developing countries, *Campylobacter* is frequently isolated from individuals who may or may not have diarrheal disease. Most symptomatic infections occur in infancy and early childhood, and incidence decreases with age. Travelers to developing countries are at risk for developing *Campylobacter* infection, with isolation rates from 0 to 39% reported in different studies (91).

CLINICAL SIGNIFICANCE

C. jejuni and *C. coli*

C. jejuni and *C. coli* have been recognized since the early 1970s as agents of gastrointestinal infection. *C. jejuni* is one of the most common causes of bacterial enteritis in the United States. *C. jejuni* and *C. coli* continue to be the most common *Campylobacter* species associated with diarrheal illness and produce clinically indistinguishable infections. Most laboratories do not routinely distinguish between these organisms.

In patients with gastroenteritis caused by *C. jejuni* or *C. coli*, symptoms range from nonexistent to severe and may include fever, abdominal cramping, and diarrhea (with or without blood or fecal white blood cells) that last several days to more than 1 week. Symptomatic infections are usually self-limited, but relapses may occur in 5 to 10% of untreated patients (11). *Campylobacter* infection may mimic acute appendicitis and result in unnecessary surgery. Extraintestinal infections and other complications have been reported following *Campylobacter* enteritis and include bacteremia, hepatitis, cholecystitis, pancreatitis, abortion and neonatal sepsis, nephritis, prostatitis, urinary tract infection, peritonitis, myocarditis, and focal infections including meningitis, septic arthritis, and abscess formation (114). Bacteremia has been reported to occur at a rate of 1.5 per 1,000 intestinal infections, with the highest rate occurring in the elderly (115). Persistent diarrheal illness and bacteremia may occur in immunocompromised hosts, such as patients with human immunodeficiency infection (102), and may be difficult to treat. Deaths attributable to *C. jejuni* infection are uncommon (34). The health burden of campylobacteriosis may be under-recognized (45).

C. jejuni infection is the most often recognized infection preceding the development of Guillain-Barré syndrome (GBS), an acute paralytic disease of the peripheral nervous system (85). Certain heat-stable (HS) serotypes appear to be overrepresented in some GBS cases, such as HS:19 and HS:41; however, other more common serotypes are frequently reported (85). The pathogenesis of *Campylobacter*-induced GBS likely involves host immune responses to ganglioside-like epitopes present in the core region of the lipooligosaccharide (36) which, in the susceptible host, mediate damage to the peripheral nerves, where ganglioside targets are highly enriched (145).

Reactive arthritis sometimes follows *Campylobacter* infection, with the onset of pain and joint swelling occurring an average of 2 weeks after the infection and lasting from a few weeks to nearly a year. Reiter's syndrome may also occur in some patients (114). The pathogenesis of *Campylobacter* infection and joint involvement is unknown, but HLA B27 positivity is strongly associated with reactive arthritis (19).

The pathogenesis of *Campylobacter* enteric infection is still not well understood. The infective dose of *Campylobacter* is not well defined, but as few as 1,000 organisms may be capable of causing illness (9). The signs and symptoms of infection suggest an invasive mechanism of disease. A variety of determinants may be important in the virulence of *C. jejuni* infection, including invasiveness (49), the presence or absence of the large virulence plasmid pVir (64), protein glycosylation (120), and motility (53). *C. jejuni* express a cytolethal distending toxin; however, the role of the toxin in pathogenesis is not understood (63). *Campylobacter* spp. do not produce a classic, cholera-like enterotoxin (142).

Campylobacter Species Other Than *C. jejuni* and *C. coli*

Campylobacter species other than *C. jejuni* and *C. coli* are increasingly isolated from cases of human infection in newer studies using culture methods that are more optimal for the non-*C. jejuni* and non-*C. coli* species.

C. fetus subsp. *fetus* is primarily associated with bacteremia and extraintestinal infections in patients with underlying diseases (11). *C. fetus* is also associated with septic abortions, septic arthritis, abscesses, meningitis, endocarditis, mycotic aneurysm, thrombophlebitis, peritonitis, and salpingitis (11). Although gastroenteritis does occur with this species, the incidence is probably underestimated because the organism may not grow well at 42°C and is usually susceptible to cephalothin, an antimicrobial agent used in some common selective media for stool cultures (135). *C. fetus* subsp. *fetus* produces a surface protein microcapsule composed of a high-molecular-weight surface array protein that is essential for virulence (126). *C. fetus* subsp. *venerealis* causes bovine venereal campylobacteriosis and is a cause of bovine infertility. It has rarely been isolated from cases of human infection (126).

C. upsaliensis is a thermotolerant species that causes diarrhea and bacteremia in humans and is also associated with canine and feline gastroenteritis (39, 135). *C. upsaliensis* is susceptible to many antimicrobial agents present in *C. jejuni*-selective media and, thus, is usually not isolated on routine primary isolation media; it can be recovered using the filtration technique described below.

C. lari is a thermophilic species first isolated from gulls of the genus *Larus* and from other avian species, dogs, cats, and chickens. *C. lari* has been infrequently reported to be isolated from humans with bacteremia and gastrointestinal and urinary tract infections. A waterborne outbreak of infection occurred in 1985 and affected more than 100 individuals (121).

Other *Campylobacter* species have been isolated from clinical specimens from patients with a variety of diseases, but their pathogenic role has not been determined. *C. jejuni* subsp. *doylei* is a nitrate-negative subspecies of *C. jejuni* that is rarely recovered from patients with upper gastrointestinal tract infections and gastroenteritis (39, 118). *C. hyointestinalis* has been occasionally associated with proctitis and diarrhea in human infection (24). *C. concisus* is associated primarily with periodontal disease but has also been isolated from patients with bacteremia, foot ulcers, and upper and lower gastrointestinal tract infections (54, 132). Although *C. concisus* has been isolated from many patients with diarrheal illness, it can also be isolated from the feces of healthy individuals, and there is no convincing evidence to date that it causes diarrhea (28, 137). *C. sputorum* biovars have been associated with lung, axillary, scrotal, and groin abscesses (66). *C. sputorum* bv. paraureolyticus, formerly referred to as catalase-negative, urease-positive campylobacter, has been isolated from patients with diarrhea, but the significance of this finding is unknown (97). *C. mucosalis* was reportedly isolated from two children with enteritis (30), but the isolates were subsequently shown to have been misidentified and were actually *C. concisus* (96). *C. helveticus* (117) has been recovered from domestic cats and dogs and has not been reported to have been isolated from human sources. *C. rectus* is primarily isolated from patients with active periodontal infections but has also been isolated from patients with pulmonary infections (107, 116) and a breast abscess (42). *C. curvus* is also isolated from patients with periodontal infections, but its role in gastrointestinal infections is unknown (28). *C. curvus* was isolated from numerous patients with diarrhea (1) and from patients with a liver abscess and pneumonia (42). *C. showae* has been isolated from the human gingival crevice (29). *C. gracilis* has been isolated from patients with appendicitis and peritonitis, bacteremia, soft-tissue abscesses, and pulmonary infections (81). A more extensive review of the clinical significance of non-*C. jejuni* and non-*C. coli* species was published previously (66).

Arcobacter

Arcobacters are aerotolerant, *Campylobacter*-like organisms frequently isolated from bovine and porcine products of abortion and feces of animals with enteritis (143). Two of the five *Arcobacter* species have been associated with human infection. *A. butzleri* has been isolated from patients with bacteremia, endocarditis, peritonitis, and diarrhea (66, 135). *A. cryaerophilus* strains have been previously classified into two DNA-related groups, 1A and 1B (60). *A. cryaerophilus* group 1B has been isolated from patients with bacteremia and diarrhea (66, 135). Group 1A has been isolated from animal sources (60). *A. butzleri* was reported by Vandenberg et al. to be the fourth most common *Campylobacter*-like organism isolated from patients with diarrhea (135) and may be underrecognized if appropriate culture conditions are not used. *A. nitrofigilis,* a nitrogen-fixing bacterium found on the roots of a small marsh plant in Nova Scotia, is not associated with human disease (77). The recently described *A. cibarius* has been isolated only from poultry carcasses; the clinical significance of this species is unknown (48). *A. halophilus* requires increased salt for growth in culture; however, the medical significance of this species is unknown (23).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Fecal Samples

Fecal specimens are preferred for isolating *Campylobacter* species from patients with gastrointestinal infections; however, rectal swabs are acceptable for culture. For hospitalized patients, the 3-day rule (rejection of specimens collected >72 h after admission) should be used as a criterion for acceptability of routine culture requests (40, 47). For routine purposes, testing of a single stool sample has high sensitivity for detection of common enteric pathogens, but testing of two samples may be desirable depending upon clinical circumstances, such as a >2-h delay in transport of the first sample, that could affect recovery (128). A transport

medium should be used when a delay of more than 2 h is anticipated and when rectal swabs are transported. Several types of transport media are useful for *Campylobacter,* including alkaline peptone water with thioglycolate and cystine (140), modified Stuart medium (2), and Cary-Blair medium (16). Transport media such as commercial Stuart medium and buffered glycerol saline do not appear to perform well (140). Modified Cary-Blair medium containing reduced agar (1.6 g/liter) appears to be the most suitable single transport medium for *Campylobacter* as well as other enteric pathogens (140). Specimens received in Cary-Blair medium should be stored at 4°C if processing is not performed immediately. Use of Cary-Blair medium supplemented with laked sheep blood may be effective for prolonged storage of stool samples and recovery of *C. jejuni* (141).

Blood

Campylobacter species, primarily *C. fetus, C. jejuni,* and *C. upsaliensis,* have been isolated from blood; however, in only a few studies have optimal conditions for isolating *Campylobacter* from blood culture systems been evaluated. Both the BACTEC system (BD, Sparks, Md.; aerobic bottles) and the Septi-Chek system (BD) appear to support the growth of the common *Campylobacter* species (59, 65, 139). Other systems, such as anaerobic broth and lysis-centrifugation, may not be as sensitive (59).

DIRECT EXAMINATION

Microscopy

Clinical microbiologists do not normally consider performing Gram stain analysis of stool samples for diagnosis of bacterial gastroenteritis; however, this is a rapid and sensitive method for presumptive diagnosis of *Campylobacter* enteritis. Campylobacters are not easily visualized with the safranin counterstain commonly used in the Gram stain procedure and are somewhat thinner than other enteric gram-negative bacteria; carbol-fuchsin or 0.1% aqueous basic fuchsin should be used as the counterstain for smears of stools or pure cultures (99, 111, 138). Because of their characteristic microscopic morphology, campylobacters may be detected by direct Gram stain examination of stools obtained from patients with acute enteritis, with sensitivity ranging from 66 to 94% and specificity above 95% (99, 111, 138). Phase-contrast and dark-field microscopy have also been used to directly detect motile campylobacters in fresh stool samples; however, the sensitivities of these approaches have not been studied widely, and in our opinion, these methods require significant microscopic expertise (56, 98).

Fecal white blood cells may be present during *Campylobacter* infection and have been reported in 25 to 80% of culture-proven cases (47). While the likelihood of infection with *Campylobacter* or other enteroinvasive pathogens may be higher in the presence of fecal leukocytes, the absence of fecal leukocytes does not rule out the diagnosis (128). Thus, routine examination of stool samples for fecal leukocytes is not recommended as a test for predicting bacterial infection or for selective culturing for *Campylobacter* or other stool pathogens (40).

Antigen Detection

A commercially available antigen detection system for *Campylobacter* in stool samples is available (ProSpecT *Campylobacter;* Alexon-Trend, Inc. [distributed through Remel, Lenexa, Kans.]). Compared with culture, this immunoassay has been shown to vary in sensitivity from 80 to 96%, and it has good specificity, >97% (21, 46, 127). *C. upsaliensis* was found to cross-react in the enzyme immunoassay (21). Antigen may be detected in stored samples at 4°C for several days (25).

Nucleic Acid Detection Techniques

Amplification techniques have been used to detect *Campylobacter* directly in stool samples (103). Molecular approaches to detecting *Campylobacter* directly may reduce the time to detection and improve abilities to identify isolates to the species level and to identify the less common *Campylobacter* species often missed by conventional culture. A commercially available molecular test for detection of *Campylobacter* spp. in fecal samples is not currently available. Molecular approaches are also more expensive than culture and do not provide an isolate for further characterization.

ISOLATION PROCEDURES

Most *Campylobacter* species require a microaerobic atmosphere containing approximately 5% O_2, 10% CO_2, and 85% N_2 for optimal recovery. Several manufacturers produce microaerobic gas generator packs that are suitable for routine use. A trigas incubator (125) or evacuation and replacement of an anaerobic jar with the approximate gas mixture may also be used for routine cultures (82). The concentration of oxygen generated in candle jars is suboptimal for the isolation of *Campylobacter,* and these jars should not be used for routine laboratory isolation procedures (72).

Some species of *Campylobacter,* such as *C. sputorum, C. concisus, C. mucosalis, C. curvus, C. rectus,* and *C. hyointestinalis,* require increased hydrogen for primary isolation and growth. These species will usually not be recovered using the conventional microaerobic conditions since the amount of hydrogen generated in properly used commercial gas packs is <2%. A gas mixture of 10% CO_2 and 6% H_2, with the balance consisting of N_2, in an evacuation-replacement jar is sufficient for isolating hydrogen-requiring species (12). A study by Vandenberg and colleagues reemphasized the requirement for increased hydrogen for isolating certain *Campylobacter* spp. (135).

A number of selective media are recommended for isolating *C. jejuni* and *C. coli.* These include blood-free media, such as charcoal cefoperazone deoxycholate agar (CCDA) (51) and charcoal-based selective medium (CSM) (57), and blood-containing media, such as Campy-CVA medium (L. B. Reller, S. Mirrett, and L. G. Reimer, *Abstr. Annu. Meet. Am. Soc. Microbiol. 1983*, abstr. C-274, p. 357, 1983) and Skirrow medium (113). Charcoal-based medium containing cefoperazone, amphotericin, and teicoplanin (CAT medium) is a selective medium for the primary isolation of *C. upsaliensis* (6). Researchers in two studies, however, did not isolate *C. upsaliensis* from any stool samples by using this medium (28, 46). *C. upsaliensis* may occasionally be recovered on some other selective media. *C. upsaliensis* isolates can also be recovered by using the filtration method, and some strains may grow better in a hydrogen-enriched atmosphere (39, 66).

To achieve the highest yield of *Campylobacter* from stool samples, use of a combination of media including either CCDA or CSM as one of the media appears to be the optimal method (26, 41) and may increase the recovery of *Campylobacter* by as much as 10 to 15% over that with the use of a single medium. If only a single medium can be used because of budgetary constraints, we suggest the Campy-CVA formulation, but a charcoal-based medium such as CCDA

works just as well. In a comparative study, CCDA medium was found to be the most sensitive for detecting *C. jejuni* and *C. coli* when compared with Skirrow medium, CAT agar, and the filtration technique (28).

Most of these selective media have one or more antimicrobial agents, mainly cefoperazone, as the primary inhibitors of enteric bacterial flora. Antimicrobial agents such as cephalothin, colistin, and polymyxin B present in some selective medium formulations are inhibitory to some strains of *C. jejuni* and *C. coli* (38, 90), and are inhibitory to *C. fetus* subsp. *fetus*. *C. jejuni* subsp. *doylei*, *C. upsaliensis*, and *A. butzleri* generally will not grow on cephalothin-containing media. We no longer recommend cephalothin-containing formulations for primary isolation of *Campylobacter* and *Arcobacter* from fecal samples.

If *Campylobacter* infection is suspected at the time blood specimens are drawn, broth media should be subcultured after 24 to 48 h to a nonselective blood agar medium and plates should be incubated under microaerobic conditions at 37°C, preferably with increased hydrogen. This will allow for the isolation of thermophilic and nonthermophilic species. Although commonly used blood culture systems should support the growth of *Campylobacter* and give appropriate signals if cultures are positive, it may be prudent to perform a blind subculture. Similarly, blood drawn in Isolator (Wampole Laboratories, Cranbury, N.J.) tubes for bacterial culture should be inoculated onto a nonselective blood agar plate and incubated under microaerobic conditions at 37°C if *Campylobacter* infection is suspected. If a curved, gram-negative rod is observed upon Gram stain examination of a positive blood culture bottle, an aliquot should be cultured on a nonselective blood agar plate and incubated under microaerobic conditions at 37°C. An alternative staining method such as the use of acridine orange may also be effective for detecting campylobacters in blood culture bottles if the Gram stain is negative.

Optimal conditions for recovery of *Arcobacter* from clinical specimens have not been determined. *Arcobacter* spp. were first isolated on semisolid media designed to isolate *Leptospira* spp. (133, 143). *Arcobacter* species are aerotolerant and have been recovered on certain selective media such as Campy-CVA (5) incubated under microaerobic conditions at 37°C and on nonselective media used in the filtration method (135). Several other media have been reported to recover *Arcobacter* species but have not been studied in clinical settings (5, 20, 133).

Enrichment Cultures

Enrichment broths formulated to enhance the recovery of *Campylobacter* from stool include Preston enrichment (13), Campy-thio (109), and *Campylobacter* enrichment broth (76). Enrichment cultures may be beneficial in instances in which low numbers of organisms may be expected due to delayed transport to the laboratory or after the acute stage of disease, when the concentration of organisms may be low, such as in the investigation of GBS following acute *Campylobacter* infection (84). The clinical effectiveness and cost-effectiveness of using enrichment cultures as part of the routine stool culture setup has not been studied adequately.

Filtration

Filtration techniques designed to isolate *C. jejuni* and *C. coli* as well as other *Campylobacter* species (28, 38, 135) and *Arcobacter* spp. (60, 135) that are susceptible to antibiotics present in most selective media (135) should be used to complement direct culture to selective plating media and not as a replacement. The filtration method is based on the principle that campylobacters can pass through membrane filters (pore sizes, 0.45 to 0.65 μm) with relative ease (because organisms are thin and highly motile) while other stool flora are retained during the short processing time. Cellulose acetate membrane filters with a 0.65-μm pore size are recommended for routine use and are available from a number of suppliers (J. G. Wells, N. D. Puhr, C. M. Patton, M. A. Nicholson, M. A. Lambert, and R. Jerris, *Abstr. Annu. Meet. Am. Soc. Microbiol. 1989*, abstr. C231, p. 432, 1989). Filtration is performed by placing a sterile 0.65-μm-pore-size cellulose acetate filter onto the surface of an agar medium such as antibiotic-free CCDA, CSM, or blood-containing medium. Ten to 15 drops of fecal suspension are placed onto the filter, and the plate is incubated at 37°C for 45 to 60 min. The filter is then removed, and the plate is incubated at 37°C under microaerobic conditions, preferably with an atmosphere containing increased hydrogen (for the hydrogen-requiring species). Stool samples containing ~10^5 CFU of *Campylobacter*/ml will be detected with this method, and thus, the filtration method is not as sensitive as primary culture with selective media (39).

Species within the genus *Campylobacter* and *Arcobacter* have different optimal temperatures for growth. The choice of incubation temperature for routine stool cultures, therefore, is critical in determining the spectrum of species that will be isolated. By convention, most laboratories use 42°C as the primary incubation temperature for *Campylobacter*. This temperature allows the growth of *C. jejuni* and *C. coli* on selective media. *C. upsaliensis* also grows well at 42°C but usually is not recovered on selective media. *C. fetus* exhibits variable growth at this temperature and may not be recovered. *Arcobacter* species are not thermophilic and will generally not be recovered at 42°C.

In contrast, most *Campylobacter* and *Arcobacter* species grow well at 37°C. Selective media, such as Skirrow medium, were devised for use at 42°C and have poor selective properties at 37°C, whereas CCDA and CSM show good selective properties at 37°C (26). Plates should be incubated for a minimum of 72 h before being reported as negative. It has been reported that incubation of CCDA medium for 5 to 6 days increases the yield of *C. jejuni* and *C. coli* compared with that obtained after 2 days of incubation (28).

Because of the expense of including several types of media and the filtration method in the initial workup for campylobacters, a practical approach is to use a single medium for isolation of thermophilic *Campylobacter* spp., such as Campy-CVA or CCDA (or equivalent) incubated at 42°C, in the workup for acute bacterial gastroenteritis. If the primary culture workup is unrevealing and if a patient has persistent diarrhea, cultures for non-*C. jejuni* and non-*C. coli* species may be appropriate. Additional stool samples should be plated on multiple selective media (e.g., CCDA and Campy-CVA), processed by the filtration method as well, and incubated at 37°C under microaerobic conditions with increased hydrogen.

IDENTIFICATION

The identification of campylobacters is made difficult because of their complex and rapidly evolving taxonomy, fastidious growth requirements, and biochemical inertness (Table 2). These problems have resulted in a proliferation of phenotypic and genotypic methods for identifying members of this group (93).

TABLE 2 Useful phenotypic properties of *Campylobacter* and *Arcobacter* species[a]

Organism	Catalase	H_2 required	Urease	H_2S (TSI)	Hippurate hydrolysis	Indoxyl acetate hydrolysis	Aryl sulfatase	Selenite reduction	Growth in 1% glycine
C. jejuni subsp. *jejuni*	+	−	−	−	+	+	V	V	+
C. jejuni subsp. *doylei*	V	−	−	−	V	+	−	−	+
C. coli	+	−	−	V	−	+	−	+	+
C. fetus subsp. *fetus*	+	−	−	−	−	−	−	V	+
C. fetus subsp. *venerealis*	V	−	−	−	−	−	−	V	V
C. lari	+	−	V	−	−	−	−	V	+
C. upsaliensis	−	−	−	−	−	+	−	+	+
C. hyointestinalis subsp. *hyointestinalis*	+	V	−	+	−	−	−	+	+
C. hyointestinalis subsp. *lawsonii*	+	V	+	+[b]	−	−	−	+	V
C. lanienae	+	−	−	−	−	−	ND	+	V
C. sputorum[b] bv. sputorum	−	+	−	+	−	−	+	V	+
C. sputorum bv. faecalis	+	+	−	+	−	−	+	V	+
C. sputorum bv. paraureolyticus	−	+	+	+	−	−	+	V	+
C. helveticus	−	−	−	−	−	+	ND	−	V
C. hominis	−	+[c]	ND	−	−	−	ND	−	+
C. mucosalis	−	+	−	+	−	−	−	V	V
C. concisus	−	+	−	V	−	−	+	V	V
C. curvus	−	+	−	V	V	V	+	−	+
C. rectus	V	+	−	−	−	+	+	−	+
C. showae	+	+	−	−	−	V	+	−	V
C. gracilis[c]	V	ND	−	−	−	V	ND	−	+
A. cryaerophilus	V	−	−	−	−	+	−	−	V
A. butzleri	V	−	−	−	−	+	−	−	+
A. nitrofigilis	+	−	+	−	−	+	−	ND	−
A. skirrowii	+	−	−	−	−	+	−	ND	V
B. ureolyticus	V	+	+	−	−	−	ND	−	+

[a] Adapted from references 29, 60, 67, 71, 105, 117, 129, 131, and 133. +, positive reaction; −, negative reaction; V, variable reaction; ND, not determined; TSI, triple sugar iron agar.
[b] Strains of *C. sputorum* and *C. hyointestinalis* subsp. *lawsonii* normally produce large amounts of H_2S in triple sugar iron agar (67).
[c] Anaerobic growth only.

Campylobacter spp. and *Arcobacter* spp.

Depending on the medium used, *Campylobacter* colonies may have different appearances. In general, *Campylobacter* spp. produce gray, flat, irregular, and spreading colonies. Spreading along the streak line is commonly seen, particularly on freshly prepared media. As the moisture content decreases, the organisms may form round, convex, and glistening colonies with little spreading observed. Thus, proper storage of media to ensure correct moisture content is important for optimal isolation and recognition of *Campylobacter* spp. Hemolysis on blood agar is not observed. *Arcobacter* colonies are morphologically similar to *Campylobacter* colonies (129, 133).

The Gram stain appearance of arcobacters may differ from that of typical campylobacters. *A. butzleri* is only slightly curved, and *A. cryaerophilus* tends to be much more helical in appearance than campylobacters. Commercial systems for identification of *Campylobacter* species were not found to be more accurate than conventional tests (52). Unfortunately, *Campylobacter* species are difficult to differentiate from *Arcobacter* species based on phenotypic tests. However, an aerotolerant species (i.e., one that grows under aerobic conditions) that grows on MacConkey agar (under microaerobic conditions) may be presumptively identified as an *Arcobacter* species. The failure to grow on MacConkey agar, however, does not rule out an *Arcobacter* species.

C. jejuni and *C. coli*

For initial analysis, a Gram stain examination of the colony should be performed along with an oxidase test. Oxidase-positive colonies exhibiting a characteristic Gram stain appearance (e.g., gram-negative, curved to S-shaped rods) isolated from selective media incubated at 42°C under microaerobic conditions can be reliably reported as *Campylobacter* spp., and only hippurate hydrolysis testing is recommended (Fig. 1). Hydrolysis of sodium hippurate is the major test for distinguishing *C. jejuni* (and also *C. jejuni* subsp. *doylei*) from other *Campylobacter* species. *C. jejuni,* the most common *Campylobacter* species, is relatively easy to identify phenotypically; strains isolated on selective media that grow at 42°C, are oxidase positive, show characteristic microscopic morphology, and give a positive result with hippurate hydrolysis should be reported as *C. jejuni* subsp. *jejuni,* and for routine clinical purposes, no other tests need to be performed. Methods for the hippurate hydrolysis test are described elsewhere (73). A large inoculum (i.e., full loop) should be used in performing this test. Occasional strains of *C. jejuni* may be hippurate negative, making them more difficult to identify. Gas-liquid chromatography for detecting benzoic acid (liberated through hydrolysis of sodium hippurate) is the most sensitive assay for hippurate hydrolysis and can be used for more definitive determination. Molecular detection of the *hip*O (hippuricase) gene or other *C. jejuni*-specific markers (see Table 3) by PCR may be useful for identifying phenotypically negative isolates (43). Except in hippuricase activity, which *C. coli* is lacking, *C. coli* and *C. jejuni* are similar biochemically (Table 2). Thus, molecular methods are needed to accurately identify *C. coli*. Tests of susceptibility (inhibition) or resistance of *Campylobacter* spp. to nalidixic acid and cephalothin have routinely been used as an aid for species identification. However, with the increasing prevalence of fluoroquinolone resistance in these species, these disk identification assays can no longer be relied upon.

For species other than *C. jejuni,* phenotypic characterization is more problematic. An algorithm for identification of the thermophilic *Campylobacter* spp. is shown in Fig. 1. The most useful tests for initial identification include those for growth at 25, 37, and 42°C; catalase; hippurate hydrolysis (73); indoxyl acetate hydrolysis (74); and production of H_2S (8). Indoxyl acetate hydrolysis is useful for differentiating some species of thermophilic *Campylobacter*. The disk method is rapid (5 to 30 min) and easy to perform. Disks are prepared by making a 20% solution of indoxyl acetate in acetone and adding 25 μl to a blank paper disk (0.6 cm). Dry the disks at room temperature and store them at 4°C in a brown tube with desiccant. Indoxyl acetate disks are also available ready made from commercial sources. Place a large loopful of growth from a plate onto the indoxyl acetate disk and add a drop of sterile distilled water. Hydrolysis of indoxyl acetate is indicated by the appearance of dark blue or blue-green. Weakly positive strains show pale blue in 10 to 30 min. Absence of color change is indicative of a negative reaction.

Additional tests can be performed to aid in the identification of *Campylobacter* spp. (Fig. 1). To obtain consistent and reproducible results, a standardized suspension and inoculum should be used for performing phenotypic tests. For growth temperature and oxygen tolerance studies, a suspension of the organism in heart infusion broth or tryptic soy broth to the turbidity of a McFarland standard of 1 should be used. A fiber-tipped swab dipped in the broth suspension should be used to make a single streak across the plate (Mueller-Hinton agar with 5% sheep blood is a suitable medium) and the plate should be incubated at the desired temperature and/or under the appropriate atmospheric conditions (8, 82).

Several commercial systems have been developed to aid in the identification of *Campylobacter* spp. to the genus level. Two immunologic reagents are currently available in the United States for culture identification, INDX Campy-JCL (Hardy Diagnostics, Santa Maria, Calif., and Fisher Scientific, Hampton, N.H.) and Dry Spot campylobacter test kit (Remel). INDX Campy-JCL was previously evaluated and does not differentiate between *C. jejuni* and *C. coli* (86). The Dry Spot campylobacter latex test is reported by the manufacturer to identify, but not differentiate among, *C. jejuni, C. coli, C. lari,* and *C. upsaliensis,* with variable results for *C. fetus* subsp. *fetus* (Oxoid USA; http://www.oxoid.com). A DNA probe (Accuprobe; Gen-Probe Inc., San Diego, Calif.) directed against *Campylobacter* rRNA sequences identifies *Campylobacter* species to the genus level and detects *C. jejuni* subsp. *jejuni, C. jejuni* subsp. *doylei, C. coli,* and *C. lari* (106, 124). However, the probe also hybridized with 2 of 17 *C. hyointestinalis* strains (106). Thus, these methods may be useful for confirming the identities of *Campylobacter* species to the genus level if other tests are not conclusive. However, cross-reactivity in these tests with closely related taxa and/or more newly described species needs to be determined.

Because many species of *Campylobacter* and *Arcobacter* are difficult to identify by phenotypic testing, tests for detection of species-specific sequences via PCR have been developed. *C. jejuni, C. coli,* and other *Campylobacter* and *Arcobacter* species have been reported to be reliably differentiated based on polymorphism in the *ceuE* gene (37), a GTPase gene (136), a 16S rRNA gene (75), a 23S rRNA gene (50), and the *glyA* gene (4). Table 3 lists some of the more commonly used published PCR assays for the identification of *C. jejuni* and *C. coli*. Recently published evaluations of some of these assays highlight the importance of validating their sensitivities and specificities (15, 94). In addition, it has been reported that the use of heated lysates rather than purified DNA may in some cases not always provide a suitable reaction template for these PCR assays (80, 94). As false

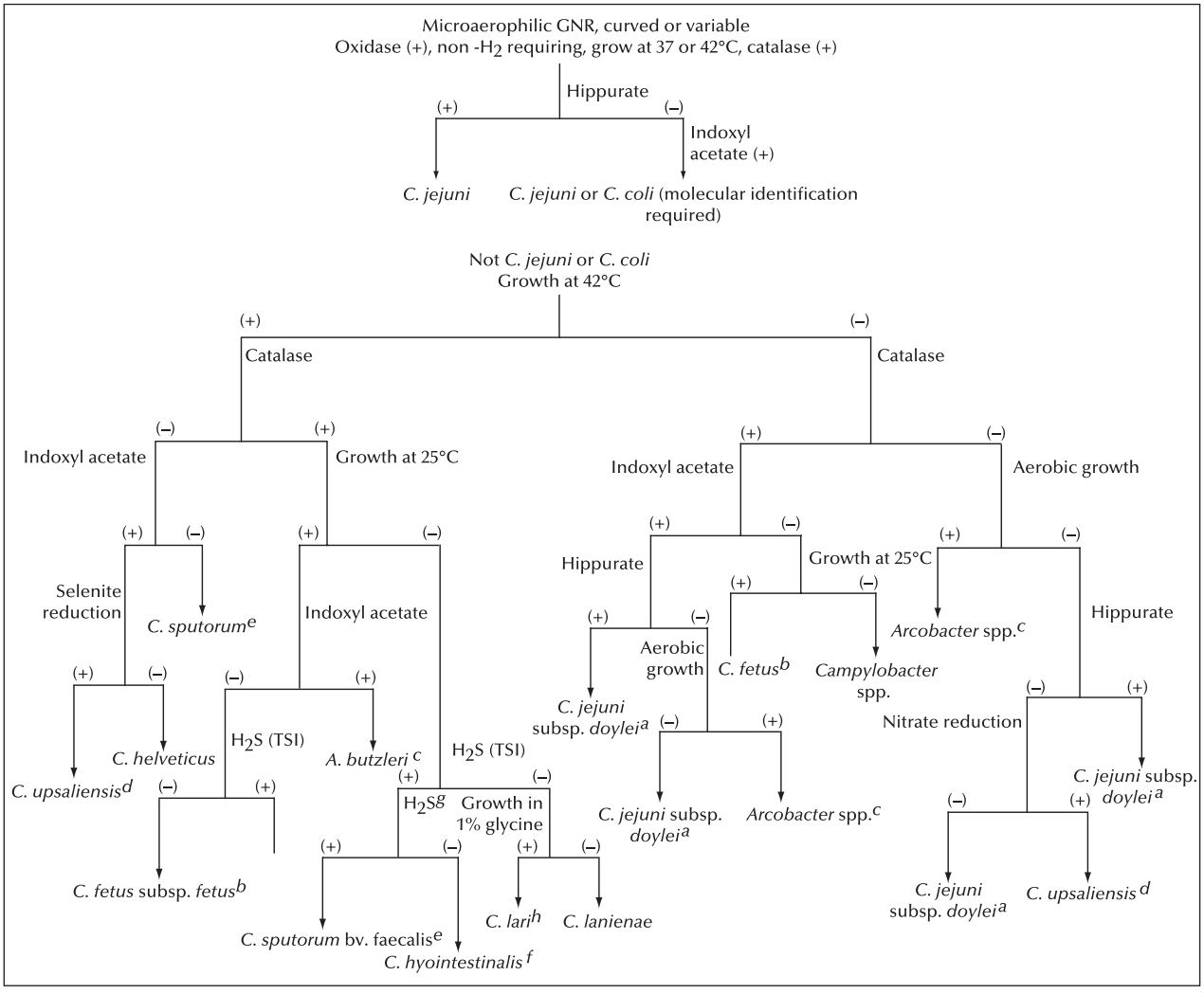


FIGURE 1 Practical algorithm for identifying *Campylobacter* and *Arcobacter* spp. that do not require increased hydrogen for primary isolation in the clinical laboratory. GNR, gram-negative rods; TSI, triple sugar iron agar. [a]*C. jejuni* subsp. *doylei* produces variable results in the catalase and hippurate tests. [b]Strains of *C. fetus* subsp. *fetus* can be variable for growth at 42°C. [c]*A. butzleri* is variable for growth at 42°C and produces variable results in the catalase test. *A. cryaerophilus* produces variable results in the catalase test. [d]*C. upsaliensis* is variable for growth at 42°C. [e]Results for the catalase test are biovar dependent; *C. sputorum* bv. faecalis is catalase positive. Some strains of *C. sputorum* may grow better in environments with increased hydrogen concentration. [f]*C. hyointestinalis* grows under microaerophilic conditions, but some strains require additional hydrogen (66). [g]Rapid H_2S test (69). [h]Urease-positive *C. lari* strains are referred to as the urease-positive thermophilic campylobacter group.

negatives or nonspecifically amplified product(s) have been noted for some of the *C. jejuni*-specific assays, a second PCR targeting another *C. jejuni*-specific gene may be necessary in some instances.

TYPING SYSTEMS

Typing systems for *Campylobacter* epidemiologic studies vary in complexities and abilities to discriminate among strains. Common phenotypic methods that have been applied include biotyping, phage typing, and serotyping (89, 100). The most frequently used system is serotyping. The heat-labile serotyping scheme originally described by Lior et al. (70) can detect over 100 serotypes of *C. jejuni, C. coli,* and *C. lari*. Uncharacterized bacterial surface antigens and, in some serotypes, flagellar antigens are the serodeterminants for this serotyping system (3). The heat-stable (HS) serotyping scheme described by Penner and Hennessy (101) detects 60 types of *C. jejuni* and *C. coli* (100). Initially thought to detect lipopolysaccharide antigenic determinants, the HS system has been shown to detect a *Campylobacter* capsular polysaccharide (55). Serotyping is performed in only a few reference laboratories worldwide because of the time and expense needed to maintain quality antisera. A serotyping reagent kit is also commercially available (Denka Seiken USA Inc., Campbell, Calif.).

TABLE 3 Differentiation of *C. jejuni* and *C. coli* by PCR

Species	Target gene	Primers[a]	Reference
C. jejuni	Unknown	F, 5′-CAT CTT CCC TAG TCA AGC CT-3′ R, 5′-AAG ATA TGG CTC TAG CAA GAC-3′	134
	*hip*O	F, 5′-GAA GAG GGT TTG GGT GGT-3′ R, 5′-AGC TAG CTT CGC ATA ATA ACT TG-3′	68
	*map*A	F, 5′-ATG TTT AAA AAA TTT TTG-3′ R, 5′-AAG TTC AGA GAT TAA ACT AG-3′	119
C. coli	Unknown	F, 5′-AGG CAA GGG AGC CTT TAA TC-3′ R, 5′-TAT CCC TAT CTA CAA ATT CGC-3′	134
	*ceu*E	F, 5′-ATG AAA AAA TAT TTA GTT TTT GCA-3′ R, 5′-ATT TTA TTA TTT GTA GCA GCG-3′	37
	Putative aspartokinase gene	F, 5′-GGT ATG ATT TCT ACA AAG CGA G-3′ R, 5′-ATA AAA GAC TAT CGT CGC GTG-3′	68

[a] F, forward primer; R, reverse primer.

The limitations of phenotypic subtyping methods and the rapid growth of molecular biology techniques in the 1990s led to the development of a range of molecular biology-based subtyping methods, such as restriction endonuclease analysis, ribotyping, PCR-based techniques, pulsed-field gel electrophoresis of macrorestricted chromosomal DNA (PFGE), and amplified fragment length polymorphism (89, 100). The development of a rapid one-day standardized PFGE protocol (108), which is used by participants of the PulseNet national surveillance network for foodborne pathogens (http://www.cdc.gov/pulsenet), has facilitated the use of the PFGE approach for investigations of campylobacteriosis outbreaks (92). However, interpretation of data can be difficult since genomic rearrangements can lead to changes in PFGE profiles (31). Advances in DNA sequencing technology have provided a means to investigate strain variation at the nucleotide level and have led to the emergence of DNA sequencing-based subtyping systems such as multilocus sequence typing (22). In addition, the generation of *Campylobacter* whole-genome sequences has led the way for the development of a new technique, genomotyping based on microarray technology (61). At present, no method alone is adequate for all applications, and a combination of methods such as serotyping and molecular methods should be used for reliably determining strain relatedness and for studying the epidemiology of *Campylobacter* infections (89).

SEROLOGIC TESTS

Serum immunoglobulin G (IgG), IgM, and IgA levels rise in response to infection, but elevated serum and fecal IgA levels appear during the first few weeks of infection and then fall rapidly (10, 123). Serum antibody assays vary in both sensitivity and specificity for detecting *Campylobacter* infection, and test performance appears to be population dependent. Patients with *Campylobacter* infection may have false-positive legionella antibody tests (14). Serologic testing appears to be useful for epidemiologic investigations and is not recommended for routine diagnosis (122).

ANTIMICROBIAL SUSCEPTIBILITY

C. jejuni and *C. coli* have variable susceptibilities to a variety of antimicrobial agents, including macrolides, fluoroquinolones, aminoglycosides, chloramphenicol, nitrofurantoin, and tetracycline (http://www.cdc.gov/NARMS) (11). Most isolates are not susceptible to cephalosporins and penicillins (11). Azithromycin and erythromycin are drugs of choice for treating *C. jejuni* gastrointestinal infections, and for susceptible organisms, ciprofloxacin or norfloxacin may also be used (35). Early therapy of susceptible *Campylobacter* infection with erythromycin or ciprofloxacin is effective in eliminating the organism from stools and may also reduce the duration of symptoms associated with infection (11).

C. jejuni is generally susceptible to erythromycin, with resistance rates of less than 5% (27). Rates of erythromycin resistance in *C. coli* vary considerably, with up to 80% of strains showing resistance in some studies (27). Although ciprofloxacin has been effective in treating *Campylobacter* infections, the emergence of ciprofloxacin resistance during therapy has been reported (104). Several in vitro studies now show significant rates of resistance to fluoroquinolones (27, 58, 87). Individuals infected with fluoroquinolone-resistant *C. jejuni* have been shown to have diarrhea for a longer duration, and thus, routine testing of isolates may be indicated (88). *C. jejuni* and *C. coli* may also produce a β-lactamase that appears to be active against amoxicillin, ampicillin, and ticarcillin; this enzyme has been reported to be inhibited by clavulanic acid but not sulbactam or tazobactam (62).

Parenteral therapy is used to treat systemic *C. fetus* infections; drugs used include erythromycin, ampicillin, aminoglycosides, and chloramphenicol, depending upon the type of infection (11). *C. gracilis* is generally susceptible to a variety of antimicrobial agents, including amoxicillin-clavulanate, cefoxitin, ceftriaxone, clindamycin, metronidazole, and piperacillin-tazobactam (80).

Agar dilution is the method recognized by the Clinical and Laboratory Standards Institute (formerly NCCLS) for testing *Campylobacter* spp., with published quality control ranges for several antimicrobial agents (18, 78). A broth microdilution method with published quality control ranges for several antimicrobial agents was also recently approved by the Clinical and Laboratory Standards Institute (79). The E test (PDM Epsilometer; AB Biodisk, Solna, Sweden) using Mueller-Hinton agar with 5% sheep blood compares favorably with the agar dilution method (7).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Campylobacter species, including the common thermophilic species *C. jejuni* and *C. coli,* should be sought in all diarrheic

stools submitted to the laboratory for routine culture. Except for epidemiological purposes, cultures of formed stools should not be performed. Isolation of *Campylobacter* from a patient with acute diarrhea is usually significant since the carrier rate in developed countries is quite low; however, in developing countries, isolation might be more difficult to interpret, especially in the presence of other enteric pathogens. In acute infection, there is usually a high number of organisms in the stool; however, the quantity of organisms is not related to the severity of infection, nor is it indicative of a carrier state. Gram stain analysis of fecal samples for organisms with typical *Campylobacter* morphology is a highly sensitive and specific test that is currently underutilized; it should be performed for rapid preliminary diagnosis of *Campylobacter* infection. Other species, such as *C. fetus* subsp. *fetus* and *C. upsaliensis*, may be important causes of diarrhea and are not isolated on routine selective media. Special methods including alternative incubation techniques are required as described in this chapter and should be performed by special request. Oxidase-positive, curved, gram-negative rods that are hippurate hydrolysis positive should be reported as *C. jejuni* without further workup. The importance of identifying other species will depend upon the clinical circumstances, but identification tests should always be performed on isolates from blood or other sterile sites since results may influence antimicrobial therapy decisions. Given that fluoroquinolone resistance is present in a significant proportion of *C. jejuni* isolates, fluoroquinolone susceptibility testing is suggested for patients who are receiving or being considered for therapy of gastroenteritis. Susceptibility testing should be performed on all isolates from sterile clinical sites.

Use of trade names is for identification only and does not imply endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

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*Helicobacter**

JAMES G. FOX AND FRANCIS MEGRAUD

60

TAXONOMY

Gastric spiral-shaped bacteria have been observed in animals and humans for more than 100 years. The first recorded observations of gastric spiral-shaped bacteria in animals were made by Rappin in 1881 and Bizzozero in 1893. In 1896, Salomon noted spiral bacteria in the stomachs of dogs, cats, and Norway rats (152), but the first observation in humans was made by Krienitz in 1906 (80). In 1982, *Campylobacter pyloridis* (later known as *Helicobacter pylori*) was successfully cultured from stomach biopsy specimens from human patients with gastritis (178). Subsequently, other spiral gram-negative bacteria have been observed in and isolated from the gastrointestinal tracts of mammals such as cats, dogs, ferrets, sea mammals, and rodents (37).

Initially, many spiral gram-negative bacteria isolated from the mammalian gastrointestinal tract were grouped as campylobacters. This classification was based on similar microscopic and ultrastructural morphologies, common microaerobic growth requirements, and similar ecologic niches (Table 1). However, partial sequencing of 16S rRNA genes provided evidence that *Helicobacter pylori* belonged in a different genus (148). The genus *Helicobacter* was formally distinguished from other gram-negative curved rods (e.g., *Campylobacter*) following extensive analysis of enzymatic activities, fatty acid profiles, growth characteristics, and nucleic acid hybridization profiles, 16S rRNA sequence analysis, and most recently, 23S rRNA analysis (20, 51, 124, 133, 173). Sequencing of the *H. pylori* (1, 169) and *Helicobacter hepaticus* (164) genomes has emphasized differences but also similarities between *Campylobacter* and *Helicobacter* organisms.

DESCRIPTION OF THE GENUS

The genus *Helicobacter* comprises 23 species formally validated by international rules of nomenclature (21, 124, 125), with at least a dozen species awaiting validation or considered to be candidate species. Four of the latter, *Helicobacter cetorum,* isolated from stomachs of cetaceans; *Helicobacter winghamensis,* isolated from diarrheic patients; and *Helicobacter marmotae* and *Helicobacter mastomyrinus,* isolated from intestines and livers of rodents, have been formally named (but not validated by the *International Journal of Systematic and Evolutionary Microbiology*) and are listed in Table 1 (44, 64, 105, 157). The genus includes spiral or curved bacilli ranging from 0.3 to 1.0 μm in width and 1.5 to 10.0 μm in length. Helicobacters are gram-negative, non-spore-forming rods that may form spheroid or coccoid bodies if they are cultured for a prolonged period or if growth conditions are not optimal (Fig. 1A and B). These bacteria are motile and usually possess either single or multiple bipolar sheathed flagella (Table 1). *H. pylori* isolates, in contrast to those of other *Helicobacter* species, have multiple monopolar sheathed flagella. Several helicobacters *(Helicobacter pullorum, Helicobacter rodentium, Helicobacter mesocricatorum, Helicobacter canadensis,* and *H. winghamensis)* have unsheathed flagella like those of the campylobacters. The animal gastric helicobacters, *Helicobacter bizzozeronii, Helicobacter felis,* and *Helicobacter salomonis,* as well as the human-animal gastric pathogen "*Helicobacter heilmannii*" (formerly *Gastrospirillum hominis*), have distinctive, tightly spiraled morphologies under light and transmission electron microscopy (Fig. 1C). *H. pylori* can be induced in liquid culture to assume the morphology of "*H. heilmannii,*" so these distinctions may not be absolute in vivo (33, 127).

Helicobacters are microaerobic and possess respiratory metabolic capabilities. Successful cultivation of these organisms requires a humid atmosphere maintained at 37°C with reduced levels of oxygen (5 to 10%) and increased levels of carbon dioxide (5 to 12%). Atmospheric hydrogen (as much as 5 to 10%) either is required or stimulates the growth of these organisms. Most *Helicobacter* species grow poorly, if at all, in routine aerobic atmospheres. Several helicobacters, including *H. felis* (88), *H. hepaticus* (40), and *H. rodentium* (156), can grow both anaerobically and microaerobically. Interestingly, *Helicobacter ganmani,* a rodent enteric organism, requires anaerobic conditions for growth (146).

Several biochemical and genetic criteria distinguish this genus, although significant intragenus variation exists with respect to each trait (Table 1). All helicobacters are oxidase positive and appear to be relatively inert with respect to carbohydrate pathways as determined by conventional methods. Nuclear magnetic resonance spectroscopy (65) and genomic (1) studies have revealed the existence of carbohydrate catabolic pathways in *H. pylori* (65). Comparative genomic analysis of *H. pylori* supports the presence of important catabolic pathways such as the pentose phosphate shunt, the Entner-Doudoroff pathway, glycolysis, and an

* This chapter contains information presented in chapter 58 by James Versalovic and James G. Fox in the eighth edition of this Manual.

TABLE 1 Habitats and phenotypic characteristics of *Helicobacter* species[a]

Helicobacter taxon	Source(s)	Primary site(s)	Catalase production	Nitrate reduction	Alkaline phosphatase	Urease	Indoxyl acetate hydrolysis	γ-Glutamyl transferase	Growth		Resistance to[b]:		Flagella
									At 42°C	With 1% glycine	Nalidixic acid	Cephalothin	
Human													
H. bizzozeronii[c]	Human, cat, dog, primate	Stomach	+	+	+	+	+	+	+	−	R	S	Bipolar
H. canis	Human, cat, dog	Intestine	−	−	+	−	+	+	+	−	S	I	Bipolar
H. canadensis	Human, goose	Intestine	+	+/−	−	−	+	−	+	+	R	R	Mono/bipolar
H. cinaedi	Human, hamster, macaque, fox, rat, dog	Intestine	+	+	−	−	−	−	−	+	S	I	Bipolar
H. fennelliae	Human	Intestine	+	−	+	−	+	−	−	+	S	S	Bipolar
H. pullorum	Human, chicken	Intestine	+	+	−	−	−	ND[d]	+	−	R	S	Monopolar
H. pylori	Human	Stomach	+	−	+	+	−	+	−	−	R	S	Monopolar
Helicobacter sp. strain flexispira taxon 8[e]	Human, dog, sheep, mouse	Intestine	+/−	−	−	+	−	+	+	−	R	R	Bipolar
H. winghamensis	Human	Intestine	−	−	−	−	+	ND	−	+	R	R	Bipolar
Nonhuman													
H. acinonychis	Cheetah	Stomach	+	−	+	+	−	+	−	−	R	S	Bipolar
H. aurati	Hamster	Stomach, intestine	+	−	−	+	+	+	+	−	S	R	Bipolar
H. bilis	Mouse, dog, rat	Intestine, liver	+	+	−	+	−	+	+	+	R	R	Bipolar
H. cetorum	Dolphin, whale	Stomach	+	−	−	+	−	+	+	ND	I	S	Bipolar
H. cholecystus	Hamster	Liver	+	+	+	−	−	−	+	+	I	R	Monopolar
H. felis	Cat, dog	Stomach	+	+	+	+	−	+	+	−	R	S	Bipolar
H. ganmani	Mouse	Intestine	+	+	−	−	−	ND	−	−	R	S	Bipolar
H. hepaticus	Mouse	Intestine, liver	+	+	−	+	+	−	−	+	R	R	Bipolar
H. marmotae	Woodchuck, cat	Intestine, liver	+	−	+	+	−	−	+	+	R	R	Bipolar
H. mastomyrinus	Mastomys, mouse	Intestine, liver	+	−	−	+	−	−	+	+	R	R	Bipolar
H. mesocricetorum	Hamster	Intestine	+	+	+	−	ND	−	+	−	S	R	Bipolar
H. muridarum	Mouse, rat	Intestine	+	−	+	+	+	+	−	−	R	R	Bipolar
H. mustelae	Ferret, mink	Stomach	+	+	+	+	+	+	+	−	S	R	Bipolar
H. pametensis	Bird, pig	Intestine	+	+	+	−	−	−	+	+	S	S	Peritrichous
H. rodentium	Mouse	Intestine	+	+	−	−	−	−	+	+	R	R	Bipolar
H. salomonis	Dog	Stomach	+	+	+	+	+	+	−	ND	R	S	Bipolar
H. typhlonius	Mouse	Intestine	+	+	−	−	−	−	+	+	S	R	Bipolar
H. trogontum	Rat	Intestine	+	+	−	+	−	+	+	ND	R	R	Bipolar

[a] Data are from references 21, 38, 40, 42, 43, 61, 69, 105, 133, 156, 160, 161, 173, 176, and 180. All *Helicobacter* species are oxidase positive and lack the ability to oxidize or ferment carbohydrates in routine reactions.

[b] Resistance is determined by disk diffusion. Isolates are incubated for several days at 37°C on blood-containing medium containing 30-μg antibiotic disks. Microaerobic conditions are typically used, and exact incubation times vary among organisms. Resistance (R) is defined as the complete absence of an inhibition zone, whereas intermediate (I; zones usually <15 mm in diameter) and susceptible (S; zones usually >20 mm in diameter) isolates have visible inhibition zones of various sizes.

[c] Likely the same as "*H. heilmannii*." "*H. heilmannii*" (formerly *Gastrospirillum hominis*) has the same phenotype as that listed here for *H. bizzozeronii*. Only a single "*H. heilmannii*" strain has been isolated by culture (4), and thus "*H. heilmannii*" has not been included in Table 1.

[d] ND, not determined.

[e] Formerly regarded as "*Flexispira rappini*," now subgrouped into 10 taxa (21).

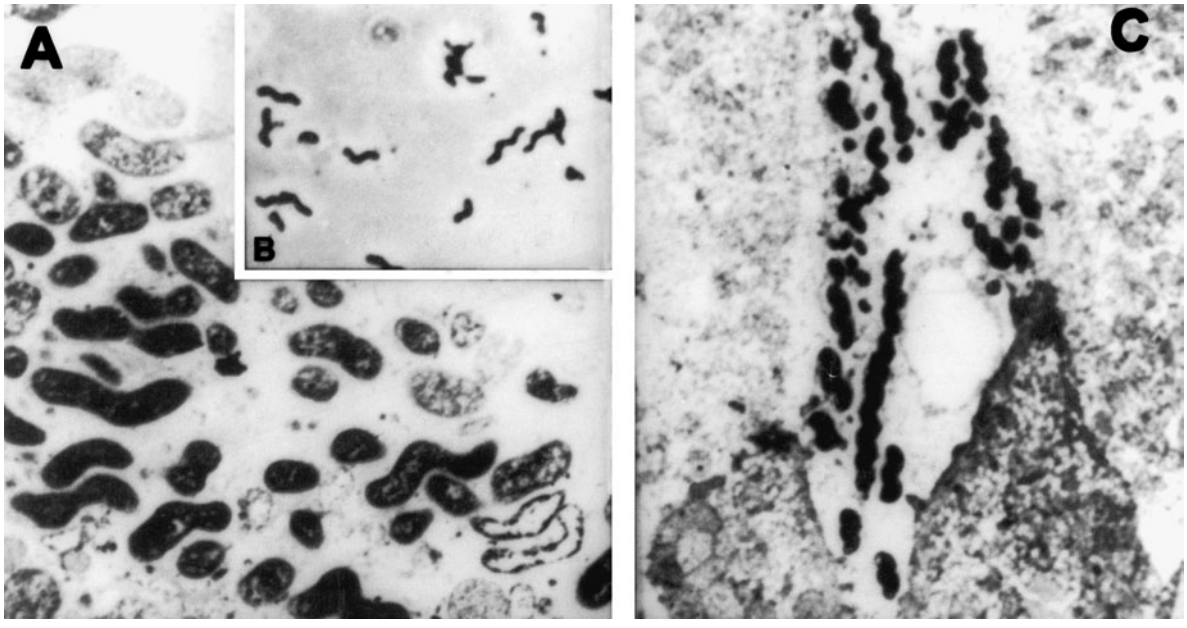


FIGURE 1 Microscopic morphology of *H. pylori* and "*H. heilmanni.*" (A) Transmission electron micrograph of a gastric biopsy specimen from an *H. pylori*-infected individual. (B) Phase-contrast micrograph of *H. pylori.* (C) Transmission electron micrograph of bacteria resembling "*H. heilmannii*" in the gastric pits of an infected cat. Adapted from reference 43 with permission.

altered noncylic version of the Krebs reaction sequence (97). Members of the genus *Helicobacter* have G+C contents ranging from 30 to 48 mol% (180), similar to those of the campylobacters.

NATURAL HABITATS

Helicobacter species have been isolated from the gastrointestinal and hepatobiliary tracts of mammals and birds (Table 1). In this chapter, we will refer to gastric (stomach) and enterohepatic (intestine and liver or bile) helicobacters in order to distinguish these organisms according to their preferred niche(s) in the gastrointestinal tract. Gastric helicobacters possess several unifying features, including phylogenetic clustering (56) and urease activity, and most have the ability to form discrete colonies on plated media (176). Gastric helicobacters inhabit primarily the stomach either within or beneath the mucus gel layer adjacent to the epithelium and rarely invade the bloodstream (in contrast to enterohepatic helicobacters). Recent data suggest that *H. pylori* may on occasion be located intracellularly (121, 154). *H. pylori* colonizes the cardia, corpus, and antrum (distal portion) of the human stomach. Gastric helicobacters may also be found transiently in areas of gastric metaplasia of the proximal small intestine (duodenum), gastric juice (vomitus), saliva, and feces.

The reservoir of *H. pylori* is essentially the human stomach, and *H. pylori* can be considered the gastric helicobacter of humans (103). However, *H. pylori* strains have been isolated occasionally from animals (cats and nonhuman primates), which were most likely infected by humans because of either close cohabitation with humans or, in the case of cats, inadvertent exposure to *H. pylori* while being maintained in a vivarium (26, 60). Conversely, "*H. heilmannii*" is an inhabitant of the stomachs of pigs, cats, dogs, wild rats, and nonhuman primates, and infection with this organism is considered to have zoonotic potential (162).

The route of transmission of *H. pylori* from one human stomach to another human stomach has not been definitely identified. There is evidence for both oral-oral and fecal-oral transmission. Fecal-oral transmission implies (i) that *H. pylori* must survive gut passage (25) which apparently occurs mainly in the case of a short transit time (diarrhea) (131), (ii) that fecal hygiene is not optimal, and (iii) that the water supply is not treated properly. These conditions usually exist in developing countries, making likely that fecal-oral transmission occurs in this context. The rationale for oral-oral transmission relies on the presence of *H. pylori* in gastric juice which has been regurgitated, thus allowing *H. pylori* to temporarily colonize the oral cavity. There are reports of isolation of *H. pylori* from dental plaque and saliva (52, 77, 131). Indeed, a recent 6-year prospective study demonstrated that dental professionals are at greater risk of being infected with *H. pylori* than are controls (98). *H. pylori* could then be transmitted to another subject via the oral-oral route. Another possibility is via vomitus since *H. pylori* can remain viable in this biological medium for hours (7, 131). In any case, the oral-oral route of transmission is probably less efficient than the fecal-oral one. This fact contributes to the lower *H. pylori* prevalence in developed countries. Transmission appears to be most frequent in intrafamilial settings during childhood and accounts for a maximum of two-thirds of the cases; however, in a third of the cases, children are infected while their parents are not (24).

In contrast to the gastric helicobacters, enterohepatic helicobacters inhabit the lower bowels and hepatobiliary tracts of mammals and birds. In humans, enterohepatic helicobacters *(H. canadensis, Helicobacter canis, Helicobacter cinaedi, Helicobacter fennelliae, "Helicobacter rappini," H. pullorum, and H. winghamensis)* have been isolated from rectal swabs and feces and in some cases from blood and other extraintestinal sites (13, 105, 160, 161). Several of these as

well as other *Helicobacter* spp. have also been identified by molecular techniques in the hepatobiliary tissues of humans (6, 37, 39).

CLINICAL SIGNIFICANCE

Gastric Helicobacters

H. pylori is the main cause of peptic ulcer disease and a major risk factor for gastric cancer (165). Warren and Marshall (178) first proposed the association of *H. pylori* with peptic ulcer disease. In February 1994, the U.S. National Institutes of Health Consensus Development Conference concluded that *H. pylori* infection is the major cause of peptic ulcer disease and stated that all patients with confirmed peptic ulcer disease associated with *H. pylori* infection should receive antimicrobial treatment (113). This statement has been systematically repeated in all consensus conferences held worldwide since then (89). In June 1994, the International Agency for Research on Cancer Working Group of the World Health Organization identified *H. pylori* as a group I, or definite, human carcinogen. Approximately 50% of the world's population is estimated to be infected with *H. pylori*. However, there is a large difference in the prevalences of *H. pylori* infection among developing and developed countries (140, 181). In developing countries, the infection occurs very early in life and most of the children are infected by the age of 10. The prevalence remains in the range of 80 to 90% for all adult age groups in developing countries. In developed countries, a progressive increase in prevalence is observed, from a low percentage of infection in children to 40 to 50% infection rates in the older age groups. This is not the consequence of a progressive acquisition of the infection but rather the result of a cohort effect (8). Indeed, the majority of subjects acquire the infection in childhood, and the infection persists lifelong. Thus, if the oldest individuals have a higher *H. pylori* infection prevalence, it is because the risk of becoming infected was higher when they were children.

Individuals infected with *H. pylori* may develop acute gastritis (abdominal pain, nausea, and vomiting) within 2 weeks following infection. *H. pylori* establishes a chronic infection in the majority of infected people, represented by chronic gastritis of different types. Prominent mucosal inflammation in chronic active gastritis is often evident in the antrum (antral predominant gastritis), predisposing to hyperacidity and duodenal ulcer disease. Many patients infected with *H. pylori* have recurrent abdominal symptoms (nonulcer dyspepsia) without ulcer disease, and there appears to be a clinical benefit in eradicating *H. pylori* in these patients (108). Inflammation of the duodenum (duodenitis) often occurs with *H. pylori* infection, and duodenal ulcers develop in as many as 16% of infected individuals. *H. pylori* infection has been associated with the majority of duodenal and gastric ulcers (3). *H. pylori*-associated multifocal atrophic pangastritis or atrophic corpus-predominant gastritis results from long-standing infection and is characterized by glandular atrophy, intestinal metaplasia, and sparse inflammatory cells, pathophysiological features which manifest in the patient as low acidity and an increased risk of gastric carcinoma (28, 62). *H. pylori* infection is an independent risk factor for the development of atrophic gastritis, gastric ulcer disease (166), gastric adenocarcinomas (118, 129), and gastric mucosa-associated lymphoid tissue (MALT) lymphomas (32). Gastric MALT lymphoma, a rare stomach cancer, is caused by *H. pylori* infection. Indeed, it is the only cancer which can be cured by antibiotics (15, 183). However, advanced cases of MALT lymphoma which involve genetic translocations do not appear to respond to antibiotic treatment (2).

"*H. heilmannii*" (formerly *Gastrospirillum hominis*) has been observed in human gastric biopsy specimens (29, 67, 158) and cultured, according to one report, from human stomach tissue (4). The species name has not been formally recognized since significant nucleic acid sequence variation exists among various human isolates, resulting in the designation of multiple genotypes (19). Infection with the large gastric spiral helicobacters has been associated with mild to moderate gastritis in cats and dogs (119), peptic ulcer disease in swine (143), and gastritis, peptic ulcer disease, and gastric MALT lymphomas in humans (66, 67, 111, 170). "*H. heilmannii*"-like organisms are relatively uncommon in humans, being present in fewer than 1% of human gastric endoscopy specimens, in contrast to *H. pylori,* which can be present in >90% in some populations. *Helicobacter* species have been observed in and isolated from the stomach tissues of other mammals, including cats, dogs, ferrets, rodents, sea mammals, and nonhuman primates (Table 1).

Enterohepatic Helicobacters

Multiple species, including *H. cinaedi* and *H. fennelliae,* have been implicated as causes of human gastroenteritis and bacteremia, particularly in immunocompromised individuals (54, 86, 144). Cases of human gastroenteritis have also been associated with infection with *H. canadensis* (38), *H. canis* (161), *H. pullorum* (43, 160), and *H. winghamensis* (105). *Helicobacter* sp. strain flexispira organisms have been divided into 10 taxonomic taxa (21). *Helicobacter* sp. strain flexispira taxon 8 includes clinically relevant isolates that have been implicated in cases of human gastroenteritis (5, 149) and bacteremia (21). In contrast to *H. pylori*, *H. cinaedi* has been identified in the blood of multiple patients with febrile bacteremia and has been associated with multifocal cellulitis or monoarticular arthritis in a subset of infected individuals (12, 75). *H. cinaedi* (previously identified in some reports as *Helicobacter* sp. strain Mainz) (174) was cultured from the blood and joint fluid of a human immunodeficiency virus type 1-infected male with septic arthritis and recurrent fever (69). *H. fennelliae* has occasionally been isolated from human blood (115) and was associated with septic shock in a non-human immunodeficiency virus type 1-infected individual (68). Presumably, these bacteremia-associated helicobacters invaded the bloodstream via colonization of the human lower gastrointestinal tract.

Enterohepatic *Helicobacter* species have been detected in the human hepatobiliary tract by using molecular methods. In Chilean patients with chronic cholecystitis, *Helicobacter bilis* and *H. pullorum* were detected in gallbladder tissue and bile specimens (39). *Helicobacter* spp. have been identified in liver specimens from patients with primary sclerosing cholangitis (117), cholangiocarcinoma (116), and hepatocellular carcinoma (6, 116) and most recently in livers (with cirrhosis and hepatocellular carcinoma) of patients with hepatitis C viral infections (147) by genus-specific PCR amplification and partial DNA sequencing. The pathogenic significance of *Helicobacter* species DNA demonstrated by PCR amplification is not clear since helicobacters have not been cultured from the human hepatobiliary tract.

Several other species of enterohepatic helicobacters have not been isolated to date from human specimens, even though they cause significant diseases in other mammals (Table 1). In addition to the intestine, enterohepatic helicobacters colonize and cause inflammation in the mammalian hepatobiliary tract. For example, the isolation of *H. cinaedi*

from the colon and liver of a 2-year-old rhesus monkey (42) and the isolation of *H. canis* from a dog with hepatitis (41) implicate these organisms as possible etiologic agents of chronic hepatitis in nonhuman primates and dogs, respectively. *H. hepaticus* infection is associated with multifocal necrotic hepatitis in several strains of barrier-maintained mice and is responsible for the development of hepatic adenomas and hepatocellular carcinomas in A/JCr mice, $B_6C_3F_1$ mice, and A × B recombinant inbred mice (40, 45, 70). Coinfection with *H. hepaticus* and *H. rodentium* as well as monoinfection with *H. bilis* has also recently been demonstrated to be a critical component in the development of cholesterol gallstones in C57L mice fed a lithogenic diet (99).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Blood Specimens

Given that *H. pylori* specifically adheres to the gastric mucosal epithelium and seldom is identified intracellularly in gastric infections, it has rarely been isolated from human blood. In contrast, enterohepatic helicobacters translocate across the intestinal barrier and cause invasive infections. If bacteremia is suspected, peripheral venous blood should be collected in commercially available aerobic and anaerobic blood culture bottles as recommended by the manufacturer. Culture conditions are described in the subsequent section. No comparative studies have been performed with the different culture bottles available commercially. However, detection of these slowly growing bacteria which do not lead to detectable turbidity is linked to careful measurement of optical density in blood being incubated in automated blood equipment.

Feces

Gastric helicobacters *H. pylori* and "*H. heilmannii*" are rarely isolated from human fecal specimens. Experimental administration of a diarrheic compound increases the probability of being able to culture *H. pylori* from stools of *H. pylori*-infected patients (130). In contrast, enterohepatic helicobacters, like campylobacters, can be cultivated routinely from fecal specimens. These organisms are likely to be underisolated due to the primary focus of diagnostic laboratories being directed at the isolation of thermophilic campylobacters (e.g., *Campylobacter jejuni*). Enterohepatic helicobacters grow at 37°C but not uniformly at 42°C, the temperature often used for *Campylobacter* species isolation. Importantly, unlike some campylobacters, enterohepatic helicobacters require 5 to 10% hydrogen for optimum growth. They also may be susceptible to antibiotics present in the primary isolation medium, and they rapidly lose their viability.

Gastric Biopsy Specimens

Gastric biopsy specimens are obtained for the direct diagnosis of *H. pylori* or "*H. heilmannii*" infection. This is achieved by the use of a rapid urease test, impression smears, histopathology, or PCR. Culture of *H. pylori* may be necessary for antimicrobial susceptibility testing, diagnostic confirmation, or molecular fingerprinting in epidemiologic studies. It is stressed, however, that the proper collection, storage, and transport of gastric biopsy specimens are important for successful culture of *H. pylori*.

Although endoscopic features of *H. pylori* infection are considered not to be specific, the new method of confocal laser endomicroscopy has allowed, for the first time, surface microscopy imaging of living tissue during ongoing endoscopy and observation of *H. pylori* (76).

Biopsy specimens are routinely obtained from the antrum and corpus by esophagogastroduodenal endoscopy. The recommended number of biopsy specimens is two from each site for histology (22), one or two from each site for culture, and one from the antrum for a urease test. Biopsy specimens for histology are fixed in buffered formalin before being paraffin embedded.

H. pylori is sensitive to desiccation, ambient atmosphere, and room temperature. Therefore, a transport medium must be used to maintain the viability of the organisms. For short-term transport (less than 4 h), saline may be sufficient; otherwise, a semisolid transport medium maintained at 4°C is preferable. Stuart's transport medium, brucella broth with 20% glycerol, or Portagerm pylori (bioMérieux, Durham, N.C.) can be used for this purpose (45, 54, 115). Transport containers which maintain a temperature below 10°C for up to 16 h (Sarstedt, Nümbrecht, Germany) can be used to send biopsy specimens by mail. If culture of *H. pylori* is not possible within 24 h, it is recommended to freeze the biopsy specimen at −70°C in a tube without any medium and to transport it in dry ice. This method has proved to be efficient.

For long-term storage, the recommendation is to freeze freshly isolated cultures at −70°C in glycerol-containing media. Strains can then be recovered decades apart (59). Short-term maintenance is possible for 1 week in glycerol-containing medium at 4°C; 81% of isolates remained viable in one study (59).

Gastric Juice

Gastric juice can be obtained by aspiration after the introduction of a nasogastric tube, but it is easier to collect this fluid with the so-called string test. This test (Enterotest; HDC, San Jose, Calif.) consists of swallowing a capsule fixed to a string. After 30 min, the capsule dissolves and the string is removed. The distal portion of the string, which is impregnated with gastric juice, is cut and introduced into a transport medium. The impregnated material can then be used for culture or PCR (137). Recently, a novel device has been designed for the rapid recovery of gastric epithelia and mucus. It consists of an extendable orogastric brush contained within a plastic tube which is swallowed by the patient. Once the tube is in the stomach lumen, the brush is extended and brushed across the mucosa. It is then retracted into the protective sleeve and withdrawn from the patient. The material from the brush can then be used for culture of *H. pylori* (53).

DIRECT EXAMINATION AND DETECTION

Fecal *H. pylori* Antigen Detection

An enzyme-linked immunosorbent assay (ELISA) can be used to detect *H. pylori* antigens in stools and has been approved in the United States by the Food and Drug Administration. The first commercially available reagent, named Premier platinum HpSA (Meridian, Cincinnati, Ohio), consisting of a polyclonal antibody fixed on microwells, proved to be sensitive and specific in a multicenter study performed in Europe (171). A systematic review of published data including results of 89 studies (10,858 patients) confirmed the value of HpSA as a diagnostic tool for pretreatment (sensitivity, 91%; specificity, 93%) as well as for follow-up posttreatment (47).

A new generation of antigen stool tests using a monoclonal antibody directed against *H. pylori* catalase has also been developed (Amplified-IDEA-HpStar; DaKo, Glostrup, Denmark). This test ensures consistent quality of the reagents and improved reproducibility (94). Monoclonal antibodies have also been used to develop an immunoenzymatic rapid test (Immunocard STAT HpSA; Meridian) (91). The most reliable test is the ELISA laboratory test using monoclonal antibodies (sensitivity, 96%; specificity, 97%). Rapid antigen stool tests using monoclonal antibodies are very promising but need further evaluation.

Some aspects with an impact on the reliability of stool antigen-based assays, such as the influence of the frequency of bowel movements, have not been studied in detail. In principle, a short transit time should favor retrieval of unaltered antigens unless there is an excessive dilution factor, and constipation may lead to antigen degradation. Experimentally, tests performed on spiked stool specimens showed decreased sensitivity after a 24-h delay (110). Antigen stool tests may be well suited for children, from whom it is common to obtain stools, in contrast to adults, who usually prefer another type of test, e.g., the urea breath test (UBT). Although these tests have been found to be quite specific, it will be interesting to ascertain whether enterohepatic helicobacters sometimes present in stools could also be detected and possibly interfere with the interpretation of test results. Up to this point, it has been considered unnecessary to screen stool specimens for enterohepatic helicobacters given the low number of reported enterohepatic helicobacter cases. However, the recent identification of enterohepatic helicobacters in appreciable numbers of samples from diarrheic and nondiarrheic individuals may require a reevaluation of their impact on *H. pylori* stool antigen assays (14, 58).

Gastric Biopsies

Smear Evaluation

Imprint cytology specimens do not require overnight formalin fixation and provide a rapid adjunct to histopathologic examination of gastric biopsy specimens. After biopsy specimens are collected with forceps, imprints are made by pressing a needle against the tissue on a glass slide or by simply rubbing the tissue over the slide. Cytology specimens may be prepared immediately following biopsy by staining the imprints with a rapid Giemsa or Gram stain, and *H. pylori* or "*H. heilmannii*" organisms may be directly visualized. This approach has been demonstrated to match or outperform conventional histology in multiple studies (17, 107, 132). When imprint smears were used, 30 of 32 biopsy specimens with positive *H. pylori* cultures yielded visible organisms by Gram staining (132). "*H. heilmannii*" organisms were detected in 11 of 100 patients with dyspepsia by imprint cytology. Imprint cytology may be more sensitive than histology for detection of this organism (17). Direct fecal smears, like fecal cultures, lack utility for the detection of *H. pylori*.

Urease Testing

H. pylori produces large amounts of extracellular urease which can be detected within hours following introduction of gastric biopsy tissue into a urea-containing medium. Urease catalyzes the hydrolysis of urea into ammonia and carbamate. The net effect of ammonia production is to increase local pH. Biopsy samples are placed in an agar gel or on a paper strip containing a pH indicator. If organisms are present in sufficient numbers in the antral biopsy sample, a color change will occur as a result of urea breakdown and ammonia production by *H. pylori* urease. Commercial rapid urease tests include the agar gel-based tests (e.g., CLOtest [Kimberly-Clark, Neenah, Wis.] and Hp*fast* [GI Supply, Camp Hill, Pa.]) and paper strip tests (e.g., PyloriTek [BARD, Murray Hill, N.J.]) (142, 184). These tests can be used in the endoscopy ward. Detection sensitivity may approach 90%, but this depends on the *H. pylori* density in mucosal biopsy specimens and the number of biopsy specimens sampled. At least one fresh biopsy specimen from the gastric angle or antrum should be submitted for rapid urease testing. The sensitivity of rapid urease testing is maximized if specimens are obtained from the gastric angle (182) and multiple specimens are obtained (100). Agar gel-based tests have their optimal sensitivity after a 24-h incubation, whereas strip tests are optimal within an hour, making them truly rapid tests. The specificities of the urease tests are usually excellent. Agar gel-based rapid urease assays represent a cost-effective and widely used approach to screen biopsy tissue for *H. pylori* infection.

The urease broth tests commonly used in the laboratory are not optimized to have sensitivities equivalent to those of commercially available kits.

Histology

The standard hematoxylin and eosin tissue staining technique is not sufficient to accurately detect *H. pylori*. Various special stains, including Giemsa and the modified Steiner silver, must be used (141). The Warthin Starry stain can also be used but necessitates trained histology personnel to perform the technique. The sensitivity for discerning the presence or absence of *H. pylori* in gastric tissue depends on the quality of the samples and is very much observer dependent. Although the specificity is usually adequate, the presence of bacteria with atypical morphologies may result in misinterpretations. Under optimal conditions, however, histological diagnosis can have a sensitivity and specificity of 95%. Immunohistological staining with specific *H. pylori* antibodies can also improve specificity. More recently, fluorescence in situ hybridization with species-specific 16S rRNA probes (Creatogen GmBH, Augsburg, Germany) has been proposed for the detection of *H. pylori* (150) and "*H. heilmannii*" (170) in human gastric biopsy specimens. *H. pylori* and "*H. heilmannii*" can be further distinguished by their respective morphologies in gastric biopsy specimens (Fig. 1).

DNA Amplification

Since rapid urease testing, histologic examination, and culture yield high sensitivities and specificities for the diagnosis of *H. pylori* infection, PCR amplification of *H. pylori* DNA in gastric biopsy specimens may not be cost-effective as a primary diagnostic strategy. Standard PCR methods which specifically detect *H. pylori* in gastric biopsy specimens have been developed and include different target genes such as 23S rRNA (93), *glm*M (92), and *vac*A (175). In a comparative study of five different target sequences, with culture of *H. pylori* as the "gold standard," the method using the *glm*M gene had the best accuracy (92). Development of real-time PCR assays based on the fluorescent resonance energy transfer method may have increased diagnostic utility because of the assays' improved sensitivities and ease of performance and the ability to detect macrolide resistance by using the same assay (see "Antibiotic Susceptibilities"). Species-specific PCR assays have been developed for other helicobacters such as *H. pullorum*

(14) and *H. hepaticus* (145). However, no PCR-based tests for enterohepatic helicobacters are commercially available at this time.

CULTURE AND ISOLATION PROCEDURES

Gastric Helicobacters

Although *H. pylori* can be routinely isolated by culture from human gastric biopsy samples, *H. pylori* infection is usually diagnosed by nonculture methods such as histology, serology, or urease testing. However, the increasing prevalence of antimicrobial resistance (27) has stimulated efforts to culture organisms for antimicrobial susceptibility testing.

For *H. pylori* culture, it is recommended to grind the gastric biopsy specimens to provide a higher yield of bacteria. This can be performed with an electric grinder and probes. However, if PCR has to be performed, disposable grinders are recommended to avoid contamination.

The biopsy suspension must be plated rapidly onto fresh media. Various agar media can be used, including brain heart infusion, brucella agar, Columbia agar, Wilkins Chalgren agar, and Trypticase soy agar supplemented with 10% blood or serum. Cyclodextrin can also be used to improve growth (123). Ideally, more than one medium should be used, one selective and one nonselective. For selectivity, Skirrow's supplement or Dent's supplement gives satisfactory results.

Incubation must be performed at 37°C in a humid microaerobic atmosphere (5% O_2, 10% CO_2, and 85% N_2) which is achieved by using a special incubator or jars. An apparatus which creates the proper atmosphere and ensures that the jar is airtight can be used (Anoxomat; MART Microbiology B.V., Lichentwoorde, The Netherlands). Another alternative is to use commercial gas-generating sachets in jars (CampyPak Plus [BD Diagnostics Systems, Sparks, Md.] and Anaxopack-Campylo [Remel, Lenexa, Kans.]). The presence of hydrogen in the atmosphere enhances growth. Smooth, circular colonies may appear after 3 days of incubation, but the plates must be kept for 10 days before concluding that the culture attempt has a negative result.

Currently, only one "*H. heilmannii*" strain has been cultured from a human gastric biopsy specimen after 7 days on a nonselective medium containing 7% lysed horse blood in a 5% O_2 and 10% CO_2 atmosphere (4).

Enterohepatic Helicobacters

Selective, enriched media and a microaerobic atmosphere are essential for the cultivation of helicobacters from feces or rectal swabs. *H. canadensis*, *H. canis*, *H. cinaedi*, *H. fennelliae*, *H. pullorum*, *Helicobacter* sp. strain flexispira taxon 8, and *H. winghamensis* have been isolated from humans with gastroenteritis (37). Fresh stool specimens should be plated directly onto selective media and incubated for a minimum of 5 days (5 to 7 days) at 37°C in a microaerobic atmosphere. We recommend selective CVA medium (Columbia agar base with 5% sheep blood and cefoperazone, vancomycin, and amphotericin B; Remel) and a defined microaerobic atmosphere in partially evacuated anaerobic jars (5 to 10% carbon dioxide, 5 to 10% hydrogen, 5 to 10% oxygen, and 80% N_2) for the successful isolation of most enterohepatic helicobacters from human as well as animal feces (37, 174). Commercial gas-generating sachets (e.g., CampyPak Plus [BD Diagnostic Systems]) have been used to isolate enterohepatic helicobacters, but because of increased atmospheric hydrogen requirements, the amount of hydrogen may be inadequate in such systems (37). Alternatively, *H. winghamensis* was isolated from human stool on nonselective Mueller-Hinton agar supplemented with 10% sheep blood in a defined microaerobic atmosphere as described above (105). Some authors from South Africa recommend a special protocol that allows the primary isolation of multiple species (84, 85). It must be cautioned, however, that most laboratories do not use the high H_2 level (40%) involved in this approach because of its flammable nature.

Blood culture isolates have been detected in aerobic and anaerobic bottles (usually aerobic bottles only) by using routine media with prolonged incubation (minimum of 6 days) in automated instruments such as the BACTEC (Becton Dickinson, Franklin Lakes, N.J.) (12) and the BacT/Alert (bioMérieux) systems (126). Identifying enterohepatic helicobacters in blood cultures may require special stains. The authors of a multicenter study, which included 22 *H. cinaedi* blood isolates, noted that these thin, gull-shaped organisms were generally not visible upon Gram staining and required acridine orange staining, dark-field microscopy, or Giemsa staining for visualization (75). A modified Gram stain with carbol (0.5% [wt/vol]) or basic fuchsin (0.1% [wt/vol]) as the counterstain is also recommended for detection. Instrument-positive blood cultures should be stained with acridine orange if Gram staining is negative.

Nonselective, blood-enriched media are preferred for the isolation of helicobacters from primary blood culture media and sterile body fluids. *H. cinaedi* isolates from positive blood cultures have been cultivated in 2 to 3 days at 37°C on nonselective blood media (e.g., brucella or Columbia agar base with 5% horse or sheep blood) in a microaerobic atmosphere (e.g., CampyPak Plus [BD Diagnostic Systems] or partially evacuated jars with defined microaerobic atmospheres as described above). Supplementation of atmospheric hydrogen (5 to 10%) permitted successful culture of *H. cinaedi* in microaerobic conditions (174). Supplementation of atmospheric hydrogen occurs by the addition of gas-generating sachets to closed containers or, ideally, by direct supplementation with gas tanks containing 5 to 10% hydrogen (37). The only documented examples of *Helicobacter* species cultivation from nonblood sterile body fluids include the isolation of *H. cinaedi* from cerebrospinal fluid in nonselective Trypticase soy broth (126) and that of *Helicobacter* sp. strain Mainz (reclassified as *H. cinaedi*) from joint fluid on nonselective blood agar at 37°C (69, 174).

IDENTIFICATION

Helicobacters yield various colony phenotypes on blood agar, ranging from discrete, gray, and translucent colonies of *H. pylori* and selected gastric helicobacters to various swarming phenotypes of intestinal helicobacters and some gastric helicobacters (e.g., *H. felis*). Enterohepatic helicobacters yield a swarming phenotype with a thin film (e.g., *H. cinaedi and H. fennelliae*) or a thick, mucoid film similar to that of campylobacters (e.g., *H. pullorum and H. canadensis*). Helicobacters have a characteristic morphology visualized by light microscopy that resembles that of other gram-negative spiral or curved bacteria (Fig. 1; Table 1). These organisms usually appear faint by conventional Gram staining and may require counterstaining with carbol fuchsin (0.5% [wt/vol]) for enhanced visualization. Most helicobacter isolates are motile if observed by phase-contrast microscopy. Helicobacters are routinely tested for oxidase, catalase, and urease activities (Table 1). All helicobacters are oxidase positive. Most *Helicobacter* species, including *H. pylori*, are catalase positive (Table 1).

Gastric Helicobacters

The morphologies of helicobacters observed in gastric biopsy specimens may differ markedly from those observed in a Gram-stained preparation of cultured organisms. For example, *H. pylori* usually appears as a curved or straight rod in culture, whereas stained tissue biopsy samples usually reveal a helical or more strikingly curved appearance. "*H. heilmannii*" (likely identical to *H. bizzozeronii;* Table 1) is usually distinguished from *H. pylori* by its larger size and more pronounced helical morphology in gastric biopsy specimens (Fig. 1) (67). Because *H. felis* cannot be distinguished from "*H. heilmannii*" or *H. bizzozeronii* by light microscopy in human gastric biopsy specimens, *H. felis* must be cultured or identified by electron microscopy (46, 87).

H. pylori infection is diagnosed by microscopic morphology (Gram or Giemsa staining) and the presence of catalase, oxidase, and urease activities from primary culture. *H. cineadi* also has been identified by DNA analysis in gastric biopsy samples that were urease negative (136). *C. jejuni*, another urease-negative relative of *H. pylori* (151), or the rare *C. jejuni* subspecies *doylei* can also be found in the stomach, highlighting the importance of performing biochemical tests. Rapid urease, indoxyl acetate hydrolysis, and hippurate hydrolysis tests distinguish *H. pylori* from *C. jejuni* (chapter 59) (139). Commercial tests for urease (Christensen's urea agar slant; Remel or BBL Microbiology Systems), indoxyl acetate (Remel), and hippurate hydrolysis (Remel) are available and convenient for rapid identification of cultured specimens. In a study of 400 clinical isolates, all *H. pylori* isolates were positive for cytochrome oxidase, catalase, and urease activities (Table 1).

Enterohepatic Helicobacters

The enterohepatic helicobacters possess several distinguishing biochemical characteristics. Most intestinal helicobacters isolated from humans, such as *H. canadensis*, *H. cinaedi*, *H. fennelliae*, and *H. pullorum*, lack urease activity. Distinguishing catalase-positive, urease-negative helicobacters from the enteric campylobacters (e.g., *C. jejuni*) may be especially challenging. Unlike *C. jejuni* and *Campylobacter coli*, *H. cinaedi* and *H. fennelliae* do not survive at 42°C and *H. cinaedi* does not hydrolyze indoxyl acetate (Table 1) (139). Indoxyl acetate hydrolysis distinguishes *C. jejuni* and *C. coli* from *H. pullorum* (Table 1). *H. pullorum* is distinguished from *Campylobacter lari* only by its resistance to nalidixic acid. A nalidixic acid-resistant *C. lari* isolate would require species-specific PCR of 16S rRNA target sequences (160) for differentiation from *H. pullorum*. *H. canadensis* was initially characterized as a separate cluster of *H. pullorum*-like isolates capable of hydrolyzing indoxyl acetate and resistant to nalidixic acid (38). Unlike most other helicobacters, *H. canis* is catalase negative and urease negative. To distinguish *H. canis* from catalase-negative campylobacters, the nitrate reduction and indoxyl acetate hydrolysis tests may be useful (Table 1). It is important to note that diarrheic or nondiarrheic stool specimens may be cocolonized with multiple *Helicobacter* spp. and *Campylobacter* spp., making a complete diagnostic evaluation challenging (84, 85, 155).

NONINVASIVE TESTS

In addition to the detection in stool samples of *H. pylori* antigens by immunoenzymatic techniques and of *H. pylori* DNA by real-time PCR as previously mentioned, there are two other noninvasive approaches that can be utilized for the diagnosis of *H. pylori* infection: the detection of specific antibodies in serum, saliva, and urine, and the UBT.

UBT

The UBT has been developed specifically for *H. pylori* detection (79). It is based on an important feature of this bacterium, production of a large amount of urease. A solution containing isotopically labeled urea is ingested by the patient. If *H. pylori* is present in the stomach, the labeled urea is broken down into labeled CO_2 and ammonia; the labeled CO_2 is then absorbed into the bloodstream and exhaled in the breath of the patient being tested.

This test comprises either a carbon radioactive isotope (^{14}C) or a nonradioactive natural isotope (^{13}C). Both types of the test have been approved by the U.S. Food and Drug Administration for the diagnosis of *H. pylori* infection. $^{14}CO_2$ can be detected by a scintillation counter 20 min after ingestion, whereas for $^{13}CO_2$, the $^{13}C/^{12}C$ ratio is determined before and 30 min after ingestion by a gas isotope ratio mass spectrometer or an infrared spectrometer. The result is then expressed as a δ value between 0 and 30 min. The standardized ^{13}C-UBT must be performed on a subject after an overnight fast. A test meal consisting of citric acid makes this test more sensitive by delaying gastric emptying but also possibly by increasing urease activity (23). An initial breath sample is then obtained by blowing into a tube with a straw. Subsequently, a 75-mg dose of ^{13}C-urea is ingested. The patient must remain inactive, with no smoking, eating, or drinking, and 30 min later, a second breath sample is obtained. If an infrared spectrometer is used, a larger amount of exhaled air must be obtained by blowing into a special balloon. The cutoff used to define a positive result is usually a δ of 4.5‰.

With regard to the ^{14}C-UBT, there has been an ongoing effort to develop an assay which would allow a decrease in the dose of radioactive isotope administered. The test can now be performed with only 1 μCi contained in a capsule (138). The cutoff to define a positive result is usually 2% of the administered dose at 20 min. In contrast to the ^{13}C-UBT, the ^{14}C-UBT cannot be used for children and pregnant women. Furthermore, its use is not allowed in certain countries. The limitation on the ^{13}C-UBT is the availability of a spectrometer, but specimens can be sent by mail.

The UBT is considered to be the best noninvasive test for patient follow-up after *H. pylori* eradication, and its use has been consistently recommended in consensus conferences (96). UBTs have reproducibly proved highly accurate with a high degree of reliability in all the studies performed (sensitivity and specificity in the range of 95%). Importantly, the results are not influenced by transport conditions (tubes of breath air can be transported and stored for weeks at room temperature) and the measurement is observer independent.

Antibody Detection

Serologic testing represents a primary screening method for the diagnosis of *H. pylori* infection. Pooled *H. pylori* antigens of high-molecular-weight surface-associated proteins, acid extracts, or whole-cell lysates (sonicates) are used in most serologic assays. Various cytosolic and cell surface-associated proteins represent immunodominant antigens recognized by serum antibodies of infected individuals (78). Infection with *H. pylori* results in a local and systemic humoral response to multiple antigens (78, 168). In contrast to serum immunoglobulin M (IgM) levels, serum IgA and IgG levels persist for months and years and correlate with active infection in

untreated individuals (11, 112, 163). Only a small percentage of individuals do not have detectable systemic seroconversion following infection (100). Anti-*H. pylori* serum IgG levels are more consistently elevated than serum IgA levels. Consequently, serum IgG immunoassays yield greater sensitivities and specificities than serum IgA assays (82).

Commercial ELISA kits detecting anti-*H. pylori* IgG in serum are the serologic tests of choice for the primary screening of patients with uncomplicated infections (82). These recommended ELISA-based tests include HM-CAP (Enteric Products, Westbury, N.Y.), *H. pylori* IgG ELISA (Wampole Laboratories, Cranbury, N.J.), Premier (Meridian), and Pyloriset EIA-GIII (Orion, Espoo, Finland) (109). Overall, the median sensitivity and specificity for all commercially available *H. pylori* serology kits are 92 and 83%, respectively (82). Performance varies significantly among commercial serologic kits, with top performers exceeding 90% in sensitivity and specificity and bottom performers failing to reach 90% in sensitivity or 80% in specificity (82). However, the determined specificity of serology may be incorrectly low in some comparative studies where the gold standard used has poor sensitivity. ELISA serologic testing has the lowest cost per correct diagnosis, but overall accuracy is lower than that of stool antigen testing or UBT (172). Patients infected with "*H. heilmannii*" are usually negative by anti-*H. pylori* IgG assays (67). Serum IgA immunoassays may be used as second-line tests for assessing equivocal or possibly false-negative anti-*H. pylori* serum IgG results. The key advantage of serum IgA studies is that follow-up testing may be performed with the same serum sample. In one study, more than 7% of samples with a negative serum IgG result were found to possess detectable anti-*H. pylori* serum IgA levels when patients had symptoms consistent with *H. pylori* infection (71). With sensitivities of 39 to 82%, serum IgA assays lack the requisite sensitivity to serve as primary screening tests but may be useful in cases when infection is strongly suspected and the serum IgG result is negative or equivocal. Another alternative diagnostic approach is to perform immunoblotting for equivocal specimens.

Although serum IgG assays remain the tests of choice for screening patient sera, whole-blood immunoassays are being used with increasing frequency in physicians' offices and point-of-care testing protocols. Qualitative point-of-care immunoassays produce rapid results (4 to 10 min) with heparinized whole blood or capillary blood specimens. The major drawback of these rapid whole-blood immunoassays is their low sensitivities (usually 80 to 90%) despite specificities comparable to those of laboratory-based serum enzyme immunoassays (30, 31). Concerns regarding interoffice variability with point-of-care whole-blood assays have been raised. Negative results must be confirmed by laboratory ELISA-based serum IgG testing, fecal antigen testing, or UBT.

Sustained immunoglobulin responses to multiple antigens of *H. cinaedi* and *H. fennelliae* have been documented (35). Little is known about the nature of immunoglobulin class and subclass responses following infection with *H. cinaedi* and *H. fennelliae*. No commercial serologic assays have been developed to monitor infection with *H. cinaedi* or other enterohepatic helicobacters.

Detection of *H. pylori* Antibodies in Urine

Specific *H. pylori* IgG antibodies are detected in urine at low concentrations. An ELISA allowing their detection has been developed (Urinelisa; Otsuka,Tokuchima, Japan) as well as a rapid immunoenzymatic test (Rapirun; Otsuka) (73, 90). A pooled sensitivity and a specificity of 92.3 and 89.7%, respectively, have been reported. While the accuracy is not dependent on the pH or the presence of bacteriuria, it may be influenced by a large amount of total IgG. Urine samples cannot be frozen because it would lead to protein precipitation. The test must be performed on fresh samples, or a preservative must be added. These tests, although very attractive because of their noninvasive nature, have not achieved the optimal sensitivity required for routine use.

Detection of *H. pylori* Antibodies in Saliva

Salivary antibodies are secreted during the immune response to infectious agents (134). A commercial kit has been developed to detect *H. pylori*-specific IgG in saliva (Helisal; Provalis, Deeside, United Kingdom). Saliva can be collected by having the patient spit into a tube, but better results are obtained using a special device (OraSure; OraSure Technologies, Inc., Bethlehem, Pa.) designed to obtain gingival transudate enriched with IgG (95). However, this assay also suffers from insufficient sensitivity.

ANTIBIOTIC SUSCEPTIBILITIES

The standard regimen to eradicate *H. pylori* consists of a macrolide (e.g., clarithromycin), a β-lactam (amoxicillin), and a proton pump inhibitor (e.g., omeprazole, lansoprazole, pantoprazole, rabeprozole, or esomeprazole). The antisecretory drug is prescribed to increase the stomach pH in order for the antibiotics to be effective. Acid antisecretories also reduce pain and accelerate ulcer healing. This regimen is recommended to last for 10 days in the United States and 7 days in other countries.

Other alternatives include (i) a triple therapy in which a nitroimidazole (e.g., metronidazole) is used instead of amoxicillin, (ii) bismuth-based therapy with bismuth salts, tetracycline, and metronidazole, and (iii) a quadruple therapy in which a proton pump inhibitor is added to the bismuth-based regimen; all of these therapies are prescribed for 7 to 14 days.

The main cause of failure to eradicate *H. pylori* with the standard antimicrobial regimen is macrolide resistance (104). The eradication rate decreases by 70% (from 88 to 18%) when the strain is resistant versus susceptible (102). This finding should stimulate routine susceptibility testing with clarithromycin. In the case of resistance, clarithromycin must be replaced with an alternate antibiotic. Using metronidazole with amoxicillin and a proton pump inhibitor for 14 days is an alternative (83), but metronidazole resistance also decreases the success rate by 25%. Other antibiotics, e.g., levofloxacin, rifabutin, and furazolidone, may also be used in association with amoxicillin (101).

The prevalence of resistance parallels the use of drugs worldwide (102, 128). For clarithromycin the resistance rate is between 0 and 20%, for metronidazole it is between 10 and 80%, and for fluoroquinolones it is between 0 and 20%. Resistance to amoxicillin and tetracycline is rarely found.

Various susceptibility testing methods such as broth microdilution, disk diffusion, the E test, and agar dilution have been used to assess antimicrobial resistance in *H. pylori* (18, 57, 63). The Clinical and Laboratory Standards Institute (CLSI) made the recommendation of an agar dilution method for testing of *H. pylori* susceptibility to clarithromycin (114). Mueller-Hinton agar base with 5% aged sheep blood and incubation for 72 h at 35°C were selected by the CLSI for susceptibility testing by agar dilution (see chapter 75). Resistance in vitro to clarithromycin by *H. pylori* is clinically relevant, and a MIC breakpoint of 1 μg per ml is recommended by the CLSI

(114). If an agar diffusion method is used, an inhibitory zone of less than 17 mm around an erythromycin disk indicates a resistant strain (55). In contrast to that for clarithromycin, in vitro susceptibility testing for metronidazole has not been standardized, although elevated MICs (>8 μg/ml) have been correlated with treatment failures (9). Given that resistance in *H. pylori* is the consequence of chromosomal mutations, molecular tests have been developed especially for the most important antibiotic, clarithromycin. Point mutations at two sites of the 23S rRNA gene which inhibit macrolide binding are essentially involved (16, 120, 177). A number of methods have been developed for their detection. One of the most widely used is PCR-restriction fragment polymorphism (135, 167, 177). Real-time PCR using the fluorescent resonance energy transfer principle has been developed which allows the detection of both *H. pylori* and its resistance to clarithromycin directly from gastric biopsy specimens. The assay has excellent sensitivity (122) and has been used as well to detect *H. pylori* from stools (153). The same approach has been used to ascertain resistance to fluoroquinolones (48). In contrast, multiple null mutations in the NADPH nitroreductase gene (*rdxA*) are the primary molecular bases for metronidazole resistance in *H. pylori* (50, 72, 81, 106), leading to the inability of cells to reduce and activate nitroimidazole compounds intracellularly.

Eradication of "*H. heilmannii*" by antimicrobial therapy also results in the resolution of gastritis and peptic ulcer disease (49, 66, 67) as well as "*H. heilmannii*"-associated primary low-grade MALT lymphoma (111). Antibiotic susceptibility testing has been described only for multiple isolates from a single patient (4). "*H. heilmannii*" isolates were susceptible to amoxicillin, ciprofloxacin, erythromycin, and tetracycline and resistant to nalidixic acid and metronidazole (4). "*H. heilmannii*" infections have been successfully treated with bismuth alone and with combination therapies that included amoxicillin, metronidazole, and omeprazole (4, 66, 67).

Bacteremias due to intestinal helicobacters in immunocompromised patients require drug combinations in prolonged intravenous treatment regimens, often including aminoglycosides. *Helicobacter* sp. strain flexispira taxon 8 bacteremias require multiagent regimens including aminoglycosides such as amikacin (159) and gentamicin (179) for the clearance of infections. Effective therapy for *H. cinaedi* infection has included ciprofloxacin, gentamicin, or tetracycline for at least 2 to 3 weeks (12, 75, 126). In vitro susceptibility testing of *H. cinaedi* (34, 74, 75) appears to be meaningful, and resistance in vitro has been correlated with treatment failures in patients medicated with erythromycin (12) or ciprofloxacin (75). Relatively limited therapeutic experience is available for *H. fennelliae* infections. Gentamicin (115) and ampicillin-sulbactam (68) have been used successfully to treat bacteremia caused by *H. fennelliae*. Neither in vitro susceptibility testing data nor treatment recommendations have been reported for cases of gastroenteritis caused by *H. canis*, *H. pullorum*, *H. canadensis*, or *H. winghamensis*. CLSI recommendations are not available for antimicrobial susceptibility testing of helicobacter organisms other than *H. pylori*.

INTERPRETATION AND REPORTING OF RESULTS

Noninvasive tests are recommended only for patients younger than 50 years and without "alarm" symptoms. The best screening test is the UBT, but the *H. pylori* fecal antigen test or ELISA-based IgG serology can also be used. Rapid whole-blood test results must be confirmed by a laboratory-based ELISA or another test.

Patients with alarm symptoms (36) such as old age, weight loss, or gastrointestinal bleeding should undergo esophagogastroduodenal endoscopy. Rapid urease testing of gastric biopsy specimens can be used to assess *H. pylori* infection status. If results are positive, they can be reported as "positive and consistent with *H. pylori* infection." Histology of gastric biopsy specimens must be interpreted by an experienced pathologist. Routine hematoxylin and eosin staining indicates the nature of gastritis or the presence of gastric adenocarcinoma or gastric MALT lymphoma. *H. pylori* organisms may be visible with Gram staining of impression smears or the use of special stains such as Giemsa or modified Steiner stain. "*H. heilmannii*" infection must be diagnosed by bacterial morphology in gastric biopsy specimens, since this organism is rarely cultured.

In order to perform antimicrobial susceptibility testing, bacteriologic culture of *H. pylori* from gastric biopsy specimens is recommended. Successful culture of *H. pylori* from biopsy specimens may be reported if organisms have the typical gram-negative morphology and are cytochrome oxidase, catalase, and urease positive. Primary biopsy specimen cultures should be incubated at least 7 days prior to a report of a negative result. Susceptibility testing for clarithromycin should be performed by using the NCCLS (CSLI) reference method (114) or a substantially equivalent method. Molecular resistance testing (mutation detection) can be used as an alternative to detect the presence of discrete mutations conferring macrolide resistance (135).

It is necessary to perform a follow-up after an eradication treatment. A delay of at least 4 to 6 weeks after antibiotic treatment is completed must be observed. The method universally recommended is the UBT because it is noninvasive and has high sensitivity. The stool test may also be used but not serology unless it is possible to test at the same time both the serum obtained before treatment and the serum obtained 6 months after treatment. A decreased antibody titer of more than 25% must be observed to claim that the antibiotic treatment has been successful in eradicating *H. pylori* (10).

Enterohepatic helicobacters such as *H. cinaedi* and *H. fennelliae* must be isolated by microbiologic culture from blood or feces for a definitive diagnosis of infection. If the laboratory isolate is negative for the Gram stain, microscopic morphology may be assessed by using a modified Gram stain, Giemsa stain, acridine orange stain, or dark-field microscopy. Appropriate biochemical tests must be performed (Table 1). Enterohepatic helicobacters are typically urease negative and may be easily confused with campylobacters. Even with supplemental tests, the distinctions can be difficult without genotypic tests such as species-specific PCR amplification or targeted DNA sequencing.

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Leptospira

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61

TAXONOMY

Serologic Classification

The genus *Leptospira* is comprised of spiral-shaped bacteria with hooked ends (32). This genus, along with the genera *Leptonema* and *Turneriella,* makes up the family *Leptospiraceae* within the order *Spirochaetales* and class "*Spirochaetes*" of the recently proposed phylum *Spirochaetes* (26). The genus was formerly divided into two species, *Leptospira interrogans,* comprising all pathogenic strains, and *Leptospira biflexa,* containing the saprophytic strains isolated from the environment (24, 32). *L. biflexa* and *L. interrogans* were differentiated by a number of biochemical tests (32).

Leptospires are divided into serovars defined by agglutination after cross-absorption with homologous antigen (17, 32, 34). Serovars are considered distinct if more than 10% of the homologous titer remains in at least one of the two antisera on repeated testing (31). Over 60 serovars of *L. biflexa* sensu lato and more than 200 serovars of *L. interrogans* sensu lato are recognized. Serovars that are antigenically related have traditionally been grouped into serogroups (34). Serogroups have no taxonomic standing, but the concept has proved useful for epidemiological understanding, particularly when interpreting the serological results from the microscopic agglutination test (MAT). The serogroups of *L. interrogans* sensu lato and some common serovars are shown in Table 1.

Genotypic Classification

The phenotypic classification of leptospires was replaced by a genotypic one, in which 17 genomospecies include all serovars of *Leptospira* (8, 53, 55, 80). Table 2 lists all species of *Leptospira*. DNA hybridization studies have also confirmed the taxonomic status of the monospecific genera *Leptonema* (8, 54) and *Turneriella* (38). The genetically defined species of *Leptospira* do not correspond to the previous two species (*L. interrogans* sensu lato and *L. biflexa* sensu lato), and both pathogenic and nonpathogenic serovars occur within some species (35). Neither serogroup nor serovar reliably predicts the true species of *Leptospira*. Moreover, genetic heterogeneity resulting from horizontal transfer of genes coding for cell surface antigens occurs within serovars (8, 25), resulting in strains of some serovars being classified in multiple species (Table 3). In addition, the phenotypic characteristics formerly used to differentiate *L. interrogans* sensu lato from *L. biflexa* sensu lato do not differentiate the genetically defined species (8, 80). Both *L. interrogans* and *L. biflexa* are retained as specific names in the genomic classification, and in this chapter, specific names refer to the genetically defined species, including *L. interrogans* sensu stricto and *L. biflexa* sensu stricto.

The molecular classification of leptospires requires the identification of both species and serovar of each isolate. Identification of *Leptospira* species is readily accomplished by 16S rRNA gene sequencing, and sequences are available in GenBank (R. E. Morey and P. N. Levett, unpublished data). Phylogenetic analysis of 16S rRNA gene sequences demonstrates three clades of leptospires, representing pathogens, saprophytes, and a group of species of uncertain pathogenicity (Fig. 1). Serovar identification is discussed below.

The concept of serogroups has no application in the taxonomy of *Leptospira* but remains a convenient tool for serologists.

DESCRIPTION OF THE FAMILY

Leptospires are tightly coiled spirochaetes, usually 0.1 μm by 6 to 20 μm. The helical conformation is right-handed, while the amplitude is approximately 0.1 to 0.15 μm and the wavelength is approximately 0.5 μm (23). The cells have pointed ends, either or both of which are usually bent into a distinctive hook (Fig. 2). Two axial filaments (periplasmic flagella), with polar insertions, are located in the periplasmic space. Leptospires exhibit two distinct forms of movement, either translational (rapid back-and-forth movements) or rotational (spinning rapidly about the long axis of the cell) (27). Morphologically, all leptospires are indistinguishable.

Leptospires are obligate aerobes with an optimum growth temperature of 28 to 30°C. The optimum pH for growth is 7.2 to 7.6. They produce both catalase and oxidase. They grow in simple media enriched with vitamins (vitamins B_2 and B_{12} are growth factors), long-chain fatty acids, and ammonium salts (32).

EPIDEMIOLOGY AND TRANSMISSION

Leptospires are ubiquitous, either free-living in water or associated with renal infection of animals. Leptospirosis is

TABLE 1 Serogroups and selected serovars of *Leptospira interrogans* sensu lato

Serogroup	Serovars
Icterohaemorrhagiae	Copenhageni
	Icterohaemorrhagiae
	Lai
Hebdomadis	Hebdomadis
	Jules
Autumnalis	Autumnalis
	Bim
	Fortbragg
Pyrogenes	Pyrogenes
Bataviae	Bataviae
Grippotyphosa	Canalzonae
	Grippotyphosa
	Ratnapura
Canicola	Canicola
Australis	Australis
	Bratislava
	Lora
Pomona	Pomona
Javanica	Javanica
Sejroe	Sejroe
	Hardjo
Panama	Mangus
	Panama
Cynopteri	Cynopteri
Djasiman	Djasiman
Sarmin	Sarmin
Mini	Mini
	Georgia
Tarassovi	Tarassovi
Ballum	Arborea
	Ballum
Celledoni	Celledoni
Louisiana	Lanka
	Louisiana
Ranarum	Ranarum
Manhao	Manhao
Shermani	Shermani
Hurstbridge	Hurstbridge

TABLE 2 Genospecies of *Leptospira*[a]

Species
L. alexanderi[b]
L. biflexa[c]
L.borgpetersenii[b]
L. broomii
L. fainei
L. inadai
L. interrogans[b]
L. kirschneri[b]
L. meyeri[b]
L. noguchii[b]
L. santarosai[b]
L. weilii[b]
L. wolbachii[c]

[a] Based on data reported by Brenner et al. (8), Ramadass et al. (55), Levett et al. (37a), and Pérolat et al. (53).
[b] These species constitute the clade of pathogenic *Leptospira* species.
[c] Currently only nonpathogenic strains of these species are known.

TABLE 3 Leptospiral serovars found in multiple species[a]

Serovar	Species
Bataviae	*L. interrogans, L. santarosai*
Bulgarica	*L. interrogans, L. kirschneri*
Grippotyphosa	*L. kirschneri, L. interrogans*
Hardjo	*L. borgpetersenii, L. interrogans, L. meyeri*
Icterohaemorrhagiae	*L. interrogans, L. inadai*
Kremastos	*L. interrogans, L. santarosai*
Mwogolo	*L. kirschneri, L. interrogans*
Paidjan	*L. kirschneri, L. interrogans*
Pomona	*L. interrogans, L. noguchii*
Pyrogenes	*L. interrogans, L. santarosai*
Szwajizak	*L. interrogans, L. santarosai*
Valbuzzi	*L. interrogans, L. kirschneri*

[a] Based on data reported by Brenner et al. (8) and by Feresu et al. (25).

presumed to be the most widespread zoonosis in the world (78). The source of infection in humans is usually either direct or indirect contact with the urine of an infected animal. The incidence is very much higher in warm-climate countries than in temperate regions, due mainly to the longer survival of leptospires in the environment under warm, humid conditions. The disease is seasonal, with the peak incidence occurring in summer or fall in temperate regions, where temperature is the limiting factor in survival of leptospires, and during rainy seasons in warm-climate regions, where rapid desiccation would otherwise prevent survival.

Animals, including humans, can be divided into maintenance hosts or accidental (incidental) hosts. A maintenance host is defined as a species in which infection is endemic, usually transferred from animal to animal by direct contact. Infection is usually acquired at an early age, and the prevalence of chronic excretion in the urine increases with the age of the animal. Other animals (such as humans) may become infected by indirect contact with the maintenance host. Animals may be maintenance hosts of some serovars but incidental hosts of others, infection with which may cause severe or fatal disease. The most important maintenance hosts are small mammals, which may transfer infection to domestic farm animals, dogs, and humans. Different rodent species may be reservoirs of distinct serovars, but rats are generally maintenance hosts for serovars of the serogroup Icterohaemorrhagiae, and mice are generally maintenance hosts for serogroup Ballum serovars. Domestic animals are also maintenance hosts; dairy cattle may harbor serovars Hardjo and Pomona; pigs may harbor Pomona, Tarassovi, or Bratislava; and dogs may harbor Canicola. Distinct variations in maintenance hosts and the serovars they carry occur throughout the world, and these associations may change over time.

Knowledge of the prevalent serovars and their maintenance hosts is essential in understanding the epidemiology of the disease. For example, canine leptospirosis is increasing in the northeastern regions of North America. Very few cases have been diagnosed by culture, but serology suggests that they are caused largely by serovar Grippotyphosa (9, 73). Epidemiological evidence implicates raccoons and possibly skunks as the reservoir. Cases are clustered particularly in newly suburbanized areas (73). Canine vaccine composition has been changed to include serovars Grippotyphosa and Pomona as a result of this change in epidemiology.

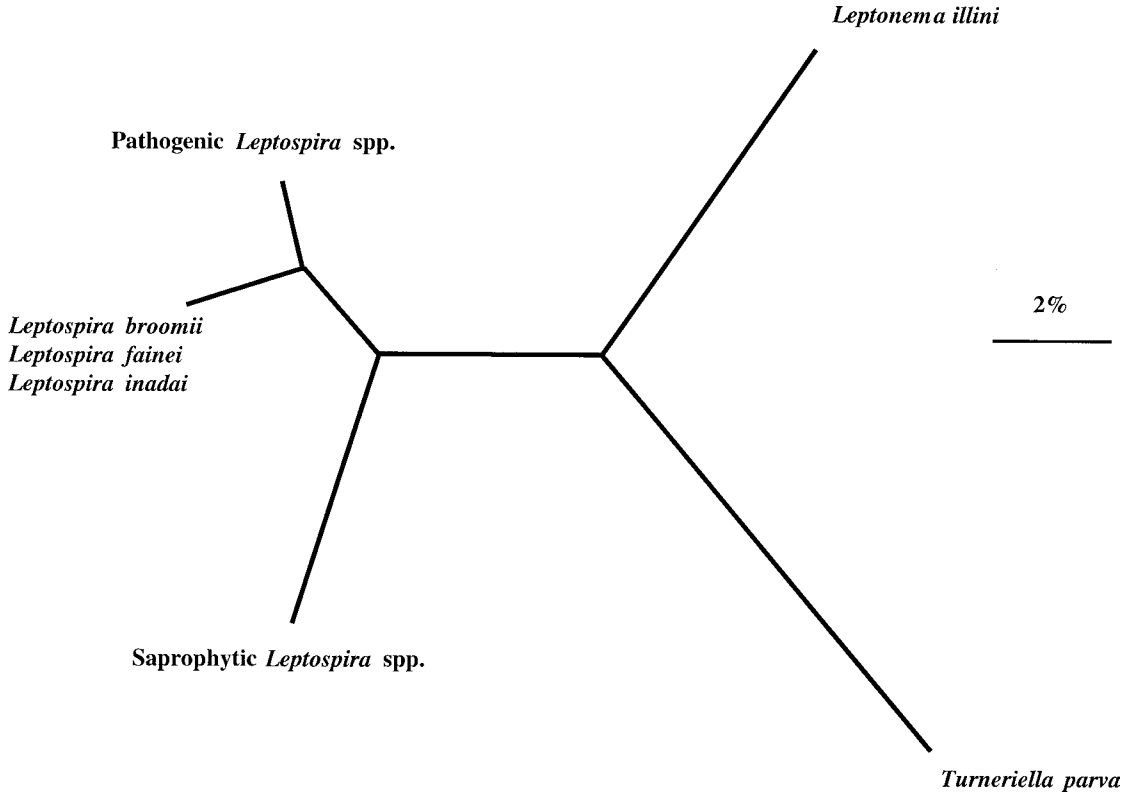


FIGURE 1 Phylogenetic relationships between the genera of the *Leptospiraceae*, based on 16S rRNA gene sequences. The scale bar represents a 2% difference in sequence.

Human infections may be acquired through occupational, recreational, or avocational exposures. Occupation is a significant risk factor for humans. Direct contact with infected animals accounts for most infections in farmers, veterinarians, abattoir workers, rodent control workers, and people in other occupations which require contact with animals, while indirect contact is important for sewer workers, miners, soldiers, septic tank cleaners, fish farmers, rice field workers, and sugar cane cutters. Livestock farming is a major occupational risk factor throughout the world. The highest risk is associated with dairy farming and is associated with serovar Hardjo and in particular with milking of dairy cattle. There is a significant risk associated with recreational exposures occurring in water sports (46, 59).

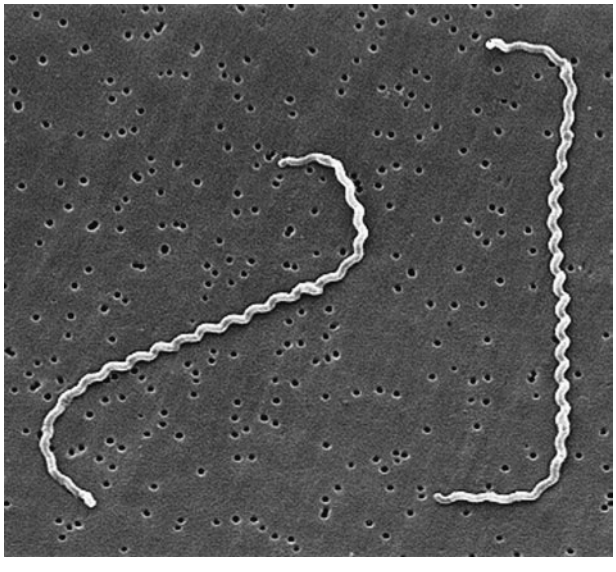


FIGURE 2 Scanning electron micrograph of leptospiral cells bound to a 0.2-μm-pore-size filter. Magnification, approximately ×3,500. Public Health Image Library.

CLINICAL SIGNIFICANCE

The usual portal of entry is through abrasions or cuts in the skin or via the conjunctiva. The great majority of infections are either subclinical or of very mild severity, and most patients will probably not seek, or be brought to, medical attention. The clinical presentation of leptospirosis is biphasic, with a septicemic phase lasting about a week, followed by the immune phase, characterized by antibody production and excretion of leptospires in the urine. Most of the complications of leptospirosis are associated with localization of leptospires within the tissues during the immune phase and thus occur during the second week of the illness.

The overwhelming majority of the recognized cases present with a febrile illness of sudden onset, the symptoms of which include chills, headache, myalgia, abdominal pain, and conjunctival suffusion. Aseptic meningitis may be found in ≤25% of all leptospirosis cases. Between 5 and 10% of all patients with leptospirosis have the icteric form

of the disease (Weil's disease), in which the clinical course is often very rapidly progressive. In addition to jaundice, patients with icteric leptospirosis may develop acute renal failure, pulmonary hemorrhage, and cardiac arrythmias. Severe cases often present late in the course of the disease, and this contributes to the high mortality rate, which ranges between 5 and 15%.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Leptospires can be isolated from blood, cerebrospinal fluid (CSF), and peritoneal dialysate fluids during the first 10 days of illness. Specimens should be collected before antibiotic therapy is initiated and while the patient is febrile. Under optimal conditions, 1 or 2 drops of blood are inoculated directly into culture medium at the bedside. Survival of leptospires in commercial blood culture media for several days has been reported (51). There are no transport media available, but blood can be collected and shipped at ambient temperature in tubes containing heparin, oxalate, or citrate (23). Citrate or EDTA-containing tubes are optimal for detection by PCR, whereas tubes containing heparin, sodium polyanethol sulfonate, or saponin are inhibitory to PCRs (39, 62).

Urine can be cultured after the first week of illness. Specimens should be collected aseptically into sterile containers without preservatives and must be processed within a short time of collection; best results are obtained when the delay is less than 1 h. Urine specimens for PCR testing may be stabilized in DNA/RNA Protect containers (Sierra Diagnostics, Sonora, Calif.), allowing for transport over long distances (39).

DIRECT EXAMINATION

Microscopy

Leptospires may be visualized in clinical material by dark-field microscopy (×100 and ×400 magnifications) or by immunofluorescence or light microscopy after appropriate staining. Dark-field microscopic examination of body fluids such as blood, urine, CSF, or dialysate fluid is both insensitive and lacking in specificity (3, 72, 76). Approximately 10^4 leptospires/ml are necessary for one cell per field to be visible by dark-field microscopy (69). Direct dark-field microscopy of blood is also subject to misinterpretation of fibrin or protein threads, which may demonstrate Brownian motion (23, 69, 76). Leptospires in tissues were first visualized by silver staining (63), and the Warthin-Starry stain is widely used for histologic examination. More recently, immunohistochemical methods have been applied (70, 81, 82). Immunohistochemical staining can be performed at the Centers for Disease Control and Prevention, Atlanta, Ga. (contact Sherif Zaki at szaki@cdc.gov).

Antigen Detection

Antigen detection is generally regarded as insensitive. An assay for detection of antigen in urine has been described (58) but has not been evaluated widely. There are no commercial antigen detection assays.

Nucleic Acid Techniques

Several primer pairs for conventional PCR detection of leptospires have been described (35). However, only two methods have been subject to extensive clinical evaluation (11, 44). Both methods were found to be more sensitive than culture, and leptospiral DNA has been amplified from serum, urine, aqueous humor, CSF, and tissues obtained at autopsy (10).

Real-time PCR assays have been developed, targeting either 16S rRNA genes or pathogenic-specific sequences (39, 45, 49, 62). These assays have yet to be evaluated in large clinical studies.

A limitation of PCR-based diagnosis of leptospirosis is the inability of most PCR assays to identify the infecting serovar. While this is not significant for individual patient management, the identity of the serovar has both epidemiological and public health value.

ISOLATION PROCEDURES

Leptospiremia occurs during the first stage of the disease, beginning before the onset of symptoms, and has usually finished by the end of the first week of the acute illness (43). Therefore, blood cultures should be taken as soon as possible after the patient's presentation. One or 2 drops of blood (approximately 50 μl) are inoculated into 10 ml of semisolid oleic acid-albumin medium (20, 33), such as Ellinghausen-McCullough-Johnson-Harris (EMJH) (Difco Laboratories, Sparks, Md.) or PLM-5 (Intergen Co., Purchase, N.Y.), containing 0.1% agar and 200 μg of 5-fluorouracil per ml, at the patient's bedside. Blood is allowed to drop onto the surface of the medium; mixing is not necessary. Care should be taken to avoid overinoculation of the medium, as blood contains inhibitors of leptospiral growth. For the greatest recovery rate, multiple cultures should be performed, but this is rarely possible. Inoculation of media with further 10-fold dilutions of blood samples may increase recovery (64). Survival of leptospires in commercial blood culture media for up to a week has been reported (51). Since the organism does not grow in conventional media, positive signals in automated systems are most unlikely. However, if a diagnosis of leptospirosis is suspected after the collection of conventional blood cultures, it is possible to detect leptospires by using PCR assays. There is insufficient experience to show the value of subculture from blood cultures into leptospira culture media, but this should be attempted if possible.

Other samples that may be cultured during the first week of illness include CSF and peritoneal dialysate. Urine cultures may yield growth from the beginning of the second week of symptomatic illness. Survival of leptospires in voided human urine is limited, so urine should be processed immediately, by neutralization of pH with sodium bicarbonate followed by centrifugation. After centrifugation in 15-ml tubes for 30 min at 1,500 × *g*, the sediment is resuspended in 1 ml of phosphate-buffered saline and 1 or 2 drops are inoculated into semisolid EMJH medium containing 5-fluorouracil as described above.

Cultures in EMJH are incubated in sealed bottles at 28 to 30°C and examined weekly by dark-field microscopy for up to 13 weeks before being discarded. Growth often develops in a discrete band, several millimeters below the surface of the medium, known as a Dinger's ring. Cultures that show growth of other bacteria may be passed through a 0.2- or 0.45-μm-pore-size filter before subculture into fresh medium.

IDENTIFICATION

Isolated leptospires are identified either by serological methods or, more recently, by molecular techniques. Traditional

methods relied on cross-agglutinin absorption (17). The number of laboratories that can perform these identification methods is very small. The use of panels of monoclonal antibodies allows laboratories which can perform the MAT to identify isolates of frequently encountered serovars with relative rapidity (66). Monoclonal antibodies are available from the WHO/OIE Leptospirosis Reference Laboratory at the Royal Tropical Institute, Amsterdam, The Netherlands. The molecular-method-based taxonomy of *Leptospira* necessitates the identification of isolates to both species and serovar. Species identification is most practically determined by 16S rRNA gene sequencing (Morey and Levett, unpublished). Sequences of some species differ by only a few bases along the entire length of the 16S rRNA gene, so for accurate identification a full-length sequence is necessary.

Typing Procedures

Because of the difficulties associated with serological identification of leptospiral isolates, there has been great interest in molecular methods for identification and subtyping (29, 66). Methods employed have included digestion of chromosomal DNA by restriction endonucleases, restriction fragment length polymorphisms, ribotyping, pulsed-field gel electrophoresis, and a number of PCR-based approaches (35). The most widely applicable molecular method for identification of serovars is pulsed-field gel electrophoresis (29, 30). This approach has been standardized, using the PulseNet model (R. Galloway and P. N. Levett, *Abstr. Int. Conf. Emerg. Infect. Dis.,* abstr. 214, 2004).

More recently, sequence-based methods such as amplified fragment length polymorphism (AFLP) and variable-number tandem-repeat methods have been developed for the study of leptospiral epidemiology (42, 71). The use of multilocus sequence typing is being investigated. The application of these powerful tools will lead to greater understanding of leptospiral epidemiology at a population level.

SEROLOGICAL TESTS

Most cases of leptospirosis are diagnosed by serology. Antibodies are detectable in the blood approximately 5 to 7 days after the onset of symptoms. The definitive serologic investigation in leptospirosis remains the MAT, in which patients' sera are reacted with live or killed antigen suspensions of leptospiral serovars. After incubation, the serum-antigen mixtures are examined microscopically for agglutination and the titers are determined. The MAT is a complex test to control, perform, and interpret (68), the use of which is limited to regional or national reference laboratories. Protocols for performing the MAT have been described in detail (2, 21, 64). An international proficiency testing scheme under the auspices of the International Leptospirosis Society has stimulated improvement in the performance of the MAT by participating laboratories (14). The range of antigens used should include serovars representative of all serogroups (21, 68) and locally common serovars (67). Titers of antibody to local isolates are often higher than titers of antibody to laboratory stock strains of serovars within the same serogroup. The wide range of antigens is used in order to detect infections with uncommon, or previously undetected, serovars. The test is read by dark-field microscopy. The end point is the highest dilution of serum in which 50% agglutination occurs. Because of the difficulty in detecting the titer at which 50% of the leptospires are agglutinated, the end point is determined by the presence of approximately 50% free, unagglutinated leptospires, by comparison with the control suspension (21). Considerable effort is required to reduce the subjective effect of observer variation, even within laboratories.

Interpretation of the MAT is complicated by the high degree of cross-reaction that occurs between different serogroups, especially in acute-phase samples. Paradoxical reactions, in which the highest titers detected are those of antibody to a serogroup unrelated to the infecting one, are also common (35). The broad cross-reactivity in the acute phase, followed by relative serogroup specificity in convalescent-phase samples, results from the detection in the MAT of both immunoglobulin M (IgM) and IgG antibodies and the presence of several common antigens among leptospires (1, 13, 40).

Paired sera are required to confirm a diagnosis with certainty. A fourfold or greater rise in titer between paired sera confirms the diagnosis, regardless of the interval between samples. The interval between first and second samples depends very much on the delay between onset of symptoms and presentation of the patient. If symptoms typical of leptospirosis are present, an interval of 3 to 5 days may be adequate to detect rising titers. However, if the patient presents earlier in the course of the disease, or if the date of onset is not known precisely, then an interval of 10 to 14 days between samples is more appropriate. Less often, seroconversion does not occur with such rapidity, and a longer interval between samples (or repeated sampling) is necessary. MAT serology is insensitive, particularly with early acute-phase specimens (4, 7, 15). Moreover, patients with fulminant leptospirosis may die before seroconversion occurs (11, 15, 56).

A presumptive diagnosis can be made by detection of a single elevated titer in association with an acute febrile illness. The magnitude of such a titer is dependent upon the background level of exposure in the population, and hence the seroprevalence. In the current CDC case definition, a titer of $\geq$200 in a patient with a clinically compatible illness is used to indicate a probable case (12).

Titers following acute infection may be extremely high ($\geq$25,600) and may take months, or even years, to fall to low levels (3, 6, 16, 41, 57). Thus, in a high-incidence population, a low cutoff titer for presumptive diagnosis is inappropriate and will generate many false-positive diagnoses. In areas of endemicity a single titer of $\geq$800 in symptomatic patients is generally indicative of leptospirosis (22), but titers as high as $\geq$1,600 have been recommended (2). Rarely, seroconversion may be delayed for many weeks after recovery, and longer serological follow-up will be necessary to confirm the diagnosis.

Formalized antigens have been used in the MAT in order to overcome some of the difficulties associated with the use of live antigens. Titers obtained with these antigens are somewhat lower, and more cross-reactions are detected (21, 50). Agglutination of formalized antigens is qualitatively different from that seen with live antigens (2). However, for laboratories without the staff or expertise to maintain live antigens, formalized and lyophilized antigens may represent an alternative. These antigens are not available commercially but may be obtained from WHO Collaborating Centers.

The MAT is the most appropriate test to employ in epidemiological serosurveys, since it can be applied to sera from any animal species and because the range of antigens utilized can be expanded or decreased as required. It is usual to use a titer of $\geq$100 as evidence of past exposure (21). Contrary to a widely held belief, the MAT is a serogroup-specific assay. However, conclusions about infecting serovars cannot be drawn without isolates; at best the MAT data can give a general impression of which serogroups are present within a population (36).

Because of the complexity of the MAT, rapid screening tests for leptospiral antibodies in acute infection have been developed. Traditional methods based upon agglutination have largely been superseded by IgM detection assays. IgM antibodies become detectable during the first week of illness, allowing the diagnosis to be confirmed and treatment to be initiated while it is likely to be most effective. IgM detection has repeatedly been shown to be more sensitive than MAT when the first specimen is taken early in the acute phase of the illness (15, 56, 75). IgM dipstick assays have been shown to be as sensitive as microtiter plate IgM-enzyme-linked immunosorbent assays (5, 28, 37). Other rapid assays include a latex agglutination assay (61) and a lateral-flow assay (60). Commercial IgM dipstick and latex agglutination assays are available from PanBio, Baltimore, Md., and bioMerieux, Durham, N.C., respectively.

ANTIBIOTIC SUSCEPTIBILITIES

Leptospires are susceptible to many antimicrobial agents, including β-lactams, macrolides, tetracyclines, fluoroquinolones, and streptomycin. Problems in the determination of susceptibility include the long incubation time required (19), the use of media containing serum (48, 79), and the difficulty in quantifying growth accurately. These constraints limited the development of rapid, standardized methods for susceptibility testing. However, broth microdilution methods that have facilitated the testing of larger numbers of isolates against a wide range of antimicrobial agents have been described recently (47, 74). Such studies will lead to the identification of potential new agents for inclusion in clinical trials.

Penicillin and doxycycline are both effective for treatment of leptospirosis and remain the drugs of choice (18); recent clinical studies have shown that expanded-spectrum cephalosporins may be equally effective (52, 65).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

A diagnosis of leptospirosis can be made by isolation of the organism or by amplification of leptospiral DNA from blood, urine, or other specimens; by demonstration of leptospires in tissues by immunohistochemical staining; or by detection of a fourfold or greater rise in titers between acute- and convalescent-phase serum samples tested by the same methodology at the same time. In populations and/or regions where leptospirosis is not endemic, MAT titers of ≥200 in a single specimen obtained after the onset of symptoms are suggestive but not diagnostic of acute or recent leptospirosis. A titer of ≥800 in the presence of compatible symptoms is strong evidence of recent or current leptospirosis (21). Delayed seroconversions are common. Assays that detect IgM antibodies give presumptive evidence of recent exposure to leptospirosis but require confirmation, since IgM titers are persistent (16). Cross-reactive antibodies, sometimes with significant seroconversion, are associated with syphilis, relapsing fever, Lyme disease, and legionellosis (5). Negative test results in the presence of compatible symptoms do not rule out the diagnosis of leptospirosis, and further samples should be examined. The isolation of leptospires, the demonstration of leptospiral DNA by molecular methods, or the detection of leptospires in tissues by immunohistochemistry confirms the diagnosis and differentiates between current infection and past exposure, which may not be clearly differentiated by serology.

Despite recent advances in molecular detection and characterization of leptospires and in the development of rapid serologic tests, there are still relatively few laboratories throughout the world with the appropriate capabilities for *Leptospira* diagnostics. Additional information regarding leptospirosis and diagnostic centers of expertise (77) is available for downloading from the International Leptospirosis Society website (http://www.med.monash.edu.au/microbiology/staff/adler/ilspage.htm).

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Borrelia

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TAXONOMY

Bacteria of the genus *Borrelia* belong to the order *Spirochaetales,* which encompasses the families *Spirochaetaceae* and *Leptospiraceae*. *Borrelia* and *Treponema* are the two genera of the family *Spirochaetaceae* that cause human disease. The type species of the genus *Borrelia* is *Borrelia anserina,* which causes borreliosis in birds. Based on 16S rRNA sequence analyses, spirochetes form a distinct entity (division D) within the eubacterial kingdom. They are neither gram positive nor gram negative. In case of the spirochetes, morphological criteria and DNA data produce concordant phylogenies, a rare trait in other bacterial groups.

DESCRIPTION OF THE GENUS

Common Characteristics

Borreliae are similar in length (8 to 30 μm) but wider (0.2 to 0.5 μm) than the two other human-pathogenic spirochetes, the treponemes and the leptospires (Fig. 1) (10). They are highly motile organisms, with corkscrew and oscillating motility. In contrast to the exoflagella of other bacteria, the flagella of spirochetes are endoflagella. The endoflagella of borreliae (7 to 20 per terminus) are localized beneath the outer membrane and insert subterminally at both ends of the protoplasmic cylinder. The protoplasmic cylinder consists of a peptidoglycan layer and an inner membrane which encloses the internal components of the cell (10). If cultivable, borreliae grow slowly under microaerophilic (9) or anaerobic (86) conditions. They require *N*-acetylglucosamine and long-chain saturated and unsaturated fatty acids and produce lactic acid through glucose fermentation (57).

Species Diversity

The causative agent of Lyme disease was first described by Burgdorfer et al. in the early 1980s (18) and named *Borrelia burgdorferi* (58). Studies published since 1992 have divided *B. burgdorferi* sensu lato into three human-pathogenic species—*B. burgdorferi* sensu stricto, *B. afzelii*, and *B. garinii* (8, 19)—and eight other species (Table 1) (108). In North America, *B. burgdorferi* sensu stricto is the only human-pathogenic species, whereas all three species have been isolated from humans in Europe. There is a strong prevalence of *B. afzelii* among human skin isolates from Europe, whereas isolates from cerebrospinal fluid (CSF) in Europe are most commonly *B. garinii* (Table 2) (19, 35, 94, 117). Studies using *ospA* PCR have shown that the strains causing Lyme arthritis are heterogeneous (Table 2) (34, 106) and not limited to *B. burgdorferi* sensu stricto. A few studies have reported the detection of other *Borrelia* species *(B. valaisiana, B. spielmanii,* and *B. lusitaniae)* in patient samples in Europe (24, 90, 91, 107). Similarly, *B. lonestari,* a species carried by a hard tick but genetically more closely related to the relapsing fever spirochetes, has been implicated in at least one case of erythema migrans (EM) (53).

The relapsing fever borreliae *B. turicatae, B. parkeri,* and *B. hermsii* are transmitted by distinct soft-bodied ticks (*Ornithordoros* species), but they may be a single species because their DNA-DNA similarity is greater than 70%. The species status of other cultivable borreliae, such as *B. anserina, B. crocidurae, B. recurrentis,* and *B. coriaceae,* has been supported by greater DNA-DNA dissimilarity findings (27, 57).

The Genome

The genome of the borreliae is composed of a small linear chromosome of approximately 1,000 kb, which is unusual among bacteria, and both linear and circular plasmids. Also atypical of most bacteria, borreliae have a low G+C content, approximately 30 mol%. The complete nucleotide sequence of the chromosome and 21 plasmids (9 circular and 12 linear) has been published for the type strain, *B. burgdorferi* B31 (20, 40). A total of 59% of the chromosomal open reading frames (ORFs) have homologs in other bacterial species; in contrast, homologs have been identified for only one-third of the plasmid ORFs. The genome encodes a basic set of proteins for DNA replication, transcription, and energy metabolism but, interestingly, lacks most cellular biosynthetic pathways. Of some surprise is the tremendous number (>150) of genes that encode putative lipoproteins, suggesting an essential role for these molecules in the life cycle of the spirochete. Recent genome analysis of another Lyme disease spirochete, *B. garinii* (strain PBi), revealed that most of the chromosome is conserved (92.7% identity on the DNA as well as the amino acid level) in the two species. Furthermore, two colinear plasmids (lp54 and cp26) seem to belong to the basic genome inventory of the *Borrelia* species that cause Lyme disease. However, the authors did not find counterparts of the *B. burgdorferi* plasmids lp36 and lp38 or their respective gene repertoires in the *B. garinii* genome (41).

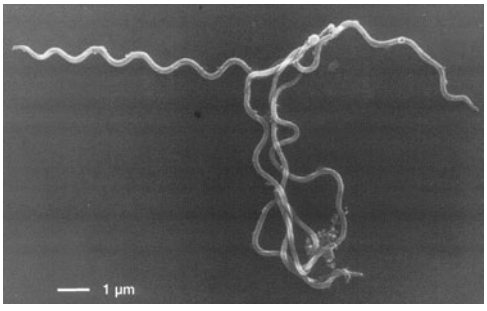


FIGURE 1 *B. burgdorferi* seen by scanning electron microscopy (provided by Gerhard Wanner, Munich, Germany).

Whole-genome microarray analysis revealed that a total of 215 ORFs, 136 of which are plasmid borne, were differentially expressed at 23 and 35°C. These findings highlight the potential importance of plasmid-borne genes in the adaptation of *B. burgdorferi* sensu lato to mammal hosts and tick vectors (74).

The linear plasmids of *B. hermsii* and *B. turicatae* contain genes encoding outer membrane lipoproteins, called variable major proteins (Vmp). These genes are silent except when they are translocated to an expression site immediately adjacent to one of the linear plasmid telomeres. Antigenic variation of Vmp-like proteins due to recombination of *vls* (Vmp-like small) gene sequence cassettes has also been described for *B. burgdorferi*. These *vls* genes have highly variable regions, as well as highly conserved sequences which encode immunogenic epitopes important for serodiagnosis (70, 123).

The large linear plasmid lp54 encodes two major outer surface proteins, OspA and OspB, which are tandemly arrayed in one operon (13). The first gene detected on a circular plasmid (cp26) was the gene encoding OspC, another major outer surface protein of *B. burgdorferi* (95, 118). Sequence analysis of the *osp*C genes from different strains suggested that gene exchange might play a role in diversity and immune evasion of Lyme disease borreliae (54).

EPIDEMIOLOGY AND TRANSMISSION

The ecological components that maintain *Borrelia* species in nature are quite diverse and spread throughout the world (Table 1).

Relapsing Fever Borreliae

Most relapsing fever borreliae are transmitted by soft-bodied ticks of the genus *Ornithodoros* (Fig. 2). The exception is *B. recurrentis,* which is vectored by the human body louse *(Pediculus humanus humanus)*. Infection in this human-specific ectoparasite is limited to the hemolymph, and spirochetes are not passed transovarially to louse progeny. Thus, humans serve as the sole reservoir of *B. recurrentis*. *B. duttonii* is vectored by the soft tick *Ornithodoros moubata,* and as is the case for *B. recurrentis,* humans serve as the only known reservoir. Notably, these human-adapted borreliae are very closely related genetically (27). The remaining relapsing fever *Borrelia* species are transmitted by other *Ornithodoros* ticks and have wild-animal reservoirs, mostly rodents (Table 1). The soft ticks feed rapidly, within 10 to 45 min, and mostly at night.

B. burgdorferi sensu lato

The Lyme disease borreliae of *B. burgdorferi* sensu lato are transmitted by hard-bodied ticks (genus *Ixodes*) (Fig. 2). *Ixodes* species feed on three different hosts depending on the developmental stage of the tick (51). The larvae and nymphs feed primarily on small rodents, whereas adult ticks feed on a variety of mammals (deer, raccoons, domestic and wild carnivores, larger domestic animals, and birds). The feeding period of *Ixodes* ticks is rather long (several days to over a week) and contributes to their geographic dispersal along with the movement of the host. Birds, particularly migratory seabirds, can transport the ticks *(Ixodes uriae)* over very long distances and thus distribute borreliae (especially *B. garinii*) worldwide (75). There appears to be an association between *B. afzelii* and small rodents and *B. garinii* and birds, likely due to different serum sensitivities of the borreliae (51) mediated by complement regulator-acquiring surface proteins (65). In unfed ticks, *B. burgdorferi* sensu lato lives in the midgut. During the blood meal from humans or mice, transcriptional changes (e.g., a switch from OspA to OspC expression) are induced in the borreliae that lead to their migration to the salivary glands (37, 100).

The role of OspC for dissemination in the tick is controversial, but its expression is required for mouse infection (38, 44, 76). Invasion from the *Ixodes scapularis* midgut takes >36 h (28). For *Ixodes ricinus,* however, spirochete migration within the tick and transmission to the mammalian host have been observed with ticks feeding for as few as 17 h (60).

CLINICAL SIGNIFICANCE

Relapsing Fever

Relapsing fever is an infectious disease with an acute onset of clinical signs and symptoms including high fever, shaking chills, severe headache, nausea, myalgias, and severe malaise. Initial physical findings often are conjunctival effusion, petechiae, and diffuse abdominal tenderness. Fever attacks of 3 to 7 days are interspersed with afebrile periods of days to weeks. More detailed descriptions have been published elsewhere for louse-borne relapsing fever (88) and tick-borne relapsing fever in North America (32, 33).

Louse-borne relapsing fever is, in general, more severe than tick-borne relapsing fever, but the number of febrile attacks is often fewer. In tick-borne relapsing fever up to 13 febrile attacks have been reported, and a rash is more often reported than in louse-borne relapsing fever (28 versus 8%). Splenomegaly, hepatomegaly, and jaundice are observed in 77, 66, and 36% of louse-borne relapsing fever cases, whereas these signs are reported in only 41, 17, and 7% of tick-borne relapsing fever cases. In louse-borne relapsing fever, 34% of the patients have respiratory symptoms and 30% have central nervous system involvement; in patients with tick-borne relapsing fever, these figures are 16 and 9%, respectively (59). Complications leading to death (mortality rate of up to 40% in louse-borne relapsing fever) are acute heart and hepatic failure and cerebral hemorrhage. Disease severity increases with compromising conditions common in many areas of endemicity. During pregnancy, relapsing fever may also lead to congenital infection and fetal death.

Lyme Borreliosis

Lyme borreliosis can be defined by early localized, early disseminated, and late-stage manifestations similar to the three stages of syphilis (104). The natural course of untreated *B. burgdorferi* infections varies considerably, and clinical manifestations can occur alone or in various combinations (102, 104, 105, 109). In the majority of cases the infection is self-limiting, but in rare

TABLE 1 Characteristics of arthropod-borne borreliae[a]

Borrelia sp.	Arthropod vector	Animal reservoir	Geographic distribution	Disease
Relapsing fever borreliae				
B. recurrentis	*Pediculus humanus humanus*	Humans	Worldwide	Louse-borne (epidemic) relapsing fever
B. duttonii	*O. moubata*	Humans	Central, eastern, and southern Africa	Tick-borne (endemic) relapsing fever
B. hispanica	*Ornithodoros erraticus*	Rodents	Spain, Portugal, Morocco, Algeria, Tunisia	Hispano-African tick-borne relapsing fever
B. crocidurae, B. merionesi, B. microti, B. dipodilli	*O. erraticus*	Rodents	Morocco, Libya, Egypt, Turkey, Senegal, Kenya	North African tick-borne relapsing fever
B. persica	*Ornithodoros tholozani*	Rodents	Western China, Kashmir, Iraq, Egypt, former USSR, India	Asiatic-African tick-borne relapsing fever
B. caucasica	*Ornithodoros verrucosus*	Rodents	Caucasus to Iraq	Caucasian tick-borne relapsing fever
B. hermsii	*Ornithodoros hermsi*	Rodents	Western United States	American tick-borne relapsing fever
B. turicatae	*Ornithodoros turicata*	Rodents	Southwestern United States	American tick-borne relapsing fever
B. parkeri	*Ornithodoros parkeri*	Rodents	Western United States	American tick-borne relapsing fever
B. mazzottii	*Ornithodoros talajé*	Rodents	Southern United States, Mexico, Central and South America	American tick-borne relapsing fever
B. venezuelensis	*Ornithodoros rudis (Ornithodoros venezuelensis)*	Rodents	Central and South America	American tick-borne relapsing fever
B. burgdorferi sensu lato				
B. burgdorferi	*Ixodes scapularis*	Rodents	Eastern and midwestern United States	Lyme borreliosis
	Ixodes pacificus	Rodents	Western United States	Lyme borreliosis
	I. ricinus	Rodents	Europe	Lyme borreliosis
B. garinii	*I. ricinus, Ixodes persulcatus*	Rodents	Europe, Asia	Lyme borreliosis
	I. uriae	Seabirds	Bipolar	?
B. afzelii	*I. ricinus, I. persulcatus*	Rodents	Europe, Asia	Lyme borreliosis
B. spielmanii	*I. ricinus*	Rodents	Europe	Lyme borreliosis (few cases)
B. japonica	*Ixodes ovatus*	Rodents	Japan	?
B. andersonii	*Ixodes dentatus*	Rabbits	United States	?
B. bissettii	*I. scapularis, I. pacificus*	Rodents	United States	?
B. tanukii	*Ixodes tanukii, I. ovatus*	Rodents	Japan	?
B. turdi	*Ixodes turdus*	?	Japan	?
B. sinica	*I. ovatus*		China	?
B. valaisiana	*I. ricinus*	?	Europe, Asia	?
B. lusitaniae	*I. ricinus*	?	Europe, North Africa	Lyme borreliosis (one case)
Other borreliae				
B. lonestari	*Amblyomma americanum*	?	United States	?
B. miyamotoi	*I. persulcatus*	Rodents	Japan	?
B. theileri	*Rhipicephalus, Boophilus* spp.	Cattle, horses, sheep	South Africa, Australia, North America, Europe	Bovine borreliosis
B. coriaceae	*O. coriaceus*	Deer?	Western United States	Epizootic bovine abortion?
B. anserina	*Argas* spp.	Fowl	Worldwide	Avian borreliosis

[a] Modified from reference 119a.

TABLE 2 Distribution of species of *B. burgdorferi* sensu lato in European isolates from CSF, skin, and synovial fluid specimens[a]

Species	% Distribution[b]		
	CSF (n = 78)	Skin (n = 560)	Synovial fluid (n = 20)
B. burgdorferi sensu stricto	12	1	33
B. afzelii	17	88	29
B. garinii	71	10	38

[a] Data from references 34, 94, 106, and 113.
[b] *B. burgdorferi* sensu lato species identifications from CSF and skin are based on culture; species identification from synovial fluid samples is based on *ospA* PCR results. Culture isolates from synovial fluid are too few to estimate *Borrelia* species distribution.

cases, *B. burgdorferi* may persist and chronic disease manifestations may develop.

EM at the site of the infectious tick bite is the most common manifestation of early (stage I) Lyme borreliosis and occurs in 60 to 90% of patients. The center of the expanding annular lesion often fades to produce a bull's-eye appearance. However, the extension, color intensity, and duration of EM vary considerably. General symptoms such as fever, myalgia, headache, and, rarely, meningismus may accompany the primary EM.

In some patients, hematogenous dissemination of spirochetes to other organs and tissues occurs within days to weeks of infection (stage II). Patients often feel quite ill and may present with fatigue, headache, fever, malaise, arthralgia, and myalgia. Multiple (secondary) erythemata are common in the United States but uncommon in Europe. Neurologic structures, including the meninges, brain, spinal cord, peripheral nerves, and nerve roots, are also potential sites of early disseminated infection. In the United States, 15 to 20% of untreated patients develop neurologic signs, most commonly facial nerve palsy (unilateral or bilateral), meningitis, and radiculoneuropathy. CSF findings in cases of Lyme meningitis almost always include a mononuclear pleocytosis (10 to 1,000 cells/μl) and elevated protein concentration. Meningitis, or even facial palsy without meningismus, is more common among children than adults. Severe encephalitis is occasionally observed in stage II. Bannwarth's syndrome is the most common neurologic manifestation of early, disseminated Lyme borreliosis in Europe. The syndrome is characterized initially by intense, migratory or focal, radicular pain, particularly at night, and by cranial nerve palsy. Pareses of the extremities and the trunk are less frequent. Further clinical manifestations of stage II may include Lyme carditis, most often with atrioventricular conduction blocks, and ophthalmic involvement. Borrelial lymphocytoma, a reddish to livid swelling of the skin in typical locations such as the earlobe, nipple, or scrotum, is manifested among some patients in Europe.

Lyme arthritis and acrodermatitis chronica atrophicans (ACA), occurring months to years after the initial infection, are the most common manifestations of late (stage III) disease. Lyme arthritis can be monoarticular or oligoarticular, typically affecting the knee, and usually takes an intermittent course. Patients with ACA initially develop an infiltrative stage, followed by alterations characteristic of the atrophic stage: creased skin with livid discolorations and plastic protrusion of vessels. ACA is observed almost exclusively in Europe, a finding highly correlated with *B. afzelii* infections. Chronic neuroborreliosis is a very rare manifestation of late (stage III) disease. Parapareses and tetrapareses are its most common symptoms. Examination of the CSF reveals a marked elevation of protein concentration with a low to moderate increase of cells in the CSF. The detection of intrathecally produced specific antibodies is currently regarded as the best marker of chronic neuroborreliosis, although there may be less frequent production of intrathecal antibodies in U.S. patients.

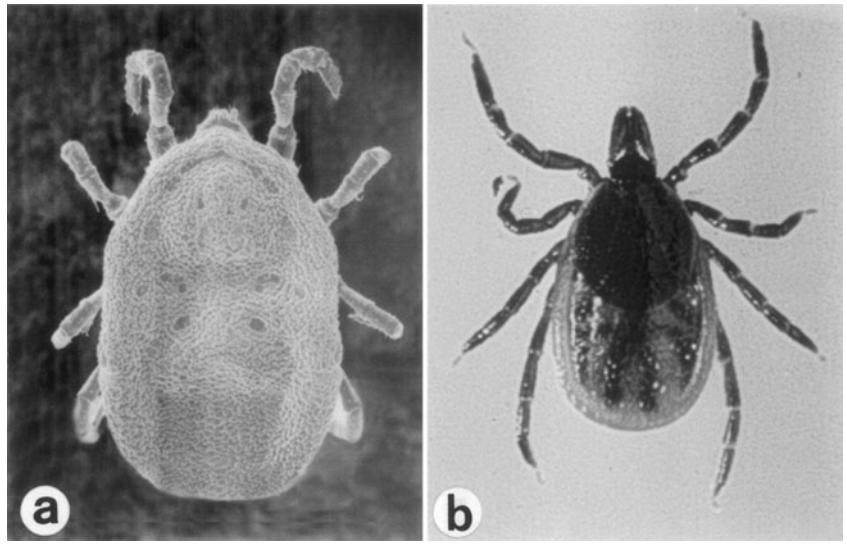


FIGURE 2 Two genera of ticks are vectors for relapsing fever and Lyme borreliosis: *Ornithodoros* (a) and *Ixodes* (b).

Early manifestations of Lyme borreliosis are observed most frequently in the spring, summer, and autumn, coinciding with tick activity. Late manifestations do not show a seasonal pattern.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Specimens for laboratory confirmation of Lyme borreliosis are presented in Table 3.

Blood and Serum

For relapsing fever, blood is the specimen of choice. During febrile attacks, borreliae may be easily detected by dark-field or bright-field microscopy of a wet-mount blood sample or a stained blood smear, respectively (see "Direct Examination," "Microscopy"). At this time, the spirochetemia may reach 10^6 to 10^8 cells ml^{-1} (57). Blood from acutely ill patients is also the best source for culture confirmation (26, 27). However, the spirochetemia diminishes with each successive relapse, and visualization or culture isolation of borreliae is often unsuccessful during afebrile periods. In contrast to relapsing fever, spirochetemia in Lyme borreliosis patients is below the level of microscopic detection. Culture and PCR of blood samples generally are not recommended, since the yields are low (43). Even when large blood volumes are cultivated, positive cultures have been derived from only 50% of patients with EM (121).

Serum is suitable for indirect (antibody) evidence of *Borrelia* exposure. Specific antibody detection tests are the most widely utilized tests for laboratory confirmation of Lyme borreliosis. Serodiagnosis of relapsing fever is performed only in a few specialized laboratories.

CSF

In suspected neuroborreliosis patients, CSF (along with serum drawn at the same time) should be obtained for determination of cell counts-cell differentials, protein content, and intrathecal immunoglobulin (immunoglobulin M [IgM] and IgG) synthesis. Laboratory confirmation of neuroborreliosis is most readily achieved by demonstration of *Borrelia*-specific, intrathecal (CSF/serum antibody index) antibody production (see "Serological Tests"). For culture or PCR, however, detection rates are only about 20% (68). Positive PCR results in CSF also seem to correlate inversely with duration of neurologic disease. Among neuroborreliosis patients, 7 of 14 (50%) with a disease duration of less than 2 weeks had a positive PCR result, compared with only 2 of 16 (13%) patients in whom the illness duration was greater than 2 weeks ($P = 0.045$) (68).

Synovial Fluid or Synovial Biopsy Specimens

Investigation of synovial fluid or a synovial biopsy specimen by PCR may be useful in special circumstances where Lyme arthritis is suspected (73). Culture is usually negative, with few exceptions. Due to the high protein permeability of the synovium, synovial fluid and serum display roughly equivalent antibody titers. Thus, it is sufficient to monitor antibody in serum.

Skin Biopsy Specimens

Skin biopsy samples are the best sources for isolation of *B. burgdorferi;* spirochetes can be isolated in most untreated cases of EM and acrodermatitis (Table 4). In cases of EM, culture success is highest (up to 86%) with biopsy samples taken close to (4 mm inside) the expanding border of the lesion (12), although this is primarily a technique for research, not for routine diagnosis. There are indications that the number of spirochetes in the skin is rather low or unevenly distributed, since an increase in the sensitivity is observed if more than one biopsy sample is investigated (124). Without treatment, *B. burgdorferi* sensu lato can persist for long periods in the skin, as shown by isolation from a 10-year-old acrodermatitis lesion (6). Biopsy samples (taken after thorough disinfection of the skin) should be sent in a small amount of sterile saline or Barbour-Stoenner-Kelly (BSK) medium (with or without rifampin) as soon as possible to the microbiological laboratory.

Other Materials

Ticks are often tested for borreliae as part of epidemiological studies to assess risk to human populations in a given geographic area. Although specialized laboratories offer diagnostic services for individual ticks, detection of spirochetes

TABLE 3 Specimen types used for the diagnosis of Lyme borreliosis

Clinical manifestation	Specimens for:	
	Direct pathogen detection (culture, PCR)	Antibody detection
Stage I (early/localized; days through weeks after tick bite)		
EM	Skin biopsy	Serum
Stage II (early/disseminated; weeks through months after tick bite)		
Multiple erythemata	Skin biopsy	Serum
Borrelial lymphocytoma	Skin biopsy	Serum
Lyme carditis	Endomyocardial biopsy	Serum
Neuroborreliosis	CSF	Paired serum/CSF[a]
Stage III (late/persistent; months through years after tick bite)		
Arthritis	Synovial fluid, synovial biopsy	Serum
ACA	Skin biopsy	Serum
Chronic neuroborreliosis	CSF	Paired serum/CSF[a]

[a] From the same day for AI determination.

TABLE 4 Sensitivity of methods for pathogen detection (PCR and culture) in Lyme borreliosis

Specimen	Sensitivity
Skin (EM, acrodermatitis)	50–70% when using culture or PCR
CSF (neuroborreliosis, stage II)	10–30% when using culture or PCR[a]
Synovial fluid[b] (Lyme arthritis)	50–70% when using PCR (culture is rarely positive)

[a] Up to 50% in patients with disease duration of less than 2 weeks, compared with only 13% in patients for whom the illness duration was greater than 2 weeks.
[b] Higher sensitivity of direct pathogen detection from synovial biopsy specimen.

within ticks by PCR or other methods has not been shown to provide clinically useful information.

General Remarks for Collection and Transport

For culture, collection and preparation of specimens under sterile conditions are of utmost importance. Body fluids should be transported without any additives, and biopsy specimens should be placed in a small quantity of sterile saline or suitable culture medium (see "Isolation Procedures"). Samples should reach the laboratory as quickly as possible (within 2 to 4 h). Before specimens are collected and transported, the laboratory should be contacted so that details of methodology can be agreed upon. If postal transport is unavoidable, overnight delivery is recommended.

DIRECT EXAMINATION

Microscopy

Direct microscopic visualization of borreliae in clinical samples is applicable only to cases of relapsing fever. During acute phases, spirochetemia often reaches 10^6 to 10^8 borreliae/ml and motile spirochetes can be visualized by dark-field microscopy from wet preparations made from a drop of blood. This simple confirmatory test is often overlooked because of the increasingly common use of automated differential blood counts. Spirochetes can be visualized by stained (e.g., Giemsa) thin- or thick-drop films (Fig. 3). Detection of low-level spirochetemias may be assisted by a microhematocrit concentration technique. The hematocrit capillary is filled 75% with citrated blood and centrifuged for 2 min. The buffy coat is then examined directly under the microscope at ×400 to ×1,000 (119a). Failure to observe spirochetes does not rule out disease, and culture isolation (see "Isolation Procedures") may be considered. In Lyme borreliosis, spirochete numbers in patient blood or other tissues are below the level of microscopic detection and microscopy is best applied to the monitoring of borrelial growth in cultured tissues or fluids.

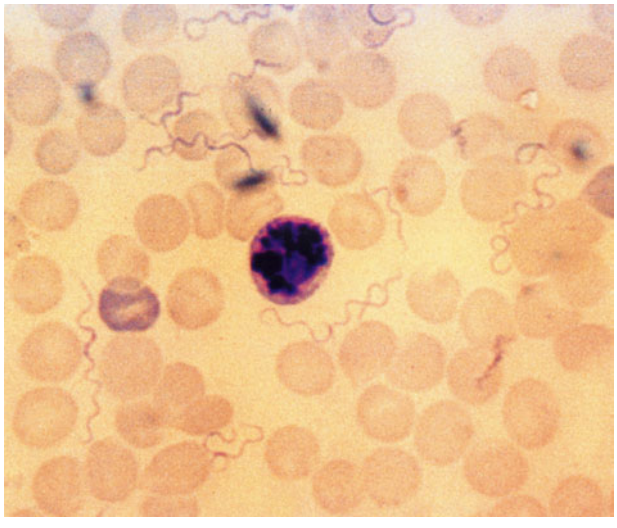


FIGURE 3 *B. hermsii* in a thin smear of rodent blood (Giemsa stain). Magnification, ×1,000.

Antigen Detection

Enzyme-linked immunosorbent assay (ELISA) and immunoblotting have been used for the detection of borrelial antigen in body fluids, including CSF and urine (25, 52). However, a commercial assay for antigen in urine was shown to lack reproducibility, and its use is not recommended (64).

Nucleic Acid Detection Techniques

Nucleic acid amplification techniques (NAT) should be restricted to experienced and specialized laboratories (92). For patients with suspected exposure outside of North America, it is imperative that selected NAT targets sensitively detect all human-pathogenic species. Species identification of amplification products is preferable (120).

Under circumstances where antibody detection and culture isolation fail to provide case confirmation, NAT can serve as a separate adjunct to clinical diagnosis. A variety of chromosomal and plasmid targets for NAT have been developed (for reviews, see references 3, 31, and 96). For PCR, an analytical sensitivity of approximately 10 to 20 borreliae per test sample has been demonstrated. Test sensitivities for both NAT and culture are greater with tissue specimens than with body fluids, except for synovial fluid, with which NAT is superior. Sensitivities of 96% (73) and 86% (14) were reported for NAT from synovial fluid from American patients with Lyme arthritis. European authors found NAT sensitivities ranging between 50 and 70% (34, 87, 106). Patients with Lyme arthritis are nearly always seropositive, so PCR of synovial samples is not used as a primary diagnostic technique. A positive PCR result after antibiotic therapy is of uncertain significance, since the presence of *B. burgdorferi* DNA does not necessarily mean that spirochetes are viable.

From skin biopsy and CSF specimens, NAT demonstrated diagnostic sensitivities of approximately 60 and 20%, respectively (15, 35, 68). A prospective study of PCR and culture detection of *B. burgdorferi* in EM biopsy samples from Slovenian patients showed comparable sensitivities (36% for culture in modified Kelly medium Preac-Mursic [MKP] medium, 24% for culture in BSK II medium, 25% for PCR, 54% with at least one positive test, and 6% with all three tests positive) (82). PCR targeting *ospA*, a plasmid-borne gene, is more sensitive than flagellin PCR, which uses a chromosomal target (79, 124). Borreliae may shed blebs containing plasmids, leading to target imbalance.

PCR amplification of *B. burgdorferi* sequences from urine has been described (89, 96) but is not recommended. Although *Borrelia*-specific DNA was demonstrated in over 70% of skin biopsy samples from patients with florid EM, parallel testing of urine samples was uniformly negative (15).

ISOLATION PROCEDURES

Many Lyme and relapsing fever borreliae are successfully cultured in artificial media. However, for diagnostic purposes, culturing is a slow, time-consuming method characterized by low sensitivity, especially from body fluids of patients with Lyme borreliosis (Table 4). For these reasons culture attempts are most often limited to research applications and performed by reference laboratories (e.g., the National Reference Center for Borreliae in Germany and the Centers for Disease Control and Prevention [CDC] in the United States).

Several media (modified Kelly medium, e.g., BSK II, BSK-H, or MKP) (9, 83, 86) are capable of supporting growth of borreliae. A commercial preparation of BSK-H is available from Sigma Chemical Co., St. Louis, Mo. It is important to verify the quality of each lot of medium by growing a reference strain from a small inoculum (<10 cells). Optimum growth (the generation time of *B. burgdorferi* is about 7 to 20 h) in these media is obtained at 30 to 33°C under microaerophilic conditions. Positive cultures from EM and synovial biopsy or fluid samples (blood and CSF) may be obtained in as few as 4 days, but most isolates require several weeks of incubation and negative cultures should be monitored by dark-field microscopy for at least 6 weeks, with blind passage into fresh medium every 2 weeks.

IDENTIFICATION

Molecular Techniques

Relapsing fever borreliae have been typed on the basis of DNA-DNA reassociation analysis and flagellin gene analysis (27, 57). *B. burgdorferi* has an arrangement of its rRNA genes (a single *rrs* and tandemly repeated *rrl* and *rrf* genes) which distinguishes it from the relapsing fever borreliae (which have single copies of each) (101). Sequencing of 5S-23S intergenic spacers is useful in typing borreliae. A number of molecular genetic methods, including pulsed-field gel electrophoresis of large restriction fragments (11), PCR, and sequence analysis or restriction fragment length polymorphism of the 16S rRNA/16S ribosomal DNA and of the 5S-23S intergenic spacer region (69, 84), have been described for species differentiation (summarized in reference 108). Analysis of certain protein-encoding genes, especially *ospA* and *ospC*, has also been utilized for *B. burgdorferi* sensu lato species identifications (35, 116, 117). However, in most cases, diagnosis and effective management of individual patients are independent of species determinations beyond the Lyme disease and relapsing fever groupings.

Immunological Techniques

Antibody responses to GlpQ, an antigen expressed by relapsing fever species but not Lyme borreliosis species, have been used to discriminate the two infections (100, 119a). For *B. burgdorferi* sensu lato, a serotyping method using monoclonal antibodies to OspA has been established. Eight different serotypes have been described among the pathogenic species for Europe, which has implications for OspA vaccine development (112, 117). Phenotypic analysis of OspC revealed 15 different serotypes (113). Some species-specific monoclonal antibodies have also been described.

SEROLOGICAL TESTS

Borrelia Antigens and Human Humoral Immune Response

In Lyme borreliosis the early antibody response is largely IgM and mainly directed against the outer membrane-associated OspC, p39 (BmpA), and p35 (BBK32, fibronectin-binding protein) and the flagellum subunits p37 (FlaA) and p41 (FlaB) (1, 4, 36, 49, 114).

The level of IgM antibody to most spirochetal antigens peaks within the first weeks of infection. However, IgM antibodies are often detected many months after effective treatment and cure (1). The IgG response increases and broadens slowly over the first weeks of disease. Among the reactive antigens to which there is an early IgG response are p35 (BBK32), p37 (FlaA), p41 (FlaB), OspC, and VlsE (1, 4, 66, 70, 77). During early disseminated (stage II) disease, IgG levels increase and reactivity against p39 (BmpA), DbpA (decorin binding protein), and p58 often appears (49) (Table 5). The late-stage immune response (stage III) is characterized by IgG antibodies to a wide variety of antigens (30, 49). Approximately 80% of the sera from European patients with late disease (arthritis and ACA) react with

TABLE 5 Reactivity of IgG antibodies with recombinant *Borrelia* proteins (DbpA, VlsE, and p58) in the line immunoblot[a]

Diagnosis	No. of sera	No. (%) of sera with reactivity							
		DbpA				VlsE			p58
		PBi (*B. garinii*)	PBr (*B. garinii*)	PKo (*B. afzelii*)	B31 (*B. burgdorferi*)	At least one DbpA[b]	PBi (*B. garinii*)	PKa2 (*B. burgdorferi*)	PBi (*B. garinii*)
EM[c]	15	0 (0.0)	2 (13.3)	4 (26.7)	1 (6.7)	5 (33.3)	12 (80.0)	6 (40.0)	1 (6.7)
Neuroborreliosis	50	19 (38.0)	20 (40.0)	17 (34.0)	6 (12.0)	39 (78.0)	44 (88.0)	41 (82.0)	27 (54.0)
Acrodermatitis	10	1 (10.0)	0 (0.0)	8 (80.0)	1 (10.0)	8 (80.0)	10 (100.0)	10 (100.0)	10 (100.0)
Arthritis	10	7 (70.0)	1 (10.0)	9 (90.0)	2 (20.0)	10 (100.0)	10 (100.0)	9 (90.0)	9 (90.0)
Controls	110	2 (1.8)	2 (1.8)	0 (0.0)	0 (0.0)	4 (3.6)	1 (0.9)	0 (0.0)	1 (0.9)

[a] Modified from reference 42.
[b] The combination of DbpA proteins from different strains (PBi, PBr, PKo, and B31) results in considerable increase of sensitivity in neuroborreliosis.
[c] Disease duration of >2 weeks.

p14, Osp17 (DbpA), p21 (not OspC), p30, p39, p43, p58, and p83/100 (homolog of p93) of *B. afzelii* strain PKo. Interestingly, IgG antibodies against OspC are detected in only 20% of these patients (49). The frequency of IgG reactivity to OspC in American patients with late disease is higher (48%) (30).

OspA is downregulated at the time of initial infection in the mammalian host. This explains why antibodies against OspA are rarely observed in patients with early disease. Among American patients with late disease, 42% were reactive to OspA (30), in contrast to 5 to 7% among European patients (49, 114). Notably, OspA antibodies are quite common in a special group of patients from the United States who suffer from therapy-refractory forms of Lyme arthritis (4). Subsequently it was shown that patients with Lyme borreliosis have a strong IgG response against a conserved epitope (C6 peptide derived from the IR6 region of VlsE) or the whole recombinant VlsE, which appears earlier during disease than immune responses to other borrelial antigens. This was observed in both American and European patients (7, 70, 97). Differences in VlsE reactivity were observed using VlsE proteins derived from different strains (42). Notably, VlsE from a *B. garinii* strain was more reactive for detection of IgM antibodies than VlsE proteins from *B. burgdorferi* sensu lato or *B. afzelii* strains.

Two-Step Approach in Serodiagnosis

For serodiagnosis of Lyme borreliosis, a two-step approach is recommended by the Association of State and Territorial Public Health Laboratory Directors (ASTPHLD) and the CDC (21, 55): "All serum specimens submitted for Lyme disease testing should be evaluated in a two-step process, in which the first step is a sensitive serological test, such as an enzyme immunoassay (EIA) or immunofluorescence assay (IFA). All specimens found to be positive or equivocal by a sensitive EIA or IFA should be tested by a standardized immunoblot procedure. Specimens found to be negative should not be tested further" (21). This procedure is also recommended in the MiQ Lyme borreliosis standard published by the German expert group on the diagnosis of Lyme borreliosis of the German Society for Hygiene and Microbiology (DGHM) (Fig. 4) (120). The concept of a two-step approach, which aims at increasing the predictive value of a positive result with each step, requires that the tests be performed in succession (56, 120). Omitting the first step, a quantitative assay, and proceeding directly with qualitative immunoblots reduces the specificity of the procedure.

IFA

For the IFA, borreliae fixed on glass slides are used as the antigen. IFA for serodiagnosis of relapsing fever, however, is challenging since expression of the major membrane proteins is variable. The specificity of IFA serodiagnosis for Lyme disease may be improved by adsorption of sera with *Treponema phagedenis* sonicate (IFA-ABS) (120). For the IgM test, pretreatment of the sera with anti-IgG-immune serum is recommended to avoid false-positive test results due to rheumatoid factor as well as false-negative results due to high IgG antibody levels. As in all antibody detection assays, it is important to verify

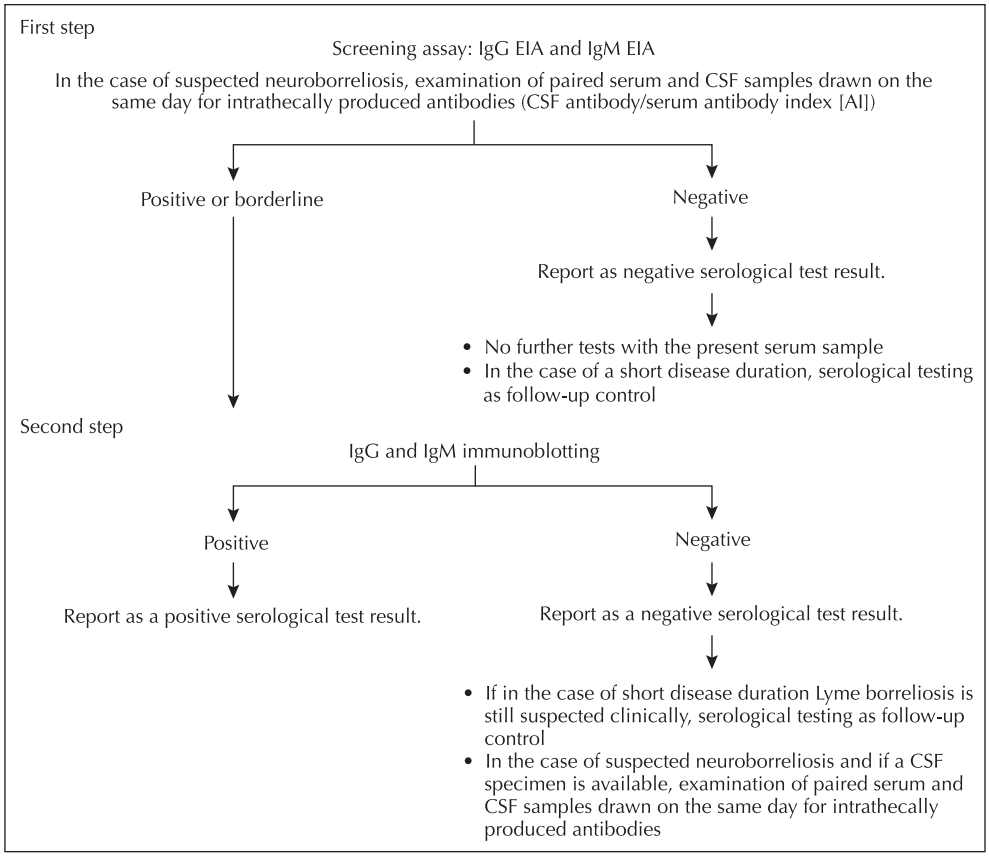


FIGURE 4 Two-step approach for serodiagnosis.

expression of OspC within the antigen source cultures. Although IFA is relatively easy to perform, it is not easy to standardize, and evaluation of test results requires expertise not always available in the routine laboratory. In general, antibody titers of ≥64 and ≥256 are regarded as positive in the IFA-ABS and unadsorbed IFA, respectively. Sera from patients with syphilis are often positive in the unadsorbed assay and are rarely positive in the IFA-ABS (120).

EIA

Different modifications of the EIA have been used for the diagnosis of Lyme borreliosis. In the indirect EIA, antigen is used to coat the plates, followed by incubation with patient serum, enzyme-labeled anti-IgM or anti-IgG, and the EIA substrate. Capture IgM-EIA (μ-capture EIA) has been specially designed to avoid false-positive reactivity due to rheumatoid factor (48). Rheumatoid factor false-positive reactivity can also be overcome by pretreatment of the sera with anti-IgG (114). EIA has the advantage of objective measurement, quantification, and high throughput. Many different antigen preparations have been used, including whole-cell sonicates (93), isolated flagella (46), detergent extracts (114), recombinant antigens (61, 67, 114), and synthetic peptides (70). Use of crude antigen preparations, such as whole-cell sonicates, often results in unacceptable specificity. Improved tests which utilize enriched, specific, or recombinant antigens are now widely used. Tests using an octyl β-D-glucopyranoside detergent extract and Reiter treponeme absorbent, isolated flagella, recombinant VlsE, or the C6 peptide of VlsE are commercially available (Dade-Behring, Marburg, Germany; Dakopatts, Copenhagen, Denmark; Diasorin, Turin, Italy; and Immunetics, Boston, Mass.). Since VlsE is not present in relevant amounts in cultivated borreliae, recombinant VlsE has been added to whole-cell extracts to increase sensitivity in some products (Dade-Behring).

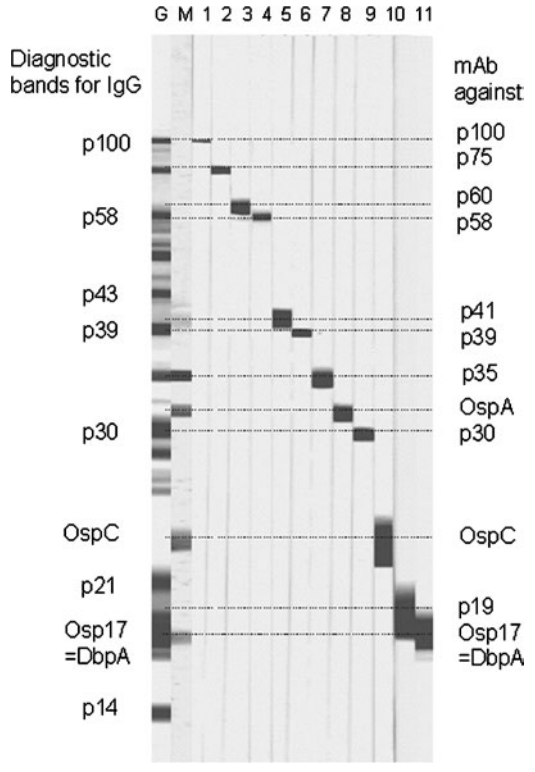


FIGURE 5 Whole-cell immunoblot for identification of diagnostic bands with monoclonal antibodies (mAb). The antigen used is *B. afzelii* strain PKo. Lane G, IgG blot from patient with late disease; lane M, IgM blot from patient with early disease; lanes 1 to 11, different monoclonal antibodies against the respective reactive proteins. (Modified from reference 49.)

Immunoblotting

Western immunoblotting enables assessment of the humoral immune response to individual protein antigens as resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The Western immunoblot is regarded as a supplementary (United States) or confirmatory (Europe) assay. This implies that it should be employed only when a screening assay is reactive (positive or indeterminate, sometimes called equivocal). Antigen preparations for Western immunoblotting include whole-cell lysates or recombinant antigen mixtures that are transferred after SDS-PAGE to blot membranes.

The advantage of the whole-cell lysate immunoblot is that antibodies against a large number (~50) of antigens can be detected. Herein also lies a significant challenge to the diagnostic laboratorian—discerning reactivity between specific and nonspecific or cross-reactive antigens that may not be clearly resolved from each other. Thus, reliable identification of the immunoreactive bands is imperative, i.e., by use of monoclonal antibodies (Fig. 5) (120). Immunoblotting for Lyme borreliosis is considered technically complex, and in order to realize its maximal performance and utility, standardization and careful quality control must be used.

Numerous immunoblot tests which use antigens of various strains or genospecies of *B. burgdorferi* sensu lato are commercially available. The ASTPHLD and the CDC, as well as the DGHM, have published recommendations for interpretation of the *Borrelia* immunoblot (21, 120). In the United States, immunoblot interpretation rules have been recommended which refer to detection of antibody against whole-cell antigens of specific *B. burgdorferi* sensu stricto strains (30, 36). The IgM immunoblot is interpreted as positive if ≥2 bands of the following proteins are reactive: OspC, 39 kDa (BmpA), and 41 kDa (FlaB). The IgG blot is interpreted as positive if ≥5 bands of the following proteins are reactive: OspC and 18, 28, 30, 39 (BmpA), 41 (FlaB), 45, 58, 66, and 93 kDa. If the immunoblot is used within the first 4 weeks of disease onset (early, stage I or II), both IgM and IgG immunoblots should be performed. A positive IgM immunoblot alone should not be used in determining infection in persons with illness duration of >1 month because the likelihood of a false-positive test for current infection is high for these individuals.

Interpretation of the antibody response among European patients is complicated by the risk of infection with different *Borrelia* species. In addition, immunoblot studies have shown that the immune response of European patients, compared with American patients, is restricted to a narrower spectrum of *Borrelia* proteins (29). Interpretive rules defined in a species- and strain-specific manner have been determined (49) and independently corroborated (62). *B. afzelii* strain PKo is preferred to PBi *(B. garinii)* and PKa2 (*B. burgdorferi* sensu stricto) strains because it permits a two-band criterion for the IgG test: at least two bands positive for p14, Osp17 (DbpA), p21, OspC, p30, p39 (BmpA), p43, p58, and p83/100 (Fig. 5) (120). According to the general Deutsches Institut für Normung (DIN) recommendations

on the immunoblot (DIN 58967, Part 40), at least a two-band criterion should be required for the positive interpretation of the IgG immunoblot. In IgM immunoblots, a detectable immune response is restricted to only a few bands. Therefore, the IgM blot is regarded as positive if there is strong reactivity to OspC (120). Specific DIN recommendations for the *Borrelia* immunoblot (DIN 58969, Part 44) have been published which include new antigens (i.e., VlsE) and the line immunoblot as a new technique.

Recombinant immunoblots with Osp17 (DbpA), OspC, p39 (BmpA), truncated p41 (FlaB), p58, and p83/100 have demonstrated sensitivity comparable to that of the whole-cell immunoblot except for patients with isolated EM (115). The recombinant immunoblot was substantially improved by the addition of several homologs of VlsE and DbpA, which considerably increased the sensitivity of antibody detection in early disease (42, 97). Recombinant blots containing DbpA, OspC, p39 (BmpA), truncated p41 (FlaB), VlsE, and p83/100 are commercially available (Mikrogen, Munich, Germany). Using the line blot technique, which allows detection of antibodies against antigens with identical molecular weights (i.e., homologs of the same *Borrelia* protein) (Fig. 6 and Table 5), the recombinant immunoblot is significantly more sensitive than the conventional sonicate immunoblot (i.e., 91.7 versus 68.8%) for patients with early neuroborreliosis (42).

For commercial immunoblot tests, the user should verify that all relevant antigens are present (must be detected by the positive control serum) and interpretation rules are established in defined patient and control populations. In immunoblot evaluation, a scored band must have an intensity equal to or greater than that of the cutoff control. Commercial Western immunoblot test kits licensed by the FDA are available from five different manufacturers. Current listings of 510K, FDA-approved serodiagnostic assays for Lyme disease can be found at http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfPMN/pmn.cfm under product code LSR.

Detection of Intrathecally Produced (CSF) Antibodies

Detection of the intrathecal *Borrelia*-specific immune response is one of the most valuable tools for the diagnosis of neuroborreliosis. Methods taking into account potential dysfunction of the blood-CSF barrier, a common finding in neuroborreliosis, are required for accurate assessment of intrathecal antibody production. Since the permeability of the blood-CSF barrier may change rapidly in inflammatory diseases like acute neuroborreliosis, CSF and serum must be obtained at the same time. Long-used procedures for detection of specific intrathecal antibody production in the diagnosis of neurosyphilis have been modified for the diagnosis of neuroborreliosis (47, 103, 119). The most frequently used method is the determination of the CSF/serum antibody index (specific antibody index [AI]).

This method requires measuring the specific antibodies in the blood and in the CSF by means of a calibrated standard curve and determination of a quotient from the antibody units and the IgG concentrations in CSF and serum:

$$\mathrm{AI} = \frac{\text{EIA units in CSF} \times \text{total IgG in serum}}{\text{EIA units in serum} \times \text{total IgG in CSF}}$$

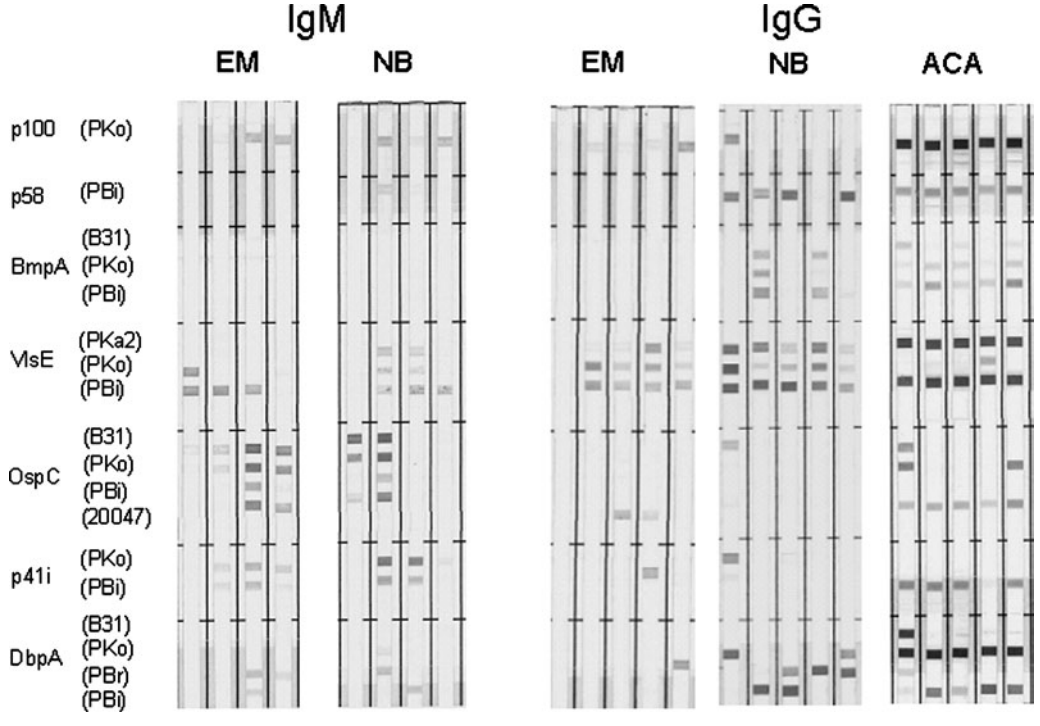


FIGURE 6 Recombinant line immunoblot. Shown are representative IgG blots and IgM blots of patient and control sera. Strains of the following species are shown: *B. burgdorferi* sensu stricto B31 and PKa2, *B. afzelii* PKo, *B. garinii* OspA type 3 (PBr), *B. garinii* OspA type 4 (PBi), and *B. garinii* unknown OspA type (20047). Sera were obtained from patients with EM, early neuroborreliosis (NB), and ACA. (Modified from reference 42.)

For the determination of the AI, EIAs allowing quantitative measurement are suitable (119). The total IgG concentrations of serum and CSF are determined by nephelometry. Alternatively, both serum and CSF can be used for EIA after adjustment to identical concentrations of total IgG (e.g., 1 mg/ dl) and the index is calculated using a simplified formula:

$$\text{AI} = \frac{\text{EIA units in CSF}}{\text{EIA units in serum}}$$

By calculating the AI, CSF and serum are compared with regard to the portion of pathogen-specific IgG antibodies in the total IgG content. An AI of ≥2.0 is considered significantly elevated (71, 120). Lower indices (e.g., ≥1.3) are also considered significant by some investigators. However, there is a higher risk of false-positive results due to technical imprecision of measurement of immunoglobulin levels and antibody titers. False-positive AI results are likely with neurosyphilis patients when tested with whole-cell or flagellum sonicates in EIA. Here EIAs with *T. phagedenis* adsorption (Dade-Behring) or recombinant antigens not cross-reacting with *Treponema pallidum* can be helpful for differential diagnosis. Other suitable methods for determination of intrathecal antibody production are the μ- or γ-capture EIA (Dakopatts) (47) and the IgG-matched immunoblot (119). The latter allows comparison of the antibody spectrum (against various *Borrelia* proteins) in serum and in CSF and thus permits conclusions as to the specificity of the intrathecal antibody response.

For cases of neuroborreliosis, intrathecally produced antibody increases dramatically over time. Antibody was detected in only 17% in the first week. Detection rates increased to 80 to 90% of cases between 8 and 41 days after onset of disease and up to 100% of cases after 41 days after onset (47). In a study with children, cases were reported where only the IgM antibody test performed on CSF showed a positive result and no specific antibodies were detected in serum samples taken at the same time (23).

Vaccination and Its Impact on Serology

In the United States, an OspA vaccine (LYMErix) against Lyme borreliosis was licensed for use in humans. In 2002, the producer withdrew the LYMErix vaccine from the market for commercial reasons. Although the OspA vaccine is no longer commercially available, it is still necessary to differentiate between antibodies induced by vaccine and natural infection. Appropriate EIAs include tests lacking the respective vaccine protein, i.e., such as those based on recombinant antigens or peptides or an OspA-deficient mutant strain. Confounding Western immunoblot reactivity that is often observed among OspA vaccine recipients to molecular weight antigens other than p31 is due to fragments of OspA (or cross-reactive OspB) that migrate independently of the intact molecule during SDS-PAGE. Despite concerns, the experienced Lyme borreliosis laboratorian is most often able to distinguish OspA vaccination from early infection using the standard two-test paradigm (2). However, the recent availability of commercial recombinant immunoblots simplifies serodiagnosis in vaccinated individuals.

Controversial Methods

A variety of diagnostic approaches have been developed as alternatives or adjuncts to the more widely practiced methods described above. T-lymphocyte proliferation assays have been used in various scientific studies to investigate the human T-cell response to *Borrelia* antigens (63). However, T-lymphocyte proliferation assays cannot be recommended as diagnostic tests due to their cumbersome nature and concerns about their specificity and standardization (22, 50, 111, 125). Antigen detection tests also are not recommended, as discussed above.

Detection of *B. burgdorferi*-specific antibodies in immune complexes has been proposed to be superior for serodiagnosis of acute Lyme disease (98) and as a marker of active infection (17). Recent work demonstrates that test results for antibodies precipitated from serum as immune complexes are highly correlated with ELISA results obtained using unprocessed serum and are not more likely to reflect active infection than standard serology (72). Transformation of *B. burgdorferi* into spheroplasts (L-forms) in vitro in response to deprivation of serum or culture in CSF has been observed (5, 16). When visualized under a microscope, spheroplasts sometimes appear to be enclosed in a sac, so they have also been called "cysts." If apparently pure L-forms are injected into mice, they are infectious (45). The clinical and diagnostic significance of L-forms has not been demonstrated but warrants further study. There are commercial tests offered that purport to specifically detect cell wall-deficient or "cystic" forms of *B. burgdorferi* by IFA (U.S. patent 6,838,247) and culture (81), but they have not been validated with appropriate controls and are not recommended.

ANTIMICROBIAL SUSCEPTIBILITIES

The antimicrobial susceptibility of *Borrelia* species has been studied intensively in vitro (57, 85). Standard methods for the determination of the minimal bactericidal concentration have not been established. However, there is general agreement on the in vitro susceptibility of borreliae to antimicrobials, as follows. *B. burgdorferi* sensu lato is sensitive to macrolides, tetracyclines, semisynthetic penicillins, and the late second- and third-generation (expanded- and broad-spectrum) cephalosporins; moderately sensitive to penicillin G and chloramphenicol; and resistant to trimethoprim, sulfomethoxazole, rifampin, the aminoglycosides, and the quinolones (57). No significant differences between the Lyme disease borreliae and relapsing fever borreliae *(B. hermsii* and *B. turicatae)* were found with regard to penicillin G, amoxicillin, ceftriaxone, erythromycin, azithromycin, doxycycline, or tetracycline (57). There is no indication for routine antimicrobial susceptibility testing in either Lyme disease or relapsing fever.

Recommendations for Antibiotic Therapy

All clinical manifestations of *B. burgdorferi* infection should be treated with antibiotics. The antibiotic, dosage, duration, and route of application depend on the clinical picture and the stage of the disease (110, 122). In cases of solitary EM, oral treatment with doxycycline, amoxicillin, or cefuroxime axetil is recommended. Disseminated infections require parenteral treatment (ceftriaxone, cefotaxime, or high-dose treatment with penicillin G). In acrodermatitis, the same antibiotics and daily doses are recommended as in EM. In arthritis, oral treatment with doxycycline may be tried first, but in cases of poor therapeutic response, patients should be treated intravenously with cephalosporins or penicillin G. Intravenous cephalosporins or penicillin G is also recommended for stage III neuroborreliosis.

INTERPRETATION AND REPORTING OF RESULTS

General Aspects

Clinical criteria (case history and clinical findings) are decisive factors in the diagnosis and ordering of microbiological laboratory testing. The predictive value of laboratory tests is directly

related to the pretest probability. It should be kept in mind that the lower the probability of the clinical diagnosis, the lower the predictive value of a positive test result. For example, a negative serological result has a high negative predictive value for Lyme arthritis, since nearly all cases are seropositive. Whether or not a positive test corresponds with the patient's presentation is a question that can only be answered by the clinician, e.g., by means of clinical case definitions applied to the various manifestations of Lyme borreliosis. Therefore, the laboratory report should not contain any therapy recommendations.

Serological Report

The serological report should contain the following points:

1. Recording of individual test results. Results of the first assay, generally an EIA, are reported as positive, indeterminate, or negative. The immunoblot results are reported as positive or negative. In the case of a positive result, the reactive diagnostic bands may be reported (120). Caution against overinterpretation of minimally reactive blots must be emphasized (e.g., IgG reactivity against p41 is expected in approximately 50% of healthy adults in the United States and Europe and is excluded from the European scoring criteria).
2. Assessment of the final result of the two-step approach regarding its immunodiagnostic significance (e.g., whether specific antibodies have been detected or not)
3. Assessment of serological findings as to the stage of the immune response, as far as test results allow pertinent statements to this effect (see below)
4. Recommendations for further reasonable diagnostic methods (PCR or culture) or for serological follow-up, if indicated

Patterns of Serological Results in Various Stages of Lyme Borreliosis

Antibody tests performed in the early stage of Lyme borreliosis may show a negative or an indeterminate result (Table 6), often due to insufficient time for the full evolution of the immune response. In some cases, seroconversion does not occur until after initiation of treatment. Serological testing of patients with EM alone is not recommended because of the low predictive value of a negative result and the highly characteristic presentation of most rashes. In the presence of a positive clinical correlation and inadequate response to therapy, serology may be warranted up to 6 weeks after onset of disease. During early-stage Lyme borreliosis, detection of IgM is consistent with an active infection. A robust IgG response in the absence of IgM may indicate an anamnestic response to reinfection except with VlsE and C6 peptide assays, where the IgG response is early and predominant (7). Usually only a few bands are detected by immunoblotting in early disease (IgG and IgM) (Fig. 6). In late disease, a positive test for IgG antibodies is mandatory for seroconfirmation. In many cases, IgM antibodies are absent. In principle, an IgM test is not useful for establishing the diagnosis of late disease. The absence of IgG rules out the diagnosis of late Lyme disease even in the presence of IgM. False-positive IgM results due to a polyclonal B-cell activation immune response in the context of herpesvirus infections or autoimmune diseases and rheumatoid disorders also need to be considered. In many cases the origin of such IgM findings remains unclear. The IgG immunoblot usually shows a broad band pattern (Fig. 6). However, positive IgG findings with a broad band pattern in the immunoblot are also compatible with past, asymptomatic, spontaneously resolved, or treated infections. Such result patterns are often found for members of high-risk groups with frequent tick exposure (for example, forest workers) who do not show any clinical manifestations.

In neuroborreliosis, apart from the serum findings, the detection of pathogen-specific, intrathecally produced antibodies is of utmost importance for the diagnosis. An elevated AI is more common in European patients than in U.S. patients, possibly owing to infection with *B. garinii* in Europe. In most cases, determination of the IgG AI will be sufficient. Neuroborreliosis in children is a notable exception to the rule because only the IgM AI may be positive, particularly in cases where disease duration has been short. In the latter circumstances, positive CSF findings may precede positive serology. Therefore, examination of paired serum and CSF specimens is mandatory in such cases. Since a positive AI may be detectable years after treatment and cure, repeated testing is not appropriate for monitoring therapy success. Diagnosis of chronic neuroborreliosis is based on demonstration of a positive AI. Detection of antibodies against *B. burgdorferi* in the serum alone is not sufficient, and direct detection of the pathogen by culture or NAT is rarely successful.

TABLE 6 Sensitivity of antibody detection methods in the diagnosis of Lyme disease

Stage	Sensitivity	Remarks
I	20–50%	Predominance of IgM
II	70–90%	Presence of IgM and IgG; in cases of long disease duration, predominance of IgG
III	Nearly 100%[a]	Usually only IgG

[a] Negative only for patients with a very short duration of symptoms.

Influence of Antimicrobial Therapy on Serodiagnosis

Clinicians are often tempted to order repeated posttreatment serologies in an effort to correlate cure and decreasing antibody titers. However, IgG antibodies against *B. burgdorferi* (especially those against whole-cell antigens) persist for a long time even after successful therapy. Significant titer changes can only be expected several months after the end of therapy; in cases of late manifestations, even years may elapse. Moreover, a decrease in antibody titer does not rule out persistence of the pathogen. Since there is practically no indication for follow-up serological tests, therapeutic success should be based on clinical criteria. A fourfold decline in titer of antibody to VlsE peptide C6 was shown to be an indicator of successful therapy for acute Lyme disease (80) but was not demonstrated for late disease (78) or posttreatment Lyme disease syndrome (39).

Sources of Error in Serodiagnosis

False results, both negative and positive, can occur from the test itself or the nature of the immune response. Seronegative results within the first days of disease are the norm. In Europe, differences between the test antigen and the species causing infection may also contribute to seronegative findings. Deficiencies in diagnostic antigen expression will compromise the sensitivity of tests (important diagnostic antigens such as OspC and DbpA are often not expressed in cultivated borreliae, and VlsE is poorly expressed in vitro). The high background reactivity of many first-generation whole-cell-based assays often results in lower specificity and therefore frequent false-positive results. Cross-reactivity with treponemes can be

largely avoided by use of Reiter treponeme adsorbent, although syphilis serology should be performed in cases where treponeme exposure cannot be ruled out. Second- and third-generation assays with improved sensitivity and specificity are preferable to the first-generation tests. Notably, the development of tests based on VlsE has considerably improved serodiagnosis in early disease. Nonetheless, critical assessment of pretest risk factors, clinical history, and presentation will provide the best guidance for laboratory test use and minimize false test outcomes of current and future diagnostic tests.

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Treponema and Other Human Host-Associated Spirochetes*

VICTORIA POPE, STEVEN J. NORRIS, AND ROBERT E. JOHNSON

63

TAXONOMY

The genus *Treponema* (order *Spirochaetales*, family *Spirochaetaceae*) includes four invasive human pathogens (Table 1) and a rabbit pathogen, a large number of oral spirochetes found in the gingival crevices in humans and other animals, and a few commensal skin organisms. The ecologic diversity of this genus was revealed recently by the discovery that *Treponema* species are also associated with bovine digital dermatitis, severe virulent ovine foot rot, and the normal gut flora of termites (25, 40, 119). In 1984, the genus taxonomy was restructured so that the species *Treponema pallidum* includes three of the human pathogens: *T. pallidum* subsp. *pallidum* (venereal syphilis), *T. pallidum* subsp. *endemicum* (endemic syphilis), and *T. pallidum* subsp. *pertenue* (yaws) (113). T. *carateum* (pinta) remains a separate species due to the lack of genetic information. The pathogenic treponemes in this group are very closely related, to the extent that they are distinguished primarily by their patterns of pathogenesis in humans and experimentally infected animals. They are morphologically indistinguishable and, where examined, have >95% DNA homology by hybridization (30), a high degree of sequence identity of known genes (84, 85), nearly identical protein profiles (see reference 90 for a review), and shared reactivity with monoclonal antibodies (82). There may be some subspecies differences, as well as differences between *T. pallidum* and *T. paraluiscuniculi,* a rabbit pathogen, in the flanking-region sequences of the 15.5-kDa lipoprotein gene (17). In addition, differences in the *tpr* and *arp* genes have been used for the molecular subtyping of *T. pallidum* strains (18, 19, 98); this approach has been shown to be useful in epidemiologic studies (100, 117). According to structure, host dependence, protein content, and DNA sequence similarities (17), *T. paraluiscuniculi* appears to be closely related to the human pathogens; however, it causes venereal spirochetosis of rabbits and is not known to cause human disease. *Brachyspira aalborgi* and *Brachyspira* (formerly *Serpulina*) *pilosicoli* are recently described human intestinal spirochetes. Because of the distinctive nature of invasive treponemal pathogens, oral spirochetes, and intestinal spirochetes, these three groups are discussed separately below.

In contrast to the close relationship among the human pathogens, *T. pallidum* subsp. *pallidum* has <5% DNA homology by hybridization with other spirochetes such as *T. phagedenis, T. refringens,* and *B. hyodysenteriae* (30). However, 16S rRNA gene sequence similarities indicate an evolutionary relationship between the members of the genus *Treponema* (93). The sequence of the 1.13×10^6-bp *T. pallidum* subsp. *pallidum* Nichols genome was published in 1998 (36), and sequencing of the 2.8×10^6-bp *T. denticola* genome was published in 2004 (112). Although the *T. denticola* genome is much larger, the predicted gene content indicates that both organisms have very limited metabolic capabilities; for example, energy production appears to be limited to glycolysis, and the pathways for synthesis of fatty acids and most amino acids are absent. Many of the additional genes in *T. denticola* are transport systems and degradative proteinases and hydrolases, consistent with a high scavenging activity for acquiring nutrients in the dense biofilm of dental plaque (36, 112).

SPIROCHETES ASSOCIATED WITH HUMAN TREPONEMATOSES

Description

T. pallidum subsp. *pallidum (T. pallidum)* and related human pathogens are spirochetes ~0.18 μm in diameter and ranging in length from 6 to 20 μm (Fig. 1). These organisms are coiled into regular helices (6 to 14 per cell) with a wavelength of 1.1 μm and an amplitude of ~0.3 μm (Fig. 1). The ends are pointed and lack the hook shape characteristic of some commensal human spirochetes. Suspensions of *T. pallidum* are best visualized by dark-field microscopy, although the bacterium can also be seen by phase-contrast microscopy. Unstained organisms are not visible by standard bright-field microscopy because of the small cell diameter. Fresh preparations of the organism exhibit rapid rotation about the axis (the characteristic corkscrew motility) due to the action of flagella inserted in both ends and extending down the cell body within the periplasmic space (Fig. 1). Flexing and reversal of rotation can occur also, but translational motion is not observed unless *T. pallidum* is in a viscous medium. The human treponemal pathogens (Table 1) cannot be distinguished at either the light or electron microscopy level. Because the diameter of the treponemes is less than 1 μm, Gram stain cannot be used to visualize any of the pathogenic *Treponema.*

* This chapter contains information presented in chapter 61 by Steven J. Norris, Victoria Pope, Robert E. Johnson, and Sandra A. Larsen in the eighth edition of this Manual.

TABLE 1 Characteristics of the human treponematoses[a]

Organism	Disease	Distribution	Predominant age of onset	Transmission	Congenital infection
T. pallidum subsp. *pallidum*	Venereal syphilis	Worldwide	Adolescents, adults	Sexual contact	Yes
T. pallidum subsp. *pertenue*	Yaws (frambesia, pian)	Tropical areas, Africa, South America, Caribbean, Indonesia	Children	Skin contact	No
T. pallidum subsp. *endemicum*	Endemic syphilis (bejel, dichuchwa)	Arid areas, Africa, Middle East	Children to adults	Mucous membrane	Rarely
T. carateum	Pinta (carate, cute)	Semi-arid, warm areas, Central and South America	Children, adolescents	Skin contact	No

[a] Data from references 2, 28, 29, 95, and 122.

Natural Habitat

The *T. pallidum* subspecies and *T. carateum* are obligate parasites of humans and are not known to have any animal or environmental reservoirs. Venereal syphilis has a worldwide distribution, with the incidence varying widely according to geographic location and socioeconomic groups (Table 1). Endemic syphilis is restricted to desert and temperate regions of North Africa and the Middle East. Yaws occurs most commonly in tropical or desert regions of Africa, South America, and Indonesia. Pinta is found primarily in tropical areas of Central and South America.

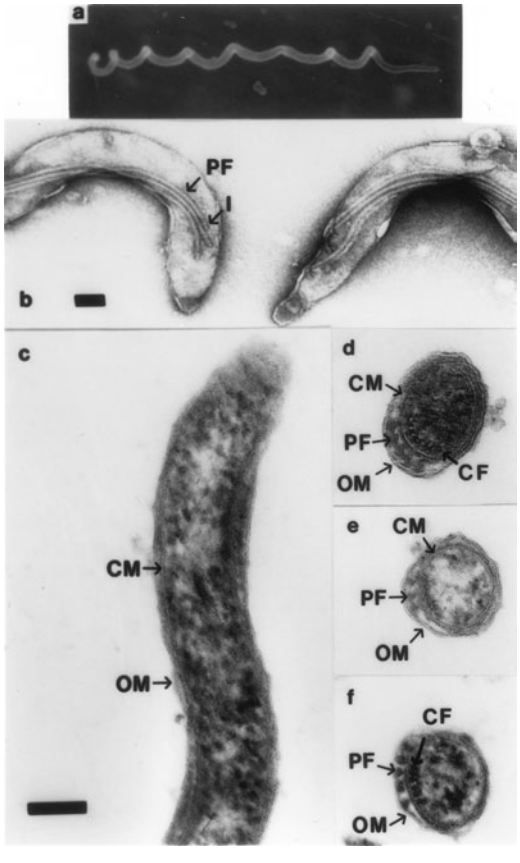


FIGURE 1 Morphology of *T. pallidum*. (a) Scanning electron micrograph showing spiral shape. (b) Negatively stained view of the tips of two organisms. Note the insertion points (I) of periplasmic flagella (PF) near the ends. (c to f) Electron micrographs of ultrathin sections, showing the outer membrane (OM), the cytoplasmic membrane (CM), periplasmic flagella (PF), and the location of the cytoplasmic filaments (CF). Bars, 0.1 μm. Reprinted from reference 88 with permission of Springer NL.

Clinical Significance

Syphilis is still a common sexually transmitted disease in many areas of the world, despite the availability of effective therapy. In 1999, the World Health Organization (WHO) estimated that the worldwide annual incidence of sexually acquired syphilis was 12 million cases (125). In the United States, 34,270 cases were reported in 2003, including 7,177 cases of primary and secondary syphilis and 413 cases of congenital syphilis in infants younger than 1 year. Rates of reported syphilis cases declined rapidly during the 1990s (Fig. 2), but transmission has persisted in certain areas, especially the Southeastern United States. In 1999, to address the increasingly focused occurrence of syphilis, the U.S. Centers for Disease Control and Prevention (CDC), in collaboration with other federal partners, launched "The National Plan to Eliminate Syphilis in the United States" using a combination of improved surveillance, strengthened community involvement, rapid outbreak response, expanded clinical and laboratory services, and enhanced health promotion (http://www.cdc.gov/stopsyphilis/Plan.pdf). The year 2010 target is 0.2 cases of primary and secondary syphilis per 100,000 population, whereas there were 2.5 cases per 100,000 population in 2003.

The male/female ratio of case rates increased from 1.6 in 1999 to 5.3 in 2003 (16). This increase in the rate of primary and secondary cases among men relative to women in the United States, along with reports from localities of outbreaks of syphilis and of high-risk sexual behavior among men who have sex with men (MSM) (24, 94), reflects an increase in sexually transmitted disease (STD) risk behavior among some MSM. Of particular concern is the high prevalence of human immunodeficiency virus (HIV) infection among syphilis cases reported in these outbreak reports and summarized in recent reviews (8, 20). Because of the increase in syphilis among MSM and because the evidence is strong that syphilis increases susceptibility to HIV infection and transmissibility of HIV infection (11, 35), federal agencies and medical organizations representing HIV care providers recommend screening for syphilis and other sexually transmitted infections among MSM (1, 13, 16).

The prevalence of nonvenereal treponematoses is uncertain due to inadequate surveillance, but in 1998 the WHO

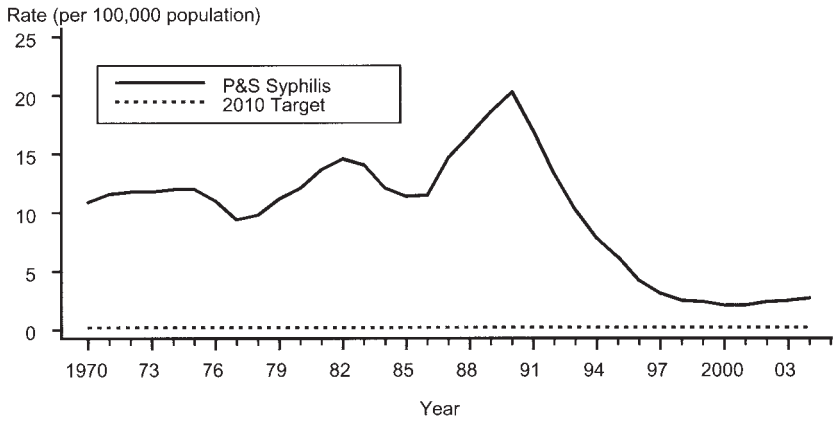


FIGURE 2 Rates of reported primary and secondary syphilis cases per 100,000 population in the United States, 1970 to 2004. The objective of ≤0.2 cases per 100,000 by the year 2010 is indicated (16).

estimated that there were 2.6 million active cases worldwide and 460,000 new cases per year (124).

Transmission of *T. pallidum* occurs through direct contact with active lesions. The transmission of *T. pallidum* subsp. *pallidum* infection, the cause of venereal syphilis, usually occurs consequent to contact of an uninfected individual with the anogenital lesions of an infected individual during sexual intercourse. Transplacental infection of the fetus also occurs in infected pregnant women.

Venereal syphilis exhibits a wide variety of clinical manifestations (Table 2). Early syphilis, defined as syphilis of <1-year duration, is itself subdivided into primary, secondary, and early latent stages. The primary stage is characterized by the appearance of one or more 0.3- to 2.0-cm indurated lesions, called chancres, which typically become encrusted or ulcerated. The lesions occur at the point of organism entry after a 10- to 90-day incubation period. The most common characteristic of secondary syphilis is the presence of skin and mucous membrane lesions. Skin lesions may be macular, papular, follicular, papulosquamous, or pustular. So-called "nickel and dime" macular lesions on the palms of the hands, soles of the feet, and other locations are a common presentation. Raised lesions called condylomata lata may occur in moist intertriginous areas, and erosions called mucous patches may appear on mucosa, such as in the mouth. Neurologic symptoms and signs can also occur during secondary-stage syphilis, and, less commonly, disease manifestations involving other organ systems. Secondary syphilis manifestations do not appear until 6 weeks to 6 months after infection. Primary and secondary syphilis manifestations may overlap, but each resolves spontaneously within a few weeks of its onset.

Numerous treponemes are often present in the ulcers of primary syphilis and the moist intertriginous and mucosal lesions of secondary syphilis. Transmission occurs as a consequence of contact with these primary- and secondary-stage lesions. Nonvenereal transmission is rare. Individuals with venereal syphilis are no longer infectious after healing of secondary syphilis lesions. However, relapses of secondary syphilis can occur in about 25% of untreated patients during the first year of infection (38).

Approximately one-third of untreated patients may develop tertiary or late syphilis following an extended period (usually years to decades) of latency during which clinical symptoms of syphilis are absent (38). The manifestations of late syphilis consist of several characteristic conditions of the central nervous system, disease of the aortic valve and thoracic aorta, and a chronic inflammatory lesion called a gumma, which usually involves the skin or bone but may occur in any organ (122). The cardiovascular and gummatous forms of syphilis are now rarely encountered, presumably because of effective therapy of early syphilis and antimicrobial therapy administered for other infections.

Viable *T. pallidum* is present in the cerebrospinal fluid (CSF) of patients with the primary and secondary stages of syphilis, indicating that central nervous system infection occurs very early in the course of disease (104). Neurologic manifestations, including syphilitic meningitis, may occur as early as 3 months postinfection; thus, neurosyphilis should not be considered solely a late manifestation of the disease (48).

Numerous case reports indicate that HIV infection may result in an increased severity of early syphilis and a poor response to therapy (103), although the results of prospective therapy trials suggest that HIV infection does not markedly affect the natural history of syphilis in most instances of coinfection (104, 106).

Congenital syphilis results from the transmission of *T. pallidum* across the placenta during pregnancy. Early congenital syphilis tends to occur when the mother has early syphilis during the course of pregnancy and may result in stillbirth or fulminant infection of the newborn (Table 2). Late manifestations of congenital syphilis are the outcome of chronic, untreated infection and can result in multiple stigmata (Table 2) that are often not obvious until the second decade of life.

Yaws, endemic syphilis, and pinta were common infections in areas of endemicity prior to the WHO eradication program beginning in 1948; in the early 1950s, it was estimated that 200 million people were exposed to yaws during their lifetimes (95). Nowadays, areas where the diseases are endemic are more restricted, but decreased surveillance has led to an increase in the incidence of yaws in many areas (2). Transmission of endemic treponematoses occurs through direct contact with early lesions or with contaminated fingers or drinking or eating utensils. Yaws and endemic syphilis commonly are transmitted among children (2 to 15 years of age), whereas pinta usually has a later onset (ages 15 to 30 years). Many of the manifestations of yaws and endemic syphilis are similar to those of venereal syphilis (Table 2); the lesions of pinta appear to be restricted to the skin. Congenital transmission of the endemic treponematoses is rare.

TABLE 2 Common manifestations of treponematoses

- Venereal syphilis *(T. pallidum* subsp. *pallidum)*
  - Primary (local): 10 to 90 days postinfection (avg, 21 days)
    - Chancre (single or multiple, skin or mucous membranes)
    - Regional lymphadenopathy
  - Secondary (disseminated): 6 wk–6 mo postinfection
    - Multiple secondary lesions (skin or mucous membranes)
    - Generalized lymphadenopathy, fever, malaise
    - Condylomata lata
    - Alopecia
    - Asymptomatic or symptomatic CNS[a] involvement (syphilitic meningitis)
  - Latent (early, ≤1-yr duration; late, >1-yr duration)
    - Reactive serologic tests for syphilis
    - Asymptomatic
  - Tertiary (late, chronic): months to years postinfection
    - Gummatous (monocytic infiltrates, tissue destruction, any organ)
    - Cardiovascular (aortic aneurysm)
    - Late forms of neurosyphilis (paresis, tabes dorsalis, optic atrophy)
  - Congenital
    - Early (onset, <2 years): fulminant, disseminated infection, mucocutaneous lesions, osteochondritis, anemia, hepatosplenomegaly, CNS involvement; may result in stillbirth
    - Late (persistence, >2 years): interstitial keratitis, bone and tooth deformities, cutaneous fissures (rhagades), eighth-nerve deafness, neurosyphilis, other tertiary manifestations
- Yaws *(T. pallidum* subsp. *pertenue)*
  - Early: onset 9–90 days postinfection (avg, 21 days)
    - Primary lesion (mother yaw): papular, nontender, often pruritic, crusted, or ulcerated
    - Disseminated lesions (daughter yaws): often resemble primary lesion, or varied appearance
    - Malaise, fever, lymphadenopathy
    - Osteitis, periostitis, other bone and joint manifestations
  - Latent: positive serologic tests, no other signs of infection
  - Late: 10% of patients
    - Destructive lesions of bone and cartilage (e.g., ulcerative rhinopharyngitis)
    - Hyperkeratotic skin lesions
- Endemic syphilis *(T. pallidum* subsp. *endemicum)*
  - Early
    - Primary: mucosal or cutaneous lesion usually not detected
    - Secondary: multiple oropharyngeal, cutaneous lesions
    - Generalized lymphadenopathy
    - Periostitis
  - Latent: positive serologic tests, no other signs of infection
  - Late: destructive skin, bone, and cartilage lesions
- Pinta *(T. carateum)*: restricted to skin
  - Early
    - Initial lesion: hyperkeratotic, pigmented papule or plaque
    - Disseminated skin lesions (pintids)
    - Regional lymphadenopathy
  - Late: pigmentary changes in skin (hyper- and hypopigmentation)

[a] CNS, central nervous system.

Collection, Transport, and Storage of Specimens

Blood and Serum

When collecting, preparing, and examining specimens, health care workers need to observe universal safety precautions (60). Serum is the specimen of choice for both nontreponemal and treponemal tests. However, the rapid plasma reagin (RPR) card test and toluidine red unheated serum test (TRUST) also may be performed with plasma samples. The technician must check the product insert to be sure that the plasma sample has not been stored for longer than the recommended storage time and that the blood was collected in the specified anticoagulant. Plasma cannot be used in the Venereal Disease Research Laboratory (VDRL) test, since the sample must be heated before testing, and plasma cannot be used in the fluorescent treponemal antibody absorption (FTA-ABS) test but can be used in other treponemal tests for syphilis such as the *Treponema pallidum* particle agglutination (TP-PA) test. Serum or plasma samples that are to be tested at a later date or shipped to another site for testing should be transferred to another properly labeled tube to prevent contamination by hemoglobin, which can interfere with the interpretation of the test results. Serum does not have to be removed from serum separator tubes. If samples are frozen prior to shipping, they should be shipped on dry ice. There is no evidence that shipping at ambient temperature is detrimental to the sample if care has been taken to avoid possible microbial contamination.

When screening for congenital syphilis, the CDC recommends testing of the mother's serum rather than cord blood. Studies comparing the reactivity of the mother's serum, cord blood, and infant's serum found that the maternal sample is the best indicator that a neonate was at risk of infection, followed by neonatal serum, with cord blood being the least reactive (21, 102). Infant's serum is the specimen of choice for the immunoglobulin M (IgM)-specific tests since cord blood specimen can be contaminated by maternal blood.

Blood collected in EDTA can be used for PCR of *T. pallidum*, but the sample must be refrigerated immediately and shipped as soon as possible on ice by overnight carrier to the testing facility if PCR is not done in-house.

CSF

Care must be taken when collecting CSF to ensure that no blood contaminates the samples. Even amounts that are too small to visibly contaminate the CSF may result in a false-positive test for neurosyphilis if the patient has detectable antibodies in the blood. When samples are to be sent to another laboratory for testing in the VDRL-CSF or FTA-ABS CSF test, shipping on ice is preferred but shipping at ambient temperature is acceptable. If CSF is being sent to the testing facility for PCR, the sample must be frozen as soon as possible after collection and shipped on dry ice to maintain the frozen state.

Tissue and Lesion Samples

Specimens collected from the active epidermal and mucosal lesions of primary, secondary, and early congenital syphilis are most useful, because the lesions tend to contain large concentrations of treponemes. The site should be cleansed and gently abraded with sterile gauze and saline until a serous exudate appears. A specimen for dark-field microscopy is collected on a glass slide and covered with a coverslip; a specimen for direct fluorescent antibody for *T. pallidum* (DFA-TP) examination of serous fluids is collected on a slide and then air dried. The specimen should consist of serous fluid free of erythrocytes, other organisms, and tissue

debris. Tissue specimens for a modification of the DFA-TP for tissue (DFAT-TP) or immunohistochemistry (IHC) should be from formalin-fixed, paraffin-embedded sections cut 3 μm thick. Several sections should be sent to the testing facility. Detection of *T. pallidum* in tissues or exudates provides a definitive diagnosis of syphilis.

Tissue or ulcer fluid sample specimens for PCR detection of *T. pallidum* DNA should be frozen immediately after collection and shipped on dry ice if the PCR is being done at another laboratory. However, check with the testing laboratory prior to sending the sample to determine what their protocol requires.

Direct Detection of *Treponema pallidum*

General Principles

Treponemes are not readily detectable with common laboratory stains but can be visualized using dark-field microscopy, DFA-TP, or IHC methods (43, 96). Currently, dark-field microscopy and DFA-TP tests are commonly used to detect *T. pallidum* in primary- or secondary-lesion exudates, while the DFAT-TP (60) and IHC are used for tissue sections. Because of the technical challenges of dark-field microscopy, DFA-TP is recommended for all but the most experienced laboratories. PCR has been introduced as a method for detecting *T. pallidum* infection (as described subsequently) but is not widely available at present.

Continuous in vitro culture of *Treponema pallidum* has not been achieved, but limited multiplication of *T. pallidum* subsp. *pallidum* has been obtained over a 10- to 20-day period in a complex tissue culture system (31, 89). Direct culture of *T. pallidum* from clinical specimens has not been accomplished, and this procedure continues to be limited to the research laboratory setting.

Dark-Field Microscopy

Dark-field microscopy (60) is appropriate for samples from genital or skin lesion exudates. When dark-field microscopy is used, the presence of morphologically similar, nonpathogenic spirochetes within and near the genitalia requires that *T. pallidum* be viewed in its "living state" in order to distinguish its characteristic morphology and motility. It is essential that exudates be examined as soon as possible (ideally within 20 min) to ensure retention of motility. *T. pallidum* is very sensitive to exposure to oxygen, heat, nonphysiologic pH, and desiccation. Wet mounts need to be thoroughly examined using a systematic scan of the entire slide before deciding that a wet mount is negative for treponemes. Because it is difficult or impossible to differentiate *T. pallidum* from other parasitic spirochetes of the gastrointestinal tract by using dark-field microscopy, this method should be used only for genital or skin lesions.

DFA and IHC

Examination of samples from lesions can be done using the DFA-TP test (60). Lesion samples for DFA-TP are air dried onto slides and therefore do not require cell viability or immediate examination. Either labeled polyclonal anti-*T. pallidum* antibody (ViroStat, Portland, Maine) or labeled monoclonal antibody (CDC, Atlanta, Ga.) can be used. Care must be taken in interpretation of the result when polyclonal antibodies are used because they are not specific for pathogenic treponemes. Polyclonal antibodies can be preabsorbed with whole Reiter treponemes prior to use to increase the specificity. Samples for DFA-TP can also be sent to the Syphilis Serology Reference Laboratory at the CDC for testing.

Tissue sections can be stained using either the IHC stain (43, 96) or the DFAT-TP test (60), as the two methods use the same specific antibody for detection. Any tissue can be used, but most frequently tissues for paraffin-embedded sections are collected from the brain, gastrointestinal tract, placenta, umbilical cord (Fig. 3), or skin. Often DFAT-TP or IHC is used to diagnose late-stage or congenital syphilis, although organism concentrations tend to be low during late adult syphilis, in asymptomatic or late congenital syphilis, and in resolving lesions of early syphilis. For tissue, IHC may offer an advantage since a fluorescent microscope is not needed and the counterstain is hematoxylin, which allows tissue structure to be observed simultaneously with the observation of treponemes (43, 96).

PCR

The newest technique for direct detection of *T. pallidum* is PCR (42, 54, 64, 85, 86, 97, 109, 123, 129). PCR as a tool for detecting *T. pallidum* is used by only a few laboratories and is considered an experimental procedure for syphilis diagnosis. However, the PCR test is available at the CDC for patients with suspected neurosyphilis (i.e., reactive serum serology, abnormal CSF or neurologic findings, and a history of syphilis) or for patients with genital ulcer disease. The test also appears useful in detecting congenital syphilis, with sensitivity approaching the "gold standard" of rabbit infectivity testing for amniotic fluid samples (42, 47, 109).

The sample source for PCR is controversial (87). Studies have found whole blood to be a useful sample (58, 68, 117) even for incubating syphilis and latent syphilis (68). Lesion samples are also useful (98, 100, 117) and may be positive for *T. pallidum* by PCR when they are negative by dark-field microscopy or DFA-TP (100). The appropriateness of serum and CSF as sample sources for PCR is still under consideration (87, 123), although CSF has been used to help determine invasion of the central nervous system by *T. pallidum* (104). Contamination with extraneous DNA from other specimens, storage, and transport of the sample prior to testing may seriously affect the results (123).

Methods using PCR and DNA amplification techniques can distinguish various strains of *T. pallidum* subsp. *pallidum* (98). These techniques are being used in some research laboratories to help determine the epidemiology of syphilis outbreaks (100, 117).

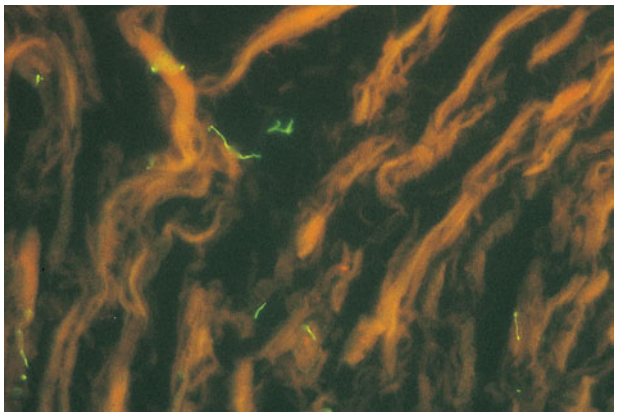


FIGURE 3 DFAT-TP of an umbilical cord section from a baby with congenital syphilis.

Serologic Tests

General Principles

Humoral antibodies produced in response to *T. pallidum* infection become detectable in the primary stage, increase in concentration during the secondary stage, and decline during latent infection. Antibody detection tests supplement the direct organism detection methods used for the diagnosis of primary and secondary syphilis and are the only practical methods of diagnosis during latent and late syphilis.

Serologic tests for syphilis are divided into nontreponemal and treponemal types. Nontreponemal tests detect so-called reaginic antibodies that react with lipoidal particles containing the phospholipid cardiolipin. These tests are commonly used for screening and are widely available, inexpensive, convenient to perform on large numbers of specimens, and useful for determining the efficacy of treatment. Limitations of the nontreponemal serologic tests include a lack of sensitivity in early primary syphilis and in late syphilis and the possibilities of false-positive results or a prozone reaction (false-negative result due to a high ratio of antibody to antigen). Treponemal tests use *T. pallidum* subsp. *pallidum* or its derivatives (e.g., recombinant proteins) as the antigen and detect anti-*T. pallidum* antibodies. In the United States, treponemal tests have been used primarily to verify reactivity in the nontreponemal tests or to confirm a clinical impression of late syphilis in which the nontreponemal test is nonreactive. However, treponemal tests such as the enzyme immunoassay (EIA) are increasingly being used to screen for syphilis. The advantages are lower cost, increased specificity and sensitivity, objective interpretation of results, and hard copies of the results generated by the EIA reader. Rapid treponemal tests, which are available internationally but not in the United States at this time, offer the advantages of specificity and rapid results, usually within 20 min. Although treponemal tests are useful for screening, they cannot be used to monitor treatment or reinfection because 85% of persons successfully treated for syphilis have test results that remain reactive for years, if not for a lifetime. Therefore, a combination of a nontreponemal test with a treponemal test is used to diagnose sexually acquired syphilis. Treponemal tests that detect IgM may be helpful in the diagnosis of congenital syphilis in the neonate. The utility of IgM-based tests to diagnose early adult syphilis or reinfection has not been fully evaluated.

Nontreponemal Tests

All available nontreponemal tests are based on antigen containing measured amounts of cardiolipin, cholesterol, and sufficient purified lecithin to produce standard reactivity (61). The nontreponemal (reagin) tests measure IgM and IgG antibodies to cardiolipin and other lipids released from damaged host cells or from treponemes (5, 70). Antilipoidal antibodies can be produced not only as a consequence of treponemal infections but also during autoimmune diseases, pregnancy, nontreponemal diseases of an acute and chronic nature in which tissue damage occurs, and other conditions (Table 3).

The standard nontreponemal tests use flocculation of lipoidal particles (antigen) to indicate reactivity. The test antigen is mixed with the patient's serum on a solid matrix by rotating at a specific speed for a specified number of minutes before reading the results. The antigen-antibody flocculant is too small to visualize without magnification unless a colored reagent (e.g., carbon particles) is added. The VDRL and unheated serum reagin (USR) tests are flocculation tests requiring microscopic examination, whereas reactions in the RPR test and TRUST can be read without magnification, because of the addition of colored particles that get caught in the antigen-antibody lattice. All of the nontreponemal tests have approximately the same sensitivity and specificity (Table 4).

TABLE 3 Causes of false-positive reactions in serologic tests for syphilis[a]

Disease or condition	Type of test affected	
	Nontreponemal test	Treponemal test
Autoimmune disease	Yes	Yes
Malaria	Yes	No
Recent immunizations	Yes	No
Dermatologic diseases	Yes	Yes
Cardiovascular disease	Yes	Yes
Tuberculosis	Yes	No
Leprosy	Yes	Yes
Intravenous drug abuse	Yes	No
Viral infections	Yes	No
Febrile illness	Yes	Yes
Pregnancy	Yes	No
HIV	Yes	No
Other STDs	Yes	No
Age	No	Yes
Multiple blood transfusions	Yes	No
Lyme disease	No	Yes
Endemic treponematoses	Yes	Yes
Transient unknown causes	Yes	Yes
Technical errors	Yes	Yes

[a] Data from references 7, 50, 61, and 66.

Any of these nontreponemal tests can be performed as a quantitative test by preparing serial twofold dilutions of the patient's serum to reach an endpoint titer. Qualitative nontreponemal tests are used to screen for possible syphilitic infection. Quantitative nontreponemal tests establish a baseline of reactivity from which change can be measured following treatment. The baseline serum sample should be drawn on the day that treatment is begun, and the same test that is used in the initial testing should be used to monitor treatment (32, 33, 34, 111). If early syphilis has been adequately treated, titers are usually nondetectable after 3 years.

The VDRL-CSF test is a modification of the VDRL test that permits detection and titer determination of reaginic antibody in CSF. Preparation of the antigen and the patient specimen differs from that of the serum VDRL test, so care should be taken to follow the appropriate procedures (60). The VDRL-CSF test is the only nontreponemal test standardized for use with CSF specimens.

Treponemal Tests

The greatest value of the treponemal tests is in distinguishing true- and false-positive nontreponemal test results and in establishing the diagnosis of late latent or late syphilis. Problems arise when these tests are used as screening procedures, because treponemal tests generally remain reactive for life following adequate treatment for syphilis and about 1% of the general population has false-positive results unrelated to past syphilis treatment. However, a reactive treponemal test result on a sample that is also reactive in a nontreponemal test is highly specific for treponemal infection. The most commonly used treponemal tests in the United States are the

TABLE 4 Sensitivity and specificity of serologic tests for syphilis

Stage	Sensitivity (%)[a]				Specificity (%) (nonsyphilis)
	Primary	Secondary	Latent	Late	
Nontreponemal tests					
VDRL	78 (74–87)	100	95 (88–100)	71 (37–94)	98 (96–99)
RPR card	86 (77–100)	100	98 (95–100)	73	98 (93–99)
USR	80 (72–88)	100	95 (88–100)		99
TRUST	85 (77–86)	100	98 (95–100)		99 (98–99)
Treponemal tests					
FTA-ABS	84 (70–100)	100	100	96	97 (94–100)
FTA-ABS DS	80 (69–90)	100	100		98 (97–100)
TP-PA	88 (86–100)	100	100		96 (95–100)
IgG EIA	92 (88–97)[b]	100	99 (96–100)	100	99 (98–100)

[a] Ranges of CDC studies are given in parentheses.
[b] Ranges of various studies.

TP-PA and the EIA tests. The number of laboratories that provide the FTA-ABS test is dwindling due to the technical difficulties of performing the test, the complexity of the test, and the lack of laboratory personnel experienced in fluorescence microscopy. As a result, the test is not discussed here, but Larsen et al. (60) give a description of the procedure.

If used appropriately as confirmatory tests, the treponemal tests have few limitations. They vary in their sensitivity for the detection of early primary syphilis and in the late stages of syphilis (Table 4). A treponemal test result should be obtained if late or late latent syphilis is suspected and the nontreponemal test result is nonreactive. The laboratory should be informed that late syphilis is suspected; otherwise, according to laboratory policy, a treponemal test may not be performed when the nontreponemal test result is nonreactive. Treponemal tests are generally 100% reactive in secondary and latent syphilis. Again, these findings should be interpreted in the light of treatment history.

TP-PA Test

The *Treponema pallidum* particle agglutination (TP-PA) test (Fujirebio Diagnostics, Inc., Malvern, Pa.) (60) has replaced the microhemagglutination assay for antibodies to *Treponema pallidum*. Instead of erythrocytes, the test uses gelatin particles sensitized with *T. pallidum* subsp. *pallidum* antigens. There is no separate absorbing step; therefore, the test is simpler to set up and requires less preparation time. The serum sample is diluted in the microtiter plate. Sensitized gelatin particles are added to the 1:40 serum dilution, making a final serum dilution of 1:80. Anti-*T. pallidum* antibodies in the serum react with the sensitized particles, forming a mat of agglutinated particles in the microtiter plate. Unsensitized particles, included as a control for nonspecific reactivity, are added to the 1:20 serum dilution (final dilution, 1:40).

Results are reported as reactive, nonreactive, or inconclusive. Reactive results are reported for a range of agglutination patterns, from a definite large ring with a rough multiform outer margin and peripheral agglutination (1+) to a smooth mat of agglutinated particles covering the entire bottom of the well (4+). Nonreactive results are reported when a definite compact button, with or without a very small hole in its center, forms in the center of the well. A button of unagglutinated particles having a small hole in the center is initially read as " ± ," and the test should be repeated. If the same pattern is again observed, then the report should be "nonreactive." Because the TP-PA test uses gelatin particles rather than sheep erythrocytes, which were used as the antigen carrier in the microhemagglutination assay for antibodies to *T. pallidum*, nonspecific agglutination of the unsensitized particles only rarely occurs. The sources for error with the TP-PA are usually associated with the use of dusty or improper plates, pipetting errors, or vibrations in the laboratory.

Enzyme Immunoassay

Several tests using the EIA format have been developed for the diagnosis of syphilis. In the United States the Captia Syphilis-G test (Trinity Biotech, Dublin, Ireland; BioRad, Richmond, Calif.; and Wampole Laboratories, Princeton, N.J.) (60, 62, 81, 110) has been cleared by the Food and Drug Administration (FDA) for use as either a confirmatory test or a screening test, while the Trep-Chek test (Phoenix Bio-Tech Corp., Mississauga, Ontario, Canada; and Sigma Chemical Company, St. Louis, Mo.) has been cleared by the FDA for use as a confirmatory test for syphilis. Initial evaluations of these EIA tests have found all to have sensitivities and specificities similar to those of the other treponemal tests (62, 81, 99, 127) (Table 4). A comparison of nine EIAs using either *T. pallidum* sonicates or recombinant antigens revealed that several EIAs had sensitivities similar to that of the IgM FTA-ABS test in the analysis of primary syphilis specimens (110). The actual procedures of the tests are very similar. An aliquot of diluted patient sample is added to a well in the plate. After an incubation period, the plate is washed and an enzyme-labeled conjugate is added. After a second incubation and wash, a substrate is added. The presence of antitreponemal antibodies is indicated by a color change that can be read spectrophotometrically or visually. Obtaining results from a spectrophotometric microplate reader has the advantages of objective interpretation and a printout of the results.

The Captia Syphilis-M test (Trinity Biotech, BioRad, and Wampole Laboratories) and the Trep-Chek M test (Phoenix Bio-Tech) are based on the use of anti-human IgM antibody to capture IgM in the patient's serum, followed by the use of a purified *T. pallidum* antigen to detect IgM anti-*T. pallidum* antibodies in the patient's serum (51). The IgM EIA is most useful in the diagnosis of congenital syphilis. One study found that the IgM capture EIA was more sensitive than the FTA-ABS 19S IgM test in detecting probable cases of congenital

syphilis (116). Another study found the IgM EIA to be equal in sensitivity to the IgM Western blot for the detection of neonatal congenital syphilis but less sensitive in delayed-onset congenital syphilis (9). The usefulness of the IgM EIA tests in adult onset syphilis has not been fully determined.

Another EIA test format being increasingly used is the Western blot for *T. pallidum* (12, 37, 60, 67). This test is performed as an experimental test in several laboratories in the United States. However, the MarBlot test (Trinity Biotech) is available outside the United States. The test uses IgG conjugate and appears to be at least as sensitive and specific as the FTA-ABS tests, and efforts have been made to standardize the procedure (37). To date, many investigators agree that the detection of antibodies to the immunodeterminants of 15.5, 17, 44.5, and 47 kDa appears to be diagnostic for acquired syphilis (90). The Western blot for *T. pallidum* has value as a diagnostic test for congenital syphilis, when an IgM-specific conjugate is used (9, 27, 63, 108, 109). The IgM Western blot for congenital syphilis appears to have greater specificity and sensitivity than the IgM EIA.

Rapid Tests

A number of rapid tests are currently on the market outside the United States, with more under development. The tests currently available in rapid-test format are treponemal tests. These tests have several advantages over currently used treponemal tests. They can all be performed within 30 min, some can use whole blood, including finger-stick specimens, as the sample, and no specialized equipment is necessary. Most of the tests on the market use a lateral-flow immunochromatographic strip methodology and follow a similar format. A nitrocellulose strip has a small filter pad at each end. One pad contains the anti-Ig antibodies, antibodies against the control strip, and an indicator such as colloidal gold. The second pad at the opposite end serves to help draw the sample through the strip. The sample (serum, plasma, or whole blood) is placed on the end of the strip that contains the antibodies and conjugate. For some tests, a buffer is added to aid in the migration of the sample up the strip. After a specified time, usually 15 to 30 min, the reaction has taken place. If two lines are present, the sample is considered positive or reactive. If only the control line is visible, the sample in nonreactive. If the control line is not visible, the results are invalid and the test needs to be repeated.

Most rapid tests have not been evaluated in studies published in peer-reviewed journals. The WHO is undertaking an evaluation of selected rapid syphilis tests to encourage the use of tests with acceptable performance in resource-poor settings. Results of the initial laboratory-based WHO evaluation of six rapid tests can be viewed at http://www.who.int/std_diagnostics. In this study, the range of reported sensitivity when using serum samples was 93.6 to 98.4% and specificity was 92.5 to 97.3%.

Interpretation and Reporting of Results

Qualitative results for the microscopic tests (the VDRL test and USR test) are reported as reactive, weakly reactive, or nonreactive. Qualitative results for the macroscopic tests (the RPR card test and TRUST) are reported as reactive (regardless of the degree of reactivity) or nonreactive. Quantitative results may be reported as the reciprocal of the dilution, for example, 4 dils, a titer of 4, or R4 rather than a 1:4 dilution. The endpoint reported is the last dilution giving a fully reactive, not weakly reactive, result. Titers for the same serum specimen may differ two- to fourfold when tested using microscopic versus macroscopic nontreponemal tests, due in part to the exclusion of weakly reactive results in the titer determination for the VDRL and USR tests. This variability underscores the need to use the same test in monitoring treatment efficacy in each patient.

Prozone reactions occur in 1 to 2% of patients with secondary syphilis, due to the high levels of antibodies. Any undiluted serum sample exhibiting a weakly reactive, atypical, or, on rare occasions, "rough negative" reaction should be diluted as in the quantitative assay. In samples exhibiting a prozone effect, reactivity increases and then decreases as the endpoint titer is approached.

Nontreponemal test results must be interpreted according to the stage of syphilis suspected as well as the population being tested and should be confirmed using a treponemal test, since the proportion of positive screening tests that are false positive increases with decreasing prevalence of syphilis. Approximately 30% of those with early primary syphilis have nonreactive nontreponemal test results on the initial visit (Table 4). In secondary syphilis, nearly all patients have nontreponemal test endpoint titers of $\geq$8. For patients with atypical lesions and/or nontreponemal test titers of $<$8, the nontreponemal tests should be repeated and a confirmatory treponemal test should be performed. Even without treatment, the nontreponemal test may be weakly reactive or even nonreactive in the late stages of syphilis.

In congenital syphilis, a paradigm commonly used in the past was that the infant was infected with *T. pallidum* if the infant's nontreponemal test titer at delivery was higher than the mother's titer. However, a lower titer in the infant's serum than in the mother's does not rule out congenital syphilis. Examination of serum sample pairs from mothers and infants with congenital syphilis indicated that only 22% of the infants had a titer higher than that of the mother (116). Passively transferred IgG antibodies reactive in nontreponemal or treponemal tests should be catabolized and undetectable among noninfected infants between the ages of 12 and 18 months.

In any form of syphilis, patients should be monitored to ensure that treatment is effective, i.e., that signs and symptoms have resolved, and that the titer of the quantitative nontreponemal test has declined fourfold, which indicates cure (32, 34, 111). A rise in titer following treatment establishes treatment failure or reinfection. For most patients treated in early syphilis, the titers decline until little or no reaction is detected after 3 years (32, 60, 61, 105). Patients who are treated in the latent or late stage and patients who have had multiple episodes of syphilis may show a more gradual decline in titer (33, 104). A low titer persists in approximately 50% of these patients after 2 years (33). As far as can be determined, this persistent seropositivity does not signify treatment failure or reinfection, and these patients are likely to remain serofast even if they are retreated (33). Recent studies indicate that HIV infection may delay the decline in antibody titer detected by nontreponemal tests for syphilis in patients with primary or secondary syphilis (104). The prognostic significance of this finding is unknown, since only 1 of 101 HIV-positive patients experienced clinical treatment failure during a 1-year posttreatment observation period. A delayed decline in antibody titer was not associated with detection of *T. pallidum* in CSF, and enhanced therapy did not appear to prevent the delay (104).

Diagnostic Criteria for Venereal Syphilis

For the laboratory diagnosis of syphilis, each stage has a particular testing requirement (Table 5) (14). In the United States, the traditional testing scheme is direct microscopic examination of lesion exudates, if present, followed by a

TABLE 5 Criteria for diagnosis of syphilis[a]

Early syphilis
- Primary
  - Confirmed (requires 1 *and* 2)
    1. One or more chancres (ulcers)
    2. Direct microscopic identification of *T. pallidum* in clinical specimens (dark field or DFA-TP)
  - Probable (requires 1 *and* either 2 or 3)
    1. A clinically compatible case with one or more chancres
    2. Reactive nontreponemal test and no previous history of syphilis
    3. For persons with a history of syphilis, a fourfold increase in titer on a quantitative nontreponemal test when results of past tests are compared with the most recent test results.
- Secondary
  - Confirmed
    - Direct microscopic identification of *T. pallidum* in clinical specimens (dark field or DFA-TP)
  - Probable (requires 1 *and* either 2 or 3)
    1. Skin or mucous membrane lesions typical of secondary syphilis
       a. Macular, papular, follicular, papulosquamous, or pustular
       b. Condylomata lata (anogenital region or mouth)
       c. Mucous patches (oropharynx or cervix)
    2. Reactive nontreponemal test titer of ≥4 and no history of syphilis
    3. For persons with a history of syphilis, a fourfold increase in the most recent nontreponemal test titer when compared with previous test results.
- Early latent
  - Probable (requires 1 *and* either 2 or 3 *and* 4, 5, or 6)
    1. Absence of signs and symptoms of syphilis
    2. No past diagnosis of syphilis and reactive nontreponemal and treponemal test results
    3. A history of syphilis therapy and a current nontreponemal test titer demonstrating fourfold or greater increase from the last nontreponemal test titer (obtained ≤1 yr ago)
    4. A history of symptoms consistent with primary or secondary syphilis during the previous 12 mo
    5. A history of sexual exposure to a partner who had confirmed or probable early syphilis (documented independently as duration of ≤1 yr)
    6. Documented seroconversion or fourfold or greater increase in titer of a nontreponemal test during the previous 12 mo

Late latent or latent of unknown duration
- Probable (requires 1 *and* either 2 or 3 *and* 4)
  1. Absence of signs and symptoms
  2. No past diagnosis of syphilis and reactive nontreponemal and treponemal test results; if a reactive treponemal screening test is followed by a nonreactive nontreponemal test, a second, different treponemal test is also reactive.
  3. A history of syphilis therapy and a current nontreponemal test titer demonstrating fourfold or greater increase from the last nontreponemal test titer (obtained >1 yr ago)
  4. No evidence of having acquired the disease within the preceding 12 mo

Late syphilis
- Benign (gummatous) and cardiovascular
  - Confirmed (requires 1 *and* 2)
    1. Clinically compatible case
    2. Observation by direct microscopic examination of treponemes in tissue sections by DFAT-TP or detection by other methods (e.g., PCR)
  - Probable (requires 1, 2, *and* 3)
    1. Clinically compatible case
    2. A reactive serum treponemal test
    3. No known history of treatment for syphilis
- Neurosyphilis
  - Confirmed (requires 1, 2, *and* either 3 or 4)
    1. Clinical signs consistent with neurosyphilis
    2. A reactive serum treponemal test
    3. A reactive VDRL-CSF on a spinal fluid sample
    4. Identification of *T. pallidum* in CSF or tissue by microscopic examination or equivalent methods
  - Probable (requires 1, 2, *and* 3)
    1. Clinical signs consistent with neurosyphilis
    2. A reactive serum treponemal test
    3. Elevated CSF protein or leukocyte count in the absence of other known causes

Neonatal congenital syphilis
- Confirmed (requires 1 *and* 2)
  1. Clinically compatible case
  2. Demonstration of *T. pallidum* by direct microscopic examination of specimens from lesions, placenta, umbilical cord, nasal discharge, or autopsy material
- Probable (requires 1, 2, *and* 3)
  1. Infant born to a mother who had untreated or inadequately[b] treated syphilis at delivery, regardless of findings in the infant
  2. An infant with a reactive treponemal test result
  3. One of the following additional criteria:
     a. Clinical sign or symptom of congenital syphilis on physical examination
     b. Abnormal CSF finding without other cause
     c. Reactive VDRL-CSF test result
     d. Reactive IgM antibody test specific for syphilis
- Syphilitic stillbirth
  1. A fetal death that occurs after a 20-week gestation or in which the fetus weighs >500 g
  2. Mother had untreated or inadequately[b] treated syphilis at delivery

[a] Adapted from reference 14.
[b] Nonpenicillin therapy or penicillin given less than 30 days before delivery.

nontreponemal test which, if reactive, is confirmed with a treponemal test (Fig. 4). In recent years, EIAs have been used for screening followed by an RPR or other nontreponemal test. This approach may eliminate many false-positive reactions due to nonspecific nontreponemal test reactivity (Table 3); however, as the rates of syphilis decline, there will be an increasing proportion of reactive results from persons with previously treated syphilis or with false-positive treponemal test results. In addition, the low sensitivity of nontreponemal tests in detecting long-duration infection is an important disadvantage to their use as confirmatory tests. The use of modified serologic screening procedures for syphilis requires careful consideration of the sensitivity and specificity of both the screening and confirmatory steps, as well as the population being tested.

There has been no reliable test for detecting incubating syphilis. However, PCR for the detection of *T. pallidum* DNA may be of some value. There is evidence that DNA can be detected in the blood of persons with incubating syphilis (68) or early syphilis, including the early latent stage (68, 117).

During the primary stage of syphilis, dark-field microscopy and the DFA-TP test (60) are the methods of choice for definitive diagnosis. DFA-TP should be used for specimens from perirectal or oral ulcers because of the inability to distinguish digestive system spirochetes from *T. pallidum* by dark-field microscopy. If the chancre is starting to heal or the patient has used either topical or systemic antibiotics, the number of treponemes may be too small to be detected or they may be nonmotile or degraded. Humoral antibodies, as detected by the current serologic tests for syphilis, usually appear 1 to 4 weeks after the chancre(s) has formed. Thus, a reactive serologic test result may be expected if the chancre is healing. Conversion to reactivity of a serologic test for syphilis or a rise in nontreponemal test titer over 1 to 2 weeks supports a diagnosis of syphilis even if a dark-field or DFA microscopic examination is negative.

By the secondary stage of syphilis, the organism has invaded every organ of the body and virtually all body fluids. All serologic tests for syphilis are usually reactive, and treponemes may be found in moist lesions.

Nontreponemal and treponemal serologic tests are consistently reactive in the early latent stage, but the patient is asymptomatic. Nontreponemal tests may become nonreactive in late latent syphilis. A reactive treponemal test in a patient with a nonreactive nontreponemal test and a history of syphilis should be confirmed by a different treponemal test. A diagnosis of early versus late latent syphilis is made by documentation of onset of infection within or prior to the past year as indicated by (i) seroconversion; (ii) symptoms of primary or secondary syphilis; or (iii) a sex partner documented to have primary, secondary, or early latent syphilis. In the absence of such documentation, the diagnosis is "latent syphilis of unknown duration."

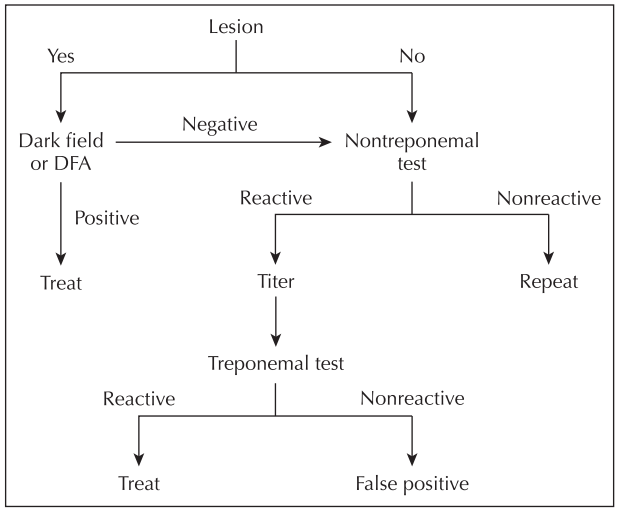


FIGURE 4 Routine screening scheme for early syphilis in the United States.

Neurosyphilis may take many forms (48). Syphilitic meningitis typically occurs during early syphilis. Meningovascular syphilis most often occurs earlier (4 to 7 years) than the classic forms of late neurosyphilis (paresis and tabes dorsalis) (48). The diagnosis of neurosyphilis is based on a combination of clinical and laboratory test criteria (Table 5). CSF examination should include the VDRL-CSF slide test and total protein and leukocyte counts. The VDRL-CSF test is considered to have high specificity but low sensitivity for neurosyphilis (48). Thus, a nonreactive VDRL-CSF test result does not rule out neurosyphilis. The FTA-ABS CSF test appears to have high sensitivity but low specificity for neurosyphilis (52). The FTA-ABS CSF test could be used to rule out neurosyphilis, especially among HIV-infected individuals who commonly have modestly elevated CSF protein and leukocyte counts in the absence of syphilis infection (69). The utility of the EIA or TP-PA in diagnosing neurosyphilis has not been adequately determined, although preliminary evaluations indicate that TP-PA may also be useful. Large-scale studies need to be carried out and the results published before any recommendations can be made. Whether CSF examination is indicated in early syphilis, especially for patients with HIV coinfection, has been controversial. CSF abnormalities and *T. pallidum* can be detected in the CSF of a substantial minority of patients with secondary syphilis (69, 104), but most such patients resolve their CSF abnormalities and appear to clear the CSF of organisms with standard therapy. A relationship between CSF abnormalities or detection of treponemes in the CSF of patients with early syphilis and the development of clinically evident neurosyphilis through enhanced therapy has not been established (69, 80, 104). Thus, routine CSF examination is not considered necessary during early syphilis in the absence of neurologic or ophthalmologic signs or for HIV-infected patients with late latent syphilis or syphilis of unknown duration (15). A nontreponemal serologic test titer of $\geq$32 or, among HIV-infected patients, a CD4 count of $\leq$350 has been associated with an increased frequency of CSF abnormalities in patients with late latent syphilis or syphilis of unknown duration (69), and some experts recommend CSF examination for this group of patients (15).

Late or tertiary syphilis is a chronic, progressive disease, and symptoms may not be evident until 10 to 20 years after the initial infection. Although rare, earlier onset of late or tertiary syphilis has been reported for individuals coinfected with HIV (55). Approximately 71% of patients with late-stage syphilis have reactive nontreponemal tests; however, treponemal tests are almost always reactive and may be the only basis for diagnosis (Table 5). Benign (gummatous) syphilis consists of granuloma-like lesions (gummas) containing lymphocytes and macrophages but few if any detectable spirochetes. Diagnosis of cardiovascular syphilis is made on the basis of findings that indicate aortic insufficiency or thoracic aortic aneurysm, reactive treponemal test results, and no known history of treatment of syphilis. Late forms of neurosyphilis include paresis (infection of the brain parenchyma, resulting

in behavioral changes including psychoses), tabes dorsalis (ataxia, paresthesias, and other manifestations resulting from posterior spinal cord and dorsal root ganglia degeneration), and optic atrophy (122).

The choice and interpretation of serologic tests for the diagnosis of syphilis are the same for HIV-infected and uninfected patients, but additional considerations apply to HIV-infected individuals (39, 53, 80, 104). The titers of nontreponemal serologic test results may be higher for patients coinfected with HIV than for those without coexistent HIV infection. The number of primary chancres may be increased (106), and case reports indicate that the course of *T. pallidum* infection may be more rapid and more virulent in a small proportion of HIV-infected individuals. Also, patients with advanced HIV immunodeficiency may become falsely seronegative for syphilis (41, 46).

Diagnostic Criteria for Congenital Syphilis

A primary stage does not occur in congenital syphilis, because the organisms directly enter the fetal circulation (128). Treponemes or the effects thereof are detectable in almost every tissue of the infant. Currently, the diagnosis of neonatal congenital syphilis depends on a combination of results from physical, radiographic, serologic, and direct microscopic examinations as well as maternal clinical history (Table 5). Clinical signs of early congenital syphilis include hepatosplenomegaly, cutaneous lesions, osteochondritis, and snuffles (nasal discharge containing large numbers of *T. pallidum*) (56, 122). Roughly one-half of live-born neonates with congenital syphilis have normal physical findings at the time of birth. If the infant is not treated, other stigmata that may develop later in life include tooth and bone malformations, deafness, blindness, and learning disabilities (Table 2).

Definitive diagnosis is based on the identification of spirochetes in tissues, lesion exudates, or secretions by dark-field examination, DFA-TP, PCR, or other means (Table 5). The standard serologic tests for syphilis, which detect reactive IgG as well as other isotypes, may reflect antibodies passively transferred from the mother to the infant rather than IgM antibodies produced by an infected fetus during gestation. IgM-specific treponemal antibody tests are available and may be useful in the diagnosis of congenital syphilis (see "Serologic Tests" above). In stillbirths, diagnosis of congenital syphilis is made by detection of the treponemes in the tissues (101).

Diagnostic Criteria for Endemic Treponematoses

Serologic tests for syphilis are uniformly reactive with yaws, pinta, and nonvenereal endemic syphilis (bejel) (6, 83, 95). Therefore, patient history, the clinical appearance and anatomic locations of the lesions, the mode of transmission, and the age of the individual are the only criteria that can be used to diagnose these infections as separate entities (6) (Table 1). PCR may be useful in distinguishing these infections in a research setting (17).

Yaws, pinta, and nonvenereal syphilis are often contracted during childhood, but the nontreponemal test titers in affected individuals may remain reactive in adulthood, presenting a dilemma in the diagnosis of subsequent cases of venereal syphilis. Adults from geographic regions in which endemic treponematoses were virtually eliminated by mass campaigns in the 1950s and 1960s would be expected to have nontreponemal test titers of <8 (3). Therefore, any titer of ≥ 8 for adults from these regions is probably indicative of venereal syphilis. Likewise, titers of ≥ 8 for children indicate a possible resurgence of yaws in the populations of these areas (2).

Treatment and Antimicrobial Susceptibilities

The CDC has published recommendations for the treatment of syphilis (15) based, in part, on detailed reviews of the literature (4, 103). Routine antimicrobial susceptibility testing of *T. pallidum* is not practical due to the lack of a suitable in vitro culture system, although a number of methods have been applied to the determination of antibiotic sensitivity in the research laboratory (88). *T. pallidum* strains appear to be uniformly susceptible to penicillin and other β-lactams. Penicillin regimens have been the mainstay of syphilis therapy since their introduction in the 1940s. Because treatment with benzathine penicillin as recommended for early syphilis yields low levels of penicillin in the central nervous system, the current recommendations include multiple doses of aqueous or procaine penicillin G for the treatment of neurosyphilis. Ceftriaxone, amoxicillin, and ampicillin are active against *T. pallidum* in vitro and also appear to be effective in the treatment of early syphilis. Ceftriaxone has a long half-life in serum and reaches high levels in the central nervous system. However, multiple doses are required, and there is a 3 to 7% risk of adverse reactions in individuals who are allergic to penicillin; in addition, the data on efficacy in patients are limited. Therefore, the use of ceftriaxone as an alternative therapy for syphilis is recommended only when better-established regimens are contraindicated.

Tetracycline has long been the second-line drug recommended for treatment of syphilis in patients allergic to penicillin. However, the in vitro activity of tetracycline against *T. pallidum* is markedly lower than the activity of penicillin and, in limited comparative trials, treatment failure appears to be more common with tetracycline. Doxycycline has been considered equivalent to tetracycline in recent treatment recommendations. In vitro studies indicate that quinolone compounds have low antimicrobial activity against *T. pallidum* (88).

Azithromycin has been used as a single dose treatment for syphilis contacts in some settings. However, recent findings indicate that a high rate of treatment failures can occur with this regimen (65). The mechanism appears to be a single base pair change in the 23S rRNA genes that confers resistance to erythromycin, azithromycin, and other macrolides (65). This mutation was present in 13, 22, 11, and 88% of clinical specimens from Seattle, San Francisco, Baltimore, and Dublin, Ireland, respectively (65). Therefore, use of macrolides for the treatment of syphilis or syphilis exposure is not recommended.

OTHER HUMAN HOST-ASSOCIATED SPIROCHETES

In addition to *T. pallidum* and related pathogens, a number of anaerobic treponemes and other spirochetes are parasites of humans. These include groups of spirochetes found in the oral cavity (particularly the gingival crevices), in sebaceous secretions in the genital region, and in the colon and rectum (Table 6). In general, these spirochetes are considered commensal organisms. However, there is evidence that spirochetes are involved in gingivitis and periodontal disease and that overgrowth of intestinal spirochetes may correlate with the occurrence of diarrhea or other bowel disorders.

Oral Spirochetes

A variety of spirochetes inhabit supragingival and subgingival plaque in humans; only a small proportion of these have been cultured in vitro and characterized (22, 26, 92). Those that have been cultivated and identified to the species level are within the genus *Treponema* (Table 6). They are spiral-shaped

TABLE 6 Other human host-associated spirochetes

Type	Habitat	Species
Oral spirochetes	Dental plaque in gingival crevices	*Treponema amylovorum*
		Treponema denticola
		Treponema lecithinolyticum
		Treponema maltophilum
		Treponema medium
		Treponema parvum
		Treponema pectinovorum
		Treponema putidum
		Treponema skoliodontum
		Treponema socranskii
		Treponema vincentii
Skin-associated spirochetes	Sebaceous secretions in genital region	*Treponema minutum*
		Treponema phagedenis
		Treponema refringens
Intestinal spirochetes	Colon, rectum, feces	*Brachyspira aalborgi*
		Brachyspira pilosicoli

organisms ranging from 0.15 to 0.30 μm in diameter and from 5 to 16 μm in length (with lengths being variable within each species). Although the species differ from one another slightly in terms of cell diameter and helical configuration, it is generally not possible to identify them on morphologic grounds; genotypic characteristics and biochemical parameters such as growth requirements, carbohydrate fermentation, and enzymatic activities are used to identify species (115).

The oral spirochetes listed in Table 6 are difficult to isolate from healthy gingiva. However, they increase both in prevalence and in number of organisms present in patients with gingivitis or periodontal disease, an inflammation of gum tissue that frequently precedes bone resorption and subsequent tooth loss. Treponemes are detected in the subgingival plaque of 88 to 97% of patients with periodontal disease (77). *T. socranskii* is the most common isolate in patients without periodontal disease, followed by *T. denticola* and *T. pectinovorum.* These organisms are isolated from a greater proportion of patients with periodontal disease than patients without the disease (77, 114, 115). Recent studies using PCR amplification of 16S rRNA gene sequences have revealed that oral spirochetes are a heterogeneous group, consisting of up to 60 different species and as yet uncultured "phylotypes" (26, 92); as many as 23 phylotypes of treponemes have been identified in the lesions of individual patients (23). All of the oral spirochetes clearly fall under the genus *Treponema* (93). Up to 415 cultured and uncultured bacterial species have been identified in human subgingival plaque (92), but treponemes represent one of the most prevalent groups (78, 79). Although it is difficult to establish a causal relationship in polymicrobial infections such as periodontitis, oral spirochetes are almost certainly involved in its etiology.

The isolation and characterization of oral spirochetes require special media and strict anaerobic conditions (75, 76, 113, 114, 115, 126) and are restricted primarily to research laboratories. At present, there is insufficient evidence linking particular oral spirochetes to pathogenic conditions to warrant their detection and identification for diagnostic purposes.

Nonpathogenic Spirochetes of the Genital Region

T. phagedenis, T. refringens, and *T. minutum* are treponemal species that inhabit the smegma (sebaceous secretions and desquamated epithelial cells) found beneath the prepuce and in other epithelial folds of the genital region. *T. phagedenis* and *T. refringens* are 0.20 to 0.25 μm in diameter, whereas *T. minutum* tends to be smaller in diameter (0.15 to 0.20 μm). Although *T. phagedenis* and *T. refringens* have been shown to have ≤5% homology with *T. pallidum* by DNA-DNA hybridization (30), comparison of 16S rRNA sequences indicates that they are related to the pathogenic treponemes. These harmless members of the normal flora could potentially be misidentified as *T. pallidum* in dark-field microscopy preparations from skin sites. Careful cleansing of the site as described in reference 60 obviates this possibility. Also, the DFA-TP test (60) is specific for pathogenic treponemes and can be used with specimens potentially contaminated with other organisms. The skin-associated treponemes may be collected with sterile moistened swabs and placed in anaerobic transport medium or directly into selective medium. They are readily cultured in peptone-yeast extract-glucose medium or in thioglycolate medium with 10% heat-inactivated rabbit serum under anaerobic conditions at 37°C (75).

Intestinal Spirochetes

The presence of spirochetes in the colon, rectum, and feces of humans has been recognized for more than 100 years (107). Since the description of "human intestinal spirochetosis" in 1967 (45), there has been a resurgence in interest in the possible involvement of intestinal spirochetes in diarrhea and other gastrointestinal diseases (44), as well as in the apparently high prevalence of these organisms in homosexual males and HIV-infected individuals (107).

Morphologically, most human intestinal spirochetes are relatively short (3 to 6 μm on average) and are 0.2 to 0.4 μm in diameter with one to two helices per cell. Longer cells may also be present. They have pointed ends and four to six periplasmic flagella attached subterminally in a single row at each end of the cell. The human intestinal spirochetes identified thus far fall into two species: *Brachyspira* (formerly *Serpulina*) *pilosicoli* and *Brachyspira aalborgi. B. pilosicoli* was initially named *Serpulina pilosicoli* (120, 121); however, this and other *Serpulina* species (e.g., *B. hyodysenteriae,* a cause of swine dysentery) were reclassified as *Brachyspira* based on phenotypic characteristics, rRNA gene sequence analysis, and DNA hybridization studies (91). *B. pilosicoli* has a broad range of natural hosts, including swine, mice, rats, dogs, and chickens. *B. aalborgi* (49) is genetically and phenotypically distinct from *B. pilosicoli* (57, 120), and thus far has been identified only in humans. Recent 16S rRNA gene analyses indicate that *B. aalborgi* can be subdivided into four different clusters or lineages, but it is not known whether this heterogeneity is associated with phenotypic differences (74).

B. pilosicoli and *B. aalborgi* are obligate anaerobes but grow in a variety of media containing blood. Until recently, *B. aalborgi* had been cultured on only one occasion (resulting in the type strain), leading to the conclusion that it was rare relative to *B. pilosicoli*. However, recent PCR analyses of human colonic biopsy specimens indicate that *B. aalborgi* is a common cause of human intestinal spirochetosis (59, 71, 72). In two series of histologically diagnosed cases of intestinal spirochetosis from Australia, Norway, and the United States, *B. aalborgi* sequences were detected in 62.5 and 82.5% of the cases, whereas *B. pilosicoli* sequences were detected in only 0 and 14.3% (72, 73). In 2000, Kraaz et al. (59) isolated a second strain of *B. aalborgi* by using a selective medium consisting of tryptose soy agar with 10% bovine blood, 400 μg of spectinomycin per ml, and 5 μg of polymyxin per ml. They found that a selective medium commonly used for the culture of intestinal spirochetes

inhibited the growth of this strain, presumably due to the inclusion of vancomycin and colistin.

When observed in association with rectal or colonic tissue, these organisms are typically attached by the tip to the apical surface of the columnar epithelial cells. Although the spirochetes can form a dense layer visible by light microscopy (using hematoxylin-and-eosin- or silver-staining techniques), signs of tissue damage or inflammation are usually absent.

A clear association between intestinal spirochetes and disease has not been established (107). Spirochetes can be present in healthy individuals as well as in those with diarrhea or other gastrointestinal symptoms. In the early 1900s, several studies examined the presence of spirochetes in human stool specimens by light microscopy. In those studies, involving more than 100 patients, the proportion of subjects with spirochetes ranged from 3.3 to 61%. Recent examinations of rectal biopsy specimens from heterosexual patients by light and electron microscopy or culture have indicated the presence of spirochetes in 1.9 to 6.9% of subjects, and helical organisms were also found to be associated with normal and inflamed appendixes. Higher proportions of homosexual males have been reported to have intestinal spirochetes. At present, there is no clear evidence for an association between intestinal spirochetosis and HIV infection.

B. pilosicoli was isolated from routine anaerobic cultures of blood from seven critically ill patients, including those with stroke, ethylene glycol intoxication, severe arteriopathy, peritonitis, and myeloma (118). The clinical significance and cause-and-effect relationship of *B. pilosicoli* bacteremia and life-threatening illness remain to be determined.

Colonic or rectal biopsy samples can serve as a source of material for culture, histologic examination for spirochetes, or PCR; fresh stool specimens or rectal swabs may also be examined by dark-field microscopy for the presence of spirochetes (107). Positive samples can be cultured under anaerobic conditions at 37°C in the presence of 5 to 10% CO_2 by being streaked onto Trypticase soy agar medium with 5 to 10% defibrinated horse or calf blood and 400 μg of spectinomycin per ml (with or without 5 μg of polymyxin B per ml) to inhibit the growth of other bacteria. After 5 to 14 days of incubation, growth appears as a thin film or as discrete, pinpoint colonies. Longer culture intervals may be required for *B. aalborgi*. Weak beta-hemolysis is typically observed. In the isolation of *Brachyspira* from anaerobic blood cultures, automated detection was not obtained uniformly and required a minimum of 5.6 to 14.9 days (depending on the strain); however, the organisms could be subcultured and observed by dark-field microscopy (10, 118). An alternative method of detection is PCR, using rRNA and/or NADH oxidase *(nox)* gene sequences as targets (59, 71); this approach can also be adapted for use with fixed, paraffin-embedded tissue specimens (71) or with human feces (73).

No commercial kits or serologic procedures for detection of human intestinal spirochetosis are available, and no data on antimicrobial susceptibility or treatment have been reported. Clearly, more information is needed to establish the potential medical importance of these organisms.

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Mycoplasma and *Ureaplasma**

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TAXONOMY

Bacteria commonly referred to as mycoplasmas ("fungus form") are included within the class *Mollicutes* ("soft skin"), which comprises 4 orders, 5 families, 8 genera, and almost 200 known species, as shown in Table 1. New species, found mainly in animals, are being identified on a frequent basis. Table 2 lists 16 species isolated from humans on multiple occasions, excluding species of animal origin that have been detected in humans from time to time, usually in immunosuppressed hosts, but that are generally considered to be transient colonizers. *Mycoplasma amphoriforme* is the most recent species to have been recognized, having been recovered from the respiratory tracts of several patients with antibody deficiencies and chronic bronchitis or bronchiectasis (121). Mollicutes are eubacteria that have evolved from clostridium-like gram-positive cells by gene deletion. The availability of species-specific PCR technology is ameliorating difficulties of both culture and identification for fastidious mollicutes. Therefore, additional noncultivable, and thus presently unknown, species are likely to be discovered.

DESCRIPTION OF *MOLLICUTES*

It should be noted that the term "mycoplasma" is often used in a trivial way to refer to any members of the class *Mollicutes,* irrespective of whether they actually belong to the genus *Mycoplasma.* It is more accurate to refer to members of the class as mollicutes and to organisms within the individual genera as mycoplasmas, ureaplasmas, and acholeplasmas, etc. Mollicutes are smaller than conventional bacteria, in cellular dimensions as well as genome size, making them the smallest free-living organisms known. Mycoplasmas and ureaplasmas associated with humans have forms ranging from coccoid cells of about 0.2 to 0.3 μm diameter, as in the case of *Ureaplasma* spp. and *Mycoplasma hominis* (87), to tapered rods of 1 to 2 μm in length and 0.1 to 0.2 μm in width in the case of *M. pneumoniae* and *M. penetrans* (122). Mollicutes are contained by a trilayered cell membrane and do not possess a cell wall. The permanent lack of a cell wall barrier makes the mollicutes unique among prokaryotes and differentiates them from bacterial L forms for which the lack of the cell wall is but a temporary reflection of environmental conditions. Lack of a cell wall also renders these organisms insensitive to the activity of beta-lactam antimicrobials, prevents them from staining by Gram stain, and is largely responsible for their pleomorphic form. The extremely small genome (<600 kb in the case of *M. genitalium*) and limited biosynthetic capabilities explain the parasitic or saprophytic existence of these organisms, their sensitivity to environmental conditions, and their fastidious growth requirements, which can complicate cultural detection. Mollicutes require enriched growth medium supplemented with nucleic acid precursors. Except for acholeplasmas, asteroleplasmas, and mesoplasmas, mollicutes require sterols in growth media, supplied by the addition of serum. Growth rates in culture media vary among individual species, with generation times of approximately 1 h for *Ureaplasma* spp., 6 h for *M. pneumoniae,* and 16 h for *M. genitalium* (54).

Typical mycoplasmal colonies vary from 15 to ≥300 μm in diameter. Colonies of some species, such as *M. hominis,* often exhibit a "fried-egg" appearance owing to the contrast between deeper growth in the center of the colony and more shallow growth at the periphery (Fig. 1), and other species, such as *M. pneumoniae,* produce spherical colonies (Fig. 2). Whereas colonies of mycoplasmal species may be observed with the naked eye, those produced by ureaplasmas are typically 15 to 60 μm in diameter and require low-power microscopic magnification for visualization (Fig. 3).

Mycoplasmas and ureaplasmas of human origin can be classified according to whether they ferment glucose, utilize arginine, or hydrolyze urea (Table 2). Except for hydrolysis of urea, which is unique for ureaplasmas, these biochemical features are not sufficient for species distinction. Anaeroplasmas and asteroleplasmas, which occur in ruminants, are strictly anaerobic and oxygen sensitive, whereas most other mollicutes are facultative anaerobes.

Attachment of *M. pneumoniae* to host cells in the respiratory tracts of humans is a prerequisite for colonization and infection. Cytadherence, mediated by the P1 adhesin and other accessory proteins, is followed by induction of ciliostasis, chronic inflammation, and cytotoxicity mediated by hydrogen peroxide, which also acts as a hemolysin. *M. pneumoniae* stimulates B and T lymphocytes and induces formation of autoantibodies which react with a variety of host tissues and the I antigen on erythrocytes, which is responsible for production of cold agglutinins (117). *M. genitalium* also possesses a terminal

* This chapter contains information presented in chapter 62 by Ken B. Waites, Yasuko Rikihisa, and David Taylor-Robinson in the eighth edition of this Manual.

TABLE 1 Classification and some distinguishing features of mollicutes

Classification of class *Mollicutes*	Distinguishing features			
	Sterol required	Genome size (kbp)	G+C content of DNA (mol%)	Other
Order I: *Mycoplasmatales*				
Family I: *Mycoplasmataceae*				
Genus I: *Mycoplasma* (120 species)[a]	Yes	580–1,350	23–40	
Genus II: *Ureaplasma* (7 species)[b]	Yes	730–1,170	25–32	Urea metabolized
Order II: *Entomoplasmatales*				
Family I: *Entomoplasmataceae*				
Genus I: *Entomoplasma* (6 species)	Yes	790–1,140	27–29	
Genus II: *Mesoplasma* (11 species)	No	870–1,100	27–30	Requires 0.04% Tween 80
Family II: *Spiroplasmataceae*				
Genus I: *Spiroplasma* (34 species)	Yes	940–2,200	25–30	Helical structure
Order III: *Acholeplasmatales*[c]				
Family I: *Acholeplasmataceae*				
Genus I: *Acholeplasma* (15 species)	No	1,500–1,650	27–36	
Order IV: *Anaeroplasmatales*				
Family I: *Anaeroplasmataceae*				Obligate anaerobes
Genus I: *Anaeroplasma* (4 species)	Yes	1,500–1,600	29–33	
Genus II: *Asteroleplasma* (1 species)	No	1,500	40	
Phytoplasma[c] (17 candidatus species designations)	Unknown	530–1,350	23–29	Noncultivable

[a] The genus *Mycoplasma* includes cell wall-less uncultivated parasitic bacteria, classified into six candidatus species designations, that attach to the surfaces of erythrocytes and also occur free in the plasma. These bacteria were previously classified in the genera *Haemobartonella* and *Eperythrozoon.* Their G+C contents and sterol requirements are unknown.

[b] *U. urealyticum* and *U. parvum,* formerly considered biovars of *U. urealyticum,* are now classified as two separate species and are the only ureaplasmas of human origin.

[c] Phytoplasmas are uncultivable mollicutes of plants and insects genetically related to the *Acholeplasmatales,* but they have not been assigned individual genus and species designations.

structure, the MgPa adhesin, which facilitates its attachment to epithelial cells (48). Factors involved in *Ureaplasma* and *M. hominis* attachment have not been characterized to the same extent as those involved in *M. pneumoniae* attachment, and *Ureaplasma* spp. and *M. hominis* do not have prominent attachment organelles. Henrich et al. (46) demonstrated the presence of the variable adherence-associated antigen (Vaa) that is believed to be a major adhesin of *M. hominis* and may also assist in evasion of host immune responses through antigenic variation. Ureaplasmas also attach to a variety of cell

TABLE 2 Primary sites of colonization by and metabolism characteristics and pathogenicity of mollicutes of human origin[a]

Species	Primary site of colonization		Metabolism of:		Pathogenicity
	Respiratory tract	Genitourinary tract	Glucose	Arginine	
M. salivarium	+	−	−	+	−
M. orale	+	−	−	+	−
M. buccale	+	−	−	+	−
M. faucium	+	−	−	+	−
M. lipophilum	+	−	−	+	−
M. amphoriforme[b]	?+	−	+	−	?
M. pneumoniae	+	−	+	−	+
M. hominis	+	+	−	+	+
M. genitalium[c]	?+	+	+	−	+
M. fermentans	+	+	+	+	+
M. primatum	−	+	−	+	−
M. spermatophilum	−	+	−	+	−
M. pirum	?	?	+	+	−
M. penetrans	−	+	+	+	?
Ureaplasma spp.[d]	+	+	−	−	+
A. laidlawii	+	−	+	−	−

[a] Symbols: +, positive for trait; −, negative for trait;?, unknown.

[b] All nine isolates reported to date have been from the lower respiratory tract, but no other sites have been sampled.

[c] The organism has been found in the oropharynx, but whether that is a common or primary location is not known.

[d] Metabolize urea.

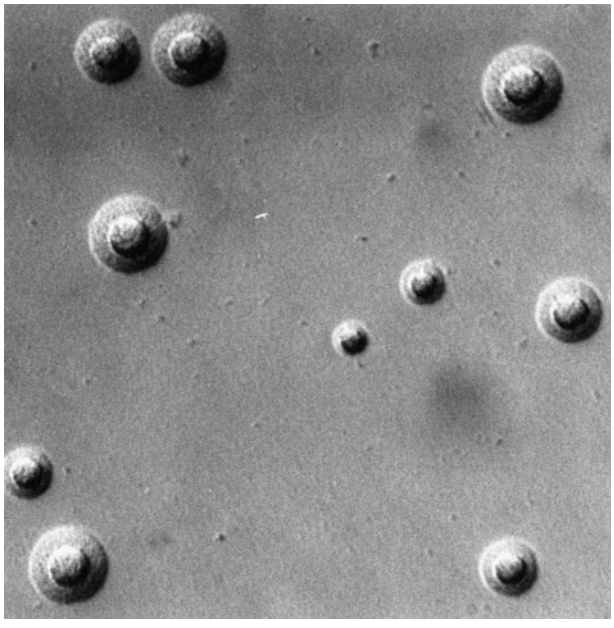


FIGURE 1 Fried-egg-type colonies of *M. hominis* of up to 110 μm in diameter. Magnification, ×132.

types, with attachment mediated by adhesin proteins expressed on the surface of the bacterial cell. The multiple-banded (MB) antigen contains serotype-specific and cross-reactive epitopes and is a prominent antigen recognized during human ureaplasmal infections (116). Ureaplasmas are known to produce immunoglobulin A (IgA) protease which may be associated with disease production, and they release ammonia through urealytic activity (116).

EPIDEMIOLOGY AND TRANSMISSION

Mollicutes are common in practically all mammalian species, as well as many other vertebrates in which they have been

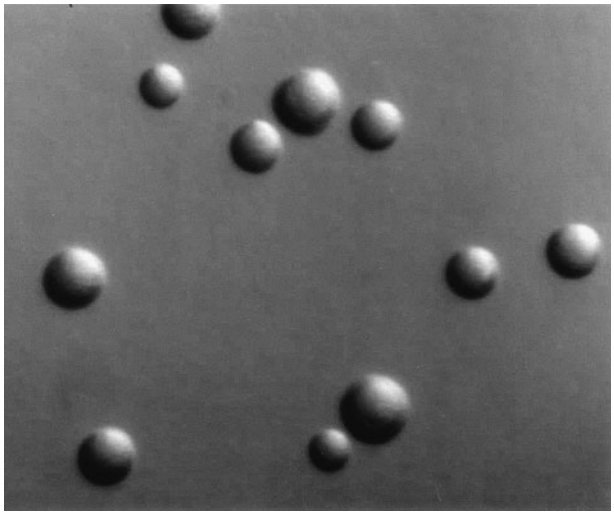


FIGURE 2 Spherical colonies of *M. pneumoniae* of up to 100 μm in diameter. Magnification, ×126.

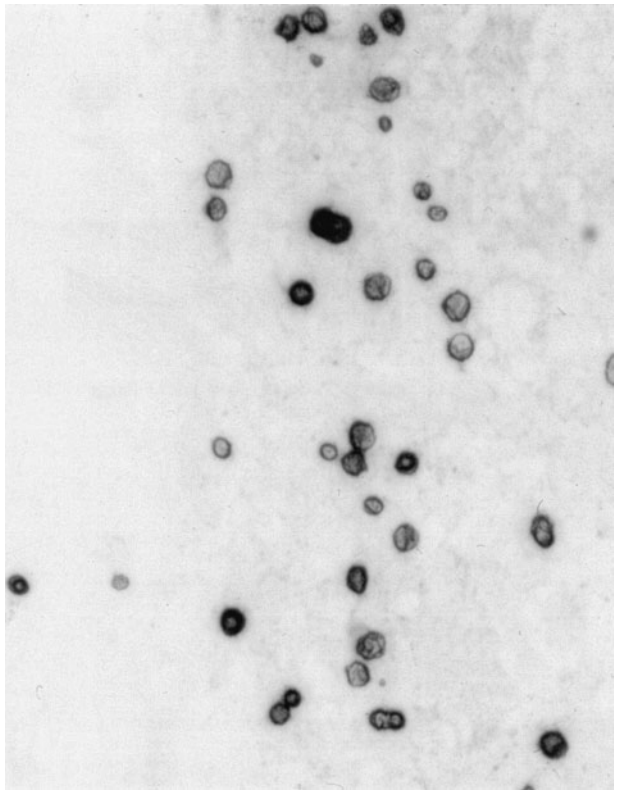


FIGURE 3 Granular, brown, urease-positive colonies of *Ureaplasma* species of 15 to 60 μm in diameter from a vaginal specimen growing on A 8 agar. Magnification, ×100.

sought. Although most mollicutes have species-specific host-organism associations, some mycoplasmas and acholeplasmas of animal origin occur in a wide variety of different animal hosts. Mollicutes in the genera *Spiroplasma, Mesoplasma, Entomoplasma,* and *Acholeplasma* can be isolated from insects and plants.

In humans, mycoplasmas and ureaplasmas are mucosally associated, residing predominantly in the respiratory or urogenital tract. They rarely penetrate the submucosa, except in cases of immunosuppression or instrumentation, when they may invade the bloodstream and disseminate to many different organs and tissues throughout the body. Many mollicutes exist as commensals in the oropharynx *(M. salivarium, M. orale, M. buccale, M. faucium, M. lipophilum,* and *Acholeplasma laidlawii)* and have been associated with invasive disease only in very rare circumstances. *M. fermentans* has been detected by culture, and more recently by means of PCR assays, in various body sites, including the urogenital tract, throat, lower respiratory tract, and other body locations, including joints (116), but its primary site of colonization and its true disease potential are incompletely understood. Oral commensal mycoplasmas may occasionally spread to the lower respiratory tract and can cause diagnostic confusion with *M. pneumoniae* infection. Frequent occurrence of pathogenic species such as *M. hominis* and ureaplasmas in the lower urogenital tract in healthy men and women has complicated complete understanding of these species' disease-producing capabilities. *M. primatum, M. spermatophilum,* and *Acholeplasma oculi* have been detected in the urogenital tract but have not been

associated with disease. PCR assays have demonstrated the frequent occurrence of *M. genitalium* in the urogenital tract in men with urethritis and in lower and upper genital tract sites in women and of *M. penetrans* in urine of homosexual males with human immunodeficiency virus infection (51, 116). Although mycoplasmas are generally considered to be extracellular organisms, intracellular localization is now appreciated for *M. fermentans, M. penetrans, M. genitalium,* and *M. pneumoniae* (11, 28). Intracellular localization may be responsible for protecting the organisms from antibodies and antibiotics, as well as contributing to disease chronicity and difficulty in cultivation in some cases. Variation in surface antigens of *M. hominis* and *Ureaplasma* spp. may be related to the persistence of these organisms at invasive sites. In humans, mycoplasmas and ureaplasmas may be transmitted by direct contact between hosts, e.g., venereally through genital-genital or oral-genital contact and vertically from mother to offspring either at birth or in utero; by respiratory aerosols or fomites in the case of *M. pneumoniae;* or even by nosocomial acquisition through transplanted tissues.

CLINICAL SIGNIFICANCE

Respiratory Infections

M. pneumoniae was first identified and described in the early 1960s. It causes approximately 20% of all community-acquired pneumonias in the general population and up to 50% of pneumonias in certain confined groups (117). Although *M. pneumoniae* has long been associated with pneumonias in school-aged children, adolescents, and young adults, in recent years this organism has also been shown to occur endemically and occasionally epidemically in older persons, as well as children under 5 years of age (117). The most typical clinical syndrome is tracheobronchitis, often accompanied by upper respiratory tract manifestations such as acute pharyngitis. Pneumonia develops in about one-third of persons who are infected. The incubation period is generally 2 to 3 weeks, and spreading throughout households is common. The organism may persist in the respiratory tract for several months after initial infection and sometimes for years in hypogammaglobulinemic patients, possibly because the organism attaches strongly to and invades epithelial cells. Disease tends not to be seasonal, subclinical infections are common, and the disease is ordinarily mild. However, severe infections requiring hospitalization and even resulting in death are known to occur (117).

Extrapulmonary complications of *M. pneumoniae* infections may include meningoencephalitis, ascending paralysis, transverse myelitis, Bell's palsy, pericarditis, hemolytic anemia, arthritis, and mucocutaneous lesions (98, 117). An autoimmune response is thought to play a role in some extrapulmonary complications. However, *M. pneumoniae* has been isolated directly from cerebrospinal, pericardial, and synovial fluids, as well as other extrapulmonary sites, and additional evidence of direct invasion by this organism has been documented by the use of the PCR assay (98). Clinical manifestations are not sufficiently unique to allow differentiation from infections caused by other common bacteria, particularly *Chlamydophila pneumoniae.* Recent data from animal models as well as clinical studies have suggested a potential role for *M. pneumoniae* and *C. pneumoniae* as etiologic or exacerbating factors in bronchial asthma (73), but additional clinical studies are required to determine the ultimate significance of these preliminary findings.

M. fermentans has been recovered from the throats of children with pneumonia, some of whom had no other etiologic agent identified, but the frequency of its occurrence in healthy children is not known (101). It has been detected in adults with an acute influenza-like illness (65) and in bronchoalveolar lavage fluids, peripheral blood lymphocytes, and bone marrow from patients with AIDS and respiratory disease (3, 4). It is apparent that respiratory infection with *M. fermentans* is not necessarily linked with immunodeficiency, but this organism may also behave as an opportunistic respiratory pathogen.

Very little is known about *M. amphoriforme* beyond what has been described in the initial reports of its detection in the lower respiratory tract by culture and/or PCR in a series of patients with antibody deficiencies and chronic bronchitis or bronchiectasis (121). Its biochemical reactivity, colonial appearance, and growth characteristics are similar to those of *M. pneumoniae,* but it is distinct genetically. Repeated isolations over time and clinical improvement after antimicrobial therapy which resulted in the elimination of the mycoplasma suggest a possible pathogenic role, but more work must be done to determine the extent of disease that may be due to this organism.

Genitourinary Infections

Ureaplasma spp. and *M. hominis* can be isolated from the lower genital tract in many healthy, sexually active adults, but there is evidence that these organisms play etiologic roles in some genital tract diseases. Results of human and animal inoculation studies and observations of immunocompromised persons are supportive of ureaplasmas being a cause of nonchlamydial, nongonococcal urethritis (NGU) in men, with further evidence supplied by therapeutic and serologic studies (31, 69, 99, 101). Since the identification of two distinct biovars of *Ureaplasma urealyticum,* biovar 2 *(U. urealyticum)* has been implicated in NGU whereas biovar 1 (*U. parvum)* has not been implicated in this manner (31). Evidence that *M. hominis* causes NGU is lacking. *M. genitalium* has been detected by PCR technology significantly more often in urethral specimens from men with acute NGU than from those without urethritis (51–54) and is now considered to be one of the causes of the disease. *M. genitalium*-positive men have been found to have symptomatic urethritis significantly more often than those infected with *Chlamydia trachomatis* (43). Antibody responses have been detected in some men with acute disease, and *M. genitalium* has also produced urethritis in nonhuman primates (101). *M. genitalium* also may be a rare cause of conjunctivitis associated with urethritis (13). *M. fermentans, M. penetrans,* and *M. pirum* were not detected in the urethras of men with urethritis by PCR assays, suggesting that these organisms are unlikely to have a pathogenic role in this condition (30). In women, there is no evidence that *M. hominis* is a cause of the urethral syndrome, but ureaplasmas may be involved (96).

M. hominis and *Ureaplasma* spp. have not been detected by culture of prostatic biopsy samples from patients with chronic abacterial prostatitis (32), and *M. genitalium* has been found rarely by using a PCR assay (61). In contrast, ureaplasmas have been recovered from an epididymal aspirate from a patient suffering from nonchlamydial, nongonococcal acute epididymo-orchitis accompanied by a specific antibody response (50) and may be an infrequent cause of the disease. *Ureaplasma* spp. produce urease and induce crystallization of struvite and calcium phosphates in urine in vitro and calculi in animal models (109). In addition, ureaplasmas have been found in urinary calculi of patients with infection-type stones

more frequently than in those of patients with metabolic-type stones, suggesting a possible causal association (45). *M. hominis* has been isolated from the upper urinary tract only in patients with symptoms of acute pyelonephritis, often with an antibody response, and may cause about 5% of cases of such disease (106). Obstruction or instrumentation of the urinary tract may be a predisposing factor. Ureaplasmas have not been associated in the same way.

Mollicutes do not cause vaginitis but are among various microorganisms that proliferate in patients with bacterial vaginosis (BV). Some studies suggest that *M. hominis* may contribute independently to BV, but evidence is lacking for an independent association of ureaplasmas with BV (116). BV may lead to pelvic inflammatory disease, and *M. hominis* has been isolated from the endometria and fallopian tubes of about 10% of women with salpingitis diagnosed by laparoscopy and accompanied by a specific antibody response (109). The significance of this mycoplasma is difficult to assess in an individual case when several microorganisms are present. Nevertheless, serologic evidence suggests that *M. hominis* may be an independent factor in tubal-factor infertility (7). *Ureaplasma* spp. have been isolated directly from affected fallopian tubes, but not alone. This finding, together with the negative results of serologic tests, studies of inoculation of nonhuman primates, and fallopian tube organ cultures (101), does not support a causal relationship for ureaplasmas in pelvic inflammatory disease. *M. genitalium,* however, may play a role as indicated by its significant association with cervicitis (42, 108) and endometritis (26). In addition, there is serologic evidence that this mycoplasma causes some cases of tubal infertility (24). That ureaplasmas may cause infertility remains speculative (99, 100).

Ureaplasmas have been isolated from internal organs of spontaneously aborted fetuses and from stillborn and premature infants more often than from fetuses from induced abortions or normal full-term infants (116). The results from some serologic and therapeutic studies have also supported a role for these organisms in fetal morbidity (21). BV is a possible confounding factor which must be considered in the association between ureaplasmas in the chorioamnion and low birth weight. Ureaplasmas at this site are directly associated with inflammation (41) and may invade the amniotic sac early in pregnancy in the presence of intact fetal membranes, causing persistent infection and adverse pregnancy outcome (19).

The notion that *M. hominis* causes fever in some women after abortion or after normal delivery is based on its isolation from the blood of about 10% of such women but not from that of afebrile women who have had abortions or that of healthy pregnant women (99). In addition, antibody responses have been detected in about half of febrile aborting women but in few of those who remain afebrile (99). Similar observations have been made for the isolation of *Ureaplasma* spp. which may be responsible for some cases of postpartum endometritis (22). It is possible that ureaplasmas play a causal role in some cases of spontaneous abortion and premature birth through elaboration of phospholipases and stimulation of cytokines which are secreted in response to the presence of the microorganisms in amniotic fluid (116). There is no evidence that *M. genitalium* is a cause of preterm labor or abortion (66, 78).

Neonatal Infections

Colonization of infants by genital mycoplasmas may occur by ascension from the lower genital tract of the mother at the time of delivery or in utero earlier in gestation and may be transient and without sequelae. The rate of vertical transmission may be 18 to 55% among infants born to colonized mothers (116). *Ureaplasma* spp. and *M. hominis* may be isolated from neonates born to mothers with intact membranes and delivered by cesarean section (116). Congenital pneumonia, bacteremia, progression to chronic lung disease of prematurity with the development of inflammatory cytokines in tracheal aspirates, and even death have occurred in very-low-birth-weight infants due to ureaplasmal infection of the lower respiratory tract (116). A meta-analysis of the literature accumulated since the 1980s supports the association of ureaplasmal infection with the development of chronic lung disease (93), but so far there has been no evidence of a reduction in the incidence of chronic lung disease or death when preterm infants have been treated with erythromycin. Both *M. hominis* and *Ureaplasma* spp. have been isolated from maternal and umbilical cord blood, as well as the blood of neonates. Both species can also invade the cerebrospinal fluid of neonates (116). Either mild, subclinical meningitis without sequelae or neurological damage with permanent handicaps may ensue. The frequency of colonization of healthy full-term infants declines after 3 months of age, and fewer than 10% of older children and sexually inexperienced adults are colonized with genital mycoplasmas (116).Vertical transmission of *M. genitalium* from mother to neonate has been reported (67), but its significance in neonates is unknown.

Routine screening of neonates for genital mycoplasmas is not clinically justified based on the available evidence that many healthy neonates may be colonized without consequence. However, if there is clinical, radiological, or laboratory evidence of pneumonia, meningitis, or overall instability, particularly in preterm neonates in whom there are no obvious alternative etiologies, infection with *M. hominis* or *Ureaplasma* spp. should be considered.

Systemic Infections and Immunosuppressed Hosts

Extrapulmonary and extragenital mycoplasmal infections probably occur more often than currently recognized. *M. hominis* is alone among pathogenic mycoplasmas of human origin which may occasionally be detected in routine bacteriologic cultures, so there have been many instances of accidental discovery when mycoplasmas were not specifically sought. Mollicutes can cause invasive disease of the joints as a result of dissemination from the genital or respiratory tract in immunosuppressed persons, especially individuals with hypogammaglobulinemia (109). Mycoplasmas should always be considered early in attempting to diagnose septic arthritis in the setting of congenital antibody deficiency. *M. hominis* bacteremia has been demonstrated after renal transplantation (89), trauma, and genitourinary manipulations, and *M. hominis* has also been found in brain abscesses, osteomyelitis lesions (74), and wound infections, having been isolated, for example, on numerous occasions from sternal wounds in recipients of heart or lung transplants (101). Numerous species, including *M. fermentans, M. salivarium,* and *Ureaplasma* spp., have been detected by culture and/or PCR in synovial fluids of persons with rheumatoid arthritis and other arthritides, although the precise contribution of these organisms to these disease conditions is still uncertain (57, 91, 92). The significance of *M. fermentans* and other mycoplasmas in persons infected with the human immunodeficiency virus, with or without AIDS, has received a great deal of attention. However, the notion that *M. fermentans* is important in AIDS disease progression lacks firm support (109). Despite the fact that mycoplasmas,

in particular *M. fermentans,* once received considerable notoriety as possible agents of Gulf War syndrome, there is no credible evidence supporting such an association or causal role (109).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Specimen Type and Collection

Body fluids appropriate for mycoplasmal culture or detection by noncultural methods include blood, synovial fluid, amniotic fluid, cerebrospinal fluid, urine, prostatic secretions, semen, wound aspirates, sputum, pleural fluid, bronchoalveolar lavage fluid, and other tracheobronchial secretions, depending on the clinical condition and organisms of interest. Swabs from the nasopharynx, cervix or vagina, wounds, and urethra are also acceptable. Tissue from biopsy or autopsy, including placenta and endometrium tissue, bone chips, and urinary calculi, can also be used. When swabs are used, care must be taken to sample the desired site vigorously to obtain as many cells as possible since mycoplasmas are cell associated. The use of urine specimens may sometimes prove more sensitive than that of urethral swabs for detection of fastidious mycoplasmas such as *M. genitalium* by PCR (52). If determination of the localization of mycoplasmas in the genitourinary tract is desired, urine specimens can be obtained at various stages during urination or after prostatic massage. Care should be taken to avoid collection of specimens that are contaminated by lubricants or antiseptics commonly used in gynecologic practice. Calcium alginate, Dacron, or polyester swabs with aluminum or plastic shafts are preferred. Wooden-shaft cotton swabs should be avoided because of potential inhibitory effects. Swabs should always be removed from specimens before transportation to the laboratory.

Successful isolation of mycoplasmas from blood can be achieved by inoculating blood, free of anticoagulant, into liquid mycoplasmal growth media at the bedside in a 1:10 ratio, using as much blood as possible (at least 10 ml for adults). Mycoplasmas are inhibited by sodium polyanethol sulfonate, the anticoagulant used in most commercial blood culture media, but the inhibitory effect can be overcome by the addition of gelatin (1% [wt/vol]) (83). Use of commercial blood culture media with or without automated blood culture instruments is not recommended for detection of mycoplasmas. None of the newer continuously monitored, nonradiometric, automated blood culture systems will flag bottles containing *M. hominis,* even when additional metabolic substrate and gelatin are added. The organism may survive in these media for several days, however (111).

Transport and Storage

Mycoplasmas are extremely sensitive to adverse environmental conditions, particularly drying and heat. Specimens should be inoculated at the bedside whenever possible by using appropriate transport and/or culture media. Specific mycoplasma media such as SP4 and Shepard's 10 B broths and 2 SP (10% heat-inactivated fetal calf serum with 0.2 M sucrose in 0.02 M phosphate buffer, pH 7.2) are acceptable transport media and can also be used for sample preparation for PCR assays. Other media available commercially for transport and storage of specimens are Stuart's medium, Trypticase soy broth with 0.5% bovine serum albumin, and Mycotrans (Irvine Scientific, Irvine, Calif.). A3B broth (Remel, Inc., Lenexa, Kans.) is available as a transport medium, whereas Remel arginine broth and 10 B and SP4 transport broths also serve as growth media. Liquid specimens do not require special transport media if cultures can be inoculated within 1 h, provided that the specimens are protected from evaporation. Tissues can be placed in a sterile container which can be tightly closed and delivered to the laboratory immediately. Otherwise, tissue specimens should be placed in transport media if delay in culture inoculation is anticipated. Specimens should be refrigerated if immediate transportation to the laboratory is not possible. If specimens must be shipped and/or if the storage time prior to processing is likely to exceed 24 h, the specimen in transport medium should be frozen at −70°C to prevent loss of viability and to minimize bacterial overgrowth. Mollicutes can be stored for long periods in appropriate growth or transport media at −80°C or in liquid nitrogen. Frozen specimens can be shipped with dry ice to a reference laboratory if necessary. Storage at −20°C is deleterious to detection, even by nonculture methods. When frozen specimens are to be examined, they should be thawed rapidly in a water bath at 37°C.

DIRECT EXAMINATION

Microscopy

Mycoplasmas, like chlamydiae and rickettsiae, cannot be clearly visualized by routine light microscopy. Lack of a cell wall precludes visualization of mycoplasmas by Gram staining, but this procedure may prove useful to exclude contaminating bacteria. *M. hominis* may occasionally appear as pinpoint colonies on bacteriologic media, and the lack of a Gram stain reaction by these colonies gives a clue as to their possible mycoplasmal identity, warranting further, more specific evaluation and subculture to mycoplasmal media. A DNA fluorochrome stain such as Hoechst 33258 (ICN Biomedicals, Costa Mesa, Calif.) or acridine orange stain may be useful when applied to body fluids such as amniotic fluid after cytocentrifugation but is not specific for mycoplasmas.

Antigen Detection

Although culture is appropriate for species which can be isolated easily and rapidly from clinical specimens, such as *M. hominis* and *Ureaplasma* spp., it is not ideal for detection of fastidious and/or extremely slow growing organisms such as *M. genitalium* and, to a considerable degree, *M. pneumoniae*. Therefore, alternate non-culture-based methods of detection should be employed even if culture is attempted for these organisms. Rapid methods for antigenic detection of *M. pneumoniae* have been developed, but these techniques are hampered by low sensitivity and cross-reactivity with other commensal mycoplasmas, and they cannot be recommended for diagnostic purposes.

Nucleic Acid Detection Techniques

DNA hybridization techniques for the diagnosis of *M. pneumoniae* infection were developed in the early 1980s. Because probes are relatively insensitive, the more recently available amplification techniques such as the PCR assay have supplanted them, and none are sold commercially in the United States or the United Kingdom.

PCR systems have been developed for all of the clinically important mollicute species that infect humans. Some examples of gene targets used in PCR assays include 16S rRNA (52, 58, 69, 80, 92, 125); other repetitive sequences such as the insertion-like elements of *M. fermentans* (120); the P1 adhesin gene (29, 55, 85), ATPase operon gene (12), and *tuf* gene of *M. pneumoniae* (68); *gap* in *M. hominis* (8); and the MgPa

adhesin gene (29, 56) and *gyrA* (15) of *M. genitalium*. Urease genes (14, 124) and the multiple-band antigen gene (59, 82) have been used as targets in *Ureaplasma* spp. Real-time PCR assays have been described for *M. pneumoniae* (72), *M. genitalium* (53, 58, 95), *M. hominis* (8), and *Ureaplasma* spp. (71). For slowly growing organisms such as *M. pneumoniae,* and especially for extremely fastidious species for which optimum cultivation techniques are not established, such as *M. genitalium* and *M. fermentans,* the use of PCR assays may be the only practical means of detecting their presence in clinical material. The sensitivity of PCR is very high, corresponding to a single organism when purified DNA is used. Other advantages are the potential ability to complete the procedure in 1 day, the requirement for only one specimen containing organisms that do not have to be viable, and the ability to detect nucleic acids in preserved tissues.

Comparison of the PCR technique with culture and/or serology, in the case of *M. pneumoniae,* has yielded varied results that are not always in agreement, and large-scale experience with this procedure is still limited. Positive PCR results for *M. pneumoniae* from culture-negative persons without evidence of respiratory disease suggest inadequate assay specificity, persistence of the organism after infection, or existence of the organism in asymptomatic carriers. Positive PCR results from serologically negative persons may be due to an inadequate immune response or to the collection of specimens before specific antibody synthesis could occur. Negative PCR results in cases of culture- or serologic-test-proven infections raise the possibility of inhibitors or other technical problems with the assay. If mycoplasmacidal antibiotics have been administered, PCR results may be negative even though serology is positive. There is some evidence suggesting that PCR inhibition may occur more commonly with nasopharyngeal specimens than throat swabs being assayed for *M. pneumoniae* (35, 86). Commercial reagents available for purification of nucleic acid can be helpful in overcoming PCR inhibition.

In addition to the PCR assays mentioned above, there have been descriptions of multiplex PCR assays to detect *M. genitalium* and other urogenital pathogens (70), a PCR-microtiter plate hybridization assay (125), and a microwell-plate-based PCR assay for large-scale screening for this mycoplasma (38). Since PCR-based assays are the mainstay for diagnosis of *M. genitalium* infection, it is essential that the procedure employed be stringently controlled and carefully monitored. Ideally, positive PCR assays should be confirmed by using a second unrelated target gene. Strong associations between serology and PCR results for *M. genitalium* have been described previously (119), but the analytic sensitivity of a single PCR assay for *M. genitalium* was questioned by Baseman et al. (10), who reported that 61% of culture-positive women tested negative by PCR, despite apparently good quality control parameters for the assay. Assuming that culture positivity was not due to cross contamination, this finding highlights the fact that reduced sensitivity of a single PCR assay may be related to the quality of the specimen or the presence of inhibitors.

PCR technology appears to be less valuable for routine diagnostic purposes in the case of infections with the more rapidly growing and relatively easily cultivable organisms, such as *M. hominis* and *Ureaplasma* spp., but this method can be valuable in clinical studies of ureaplasmal infections. The newest PCR-based techniques permit identification of *Ureaplasma* species as well as limited serotype determination (33, 59, 60, 82). In addition, real-time PCRs that differentiate between *U. urealyticum* and *U. parvum* have been described previously (71).

PCR is also a good tool for identification of an unknown mycoplasma previously obtained by culture. It can be used for characterization of strains within a species and for detection of a specific feature, such as the presence of an antibiotic resistance determinant. Presently, PCR detection for mycoplasmas and most other microorganisms of clinical significance is still too expensive and complex to be carried out routinely in most clinical microbiology laboratories. Some drawbacks must still be corrected, such as the problem of contamination and the presence of inhibitors. Development of commercial PCR kits may bring about better standardization of the technique, and if available at a reasonable cost, PCR could become a major method for the diagnosis of mycoplasmal and ureaplasmal infections.

ISOLATION PROCEDURES

Biosafety Considerations

M. pneumoniae, M. hominis, and ureaplasmas are considered to be category 2 pathogens. Work with these microorganisms and other mycoplasmas of human origin can be undertaken on the laboratory bench and/or in a class 2 safety cabinet.

Growth Media and Inoculation

Growth of mycoplasmas and ureaplasmas pathogenic for humans requires the presence of serum, growth factors such as yeast extract, and a metabolic substrate. No single formulation is ideal for all pertinent species due to different properties, optimum pHs, and substrate requirements. SP4 broth and agar (pH 7.5) are the best media overall and can be used for both *M. pneumoniae* and *M. hominis*, provided that arginine is added for the latter. Shepard's 10 B broth (pH 6.0) can be used for *M. hominis* and *Ureaplasma* spp., with A 8 as the corresponding solid medium. Penicillin G or a broad-spectrum semisynthetic penicillin should be added to minimize bacterial overgrowth. Addition of a pH indicator, such as phenol red, is important for detection because mycoplasmas usually do not produce turbidity in broth culture owing to their small cell size. Compositions of these media are provided elsewhere (110).

Lack of commercially prepared media in the past has effectively prevented many laboratories from offering on-site mycoplasma detection. For self-prepared media, quality control is crucial for each of the main components. These controls must consist of the quantitative growth of a mycoplasma strain(s) in two media that differ only in the component to be tested. New lots or batches of broth are considered satisfactory if the numbers of organisms that grow are within 1 10-fold dilution of the reference batch. Agar plates should ideally support growth of at least 90% of the colonies that are supported by the reference media. Sterility of commercially purchased medium components, such as horse serum, must be confirmed prior to their use. If a reference laboratory is to be used for mycoplasma testing, inquiry should be made as to whether the medium used is self-prepared or purchased from a manufacturer and there should also be verification of the type of quality control procedures performed. Quality control test organisms should include type strains and low-passage-number clinical isolates of the species of interest. For testing of ureaplasmas, it is recommended to include at least one representative from each of the two species. Testing inhibitory properties of media against growth of various other organisms likely present in specimens from nonsterile sites may also be worthwhile to prevent loss of mycoplasmas due to overgrowth of contaminating organisms.

Specimens should always be mixed well before inoculation of media, fluids should be centrifuged ($600 \times g$ for 15 min), and the pellet should be inoculated. Urine can be filtered

through a 0.45-μm-pore-size filter if bacterial contamination is suspected. Furthermore, it is wise to mince, not grind, tissues in broth prior to diluting. Serial dilution of specimens in broth to at least 10^{-3} with subculture of each dilution onto agar is an extremely important step in the cultivation process since it will help overcome possible interference by antibiotics, antibodies, and other inhibitors, including bacteria, that may be present in clinical specimens. Omission of this critical dilution step may be one reason why some laboratories have difficulty in recovering the organisms. Dilution also helps to overcome the problem of rapid decline in culture viability, which is particularly common with ureaplasmas, and it also provides information about the number of organisms present.

Incubation Conditions and Subcultures

Broths should be incubated at 37°C under atmospheric conditions. Agar plates yield the best growth if they are incubated in an atmosphere of room air supplemented with 5 to 10% CO_2 or in an anaerobic environment of 95% N_2 plus 5% CO_2. A candle jar or anaerobe jar with a GasPak catalyst is adequate if dedicated incubators are not available. The relatively rapid growth rates of *M. hominis* and *Ureaplasma* spp. make identification of most positive cultures possible within 2 to 4 days, whereas *M. pneumoniae* usually requires 21 days or more. Several mycoplasmal species of human origin can produce similar biochemical reactions, and identification can be accomplished only by using specific tests once the organisms are isolated. All broths that have changed color should be subcultured into a fresh tube of the corresponding broth (0.1 ml into 0.9 ml) and onto agar (0.02 ml). Subcultures must be performed soon after the color change occurs, particularly if the organism is a *Ureaplasma* species, because the culture can lose viability within a few hours. Subculture also increases the diagnostic yield since some strains may not grow sufficiently from the original specimen inoculated initially onto solid media. Blind subculture periodically during incubation may improve the yield of *M. pneumoniae* and other mycoplasmas since a color change may not always be evident, even if growth occurs. Cultures should be incubated for at least 7 days before being designated negative for genital mycoplasmas and 4 weeks for *M. pneumoniae*. The growth rate of *M. fermentans* is similar to that of *M. pneumoniae.* However, for *M. fermentans, M. genitalium,* mycoplasmas of human origin other than *M. pneumoniae* and *M. hominis,* and *Ureaplasma* spp., cultivation conditions are not well established. Due to the advent of PCR assays for use in research and reference laboratories, the need to refine culture techniques for these slowly growing and fastidious organisms is less critical and cultivation methods for them will not be discussed.

Development of Colonies

Broth cultures for *Ureaplasma* spp. should be examined for color change resulting from hydrolysis of urea twice daily for up to 7 days because of the rapid decline in viability of these organisms in culture. This timing is less critical for *Mycoplasma* spp., for which once-daily inspection of broth cultures is sufficient. Agar plates should be examined by using a stereomicroscope at a magnification of ×20 to 60 daily for *Ureaplasma* spp., at 1- to 3-day intervals for *M. hominis,* and every 3 to 5 days for *M. pneumoniae* and other more slowly growing species. *Ureaplasma* colonies (Fig. 3) can be identified on A 8 agar by urease production in the presence of a $CaCl_2$ indicator contained in the medium. The larger *M. hominis* colonies are urease negative and often have the typical fried-egg appearance (Fig. 1). Other species, such as *M. pneumoniae* and *M. genitalium,* will produce much smaller spherical colonies which may or may not demonstrate the fried-egg appearance (Fig. 2). Methylene blue stain applied directly to the agar plate to turn the colonies blue is sometimes useful if there is uncertainty about whether or not mycoplasmal colonies are present. *M. hominis* is the only pathogenic mycoplasma of humans cultivable on bacteriologic media such as chocolate agar and blood agar. However, the pinpoint translucent colonies are easily overlooked and routine bacterial cultures may be discarded before the time needed for *M. hominis* colonies to develop, which may be 4 days or more in some cases. Occurrence of suspicious colonies warrants subculture to appropriate mycoplasma media.

Commercial Media and Culture Kits

In response to the growing desires of many independent or hospital-based clinical laboratories to offer mycoplasmal culturing on-site, numerous companies have developed various transport and growth medium systems. A variety of kits for detection, quantitation, identification, and antimicrobial susceptibility testing of *Ureaplasma* spp. and *M. hominis* from urogenital specimens are available in several European countries but not in the United States. A more complete description of these commercial kits sold in Europe is provided elsewhere (118).

Mycoscreen GU (Irvine Scientific) is a broth-based culture system for screening clinical specimens for genital mycoplasmas. Vials showing evidence of growth as indicated by color change must be subcultured to the Mycotrim GU Triphasic flask system, Mycotrim GU agar, or other solid medium for isolation and identification. A comparable system, Mycotrim RS, has been adapted for detection of *M. pneumoniae* in respiratory specimens. Remel, Inc., has developed several formulations of transport and growth media, including 10 B broth, A 7 agar, A 8 agar, SP4 broth, and SP4 agar.

Some kits and other commercial products and media have been evaluated to a limited degree by independent investigators (1, 16, 23, 25, 81, 94, 123). Commercial products and kits may be of particular value if the need to detect mycoplasmas arises infrequently in laboratories which do not specialize in mycoplasma detection, but users should be aware of the potential limitations of existing products. If commercially prepared media are to be utilized, it is advisable that laboratories perform internal quality control tests.

IDENTIFICATION

Even though the numerous large-colony mycoplasmal species which may be isolated from humans cannot be identified based on colonial morphology or a particular biochemical profile, the body site of origin and rate of growth, in conjunction with biochemical features, give some clues. Biochemical properties such as glucose, arginine, or urea hydrolysis are determined based on color change in the absence of turbidity in the appropriate broth. Utilization of glucose by a mycoplasma in SP4 broth will produce an acidic shift (red to yellow), whereas utilization of arginine will produce a change from red to deeper red in this broth in the presence of the phenol red pH indicator. Urea or arginine hydrolysis in 10 B broth causes an alkaline shift from orange to deep red. Thus, a slowly growing glycolytic organism grown from the respiratory tract that produces spherical colonies on SP4 agar after approximately 5 to 20 days of incubation and exhibits hemolytic activity and hemadsorption with guinea pig erythrocytes is most likely to be *M. pneumoniae.* An alkaline color change which occurs after overnight incubation without turbidity in 10 B broth containing urea is almost certainly due to *Ureaplasma* spp.,

whereas a urogenital specimen that produces an alkaline reaction within 24 to 72 h in broth supplemented with arginine is likely to contain *M. hominis*. Examination of colonial morphology is sufficient to identify *Ureaplasma* spp., and it is important to keep in mind that these organisms often coexist with *M. hominis* in urogenital specimens.

In order to identify a large-colony mycoplasma completely to the species level, a number of different techniques are available, although they are more appropriate for a reference laboratory than for a hospital microbiology laboratory because of their complexity and the lack of commercial availability of the reagents required. PCR appears to be the best overall choice for mycoplasma species identification since it is much simpler to perform than other methods and it does not require immunologic reagents that may not be readily available. The PCR assay is also less subjective to interpretion than some of the other methods such as epi-immunofluorescence.

TYPING SYSTEMS

Several methods for typing of mollicutes have been described previously and used as a means to study epidemiology in the case of *M. pneumoniae* and differential pathogenicity in the case of the 2 genomic clusters and 14 serotypes of *Ureaplasma* spp. Typing methods have been much less extensively evaluated for other human mycoplasmal species.

Techniques employed initially to serotype ureaplasmas from clinical specimens have included use of polyclonal antibodies (39), immunofluorescence (76), immunoperoxidase (84), and agar growth inhibition (103, 110). Results of earlier studies have been varied and inconsistent due to the inefficient and imprecise methods available, the occurrence of multiple cross-reactions, and the fact that many persons may harbor more than one serotype in their urogenital tracts in the presence or absence of disease. Development of monoclonal antibodies enabled identification of MB antigens responsible for ureaplasma serotype specificity on the cell surface (126). PCR-based assays enabled the more accurate characterization of the two genomic clusters of *Ureaplasma* spp. that led to their designation as two separate species. Pulsed-field gel electrophoresis has been applied to *Ureaplasma* spp. to determine the sizes of the genomes (77, 88), and preliminary information suggests that pulsed-field gel electrophoresis can distinguish among the majority of the 14 *Ureaplasma* serotypes and detect differences within serotypes (K. B. Waites, unpublished data).

Restriction fragment length polymorphism, multilocus sequence typing, Western blotting, two-dimensional gel electrophoresis, and PCR assays have been used to characterize *M. pneumoniae* clinical isolates (27, 34, 37, 49, 79, 90). Most evaluations have determined that there are two genomic groups or subtypes distinguishable by analysis of the P1 adhesin gene, ORF6 gene, P65 gene, and typical DNA restriction fragment pattern. Typing of human mycoplasmas or ureaplasmas for diagnostic or epidemiological purposes is not recommended, and the methods are unavailable except in specialized research or reference laboratories.

SEROLOGIC TESTS

M. pneumoniae Respiratory Disease

Historically, serology has been the most common laboratory means for diagnosis of *M. pneumoniae* respiratory tract infections. Although culture and PCR are also used to detect the presence of *M. pneumoniae* in respiratory specimens, persistence of the organism for variable lengths of time following acute infection makes it difficult in some cases to assess the significance of a positive culture or PCR assay without additional confirmatory tests such as seroconversion.

M. pneumoniae has both lipid and protein antigens which elicit antibody responses that can be detected after about 1 week of illness and peak at 3 to 6 weeks, followed by a gradual decline, allowing the application of several different types of serologic assays based on different antigens and technologies. Serology is a very useful epidemiologic tool in circumstances where the likelihood of mycoplasmal disease is high, but it is less suited for assessment of individual patients in a timely manner. Its main disadvantage is the need for both acute- and convalescent-phase paired sera collected 2 to 3 weeks apart that are tested simultaneously for IgM and IgG to confirm seroconversion. This is especially important for adults over 40 years of age who may not mount an IgM response, presumably because of reinfection (117). Moreover, IgM antibodies can sometimes persist for several weeks to months, making it risky to base diagnosis of acute infection on a single assay for IgM (117). Antibody production may also be delayed in some infections or even absent if the patient is immunosuppressed. False-negative tests for IgM can also occur if serum is collected too soon after the onset of illness. Since *M. pneumoniae* is a mucosal pathogen, IgA is typically produced early in the course of infection. Measurement of IgA in serum may therefore be a better approach for diagnosis of acute infection because of IgA's rapid rise and decline, but very few commercial assays include reagents for IgA detection.

Complement fixation (CF) was the primary method for serologic testing for *M. pneumoniae* in the past. Although CF measures mainly the early IgM response, the test does not differentiate among antibody classes. Cross-reactions with other organisms, most notably *M. genitalium* (63), are well recognized, and false-positive results due to cross-reactive autoantibodies induced by acute inflammation from other unrelated causes may occur. In most clinical laboratories, CF has been replaced by alternative techniques. Table 3 summarizes various assays used to detect *M. pneumoniae* infection in the United States. More detailed descriptions of individual assay kits are available in other reference texts (see, e.g., reference 118).

Immunofluorescent-antibody (IFA) assays, direct and indirect hemagglutination using IgM capture, and other particle agglutination antibody assays (PAs) have been developed to detect antibody to *M. pneumoniae* (5, 40, 62). IFA assays consist of *M. pneumoniae* antigen affixed to microscope slides and measure IgM and IgG separately. This type of assay is technically simple to perform but is subjective in interpretation and requires a fluorescent microscope, and the presence of *M. pneumoniae*-specific IgG may interfere with IgM results (5). Qualitative and semiquantitative PAs using either latex beads or gelatin that detect IgM and IgG simultaneously can be technically easy to perform, but they do not offer any significant advantages over enzyme immunoassays (EIAs). Though commonly used in some countries because of their ease and simplicity of performance, PAs have not been widely used for serologic diagnosis of *M. pneumoniae* infections in the United States.

EIAs, developed in the 1970s, have become the most widely used methods for detection of *M. pneumoniae* antibody in the United States. All are classified as having either moderate or high complexity according to the Clinical Laboratory Improvement Amendments. They may be qualitative or quantitative and may or may not require specialized

TABLE 3 Examples of test kits sold in the United States for detection of serum antibodies to *M. pneumoniae* in serum[a]

Product name	Manufacturer	Antibody(ies) measured	Assay format and description	No. of tests/ kit	Specimen throughput	Assay time	
						Start to finish	Hands on
M. pneumoniae antibody (MP) test system	Zeus Scientific, Inc., Branchburg, N.J.	IgM and IgG separately	Indirect immunofluorescence assay available as a slurry of *M. pneumoniae* antigenic substrate or a Crown Titer of *M. pneumoniae* colonies affixed to microscope slides	100	Each slide contains 10 wells	2.5 h	30 min
ETI-MP IgM or IgG	Savyon Diagnostics, Ltd., Ashdod, Israel	IgM and IgG separately	EIA using a 96-well microtiter plate coated with a membrane preparation containing *M. pneumoniae* P1 protein	192	Strips of 8 wells	2.5 h	15–20 min
ImmunoCard	Meridian Diagnostics, Cincinnati, Ohio	IgM only	Qualitative, membrane-based EIA in a single-sample-card format consisting of a test port containing *M. pneumoniae* antigen and a control port containing immobilized human IgM	30	1 specimen per card	12 min	10 min
M. pneumoniae IgG-IgM antibody test system	Remel, Inc., Lenexa, Kans.	IgM and IgG simultaneously	Qualitative, membrane-based enzyme immunobinding assay consisting of a single sample test containing inactivated *M. pneumoniae* cytadhesin protein	10 or 40	1 specimen per card	10 min	2–3 min
Mycoplasma IgG and IgM enzyme-linked immunosorbent assay system	Zeus Scientific, Inc.	IgM and IgG separately	Qualitative EIA with multiwell break-away strips containing partially purified inactivated *M. pneumoniae*	96	Strips of 8 wells	50 min	5–10 min
GenBio Immuno-WELL *M. pneumoniae* IgM or IgG	Alexon-Trend Inc., Ramsey, Minn.	IgM and IgG separately	EIA using a 96-well microtiter plate coated with purified glycolipid antigen from *M. pneumoniae* (strain FH; ATCC 15531)	96	Strips of 8 wells	IgG, 2.35 h; IgM, 2.75 h	15–20 min

[a] This table does not include all commercially available test kits sold in the United States for serologic diagnosis of *M. pneumoniae* infections. It provides descriptions of tests representing various formats and is limited to those products that have been evaluated in independent studies.

equipment. EIAs are more sensitive than CF or culture, have good specificity, and can be performed with very small volumes of serum. The need for acute- and convalescent-phase sera has remained the obvious limitation for prompt point-of-care diagnosis. However, a membrane-based EIA specific for IgM, the ImmunoCard (Meridian Diagnostics, Cincinnati, Ohio), has been developed for rapidly detecting an acute *M. pneumoniae* infection by using a single serum specimen. The ImmunoCard has the advantages of being technically much simpler and quicker (10 min) to perform than other types of assays. It has proved highly reliable for children but has the potential disadvantage of a lack of sensitivity for detection of *M. pneumoniae* infections in some adults in whom the IgM response may be minimal. The Remel EIA is another rapid point-of-care qualitative serologic assay that detects both IgM and IgG simultaneously in an easy-to-read format without the need for any instrumentation. This test has shown good sensitivity and specificity compared to other EIAs, IFA assays, and CF tests. Several comparison studies have been performed, individually evaluating each of the EIA kits listed in Table 3 and several others (5, 6, 9, 40, 62, 97, 104, 105). The best commercial EIA for individual patient diagnosis depends on the age of the patient being tested, the timing of serum collection, the availability of paired sera, the equipment available, and the experience of the laboratory personnel who will perform the test. However, maintaining a large variety of different assays within one laboratory is not practical or cost-effective. According to a recent study (97), the rapid Remel and Meridian tests are better able to detect seropositive samples than the plate type EIAs. Among the latter assays, the Zeus and Savyon kits were more sensitive than the GenBio assay.

Cold agglutinins, detected by agglutination of type O, Rh-negative erythrocytes at 4°C, occur in association with *M. pneumoniae* infection in about 50% of cases (20). Titers of 64 to 128 or a fourfold or greater rise in titer suggests a recent *M. pneumoniae* infection, but the test is nonspecific. Detection of cold agglutinins is not recommended for serologic diagnosis of *M. pneumoniae* infection.

Infections Due to Genital Mycoplasmas

Serologic tests for *M. hominis* and *Ureaplasma* spp. using the techniques of microimmunofluorescence, metabolism inhibition, and EIA have been described previously (17, 18, 64, 102, 103). A microimmunofluorescence assay for *M. genitalium* has also been developed (44) and has been shown to detect antibody responses in men with NGU (99) and women with salpingitis (75). This method is rapid, gives reproducible results, and is quite sensitive and specific, with less cross-reactivity with *M. pneumoniae* than is seen with other methods. A sensitive and specific serologic assay for *M. genitalium* using lipid-associated membrane proteins as antigens has also been developed, and this technique has been used in combination with Western immunoblotting to assess immunoreactivities of women regarded as being culture positive for *M. genitalium* (10). No serologic tests for genital mycoplasmas have been standardized and made commercially available for diagnostic use in the United States. Therefore, they cannot be recommended for routine diagnostic purposes.

ANTIBIOTIC SUSCEPTIBILITIES

Methods Used for Testing

Several methods of susceptibility testing used for conventional bacteria have been employed for testing of mycoplasmas. Agar dilution has been used extensively as a reference method (12, 51, 64). It has the advantages that the end point is relatively stable over time, the inoculum size does not have a great effect, and the method allows detection of mixed cultures readily. However, this technique is not practical for testing small numbers of strains or occasional isolates which may be encountered in diagnostic laboratories. Agar disk diffusion is not useful for testing mycoplasmas since there has been no correlation between inhibitory zones and MICs, and the relatively slow growth of some of these organisms further limits this technology. Broth microdilution to determine MICs is the most practical and widely used method. It is economical and allows several antimicrobials to be tested on the same microtiter plate, but it has numerous disadvantages in that preparation of antimicrobial dilutions is labor-intensive and the end point tends to shift over time. Limited comparisons of agar dilution and broth microdilution indicate that the two methods provide similar results for various antimicrobials tested against *Ureaplasma* spp. and *M. hominis* (47, 115).

Studies using the E test (AB BIODISK, Solna, Sweden) agar gradient diffusion technique for detection of tetracycline resistance in *M. hominis* yielded results comparable to those of broth microdilution (113). Additional comparative studies have also validated this method for determination of in vitro susceptibilities of *M. hominis* to fluoroquinolones (112) and susceptibilities of ureaplasmas to various antimicrobials (36). The E test has the advantages that agar-based testing is relatively simple to perform, the end point does not shift over time, the inoculum size does not have a large effect, and the method can easily be adapted for testing of single isolates.

There are no universally accepted standards for pH, media, incubation conditions, or duration of incubation for performing mycoplasmal or ureaplasmal susceptibility tests. No MIC breakpoints specific for these organisms are endorsed by any regulatory agency. Lack of specific guidelines for susceptibility testing methods, quality control reference MIC ranges, and interpretation of results has led to diverse and often inconsistent susceptibility profiles, especially for the genital mycoplasmas.

MIC assays must include control strains for validation purposes. A control strain may be a commercially purchased type strain or a well-studied clinical isolate for which MIC values are reproducible. An inoculum of 10^4 to 10^5 CFU/ml has been recommended as the optimum inoculum for broth-based testing (110, 114). Nonstandardized conditions at a low pH (6.0) can affect MICs, especially those of macrolides, but such conditions may be required for adequate growth of *Ureaplasma* spp. Step-by-step procedures for performance of in vitro susceptibility tests for mycoplasmas and ureaplasmas of human origin have been published previously (114). Bactericidal activity can be tested directly from the wells in broth microdilution MIC assays by removing the mixture of organisms and antibiotic, diluting it to subinhibitory concentrations in fresh medium, and observing for a color change as evidence of growth (110, 114).

Although some reference laboratories have adopted MIC breakpoints for other bacteria for use in interpretation of MICs when testing mycoplasmas, this practice should be used with caution, and it may be preferable merely to report MICs and allow clinicians to draw their own conclusions about the suitability of a particular agent for use in the treatment of a specific infection. Tetracycline-resistant *M. hominis* and *Ureaplasma* spp. can usually be distinguished by broth- or agar-based methods since MICs for the resistant strains are generally 2 to $\geq$64 μg/ml (113, 116).

Commercial MIC panels in kits have been available in Europe for several years. Details on these products are provided in reference texts (118).

Susceptibility Profiles and Antimicrobial Resistance

The availability of information on in vitro susceptibilities is greatest for *M. pneumoniae, M. hominis,* and *Ureaplasma* spp., but in some recent studies the activities of numerous antimicrobials against *M. fermentans* and *M. genitalium* have been evaluated. Though incompletely studied, *M. genitalium* has susceptibilities generally similar to those of *M. pneumoniae,* and *M. fermentans* has susceptibilities generally similar to those of *M. hominis,* with some exceptions. A comparison of MICs of several antimicrobial agents is shown in Table 4.

Susceptibility Testing and Treatment of Mycoplasmal Infections

Mollicutes are innately resistant to all beta-lactams. Sulfonamides, trimethoprim, and rifampicin are also inactive. Resistance to macrolides and lincosamides is variable according to species, with *M. hominis* being resistant to erythromycin but susceptible to clindamycin. For *Ureaplasma* spp., the reverse is true. Newer macrolides, azalides, and ketolides have shown in vitro activity comparable to that of erythromycin against *M. pneumoniae.*

M. pneumoniae is susceptible to newer fluoroquinolones, tetracyclines, and macrolides so that susceptibility testing has not been recommended except for the in vitro evaluation of new and previously untested agents. However, a recent study from Japan found that macrolide resistance in *M. pneumoniae* due to transition mutations in domain V on the 23S rRNA gene occurred in approximately 6% of 195 clinical isolates from patients with acute respiratory infections (75a). The clinical significance of this resistance is uncertain, but it suggests the need to monitor clinical isolates. Tetracycline resistance has been well documented in recent years for both *M. hominis* and *Ureaplasma* spp. and is mediated by the *tet*(M) determinant which codes for a protein

TABLE 4 Ranges of MICs of various antimicrobials for *M. pneumoniae*, *M. hominis*, *M. fermentans*, *M. genitalium*, and *Ureaplasma* spp.[a]

Antimicrobial	MIC (μg/ml) for:				
	M. pneumoniae	*M. hominis*	*M. genitalium*	*M. fermentans*	*Ureaplasma* spp.
Tetracycline	0.63–0.25	0.2–2[b]	≤0.01–0.05	0.1–1	0.05–2[b]
Doxycycline	0.02–0.5	0.1–2[b]	≤0.01–0.3	0.05–1	0.02–1[b]
Tigecycline	0.06–0.25	0.125–0.5	ND	ND	1–16
Erythromycin	≤0.004–0.06	32–>1,000	≤0.01	0.5–64	0.02–16
Roxithromycin	≤0.01	>16	0.01	32–64	0.1–2
Dirithromycin	≤0.015–0.5	>64	≤0.015–0.125	≥64	0.25–4
Clarithromycin	≤0.004–0.125	16–>256	≤0.01	1–64	≤0.004–2
Azithromycin	≤0.004–0.01	4–64	≤0.01	≤0.003–0.05	0.5–4
Josamycin	≤0.01–0.03	0.05–2	0.01–0.03	0.1–0.5	0.03–4
Clindamycin	≤0.008–2	≤0.008–2	0.2–1	0.01–0.25	0.2–64
Lincomycin	4–8	0.2–1	1–8	ND	8–256
Pristinamycin	0.02–0.05	0.1–0.5	≤0.01–0.02	ND	0.1–1
Spiramycin	≤0.015–0.25	32–>64	0.125–1	2–4	4–32
Telithromycin	≤0.008–0.06	2–32	≤0.015	0.06–0.25	≤0.015–0.25
Cethromycin	≤0.001–0.016	≤0.008–0.031	ND	≤0.008	≤0.008–0.031
Chloramphenicol	2	2–25	ND	0.5–10	0.4–8
Gentamicin	4	2–16	ND	0.25–>500	0.1–13
Ciprofloxacin	0.5–2	0.1–4	2	0.02–>64	0.1–16
Ofloxacin	0.05–2	0.1–64	1–2	0.02–25	0.2–25
Levofloxacin	0.5–1	0.1–2	0.5–1	0.05–1	0.2–2
Sparfloxacin	≤0.008–0.5	≤0.008–0.1	0.05–0.1	≤0.01–0.05	0.003–1
Gatifloxacin	0.031–1	0.016–0.25	0.125	≤0.008–0.25	0.125–2
Moxifloxacin	0.06–0.125	0.06–0.125	0.03–0.06	≤0.015–0.06	0.125–1
Garenoxacin	≤0.008–0.125	≤0.008–0.063	0.06–0.125	≤0.008–0.015	0.016–1
Gemifloxacin	0.05–0.125	0.0025–0.01	0.05–0.125	0.001–0.01	0.03–0.5
Rifampin	>8	>1,000	ND	25–>50	>1,000
Quinupristin-dalfopristin	0.008–0.06	0.03–8	0.05	0.1–0.5	0.03–0.5
Linezolid	≥64	2–8	ND	ND	>64

[a] Data were compiled from multiple published studies in which different methodologies, and often different antimicrobial concentrations, were used. ND, no data available.
[b] Tetracycline-susceptible strains only. MICs for isolates carrying *tet*(M) are generally 2 to >64 μg/ml.

that binds to the ribosomes, protecting them from the actions of this type of drug. The extent to which tetracycline resistance occurs in genital mycoplasmas varies geographically and according to prior antimicrobial exposure in different populations but may approach 40 to 50%. Information about tetracycline resistance in *M. genitalium* does not exist, but *M. genitalium*-positive men with NGU and women with cervicitis may respond better to azithromycin than they do to a tetracycline, possibly because of the lower MICs. There are very little outcome data documented in a controlled setting with microbiological studies to demonstrate the efficacy of antimicrobial treatment of *M. genitalium* infections. Such information is needed in view of the growing importance of this mycoplasma in sexually transmitted disease. Other agents such as streptogramins, aminoglycosides, and chloramphenicol may show in vitro activity against some mollicute species. Though data are very limited, oxazolidinones such as linezolid appear to be inactive against mycoplasmas.

Extragenital infections, often in immunocompromised hosts, may be caused by multidrug-resistant mycoplasmas and ureaplasmas, making guidance of chemotherapy by in vitro susceptibility tests important in this clinical setting. Eradication of infection under these circumstances can be extremely difficult, requiring prolonged therapy, even when the organisms are susceptible to the expected agents. This difficulty highlights the facts that mollicutes are inhibited but not killed by most commonly used bacteriostatic antimicrobial agents in concentrations achievable in vivo and that a functioning immune system plays an integral part in their eradication. Fluoroquinolones such as levofloxacin, moxifloxacin, and gatifloxacin tend to have greater in vitro activity against mollicutes than older drugs such as ciprofloxacin, but resistant strains have been reported (116). Treatment of mycoplasmal and ureaplasmal infections has been reviewed elsewhere (98, 116, 117).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Tests offered through diagnostic microbiology laboratories should focus on the species known to cause human disease and for which cultivation techniques are best defined. Unusual organisms, or those for which cultivation conditions are not established, may be detectable by PCR technology offered through a few specialized research or reference laboratories. Such organisms should be sought only after consultation with clinicians and personnel from the reference laboratory. Except for *Ureaplasma* spp., which can be identified by urease production and distinct colonial morphology, until species identification can be confirmed, a preliminary report of "large-colony *Mycoplasma* species" is appropriate. In many instances, as in culturing of specimens from the lower genital tract, this information may be sufficient. Isolates from

normally sterile sites and/or from immunosuppressed persons should be identified to the species level.

M. pneumoniae

Detection of *M. pneumoniae* in culture is time-consuming and not overly sensitive. However, isolation of the organism from respiratory tract specimens is clinically significant in most instances and should be correlated with the presence of clinical respiratory disease since a small proportion of asymptomatic carriers may exist. Detection by PCR is becoming more widely available through reference laboratories, but a positive result must still be correlated with clinical events. Reliable serology remains critical for accurate diagnosis of acute *M. pneumoniae* respiratory disease. Detection of IgG and IgM antibodies by EIA is the method of choice. A fourfold rise in antibody titer between acute- and convalescent-phase sera is considered diagnostic of acute infection. For children, adolescents, and young adults, a single positive IgM result obtained by using appropriate immunoglobulin class-specific reagents may be considered diagnostic of acute infection in most but not necessarily all cases because of the possibility of prolonged IgM elevation. Mild respiratory infections due to *M. pneumoniae* do not merit a costly and time-consuming microbiological work-up since empiric treatment will be effective in most instances.

M. hominis

M. hominis can be detected in culture within a few days. It may occasionally be discovered in routine bacteriologic media from appropriate clinical material, but such detection should not be relied upon. Its isolation in any quantity from normally sterile body fluids or tissues is significantly associated with disease, but quantitation of organisms may be of value in other circumstances. When mycoplasmas are detected in nonsterile sites such as the female lower genital tract in numbers exceeding 10^5 organisms, the mycoplasmas are most likely to be associated with BV.

Ureaplasma Species

Ureaplasma species can be detected in culture within 24 to 48 h. The characteristic colonial morphology and urease production are sufficient for identification at the genus level. Isolation in any quantity from normally sterile body fluids or tissues is significantly associated with disease. The detection of fewer than 10^4 organisms in the male urethra is unlikely to be significant. Whether this is true for each species now designated within the genus, i.e., *U. urealyticum* and *U. parvum*, is not clear. Distinguishing between the two species of *Ureaplasma* by PCR may become more important in view of possible differences in pathogenicities in some circumstances (2, 31, 33).

M. genitalium

Growing evidence for the role of *M. genitalium* as a urogenital pathogen has generated interest in the development of diagnostic methods for its detection, though no molecular biology-based assays for direct detection or serology test kits are sold commercially. Even though cultivation techniques for *M. genitalium* have been described previously (10, 54, 107), relatively few clinical isolates have actually been obtained since the initial description of this mycoplasma in the early 1980s. Slow growth, requiring 6 weeks or longer even in enriched SP4 media, and poor sensitivity make culture impractical. The potential importance of this organism in sexually transmitted urogenital infections underscores the need for improved and standardized methods for its detection. At present, noncommercial, nonstandardized PCR-based assays are all that are available. Results of these assays should be used with caution if they are adapted and employed for diagnostic purposes. When *M. genitalium* is detected in clinical specimens from a urogenital tract site such as the male urethra or the female cervix in persons with clinical evidence of urethritis or cervicitis, it should be considered medically significant.

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Chlamydia and *Chlamydophila**

ANDREAS ESSIG

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TAXONOMY AND NOMENCLATURE

Chlamydia and *Chlamydophila* are nonmotile, obligate intracellular prokaryotic pathogens characterized by a unique biphasic developmental cycle bearing two chlamydial forms that differ essentially in terms of morphology and function. According to the *Approved List of Bacterial Names,* published in 1980, the *Chlamydiaceae* contained one genus with just two species, *Chlamydia trachomatis* and *Chlamydia psittaci,* which were separated by their capability to accumulate glycogen in inclusions (Fig. 1A) and their susceptibility to sulfadiazine. In the 1990s, the application of DNA-based classification methods contributed to the recognition of the emerging human pathogen *Chlamydia pneumoniae* (35) and of *Chlamydia pecorum* (31), a pathogen of ruminants, as new species of the *Chlamydiaceae*. Phylogenetic analyses of the 16S and 23S rRNA genes were the rationale for the proposal of an emended description of the order *Chlamydiales,* and a revised taxonomy of the family *Chlamydiaceae* in 1999 (25). According to this proposal, members of the order *Chlamydiales* are obligately intracellular bacteria that have the unique chlamydia-like developmental cycle and more than 80% sequence identity with chlamydial 16S rRNA genes and/or 23S rRNA genes. The emended order now includes four families: *Chlamydiaceae, Parachlamydiaceae, Simkaniaceae* (49), and *Waddliaceae* (87).

Early divergence of *C. trachomatis*-like strains in the *Chlamydiaceae* was postulated on the basis of sequence data from the ribosomal genes and supported by other data such as genome size, glycogen production, and *ompA* sequence analysis. This led to the proposal to divide the family *Chlamydiaceae* into two genera, *Chlamydia* and *Chlamydophila* (25). As a consequence *Chlamydia pneumoniae, Chlamydia psittaci,* and *Chlamydia pecorum* were placed into the new genus *Chlamydophila*. However, the newly proposed nomenclature was controversial. The division into two genera was especially objected to by experts in the field who argued that the new genus designations ignore the unique, highly conserved biology shared by these organisms that was recognized when they were in a single genus (92). Although the new taxonomy was validly published, the ongoing debate resulted in simultaneous use of the former and the new nomenclature in the literature.

* This chapter contains material presented in chapter 63 by James B. Mahony, Brian K. Coombes, and Max A. Chernesky in the eighth edition of this Manual.

DESCRIPTION OF THE *CHLAMYDIACEAE*

The *Chlamydiaceae* contain the known human pathogens *C. trachomatis, C. pneumoniae,* and *C. psittaci* as well as organisms such as *C. abortus* and *C. felis* that have been only rarely associated with human infections. Members of the *Chlamydiaceae* show less than 10% overall 16S rRNA gene diversity and less than 10% overall 23S rRNA gene diversity. The 16S rRNA gene of the *C. abortus* strain B 577 (accession no. D85709) has been suggested as a consensus sequence for the family (25). The genome sizes of the *Chlamydiaceae* range from 1.0 to 1.24 Mbp with a G+C content of about 40%.

The cell wall harbors a common lipopolysaccharide (LPS) that differs from LPS of other bacteria in its relatively low endotoxic activity. This has been related to its lipid A, which has unusual long-chain fatty acids (12). Accounting for about 60% of the protein mass, the 40-kDa chlamydial major outer membrane protein (MOMP), encoded by the *ompA* gene, is an important structural component of the organisms' outer membrane. The variable domains (VD1 through 4) lead to multiple *C. trachomatis* serovars associated with different clinical manifestations of oculogenital infections. In contrast, *C. pneumoniae* isolates possess a strikingly high MOMP homology (45) and serovars of *C. pneumoniae* have not been described. The 60-kDa cysteine-rich OMP 2 (OmcB) is also highly conserved and could account for the osmotic stability of elementary bodies (EBs) by forming a disulfide–cross-links network with the periplasmic MOMP domains and other proteins (26). OmcB binds heparin, and this may be related to mammalian host cell adhesion and entry (102).

The unique developmental cycle differentiates chlamydiae from all other bacteria, leading to important consequences in laboratory diagnosis, clinical course, and antibiotic therapy. So-called EBs of chlamydiae infect eukaryotic host cells and can survive for only a limited period of time outside the host cell. Once inside the host cell, they differentiate to metabolically active reticulate bodies (RB) that multiply by binary fission (Fig. 1D) within vacuoles that are continuously growing and that develop into large intracytoplasmic inclusions (Fig. 1C). Reticulate bodies reorganize back to EBs at the end of the chlamydial developmental cycle (Fig. 1D). After 48 to 72 h, hundreds of EBs are released from the host cell to perpetuate the infectious cycle. Genomic transcriptional analysis of the chlamydial developmental cycle revealed

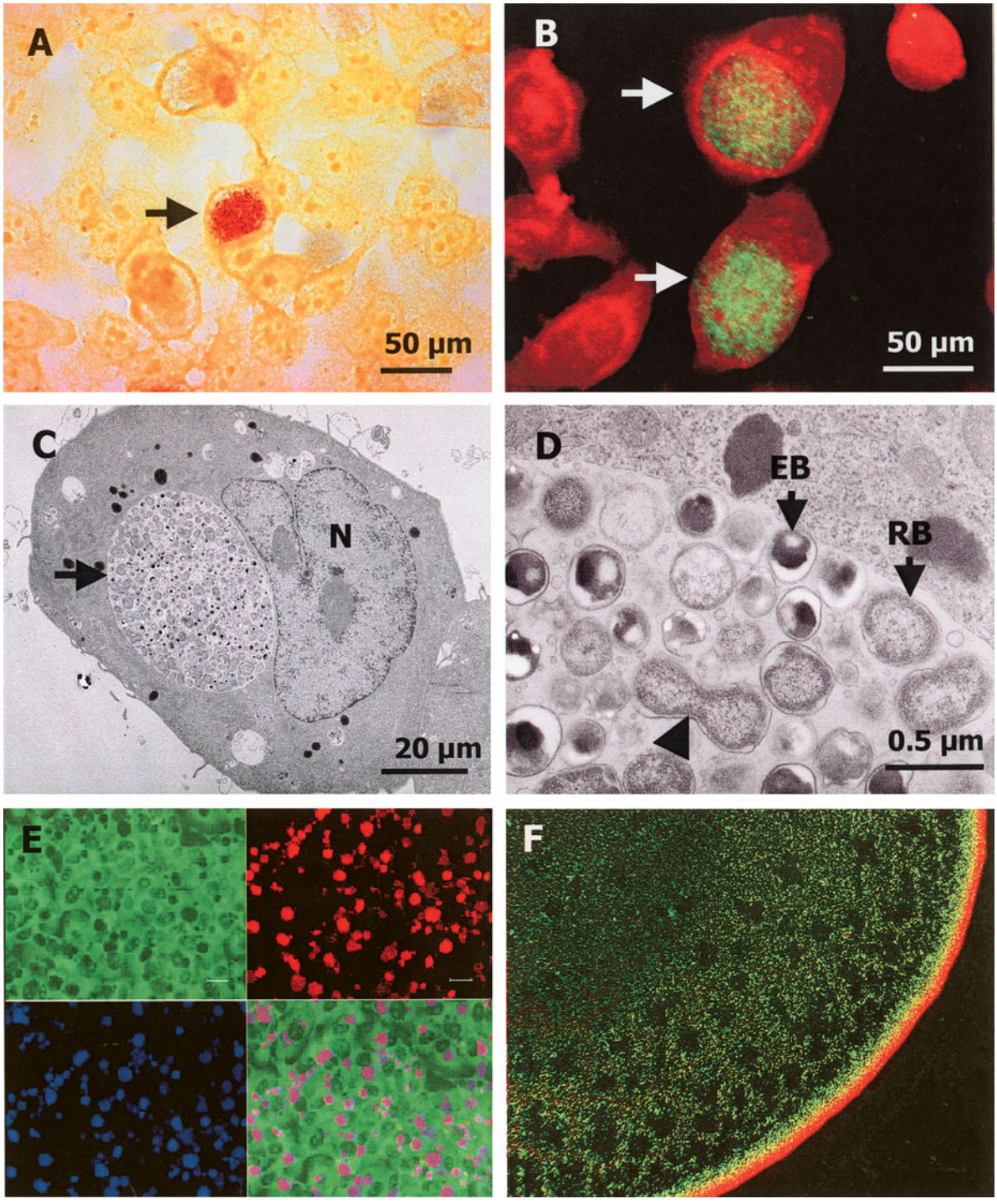


FIGURE 1 Identification of *C. trachomatis* by staining intracytoplasmic inclusions with iodine (A) and an FITC-conjugated monoclonal antibody directed at the MOMP (B). Transmission electron microscopy of a *C. pneumoniae*-infected cell shows an intracytoplasmic inclusion impressing the cell nucleus (C) and filled with EBs and RBs (D) at 60 h postinfection; the arrowhead shows a dividing RB. Identification of culture-grown *C. pneumoniae* by fluorescence in situ hybridization using rRNA targeted oligonucleotide probes (E). Simultaneous use of a Cy5-labeled probe that targets a chlamydial 16S rRNA sequence common to all members of the *Chlamydiaceae* (blue) and a Cy3-labeled probe specific for *C. pneumoniae* (red). Due to the overlap of colors, *C. pneumoniae* appears purple in the composite image; host cells are counterstained by a 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester (FLUOS)-labeled eukaryotic probe (green). IgG-MIF image (magnification, ×400) showing bright homogeneous fluorescence of *C. pneumoniae* EBs at a serum dilution of 1:512 (F). (Photographs courtesy of Sonja Maier, Sven Poppert, and Ulrike Simnacher, Department of Medical Microbiology and Hygiene, University of Ulm; and Matthias Horn, Division of Microbial Ecology, University of Vienna.)

a small subset of genes that control the differentiation stages of the cycle and have evolutionary origins in eukaryotic lineages (6).

Evidence is accumulating that factors including gamma interferon, antibiotics, and nutrient deprivation may drive chlamydiae into a state of persistence. Persistent chlamydial forms are morphologically characterized by aberrant enlarged RBs located within small intracellular inclusions that are arrested in a viable but noninfectious state (40). It was proposed that persistence is an alternative life cycle used by chlamydiae to avoid the host immune response (5). As a consequence, chronic infections have been attributed to chlamydial persistence (20). However, the clinical significance of chlamydial persistence is still a matter of debate because diagnostic tools to detect persistence in the human host are lacking.

The chlamydiae can elicit the induction of apoptosis under some circumstances and actively inhibit apoptosis under others (27, 29, 75, 116). This points to an important strategy that chlamydiae have evolved to promote their survival through the modulation of programmed cell death pathways in infected host cells.

Sequence information is now available for *C. trachomatis* (101), *C. pneumoniae* (51, 96), *C. caviae* (83), *C. abortus,* and a *Chlamydia*-like endosymbiont of *Acanthamoeba* (41). Genome analysis of this environmental chlamydial strain showed that about 700 million years ago the last common ancestor of pathogenic and symbiotic chlamydiae was already adapted to intracellular survival in early eukaryotes and contained many virulence factors found in modern pathogenic chlamydiae, including a type III secretion system (41). Comparison of the *C. pneumoniae* genome with the *C. trachomatis* genome has provided an understanding of the common biological processes required for infection and survival in mammalian cells. Prominent comparative findings include expansion of a novel family of 21 sequence-variant OMPs, conservation of a type-III secretion virulence system, three serine/threonine protein kinases and a pair of paralogous phospholipase D-like proteins, additional purine and biotin biosynthetic capability, a homologue for aromatic amino acid (tryptophan) hydroxylase, and the loss of tryptophan biosynthesis genes (51).

CLINICAL SIGNIFICANCE, EPIDEMIOLOGY, AND TRANSMISSION

C. trachomatis

Based on the antigenic reactivity of the MOMP, *C. trachomatis* is currently divided into 18 serovars. Serovars A, B, Ba, and C can be isolated from patients with clinical trachoma in areas of endemicity in poor countries in Africa, the Middle East, Asia, and South America. Acute manifestations of trachoma primarily include a follicular keratoconjunctivitis, while late-stage manifestations include tarsoconjunctival scarring with trichiasis, entropium, and subsequent loss of vision (97). According to estimates of the World Health Organization (WHO), approximately 1.3 million people in the world suffer from preventable blindness due to trachoma. Trachoma is transmitted under poor hygienic conditions between members of the same family or between families with shared facilities via discharges from the eyes of infected patients. Flies feeding from the mucopurulent eye discharges of infected and weakened humans may carry the organisms on their legs from one person to another across relatively long distances.

The *C. trachomatis* serovars D through K including the serovars Da and Ia and the genovariant Ja are associated with genital tract disease and are among the most common sexually transmitted bacterial organisms in industrialized countries. According to surveillance data of the Centers for Disease Control and Prevention (CDC), these organisms are responsible for approximately 1 million reported infections in 2004 in the United States and typically cause nongonococcal urethritis in men and cervicitis in women. Infection of the urethra and the lower genital tract may cause dysuria, whitish or clear urethral or mucopurulent vaginal discharge, and postcoital bleeding. Urethritis and the rarer manifestations proctitis and conjunctivitis are observed in both men and women. The bulk of infections are asymptomatic and therefore remain undetected (77). This may result in ascending infections such as epididymitis in men and endometritis, salpingitis, pelvic inflammatory disease, and perihepatitis (Fitz-Hugh-Curtis Syndrome) in women. Manifestations of upper genital infection in women are irregular uterine bleeding, pelvic discomfort, or abdominal pain. Salpingitis may lead to tubal scarring and therefore to severe reproductive complications such as tubal-factor infertility and ectopic pregnancy. Tubal-factor infertility attributable to *C. trachomatis* is the most frequent form of infection-induced infertility. *C. trachomatis*-infected pregnant women may transmit the organisms during delivery to the infants, who are therefore at risk to develop conjunctivitis and/or pneumonia. Sequelae of *C. trachomatis* infection in both men and women may involve HLA-B27-associated reactive arthritis, presenting most frequently as an acute asymmetric oligoarthritis with or without enthesiopathic and extramusculoskeletal symptoms (19, 121). The young age of sexually active people is strongly associated with infection. Additionally, sex workers, persons with a new sex partner, or persons who have had several sex partners are at increased risk of infection. Screening women who are at risk for *C. trachomatis* infection can prevent serious complications such as pelvic inflammatory disease (93). Consequently, screening programs have been established in some European countries and the United States to identify and treat infections of asymptomatic individuals and their partners.

The *C. trachomatis* serovars L1, L2, L2a, and L3 including the newly identified variant L2b cause lymphogranuloma venereum (LGV), a systemic sexually transmitted disease that is endemic in parts of Africa, Asia, South America, and the Caribbean but rare in industrialized countries. However, recent reports about outbreaks in Europe and the United States show that health care providers should be vigilant for LGV (73, 99), especially among men who have sex with men. The primary lesion, a small, painless papule that tends to ulcerate at the site of inoculation, often escapes attention. Proctitis is more common in people who practice receptive anal intercourse, and elevated white blood cell counts in anorectal smear specimens may predict LGV in these patients (110). Ulcer formation may favor transmission of human immunodeficiency virus and other sexually transmitted and bloodborne diseases. The cardinal feature of LGV is the presence of painful inguinal and/or femoral lymphadenopathy (82). Complications of LGV include development of coalescing fluctuant lymph nodes (buboes) that result in discharging sinuses and fistula formation. If untreated, fibrosis can lead to lymphatic obstruction causing elephantiasis of the genitalia.

C. pneumoniae

C. pneumoniae causes infections of the upper and lower respiratory tract such as sinusitis, pharyngitis, bronchitis, and pneumonia (54). *C. pneumoniae* was identified as the causative

agent in 10 to 15% of cases of community-acquired pneumonia in adults (57) as well as in children (81). However, data from studies yielding prevalence rates under 1% for *C. pneumoniae* pose the question whether its role in community-acquired pneumonia is overestimated (104). Severe and life-threatening *C. pneumoniae* infections have been described in patients with acute leukemia and treatment-induced neutropenia (38). Chronic infection with *C. pneumoniae* was reported among patients with chronic obstructive pulmonary disease and may also play a role in the natural history of asthma, including exacerbations. The clinical symptoms of *C. pneumoniae* infection are nonspecific and do not differ significantly from those caused by other atypical organisms such as viruses and *Mycoplasma pneumoniae*. Persistent cough seems not to be strongly associated with *C. pneumoniae* (115). Primary infection occurs mainly in school-age children, while reinfection has been observed in adults. Seroprevalence rates from 40 to 70% show that *C. pneumoniae* is a widely spread organism in industrialized as well as developing countries.

Atherosclerosis has been recognized as a chronic inflammatory disease of the artery vessel wall (86). The role of *C. pneumoniae* in the etiology of atherosclerosis has been discussed since 1988, when Saikku and coworkers presented serological evidence of an association of *C. pneumoniae* with coronary heart disease and acute myocardial infarction (88). In subsequent studies, the organisms were identified in atherosclerotic lesions of patients by culture, PCR, immunohistochemistry, and transmission electron microscopy; however, the discrepancies of study results (120) including those of animal studies and the failure of large-scale treatment studies (14, 36, 74) have raised skepticism about the organism's role in atherosclerosis (44). In addition, a heterogeneous spectrum of extrapulmonary diseases has been linked to *C. pneumoniae* including multiple sclerosis (100), Alzheimer's disease (3), and chronic fatigue syndrome (17); however, a causal relationship between these diseases and *C. pneumoniae* infection has not been substantiated.

C. psittaci

Psittacine birds and a wide range of other avian species may act as natural reservoirs for *C. psittaci*. In the recently proposed new *Chlamydophila psittaci* taxon (25), only the avian chlamydial strains previously designated *Chlamydia psittaci* are retained. Persons at risk for infection with *C. psittaci* include mainly those who have contact with pet birds and those who are employed in the poultry industry or in slaughterhouses. Infectious forms of the organisms are shed from symptomatic and from apparently healthy birds and may remain viable for several months. *C. psittaci* can be readily transmitted to humans following inhalation of aerosols from nasal discharges and from infectious fecal or feather dust. Symptomatic *C. psittaci* infection in humans may present as a severe chronic pneumonia (24), although mild illness and asymptomatic infections in persons exposed to infected birds have also been observed (70). Typical symptoms include fever, chills, muscular aches and pains, severe headache, hepato- and/or splenomegaly, and gastrointestinal symptoms. Cardiac complications may involve endocarditis and myocarditis. Fatal cases were common in the preantibiotic era. Due to quarantine of imported birds and improved veterinary-hygienic measures, outbreaks and sporadic cases of psittacosis are rarely observed nowadays.

C. abortus

Chlamydiae associated with ruminant abortion and formerly contained within the *Chlamydia psittaci* taxon were transferred to a new species: *Chlamydophila abortus* (114). *C. abortus* has been acknowledged as a cause of abortion and fetal loss in sheep and has also been broadly detected in calves (46). There are a number of reports of pregnant women who have had spontaneous abortions following exposure to animals infected with *C. abortus* (80, 117). The incidence of this animal-acquired infection is not known, but sheep and goats during the birthing season represent a potential risk to pregnant women. Obstetricians should consider this diagnosis along with early antibiotic treatment and cesarean section delivery in the context of the patient's case history.

Environmental Chlamydiae

The host range of chlamydiae was further broadened with the discovery of *Chlamydia*-related endosymbionts in free-living amoebae (1, 18, 30). The so-called environmental chlamydiae that have been placed in the family *Parachlamydiaceae* share the chlamydial developmental cycle and represent an evolutionary early-diverging sister of the pathogenic chlamydiae (41). Environmental chlamydiae were discussed as potential emerging pathogens; however, clinical evidence for their importance in human infection is still pending. *Simkania negevensis*, currently the only member of the *Simkaniaceae,* is a recently discovered *Chlamydia*-like intracellular agent which has been associated with respiratory infections in infants (49). The natural host of *Simkania* is not known; however, the organisms were successfully grown in various cell lines as well as in free-living amoebae and were identified in drinking water and in reclaimed wastewater (50).

COLLECTION, TRANSPORT, AND STORAGE

General Comments

Since chlamydiae are obligate intracellular pathogens, the objective of specimen collection should usually be to include the host cells that harbor the organisms (8). Outside their host, chlamydiae survive only briefly, and efforts must be undertaken to maintain the organisms' viability for successful culture. Commercial diagnostic nonculture assays do not require the presence of viable chlamydiae in the specimen; nevertheless, the instructions of the manufacturers given in the package insert should be followed for appropriate collection, transport, and storage of specimens. This includes the use of swabs and transport media specified by the manufacturer.

For successful culture of chlamydiae, the time between collection and processing of the specimens in the laboratory should be minimized while keeping specimens cold (4 to 8°C). Specimens should be forwarded to the laboratory within 24 h in a special chlamydial transport medium such as 2-sucrose phosphate or sucrose phosphate glutamate supplemented with fetal calf serum (5 to 10%), gentamicin (10 μg/ml), vancomycin (25 to 100 μg/ml), and amphotericin B (2 μg/ml) or nystatin (25 U/ml). Tetracyclines, macrolides, and penicillins cannot be used in the transport media since they have activity against chlamydiae. If specimens cannot be processed within 24 h, storage at −70°C in transport media is acceptable. Specimens for culture should not be stored at −20°C or in frost-free freezers. To test the adequacy of specimen transport, periodic transport of specimens with known numbers of inclusion-forming units should be conducted. Swab specimens should be collected on swabs with a Dacron tip and an aluminum or plastic shaft. Swab tips made of calcium alginate and swabs with wooden shafts may inhibit the growth of chlamydiae. It is recommended to check new lots of swabs that are used to collect specimens

for culture of chlamydiae for possible inhibition of chlamydial growth (21, 47).

C. trachomatis

The type and anatomical site of specimen collection for laboratory diagnosis of *C. trachomatis* infection depend on both the clinical picture and the laboratory test selection as comprehensively reviewed elsewhere (8, 47, 60, 97). Noninvasively collected specimens such as first-void urine (FVU; first 10 to 30 ml of urine) and vulvovaginal swab specimens are excellent for diagnosis of *C. trachomatis* genital tract infection by nucleic acid amplification techniques (NAATs). Sensitivity and specificity of NAATs for *C. trachomatis* on noninvasively collected specimens are similar to those obtained on samples collected directly from the cervix or urethra (90, 94, 111, 112). Patients and clinicians may prefer self-sampling to the standard collection methods (42, 85). FVU specimens should be obtained at least 2 h after the last micturition. Ambient-temperature storage of fresh unprocessed urine should not exceed 24 h to avoid denaturation of chlamydial DNA. Subsequent processing of the urine specimens for NAAT varies depending on the manufacturers' instructions. Both urine and (self-collected) vulvovaginal specimens are not recommended for testing by culture and nonamplification assays such as enzyme immunoassay (EIA), direct fluorescence assay (DFA), and nucleic acid hybridization (NAH) because of their relatively low sensitivity (47).

Traditional sites for specimen collection in *C. trachomatis* genital tract infection involve the endocervix in females and the urethra in males. Proficient specimen collection including vaginal speculum examination in females is required to obtain appropriate samples that contain sufficient columnar or squamocolumnar cells. Purulent discharges have to be cleaned before a swab is inserted 1 to 2 cm into the cervical os past the squamocolumnar junction, rotated more than two times, and removed without touching the vaginal mucosa (8). Urethral specimens from males are collected by placing a dry swab 3 to 4 cm into the urethra and rotating prior to removal. Urination prior to specimen collection may reduce test sensitivity by washing out infected columnar cells. *C. trachomatis* also infects the female urethra, and recovery rates may be improved by collecting a specimen from the urethra as well as from the cervix and sending both to the laboratory. In women with salpingitis, samples may be collected by needle aspiration of the involved fallopian tube. Endometrial specimens have also yielded chlamydiae. Further appropriate sites include the conjunctiva in chlamydial eye infection (trachoma, inclusion conjunctivitis, and newborn conjunctivitis) and the nasopharynx and deeper respiratory tract of infants in newborn pneumonia. For men who have sex with men, screening of rectal and pharyngeal specimens is recommended since early reports support the utilitity of commercial NAATs as a screening test for this population (53, 59). In cases of suspected LGV, ulcer swabs, aspirates of bubo fluid, and rectal or urethral swabs should be collected in transport medium. Buboes of LGV may contain only small amounts of thin milky fluid, and it may be necessary to inject 2 to 5 ml of sterile saline to obtain any fluid by aspiration (60).

C. pneumoniae

The optimal sites for specimen collection in *C. pneumoniae* infection are poorly defined. Respiratory specimens from which the organisms were cultured include sputum, bronchoalveolar lavage fluid, nasopharyngeal aspirates, throat washings, and throat swabs (tonsil area). Swab specimens should be collected using a Dacron tip and an aluminum or plastic shaft (21) and placed immediately in transport medium. Specimens need to be kept at 4 to 8°C in chlamydial transport medium, since the organisms are inactivated rapidly at room temperature. Rapid freezing or freezing and thawing of specimens should be avoided (54). Liquid specimens are collected in transport medium at a specimen-to-medium ratio of 1:2 (21). Testing of vascular tissue specimens and blood samples, except for research studies, is of questionable value.

C. psittaci

C. psittaci strains seem to be the most stable organisms among the pathogenic chlamydiae. Nevertheless, specimens should be collected in chlamydial transport medium. Appropriate specimens include sputum, bronchoalveolar lavage fluid, pleural fluid, blood, and tissue biopsy specimens from various anatomical sites.

DIRECT EXAMINATION

NAAT

C. trachomatis

Due to their high sensitivity and specificity, NAATs are the tests of choice for diagnosis of genital *C. trachomatis* infections in routine clinical laboratories. NAATs can be used to detect *C. trachomatis* without a pelvic examination or intraurethral swab specimen by testing self- or clinician-collected vaginal swabs or urine, respectively (16, 42, 90, 94, 111, 112). This facilitates the establishment of screening programs in asymptomatic individuals and may enhance the compliance for testing asymptomatic contact persons of infected individuals. Increasing experience is available for the use of NAATs in conjunctival, oropharyngeal, and rectal samples (53, 59) and in LGV (73, 105). For research studies of trachoma patients, NAATs have been recommended as the "gold standard"; however, the commercial assays presently available are too expensive and too complex for use in national trachoma programs (97). In many evaluations, NAATs detected 20 to 30% more positive specimens than could be detected by earlier technologies. However, NAATs are not a perfect gold standard. An expansion of the gold standard has evolved as multiple specimens from each patient have been tested by more than one FDA-cleared NAAT, enabling the gold standard to be redefined for identification of an infected patient. An infected-patient gold standard for evaluation of new diagnostic tests for *C. trachomatis* infection has been recently proposed consisting of (i) two NAATs different from the test under evaluation; (ii) three samples from each patient including cervical swabs, male urethral swabs, and urine from both men and women; and (iii) two positive results among the three samples (65).

Licensed NAATs for detection of *C. trachomatis* include (in the order of their introduction) the PCR-based Roche Amplicor (Roche Diagnostics, Basel, Switzerland), the APTIMA transcription-mediated amplification (Gen-Probe, Inc., San Diego, Calif.) and the BD ProbeTec strand displacement amplification (SDA) (Becton Dickinson and Company, Diagnostic Systems, Franklin Lakes, N.J.). The formerly frequently used Abbott LCx ligase chain reaction was withdrawn from the commercial market by the manufacturer in 2003. Both the PCR and SDA assay amplify nucleotide sequences of the 7.5-kbp cryptic plasmid of *C. trachomatis,*

which is present in an average copy number of about four plasmids per chromosome in EBs and up to seven plasmids per chromosome in replicating RBs (78). *C. trachomatis* strains that do not harbor the cryptic plasmid have been sporadically isolated from urethral specimens (28, 103). The transcription-mediated amplification-based assays target specific sequences of the 23S rRNA, which is also present in multiple copies. Each of the three commercially available NAAT systems offers the option for combination testing of *C. trachomatis* and *Neisseria gonorrhoeae* in the same specimen.

Considering the multiplicity of target sites for the amplification procedures being used, NAATs should be able to produce a positive signal from less than one EB; however, the actual sensitivity in clinical specimens is lower because of sampling variability and inhibition of amplification. Since inhibitor problems of NAATs can be reduced by dilution of specimens, heating, freeze-thaw cycles, or overnight storage at 4°C, the use of internal inhibitor controls of the amplification assays (as supplied by the manufacturers of PCR and SDA) is helpful for identification of clinical specimens containing inhibitory factors (62). All these assays are highly specific if problems with cross-contamination, labeling errors, and mistakes in specimen collection can be avoided. Confirmatory testing of positive specimens is recommended if a low positive predictive value can be expected (<90%) or if a false-positive result would have serious psychosocial or legal consequences (47).

Clinical evaluations of the NAATs have shown that they are more sensitive than culture and other nonculture methods including microscopy, antigen detection, and nucleic acid hybridization assays. As a consequence, less sensitive diagnostic tests cannot be used to confirm positive results of the more sensitive NAAT assays (47). Even among NAATs, the assays employed for confirmatory testing should have equivalent sensitivities (89). Concerns regarding consistency of test performance and reproducibility are a matter of ongoing debate. Mishandling of specimens can lead to incorrect results. Therefore, laboratory implementation of NAATs requires close attention to quality control measures such as manufacturer- or expert-based training of laboratory staff, participation in proficiency testing programs, and standard operating procedures based on the manufacturers' instructions in the package inserts (47).

In settings where resources are limited, including developing countries, the concept of pooling to detect *C. trachomatis* by NAATs has proved to be a simple, accurate, and cost-effective procedure compared to individual testing (58). Specimen pools may consist of aliquots from five processed specimens (FVU or genital swab) combined into one amplification tube. Subsequent testing of individual samples is required only if the pooled sample gives a positive result. Following this strategy, considerable savings of reagent costs can be obtained, especially in low-prevalence populations.

C. pneumoniae

A vast number of PCR-based protocols using different formats and target genes have been developed in research laboratories for detection of *C. pneumoniae* in both respiratory and nonrespiratory samples. However, the lack of a reliable gold standard for *C. pneumoniae* infection has made it difficult to evaluate the published protocols thoroughly. Broad application of NAATs for diagnosis of *C. pneumoniae* infection has been hampered because many PCR protocols are not reliable or robust enough to provide reproducible results in routine clinical laboratories. Even in specialized laboratories, there seems to be a substantial interlaboratory variation in the performance of *C. pneumoniae* NAATs, and the need for standardization of these assays has been recognized (21). Subsequently, specific recommendations for standardizing *C. pneumoniae* PCR assays were made, and it was suggested to compare the performance of newly developed PCR protocols with at least one of four recommended assays that target the *PSTI* fragment (13), the *ompA* gene (109), or the 16S rRNA gene (32, 61). However, all of these assays must be considered research tools (21), because commercial FDA-cleared assays are currently not available. Real-time PCR technology provides promising results that warrant further evaluation of this approach for detection of *C. pneumoniae* infection (2, 55, 84, 108).

C. psittaci

NAATs could be helpful for detection of avian *C. psittaci* strains from clinical samples since culture of these organisms is dangerous and requires biosafety level 3 (BSL-3) facilities. Some PCR-based assays have been developed for diagnosis of human ornithosis (24, 61, 67, 109). Due to the rarity of the disease, the performance characteristics of these assays have been only poorly evaluated in clinical specimens.

NAH

Two NAH tests are commercially available for detection of *C. trachomatis*. The Gen-Probe PACE 2 test (Gen-Probe Inc.) hybridizes to a species-specific sequence of chlamydial 16S rRNA that is present in a high copy number in replicating chlamydiae. Available data suggest that it is about as sensitive as the better antigen detection and cell culture methods and is relatively specific. However, it was shown that commercial NAATs improved the detection of infections in women by 17 to 38% compared to PACE 2 (9). The second NAH test, the Digene Hybrid Capture II, is a nucleic acid probe-signal amplification assay (Digene Corp., Gaithersburg, Md.) that uses RNA hybridization probes for DNA sequences encoding both genomic and cryptic plasmid sequences of *C. trachomatis*. This assay was shown to reach the sensitivity of a commercial NAAT when cervical specimens were investigated (113). NAHs are considered highly robust test methods for detection of *C. trachomatis*. NAH tests have been recommended for endocervical swabs or urethral swabs from men when a NAAT is not available or not economical. As is the case with other non-NAATs, NAH tests have not been recommended for use in noninvasive-collection specimens such as urine and vulvovaginal swabs (47). Both NAH systems also offer a test format that enables detection of *C. trachomatis* and *N. gonorrhoeae* in a single specimen.

DFA

The presence of typical intracytoplasmic inclusions in epithelial cells of the conjunctiva, urethra, or cervix of infected patients can be demonstrated when air-dried smears are fixed on a slide with absolute methanol and stained with Giemsa. Cytological testing was particularly useful in diagnosing acute inclusion conjunctivitis of the newborn, but the more sensitive immunofluorescence procedures have largely replaced this method. DFAs use fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies that are directed at a *C. trachomatis*-specific epitope of the MOMP (*Chlamydia CEL;* Cellabs, Brookvale, Australia; Pathfinder; Bio-Rad Laboratories, Redmond, Wash.). DFAs are based on detecting EBs in smears, although staining of inclusions can also succeed if intact

infected host cells are collected. Checking for the presence of columnar cells allows assessment of the adequacy of the sample. The procedure offers rapid diagnosis, taking only 30 min to perform, making DFA tests useful especially for laboratories that test only a limited number of specimens. However, this method requires an experienced microscopist who can distinguish between fluorescing chlamydial particles and nonspecific fluorescence. The DFA test has approximately 75 to 85% sensitivity and 98 to 99% specificity compared with culture and a lower sensitivity than NAATs (11, 72). DFA tests may be another alternative for testing endocervical swabs from females or urethral swabs from males when a NAAT is not available or not economical. In addition, DFAs have been recommended for use with conjunctival specimens and for testing of individuals with possible rectal and pharyngeal exposure to *C. trachomatis*, if a *C. trachomatis* MOMP-specific stain is used (47). Nontrachomatis chlamydial conjunctivitis should be considered if DFA testing reveals the presence of chlamydial LPS but not *C. trachomatis*-specific MOMP.

EIA

EIA-based tests for detection of *C. trachomatis* use either monoclonal or polyclonal antibodies to detect chlamydial LPS, which is more soluble than MOMP. Although they can theoretically detect all chlamydiae, EIAs have not been well evaluated for the diagnosis of infections with *C. pneumoniae* or *C. psittaci*. The performance characteristics of EIAs for laboratory diagnosis of *C. trachomatis* have been reviewed comprehensively elsewhere (8). Using cultures as reference standards, the sensitivities of EIAs applied to endocervical swabs were in a range from 62 to 72% (72). Although in single studies specificities of >99% have been reported, EIA methods that detect chlamydial LPS harbor a risk for false-positive results caused by cross-reaction with LPS of other microorganisms. Therefore, a positive EIA screening test should be verified, especially in low-prevalence populations (47). To overcome logistical and economic challenges associated with confirmatory testing, a blocking antibody format has been introduced, increasing the test specifity of some EIAs to about 99.5%. EIAs are not recommended for testing of noninvasively collected specimens such as urine and vulvovaginal swabs. EIAs are preferred in laboratories where cost is a major factor and large numbers of specimens require bulk processing, but results should note the low sensitivity of this method compared to that of NAATs.

Rapid or point-of-care tests designed for office- or clinic-based settings have been developed that provide test results in less than 30 min for *C. trachomatis* infection in women. Similar to EIAs, they also use antibodies against chlamydial LPS with the potential to yield false-positive results due to cross-reaction with other gram-negative bacteria. Point-of-care tests have not been recommended in laboratory settings because sensitivity and specificity are lower, quality controls are less rigorous, and costs are higher than for tests designed for laboratory use (47).

ISOLATION PROCEDURES

Biosafety Considerations

C. pneumoniae and *C. trachomatis* are BSL-2 organisms, whereas *C. psittaci* is a BSL-3 organism. Transmission of the organisms from patient specimens or infected cell cultures may occur through aerosols, splashes onto the mucous membranes of the eyes, and hand-to-face actions. In recent years, fewer laboratory-acquired infections have been reported probably due to the common usage of class II biosafety cabinets in laboratories that work with *Chlamydia*-infected cell cultures. Use of a class II biosafety cabinet protects laboratory staff from exposure to aerosols as well as specimens and cell cultures from contamination. Further important means for preventing laboratory-acquired infection include the use of gloves, alcohol-based hand disinfectants, safety centrifuge caps, and face protection, if appropriate. Laboratory infections with *C. trachomatis* usually manifest as follicular conjunctivitis. The LGV strains are more invasive, and severe cases of laboratory-associated pneumonia and lymphadenitis are reported. *C. psittaci* must be considered a potentially dangerous organism, requiring appropriate BSL-3 facilities. Laboratory-acquired *C. pneumoniae* infections might be underestimated since the mild clinical course may not prompt infected laboratory workers to seek medical attention.

Specimen Processing

Ocular and Genital Tract Specimens

For culture of chlamydiae from ocular and genital tract sites, only swabs that are rapidly forwarded to the laboratory in a special chlamydial transport medium are acceptable (see above). Specimens to be assayed by commercial EIA, DFA, NAH, or NAAT should be processed as directed by the manufacturer.

Bubo Pus

To prepare bubo pus, the aspirate fluid of fluctuant lymph nodes is ground and then suspended in nutrient broth or cell culture medium to at least 20% by weight. Even when the pus is not viscous, dilution is advisable. The material should be tested for bacterial contaminants and inoculated onto monolayer cultures of McCoy or HeLa 229 cells.

Blood

Blood samples may be helpful for diagnosis of *C. psittaci* endocarditis (95). The blood clot should be ground, and cell culture medium should be added to make a 10% solution. The suspension is inoculated directly into cell culture by using serial dilutions (from 1:2 to 1:10), since the concentrated material may be toxic to the cells. Collection in EDTA-treated tubes may be appropriate if fractionated blood is to be investigated. Processing of peripheral blood mononuclear cells (PBMCs) for detection of *C. pneumoniae* in cardiovascular patients should be confined to clinical trials. Protocols for isolation and processing of PBMCs have been published elsewhere (10).

Sputum, Throat Washings, and Other Secretions from the Respiratory Tract

Sputum and other respiratory samples are suspended in antibiotic-containing transport medium or cell culture medium at a ratio of specimen to medium of 1:2 to 1:10 depending on specimen consistency. Specimens are homogenized by adding sterile glass beads to the sample and vigorous vortexing for 1 to 2 min in a tightly stoppered container. Extracts should be centrifuged for 20 to 30 min at $100 \times g$ to remove coarse material before the supernatant fluid is inoculated onto cell monolayers. Serial dilutions may be required if the inoculum is toxic to cells.

Fecal Samples

Human rectal swabs for *C. trachomatis* and avian material for *C. psittaci* are suspended in chlamydial transport medium or

antibiotic-containing cell culture medium. The suspension is shaken thoroughly and centrifuged at 300 × *g* for 10 min, and the supernatant is removed. It may be further diluted (1:2 and 1:20) with medium before being inoculated into cell culture. Rectal swabs for commercial NAAT are processed in accordance with the corresponding protocol of the manufacturer.

Tissue Samples

Frozen tissue is thawed in a refrigerator at 4°C. The specimen is weighed, minced with sterile scissors or a scalpel, and ground with a mortar and pestle or homogenizer. A volume of cell culture medium required to make a 10 to 20% suspension is added, and the suspension is thoroughly mixed. For tissue specimens, serial dilutions (1:10 to 1:100) are often required for inoculation to prevent toxicity.

Isolation

Cell culture was considered the gold standard for diagnosis of genital *C. trachomatis* infection because its sensitivity and specificity were thought to be close to 100%. Problems associated with cell culture isolation of chlamydiae including technical complexity and long turnaround time and stringent requirements related to collection, transport, and storage of specimens have driven the development of commercially available noncultural methods that have found widespread application in many routine laboratories. With the advent of antigen detection methods, it became clear that the sensitivity of culture was substantially lower than previously thought, most probably due to the presence of nonviable chlamydiae that died during transport and processing. Culture for detection of chlamydiae in clinical specimens is generally now performed only in specialized laboratories (8). Culture is strongly recommended in treatment failures (when a viable isolate is needed for susceptibility testing) and in cases related to possible sexual assault for medicolegal reasons (47).

Historically, chlamydiae were cultivated in the yolk sac of embryonated eggs. The yolk sac method (for details, see reference 91) is still used for preparing antigens for the microimmunofluorescence test (MIF). For isolation of chlamydiae from clinical specimens, appropriately collected and transported samples are inoculated onto preformed cell monolayers. A number of susceptible permanent cell lines, including McCoy, HeLa 229, HEp-2, HL, BGMK, Vero, and L cells, have been used. Clinical samples are centrifuged onto monolayers to enhance infection. Strains of *C. psittaci* and LGV biovars are capable of serial growth in cell culture without centrifugation. Cultures are incubated for 48 to 72 h in the presence of the host cell protein synthesis inhibitor cycloheximide. McCoy and HeLa 229 cells are most commonly used for *C. trachomatis*. HL and HEp-2 cells seem to be more sensitive for recovery of the fastidious *C. pneumoniae* from clinical specimens. Visualization of cell culture-grown chlamydiae is achieved by immunostaining of inoculated cell monolayers for intracytoplasmic inclusions. A positive culture shows one or more typical intracellular inclusions (Fig. 1B).

Cell culture methods may vary among laboratories. Host cells are plated either onto 12-mm glass coverslips contained in 15-mm-diameter (1 dram [1 dram = 3.697 ml]) disposable glass vials (shell vial method) or in 6-, 12-, or 24-well tissue culture plates. The cells are seeded in concentrations of 1×10^5 to 2×10^5 cells/ml to give a healthy and confluent monolayer after 24 to 48 h of incubation. For optimal results, cell monolayers should be inoculated with patient specimens within 24 h after reaching confluency. Clinical specimens are thoroughly vortexed with glass beads in tightly closed screw-cap vials to facilitate release of chlamydiae before inoculation. The cell culture medium of the cell monolayers to be inoculated is discarded and replaced by a volume of 0.2 to 2 ml of the vortexed specimen. The inoculated specimen is centrifuged onto the cell monolayers at 900 to 3,000 × *g* for 1 h at 22 to 35°C. Cells are incubated at 35°C for 1 to 2 h to allow uptake of chlamydiae before the medium is replaced with chlamydial isolation medium consisting of the cell culture medium supplemented with fetal calf serum (10%), L-glutamine (2 mM), cycloheximide (1 to 2 μg/ml), gentamicin (10 μg/ml), vancomycin (25 μg/ml), and amphotericin B (2 μg/ml). Cultures are incubated at 35°C in 5% CO_2 for 48 to 72 h. Then, one coverslip per specimen is removed for immunostaining of inoculated monolayers. Both cell detritus and toxic effects of the inoculum may make it difficult to read slides. Dilution of cell-rich material (bubo pus, sputum, tissue samples, and rectal swabs) and blind performance of subpassages can be helpful for microscopic interpretation of slides.

If a blind subpassage or passage of positive material is to be performed, the corresponding cell monolayers of duplicate wells are scraped and disrupted by vortexing with glass beads. Cell debris of harvested material is removed by low-speed centrifugation (300 × *g*) for 10 min, and the supernatant is passed onto preformed cell monolayers as described above. For *C. pneumoniae,* most laboratories agree that at least two passages are needed to maximize the recovery of the organisms from respiratory specimens. Modifications of the standard procedure including use of serum-free culture medium, pretreatment of cell monolayers with polyethylene glycol or diethylaminoethyl-dextran, and extension of culture times have not been sufficiently tested to warrant their routine recommendation (21). Laboratories processing large numbers of specimens may use flat-bottom 48- or 96-well microtiter plates onto which cells are plated directly. Processing and incubation are as described above, but microscopy is modified because cells are stained directly in the well, requiring use of inverted microscopes and long working objectives.

Continuous quality control is important for maintaining a sensitive and specific culture system. Because of its technical complexity, there are multiple opportunities to modify factors in the culture system that may impact the isolation efficiency (97). Therefore, positive controls with a known number of inclusion-forming units should be run routinely to check the sensitivity of the culture system. Negative controls with uninfected human cells may help to evaluate episodes of cross-contamination as a result of handling positive patient specimens or positive controls. Routine testing of cell culture systems for *Mycoplasma* contamination has been recommended because *Mycoplasma* contamination may impair the growth of chlamydiae and may decrease the sensitivity of the culture system (21). In addition, *Mycoplasma* testing should be done with all chlamydial strains handled in the laboratory, since contamination of chlamydial isolates including strains provided by ATCC has been reported (43).

IDENTIFICATION

The basic procedure for detection of isolated chlamydiae involves demonstration of intracytoplasmic inclusions by fluorescent-antibody staining that provides both morphological and immunological identification of chlamydiae. Screening of cultures can be performed with a commercially available FITC-conjugated monoclonal anti-LPS antibody (Pathfinder

Bio-Rad), which recognizes all chlamydiae known to cause infections in humans. Confirmation of positive genital cultures can be done by the use of a *C. trachomatis* MOMP-specific monoclonal antibody (Fig. 1B). For respiratory cultures, a *C. pneumoniae*-specific monoclonal antibody may additionally be appropriate (69). Monoclonal antibodies specific for *C. psittaci* are not commercially available. Using DFA procedures, inclusions of *C. trachomatis*-infected cells are visible at 24 h postinfection. Less expensive but also less sensitive methods that were commonly used before the advent of monoclonal antibodies include Giemsa staining (which needs an experienced and well-trained microscopist for interpretation) and iodine staining for identification of glycogen-containing inclusions that are produced by *C. trachomatis* but not by *C. psittaci* or *C. pneumoniae* (Fig. 1A).

Identification of replicating chlamydiae can also be done by fluorescence in situ hybridization using fluorescently labeled oligonucleotide probes complementary to order-, genus-, and species-specific target sites on the chlamydial 16S rRNA (79). The risk of false-positive signals caused by nonspecific binding of the fluorescent dyes to nontarget organisms or structures of the host cells can be minimized by the simultaneous application of multiple probes with hierarchical specificity labeled with different dyes leading to a characteristic hybridization pattern (Fig. 1E).

TYPING SYSTEMS

Serotyping and genotyping procedures are important tools of epidemiological studies. They are of clinical use if medicolegal issues are involved or if lymphogranuloma venereum is suspected. The most convenient method for serotyping of *C. trachomatis* isolates appears to be the microwell typing system (107), in which inclusions in microtiter plates are stained with pools of monoclonal antibodies (available at Washington Research Foundation, Seattle, Wash.) that recognize serovar- and subspecies-specific epitopes of the MOMP. Genotyping of *C. trachomatis* isolates usually involves either restriction fragment length polymorphism analysis of the MOMP-encoding *ompA* gene or sequence analysis of the variable domains in the *ompA* gene. These variable regions include the peptides responsible for species, serovar, and serogroup specificities. PCR amplification of *ompA* using extracted DNA from patient specimens such as urine or genital samples allows direct genotyping from *C. trachomatis*-positive individuals without isolation of the organisms (4, 48). *ompA* sequencing is currently the only tool to identify all known and additional new genotypes of avian *C. psittaci* strains (34). Different serotypes or genotypes of *C. pneumoniae* have not been described.

SEROLOGICAL TESTS

Serological testing may be helpful in the diagnosis of human ornithosis, lymphogranuloma venereum, neonatal pneumonia caused by *C. trachomatis,* and respiratory *C. pneumoniae* infections. Serological testing for diagnosis of uncomplicated genital infections of the urethra and the lower genital tract as well as for *C. trachomatis* screening in asymptomatic individuals is not recommended (47). Since a reference standard has not been defined, the diagnostic value of some serological assays for detection of chronic or persistent chlamydial infections is difficult to estimate. General problems of chlamydial serodiagnosis arise from the difficulty in obtaining paired serum samples, the high seroprevalence of *C. pneumoniae* in adult populations, and the lack of standardized species-specific test methods. The most commonly used serological assay formats include the complement fixation (CF) test, the MIF test, and the EIA to detect immunoglobulin M (IgM), IgA, IgG, or total classes of antibodies with either family, species, or serotype specificity. Some of these assays have been commercialized and are being used by clinical laboratories, although their performance characteristics have been evaluated only in a limited number of studies. The surface-associated chlamydial macromolecules including the MOMP, OmcB, and LPS may induce a strong antibody response in infected individuals. However, these molecules share common epitopes among the different chlamydial species that make them prone to cross-reactive antibody responses. In an effort to identify species-specific immunoreactive antigens, protein profiles have been described, but interpretation is difficult and Western blot methods using whole chlamydial elementary bodies as antigens are not ready for routine use in clinical laboratories (23).

CF Test

The CF test is based on antibody reactivity to the chlamydial LPS antigen common to all members of the *Chlamydiaceae*. The CF test may be useful in diagnosing LGV in patients who present compatible clinical symptoms. A titer of $\geq$256 strongly supports the clinical diagnosis, while a titer of $<$32 rules it out except in the very early stages of the disease (60). In addition, the CF test is useful for diagnosis of psittacosis; however, in the absence of a typical patient history (exposure to birds), *C. pneumoniae* infection should be considered in patients with positive test results. Due to its potential for cross-reactivity and its low sensitivity for reinfection, CF is not recommended for serodiagnosis of *C. pneumoniae* infections (21). The CF test also lacks sensitivity for the diagnosis of trachoma, inclusion conjunctivitis, and uncomplicated genital infections caused by *C. trachomatis*.

MIF Test

The MIF test developed by Wang and Grayston in the early 1970s is still considered the method of choice for serodiagnosis of chlamydial infections. With this procedure, species- and serovar-specific antibody responses in human chlamydial infection can be detected. The MIF test allows quantitative detection of IgM and IgG antibodies that may be helpful in distinguishing recent from past infections.

The MIF is the diagnostic test of choice for *C. trachomatis* pneumonitis in infants because elevated levels of IgM antibodies are regularly associated with disease (8). A single IgM titer of $\geq$32 may support the diagnosis of neonatal pneumonia caused by *C. trachomatis*. IgG antibodies are less useful because infants may present with typical symptoms when they still have a high level of maternal IgG. In LGV-infected individuals, a MIF IgG titer of $\geq$128 strongly supports the clinical diagnosis, although invasive genital infection with *C. trachomatis* serovars D through K, such as pelvic inflammatory disease, salpingitis, or epididymitis, can also give rise to high serum titers of antichlamydial antibody (60). The MIF test may be useful in diagnosis of psittacosis and is the serological testing method of choice for diagnosis of acute *C. pneumoniae* infection. Criteria for acute infection of *C. pneumoniae* generally include paired sera demonstrating at least a fourfold rise in titer and single serum samples with IgM titers of $\geq$16 and/or IgG titers of $\geq$512. However, single IgG titers of $\geq$512 should be interpreted with caution because elevated IgG titers may persist for several years in the absence of clinically apparent disease (21). IgG titers in the range of 1:16 to 1:256 are suggestive of past infection.

The usefulness of IgA as a diagnostic marker in acute or chronic *C. pneumoniae* infections has not been substantiated.

The MIF assay is performed using purified formalinized EBs of representative strains or serovars of *C. trachomatis, C. psittaci,* and *C. pneumoniae* that are dotted in a specific pattern onto glass slides. MIF antigens are commercially available from the Washington Research Foundation. Serial dilutions of patient sera are placed over the fixed antigen dots and incubated, and bound antibody is detected with fluorescein-conjugated anti-IgG or anti-IgM antibody (Fig. 1F). A more detailed description of the MIF procedure has been summarized elsewhere (118). In addition, recommendations for standardizing the MIF assay in terms of antigen preparation, testing, interpretation of results, and quality assurance should be followed (21).

The MIF assay format is technically demanding, time-consuming, and less useful for higher volume testing. In addition, subjective reading of titers may contribute to intra- and interlaboratory variation in MIF assay results (76). For these reasons, well-trained and experienced laboratory staff are needed. A few standardized kits based on the MIF format have been developed and marketed (Focus Diagnostics, Cypress, Calif.; Labsystems OY, Helsinki, Finland; Savyon Diagnostics Ltd., Ashdod, Israel). Initial studies suggest that their performance characteristics are similar and seem to correspond well to those of the classical MIF method (7, 66). However, at the time of writing, none of these assays are cleared by the FDA for use in the United States for the diagnosis of *C. pneumoniae* or *C. trachomatis* infections.

EIA

To overcome the problems associated with MIF testing, EIAs have been developed that offer a more automated workflow and objective end points for serodiagnosis of chlamydial infections. EIAs based on synthetic peptides from the variable domain 4 (VD4) of the *C. trachomatis* MOMP have been marketed for detection of *C. trachomatis*-specific IgG and IgA antibodies (CT-EIA [Labsystems OY]; SeroCT [Savyon Diagnostics Ltd.]; CT pELISA [Medac, Wedel, Germany]). These assays performed as well as the MIF assay in a few studies (71); however, little is known regarding how long specific antibodies may persist in individuals with resolved infections. For these reasons they cannot reliably differentiate current and past infections. This makes them of little use in *C. trachomatis* infections of the lower genital tract where adequate specimens for direct detection of the organisms can be noninvasively obtained. Further studies are needed to clarify if *C. trachomatis* species-specific antibody tests are convenient tools for diagnosis of upper genital tract infections.

The major antigenic determinants of *C. pneumoniae* that are broadly immunodominant among infected individuals are elusive. Commercial assays designed for specific diagnosis of *C. pneumoniae* infection are based on either whole elementary bodies (Savyon Diagnostics Ltd.) or (to obtain more specificity) on LPS-extracted EB preparations (Labsystems OY and Medac). Most kits have been compared only to MIF (39), but none was evaluated adequately with sera from culture- or PCR-positive patients. Thus, their diagnostic value for acute *C. pneumoniae* infections remains to be determined (21).

ANTIMICROBIAL SUSCEPTIBILITIES AND TREATMENT

Evaluation of antimicrobial resistance and potential clinical treatment failure in chlamydial infection is hampered by the lack of standardized antimicrobial susceptibility tests and the fact that in vitro resistance does not correlate with the patient's clinical outcome (106). For these reasons, antimicrobial susceptibility testing of *Chlamydia* organisms has little clinical utility and is currently performed only in some research laboratories. Antimicrobial susceptibility testing in chlamydiae requires growing the organisms in epithelial cells cultured in medium containing increasing concentrations of antibiotics. Cells are stained with an FITC-labeled anti-chlamydial antibody, and the lowest concentration of antibiotic that inhibits inclusion formation after 48 h of incubation is reported as the MIC (106, 119). The minimum chlamydicidal concentration has been defined as the lowest concentration of antibiotic producing no viable bacterial progeny as determined after passage from antimicrobial-containing medium to antimicrobial-free medium. However, variation of antimicrobial susceptibility results is common because they depend on factors including the cell type used, the inoculum size, and the time between infection and the addition of an antimicrobial.

Tetracyclines, macrolides, fluoroquinolones, and rifampin are commonly used for antibiotic treatment of chlamydial infections. A single dose of azithromycin has been shown to be as effective for the treatment of uncomplicated genital *C. trachomatis* infections in adults as a standard 7-day course of doxycycline (56, 64). Alternative regimens include a 7-day course of erythromycin, ofloxacin, or levofloxacin (15). Cotreatment or testing for chlamydiae should be considered among gonorrhea-infected patients because of the frequency of coinfection. Systemic treatment with erythromycin has been recommended for ophthalmia neonatorum as well as for infant pneumonia caused by *C. trachomatis*. In the treatment of adult inclusion conjunctivitis, a single 1-g azithromycin dose was as effective as a standard 10-day treatment with doxycycline (52). Recommended treatment regimens for both bubonic and anogenital LGV include tetracycline, doxycycline, or erythromycin for 14 days (60). Doxycyline, azithromycin, erythromycin, levofloxacin, and newer macrolides such as clarithromycin and roxithromycin have been recommended for treatment of *C. pneumoniae* infection; however, evidence from clinical trials supporting their use is limited.

Chlamydial resistance to recommended antimicrobial agents appears to be rare and confined to only a few clinical isolates of *C. trachomatis* and has not yet been reported for *C. pneumoniae* or *C. psittaci* infections. Nevertheless, concern has been raised about resistance because recurrent or persistent chlamydial infections were observed in women adequately treated for *C. trachomatis* infection and in a few cases of *C. pneumoniae* infections (37). Treatment failure in genital tract infection has been associated with isolation of *C. trachomatis* variants resistant to tetracyclines, macrolides, or fluoroquinolones (98). Mutations in the 23S rRNA gene have been linked to resistance of clinical *C. trachomatis* isolates to macrolides (68). Resistant clinical isolates demonstrate a heterotypic pattern, in which a small proportion of organisms survive antimicrobial concentrations well above the MIC (106), and most isolates lose their resistance properties after prolonged culture in antibiotic-free medium. However, heterotypic resistance was also identified in *C. trachomatis* strains isolated from patients with single-incident infections (106), suggesting that the results of susceptibility testing may not predict the microbiological efficacy in vivo.

In vitro, chlamydial resistance to fluoroquinolones, macrolides, tetracyclines, and rifampin can be induced with large numbers of organisms cultured in the presence of antimicrobials. In an animal model, persistence of *C. pneumoniae*

after antimicrobial therapy has been demonstrated (63). The emergence of *Chlamydia suis* strains isolated from livestock and displaying a chromosomally stable *tet*(C) resistance gene raises the issue of antibiotic use in animal feeds (22).

INTERPRETATION AND REPORTING OF RESULTS

The laboratory diagnosis of chlamydial infections is still challenging; however, commercial NAATs enable the reliable detection of genital *C. trachomatis* infection even from noninvasively obtained specimens. Reporting of test results for chlamydiae should include the type of test used and a clinical interpretation if possible. Apart from presumptive clinical treatment failure, test of cure is recommended for pregnant women because antibiotic treatment regimens for *C. trachomatis* infections during pregnancy may not be highly efficacious. Due to the presence of nonviable bacteria, nonculture tests for *C. trachomatis,* especially NAATs, may remain positive when performed <3 weeks after completion of therapy. In cases of treatment failure and sexual assault, isolation should be attempted and specimens should be forwarded to a specialized laboratory. Sexual partners of infected patients should be notified, examined, and treated for *C. trachomatis*. Patients and their partners should be instructed to abstain from sexual intercourse until therapy is completed.

Interpretation of serological results is particularly challenging in chlamydial infections. A reliable serologic marker for chronic or persistent chlamydial infection is not available. Especially in *C. pneumoniae,* there is poor agreement between the presence of chlamydial antibody and direct markers of current infection, such as culture or PCR (33). Single-point serology for diagnosis of *C. pneumoniae* infection is discouraged, except when specific IgM antibodies are positive. Paired sera should be tested in the same assay on the same day, and seroconversion or a fourfold rise or fall in titer is diagnostic for a recent infection. Obviously, there is a general lack of reliable and standardized assays for laboratory diagnosis of *C. pneumoniae* and this basically hampers the current understanding of the organism's true prevalence and role in respiratory infections as well as in extrapulmonary diseases.

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Rickettsia and *Orientia*

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66

TAXONOMY

The family *Rickettsiaceae* comprises two genera of small, obligately intracellular bacteria that reside free within the cytosol, namely, *Rickettsia* and *Orientia*. Although the number and diversity of strains in each genus are similar, the practices of species designation differ remarkably. The second edition of *Bergey's Manual of Systematic Bacteriology* lists 20 validated names of *Rickettsia* species, and others have been proposed (84). *Orientia tsutsugamushi*, the single species of the genus, has 0.8% divergence of the *rrs* (16S rRNA) gene. Similarly, other obligately intracellular bacteria have 0.5% *rrs* divergence within a species (e.g., *Ehrlichia chaffeensis, Coxiella burnetii,* and *Chlamydia trachomatis*). A recent proposal of criteria for the limits of divergence of *Rickettsia* species would allow different species to be as closely related as 0.2% divergence for *rrs*, 0.8% for citrate synthetase *(gltA)*, 1.2% for outer membrane protein A *(ompA)*, 0.8% for outer membrane protein B *(ompB)*, and 0.7% for surface protein 4 *(sca4)* (15). There are no common or universal concepts that can be utilized to delineate prokaryotic species as there are with eukaryotes.

The typhus group (TG) and spotted fever group (SFG), defined originally by their distinctive lipopolysaccharide antigens, constitute the genus along with other ancestral species such as *R. bellii* and *R. canadensis*. The TG consists of only two members, *R. prowazekii* and *R. typhi*, whereas the SFG contains bacteria that are generally recognized as human pathogens *(R. rickettsii, R. akari, R. conorii, R. africae*, *R. sibirica, R. japonica, R. honei, R. felis, R. parkeri, R. slovaca*, *R. aeschlimannii,* and *R. australis)* as well as a steadily growing list of organisms identified only in arthropods (3, 50, 55, 84). Most of these species of undetermined pathogenicity, including *R. montanensis, R. bellii, R. peacockii,* and *R. rhipicephali,* are much more prevalent in U.S. ticks than is pathogenic *R. rickettsii*. In Europe, *R. helvetica* and *R. massiliae* have been proposed as human-infecting species based on limited evidence (5, 14). SFG isolates from Israel and the Astrakhan region of Russia, which have wider geographic distributions, are genetic variants of *R. conorii.* Similarly, SFG isolates recovered from *Hyalomma* ticks in Inner Mongolia and Africa and from patients in France and Africa have been designated the mongolotimonae strain of *R. sibirica*.

Orientia tsutsugamushi diverges from *Rickettsia* by approximately 10% in the *rrs* gene and differs greatly in its cell wall structure, containing completely unrelated proteins and lacking lipopolysaccharide and peptidoglycan (Table 1). Phylogeny using *groEL* reveals similar genetic diversity for the unispecies genus *Orientia* as for the *Rickettsia* genus, for which some believe that too many species have been created (Fig. 1) (35). *O. tsutsugamushi*, originally classified serologically, has subsequently been analyzed genetically (51). In each geographic area there are several genetic variants, and they differ from the genetic variants in other regions. Clonal evolutionary divergence has produced an enormous number of genotypes. Genetic variants of *O. tsutsugamushi* correspond to particular arthropod hosts in which divergence most likely occurred.

DESCRIPTION OF GENERA

Species of *Rickettsia* are small (0.3 to 0.5 μm by 1 to 2 μm), obligately intracellular bacteria of the α-*Proteobacteria* with a gram-negative cell wall structure that contains lipopolysaccharide, peptidoglycan, a major 135-kDa S-layer protein (OmpB), a 17-kDa lipoprotein, and for SFG rickettsiae a surface-exposed protein (OmpA) containing a variable number of nearly identical tandem repeat units (71). *Rickettsia* organisms have small (1.11 to 1.27 Mb), A+T-rich genomes resulting from reductive evolution with a high proportion (19 to 24%) of noncoding sequence and remarkable synteny (39). The lack of genes for enzymes for sugar metabolism, lipid biosynthesis, nucleotide synthesis, and amino acid synthesis and the presence of genes encoding enzymes for the complete tricarboxylic acid cycle and several copies of ATP/ADP translocase suggest both independent synthesis of ATP and acquisition of host ATP and rickettsial utilization of host sources for nutrition and building blocks. Rickettsiae adhere to the host cell receptor Ku70 by OmpB (and also by OmpA to an unknown receptor for SFG rickettsiae), trigger signaling pathways leading to recruitment and activation of induced phagocytosis, and escape from the phagosome by membranolytic activities of rickettsial phospholipase D and TlyC (36, 37, 82). *O. tsutsugamushi* (0.3 to 0.5 μm by 0.8 to 1.5 μm) has a major surface protein of 54 to 58 kDa as well as 110-, 80-, 47-, 42-, 35-, 28-, and 25-kDa surface proteins but lacks muramic acid, glucosamine, 2-keto-3-deoctulonic acid, and hydroxy fatty acids, suggesting the absence of lipopolysaccharide and peptidoglycan. Compared with *Rickettsia* spp., *Orientia* has a more plastic gram-negative

TABLE 1 Characteristics of *Rickettsia* spp. and *Orientia tsutsugamushi*[a]

Organisms	LPS	PG	OmpA	OmpB	17-kDa lipoprotein	56-kDa protein
SFG	S	+	+	+	+	0
Typhus group	T	+	0	+	+	0
R. canadensis	T	+	+	+	+	0
O. tsutsugamushi	0	0	0	0	0	+

[a] Abbreviations and symbols: LPS, lipopolysaccharide; S, spotted fever group lipopolysaccharide present; T, typhus group lipopolysaccharide present; PG, peptidoglycan; +, present; 0, absent.

cell wall with a thicker outer leaflet and thinner inner leaflet of the outer envelope and does not have a slime layer.

EPIDEMIOLOGY AND TRANSMISSION

Rickettsia spp. reside in an arthropod host (tick, mite, louse, flea, or other insect) for at least a part of their life cycle, during which they are maintained by transovarian transmission and/or cycles involving horizontal transmission to mammalian hosts (38) (Table 2). *Orientia* resides free in the cytosol and is maintained in nature by transovarian transmission in trombiculid mites, which transmit the infection to humans during feeding as the larval stage (Table 2).

CLINICAL SIGNIFICANCE

In addition to Rocky Mountain spotted fever (RMSF), rickettsialpox, murine typhus, flying squirrel-associated *R. prowazekii* infection, flea-borne spotted fever, and *R. parkeri* infection, which are indigenous to the United States, the potential for imported cases is significant for African tick bite fever, boutonneuse fever, murine typhus, and scrub typhus (Table 2) (10, 27, 28, 48, 54, 62, 75, 80). Other rickettsioses, either because of their geographic distribution and the infrequency of travelers' exposure to them or because of their incidence, are unlikely to be imported. RMSF, louse-borne typhus, and scrub typhus are life-threatening illnesses even for young, previously healthy persons. Murine typhus, boutonneuse fever, and North Asian tick typhus can have a fatal outcome in patients who are elderly or have underlying diseases or other risk factors. Although *R. felis, R. africae,* and *R. parkeri* cause milder, usually nonfatal rickettsioses, their importance has increased, with diagnoses over a wide geographic distribution (18, 45, 47, 85).

An average of 7 days after tick bite inoculation of rickettsiae, patients with RMSF develop fever, severe headache, malaise, and myalgia, frequently accompanied by nausea, vomiting, and abdominal pain and sometimes cough (27). A rash typically appears only after 3 to 5 days of illness. Rickettsiae infect endothelial cells, frequently leading to increased vascular permeability and focal hemorrhages (21). In severe cases, noncardiogenic pulmonary edema and rickettsial encephalitis

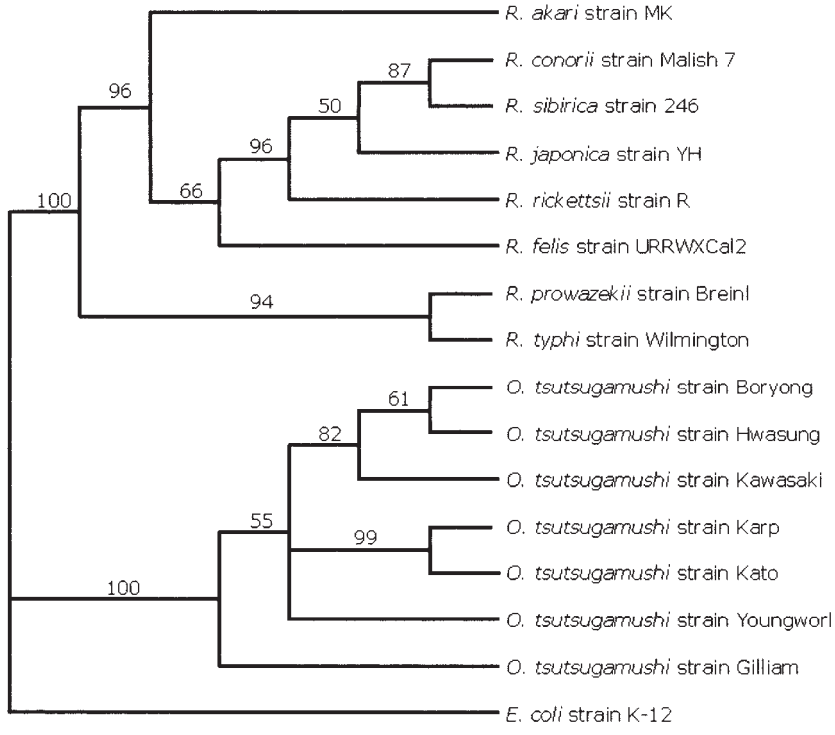


FIGURE 1 Phylogeny of *Rickettsia* and *Orientia* as determined by unweighted maximum parsimony analyses of *groEL* gene sequences prepared by PAUP 4.0 software with *Escherichia coli* as the outgroup. Numerical values on the branches represent the quantity of genetic divergence from the nearest node.

TABLE 2 Etiology, epidemiology, and ecology of rickettsial diseases

Organism	Disease	Geographic distribution	Typical mode of transmission to humans	Natural cycle
Spotted fever group				
R. rickettsii	Rocky Mountain spotted fever	Western Hemisphere	Tick bite	Transovarian in ticks and rodent-tick cycles
R. akari	Rickettsialpox	United States, Ukraine, Croatia, Korea	Mite bite	Transovarian in mites and mite-mouse cycles
R. conorii	Boutonneuse fever	Southern Europe, Africa, Middle East	Tick bite	Transovarian in ticks
R. africae	African tick bite fever	Sub-Saharan Africa, Caribbean	Tick bite	Transovarian in ticks
R. parkeri	American tick bite fever	North and South America	Tick bite	Transovarian in ticks
R. sibirica	North Asian tick typhus	Asia, Europe, Africa	Tick bite	Transovarian in ticks
R. japonica	Japanese spotted fever	Japan	Tick bite	Ticks
R. australis	Queensland tick typhus	Australia	Tick bite	Ticks
R. honei	Flinders Island spotted fever	Australia, Thailand	Tick bite	Transovarian in ticks
R. slovaca	Tick-borne lymphadenopathy	Eurasia	Tick bite	Unknown
R. felis	Flea-borne spotted fever	North and South America, Europe, Africa	Not known	Transovarian in cat fleas
Typhus group				
R. prowazekii	Primary louse-borne typhus	Worldwide	Infected louse feces rubbed into broken skin or mucous membranes or inhaled as aerosol	Human-louse cycle; flying squirrel-flea and/or louse cycle
R. prowazekii	Brill-Zinsser disease	Worldwide	Recrudescence years after primary attack of louse-borne typhus	
R. typhi	Murine typhus	Worldwide	Infected flea feces rubbed into broken skin or mucous membranes or inhaled as an aerosol	Rat-flea cycle; opossum-flea cycle
Scrub typhus group				
O. tsutsugamushi	Scrub typhus	Japan, eastern Asia, northern Australia, west and southwest Pacific	Chigger bite	Transovarian in mites

with coma and seizures are grave conditions that often presage death (74).

Rickettsialpox has been recognized mainly as a nonfatal urban disease with disseminated vesicular rash and an eschar at the location of rickettsial inoculation by the feeding mite (28, 31). The complete spectrum of clinical manifestations of *R. felis* infections has yet to be determined. This disease suffers from diagnostic neglect despite its widening recognized geographic distribution and the prevalence of cat flea exposure (62, 85).

Murine typhus causes a rash in only slightly more than one-half of patients, cough and chest radiographic infiltrates suggesting pneumonia in many patients, and severe illness with seizures, coma, and renal and respiratory failure necessitating intensive care unit admission in 10% of hospitalized cases (10).

Travelers who have returned from Africa and develop fever, one or more eschars, and in some cases regional lymphadenopathy and a maculopapular or vesicular rash are very likely infected with *R. africae* (54).

Rickettsia prowazekii, R. rickettsii, R. typhi, and *R. conorii* are bioterror threats via aerosol exposure to organisms that are stable and infectious at a low dose (72).

Scrub typhus caused by *O. tsutsugamushi* occurs in the geographic area that is bordered by Japan, Korea, and Russia on the north, Australia on the south, Pakistan and Afghanistan on the east, and the Philippines and Micronesia on the west (51, 63, 67). Clinical symptoms of the disease include fever, headache, maculopapular rash, eschar, lymphadenopathy, and central nervous system involvement (24, 58, 65). Without treatment, mortality can reach up to 30% (24).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Blood should be collected as early as possible in the course of illness. For the isolation of rickettsiae, blood should be obtained in a sterile heparin-containing vial prior to the administration of antimicrobial agents that are active against rickettsiae (27, 33). For isolation and immunocytologic diagnosis, blood should be stored temporarily at 4°C and processed as promptly as possible. If inoculation of cell culture or animals must be delayed for more than 24 h, plasma, buffy coat, whole blood, or biopsied tissue should be frozen rapidly and stored at −70°C or in liquid nitrogen. EDTA- or sodium

citrate-anticoagulated blood collected in the acute state has been used effectively for the diagnosis of murine typhus, epidemic typhus, Japanese spotted fever, scrub typhus and, with lower sensitivity, RMSF and African tick bite fever, by PCR (17, 54, 61, 62, 64, 68, 69). PCR provides a higher diagnostic yield when applied to biopsy specimens of rickettsia-infected lesions, particularly eschars (44, 45). If whole blood, plasma, buffy coat, or tissue cannot be processed for PCR within several days, it should be stored at −20°C or lower.

For serologic diagnosis, blood is collected as early in the course of disease as possible, a second sample is collected after 1 or 2 weeks, and, if a fourfold rise in antibodies has not occurred, a third sample is collected 3 or 4 weeks after onset. The serum may be stored for several days at 4°C but should be stored frozen at −20°C or lower for longer periods to avoid degradation of the antibodies. However, blood samples collected by fingerstick on appropriate blotting paper in remote areas and sent by ordinary mail can be eluted for serologic diagnosis (13).

A 3-mm-diameter punch biopsy specimen of a skin lesion, preferably a maculopapule containing a petechia or the margin of an eschar, should be collected as soon as possible (41, 73). Although treatment should not be delayed, it is best to perform the biopsy prior to the completion of 24 h of treatment with a tetracycline or chloramphenicol. For immunohistologic detection of SFG or TG rickettsiae, the specimen can be snap-frozen for frozen sectioning or fixed in formaldehyde for the preparation of paraffin-embedded sections (21, 28, 41, 73–75, 77). The former approach yields an answer more rapidly, but freezing artifacts distorts the architecture of the tissue, and fixed tissue is more convenient for shipping to a reference laboratory (44). Aseptically collected autopsy tissues, e.g., spleen and lung, are useful for rickettsial isolation, ideally inoculated fresh, or held for 24 h at 4°C or stored frozen at −70°C for longer periods, if the specimen must be shipped to a public health or reference laboratory. Autopsy tissues can also be examined for rickettsiae by immunohistochemistry or PCR.

Body lice *(Pediculus humanus corporis)* removed from patients suspected of having epidemic typhus can be examined for the presence of rickettsiae. Body lice acquire rickettsiae and remain infected for life, thus providing a useful specimen for PCR diagnosis even after a prolonged period of shipping at ambient temperature and humidity, which do not ensure survival of the lice (60).

ISOLATION PROCEDURES

Due to their high infectivity and low dose capability, rickettsial isolation is performed in biosafety level 3 laboratories. Cumbersome historic methods such as inoculation of adult male guinea pigs, mice, or the yolk sac of embryonated chicken eggs have been supplanted by cell culture methods, except for isolation of *O. tsutsugamushi,* which is achieved by intraperitoneal inoculation of mice (27, 33). Vero, L-929, HEL, and MRC5 cells have been used in antibiotic-free media to isolate rickettsiae. The best results reported are achieved with heparin-anticoagulated plasma, buffy coat, or skin lesion biopsy specimen collected prior to administration of antirickettsial therapy.

Samples containing 0.5 ml of triturated clinical material mixed with 0.5 ml of tissue culture medium are inoculated as promptly as possible onto 3.7-ml shell vials with 12-mm-diameter round coverslips having a confluent layer of cells and centrifuged at 700 × *g* for 1 h at room temperature to enhance attachment and entry of rickettsiae into host cells (2, 33). After removal of the inoculum, the shell vials are washed with phosphate-buffered saline and incubated with minimal essential medium containing 10% fetal calf serum in an atmosphere containing 5% CO_2 at 34°C. At 48 and 72 h, a coverslip is examined by Giemsa or Gimenez stain or by immunofluorescence with antibodies against SFG and TG rickettsiae. Detection of four or more organisms is interpreted as a positive result. This method has yielded a diagnosis in 59% of samples from patients with boutonneuse fever who had neither been treated nor developed antibodies to *R. conorii* prior to collection of the sample (33). Rickettsiae were detected at 48 h of growth in 82% of the positive samples. Universal precautions should be exercised, and work should be performed in a laminar flow biosafety hood with use of gloves and gown. Although the quantity of rickettsiae in the cell culture is relatively low, avoidance of aerosol, internal, or contact exposure should be taken as for mycobacteria, fungi, and viruses.

IDENTIFICATION OF *RICKETTSIA* AND *ORIENTIA* ISOLATES

Rickettsiae isolated in cell culture can be identified by indirect immunofluorescence with group-, species-, and strain-specific monoclonal antibodies. In an increasing number of laboratories, rickettsial isolates are identified by molecular methods such as PCR amplification of genes that are genus specific (17-kDa protein, citrate synthase, or OmpB) or SFG specific (OmpA) (57). Determination of DNA sequences identifies unique isolates. *O. tsutsugamushi,* being more distantly related to *Rickettsia* spp., lacks the above cell wall genes but can be identified by PCR of the gene encoding the major immunodominant 56-kDa surface protein.

Identifying the species of rickettsial isolates by microimmunofluorescence serotyping requires intravenous inoculation of mice with rickettsiae on days 0 and 7 and collection of sera on day 10 (49). These high-titer antibodies react with conformational species-specific epitopes of OmpA and OmpB. Antibodies against group-specific lipopolysaccharide develop later in the murine immune response to *Rickettsia*. This rather cumbersome and expensive method requires propagation of large quantities of the isolate and of the prototype strains for immunofluorescence titration as well as for development of the typing sera. Genetic analysis of a larger portion of the genome is currently favored for identification of isolates. An isolate that is known to be a *Rickettsia* should be identified in a biosafety level 3 laboratory, and isolates of *R. rickettsii* and *R. prowazekii* must be handled as required by U.S. federal regulations for select agents.

DIRECT DETECTION OF THE ETIOLOGIC AGENT IN CLINICAL SAMPLES BY IMMUNOLOGIC AND GENETIC METHODS

The diagnoses of RMSF, boutonneuse fever, murine typhus, louse-borne typhus, and rickettsialpox have been established by immunohistochemical detection of rickettsiae in formalin-fixed, paraffin-embedded sections of biopsy specimens of rash and eschar lesions (21, 27, 28, 41, 73, 75, 77) (Fig. 2, left). Monoclonal antibodies that are specific for lipopolysaccharides of either SFG or TG rickettsiae have been used to detect rickettsiae by immunoperoxidase staining (Fig. 2, right). There is no antibody commercially available (75, 76). The sensitivity and specificity of immunohistochemical detection of *R. rickettsii* in cutaneous biopsy specimens are 70 and 100%, respectively (27, 73). Eschar biopsies yield

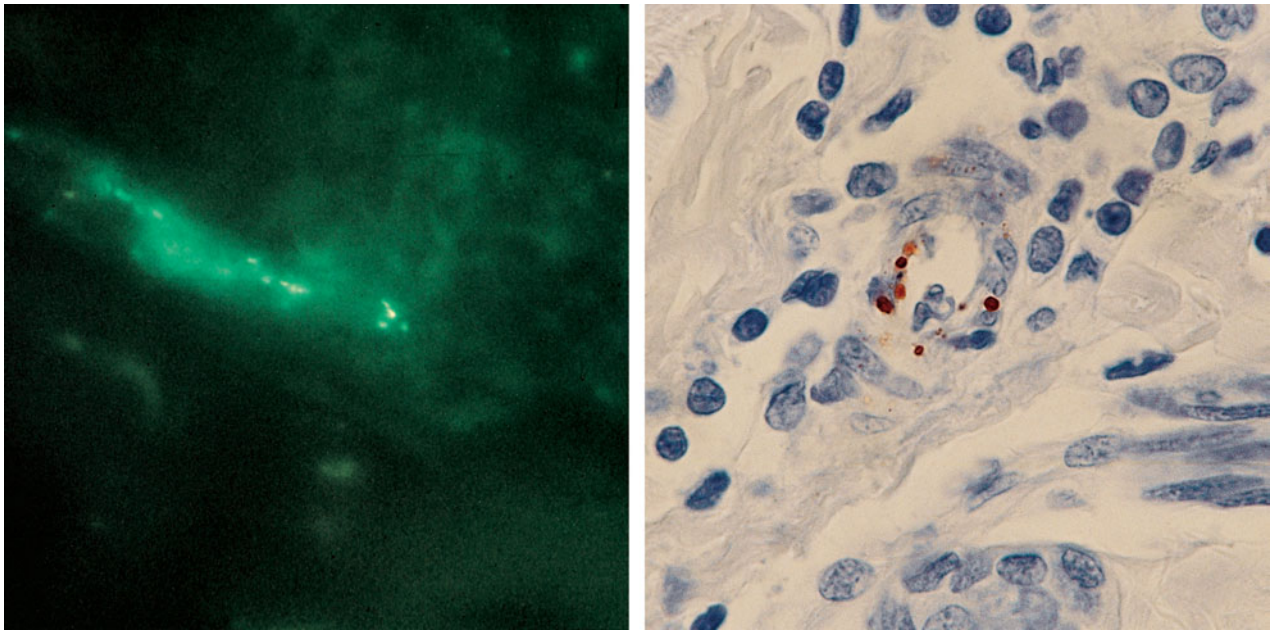


FIGURE 2 (Left) Direct immunofluorescence staining of skin biopsy specimens with anti-SFG *Rickettsia* antibodies facilitates rapid diagnosis. Rickettsiae are present in the vessel wall. (Right) Demonstration of rickettsial organisms in the microvasculature of the dermis in a patient with a history of Rocky Mountain spotted fever. Rickettsiae are seen in the vessel wall. An immunoperoxidase stain, using monoclonal antibodies directed against spotted fever group lipopolysaccharide, was used. Skin biopsy magnification, ×800.

sensitive specimens for the diagnosis of SFG rickettsioses that manifest that lesion and should be considered for diagnostic evaluation in patients suspected to have rickettsialpox, boutonneuse fever, or African tick bite fever.

Immunocytochemical detection of *R. conorii* in circulating endothelial cells has been accomplished by capture of the endothelial cells from blood samples using magnetic beads coated with a monoclonal antibody to a human endothelial cell surface antigen followed by immunofluorescent staining of the intracellular rickettsiae (33). This method has a sensitivity of 50% and a specificity of 94%. Rickettsiae are detected in 56% of untreated patients and in 29% of patients receiving antirickettsial treatment. *O. tsutsugamushi* has been identified using immunohistochemistry in human endothelial cells, macrophages, and cardiac myocytes (42). In situ hybridization has not been reported for the detection of rickettsiae in tissue samples.

PCR has been applied to the amplification of the DNA of *R. rickettsii*, *R. conorii*, *R. japonica*, *R. typhi*, *R. prowazekii*, *R. africae*, *R. felis*, *R. akari*, *R. slovaca*, and *O. tsutsugamushi*, usually from peripheral blood, buffy coat, or plasma, but occasionally from fresh, frozen, or paraffin-embedded tissue or arthropod vectors from patients (8, 12, 17, 44, 54, 59-62, 64, 66, 68–70). Nested PCR applied to skin biopsy specimens, particularly of eschars prior to treatment, has a sensitivity of 78% (16). For all pathogenic *Rickettsia* spp., the 17-kDa lipoprotein gene is the principal target, employing the primers CATTACTTGGTTCTCAATTCGGT and GTTTTATTAGTGGTTACGTAACC, which amplify a 231-bp DNA fragment (62). The *glt*A, *rrs*, and *omp*A genes have also been amplified diagnostically, with the *Rickettsia* being identified through either restriction fragment length polymorphism analysis using AluI and XbaI or sequencing of the PCR product (62). The availability of rickettsial genome sequences offers the possible design of an enormous number of primer sets. The approach of using primer sets on a single occasion to reduce the chances of amplicon contamination and false-positive results seems impractical. With batch processing being done annually, the delay in laboratory results reduces the clinical value (16). The single use of primers requires that their utility and sensitivity be unknown. The potential for amplicon contamination originating from a positive patient sample remains even if there is no positive control. Recent advances in technology such as real-time PCR allow for increased sensitivity in the detection of rickettsiae (12, 32, 43, 59, 70). The advantage of real-time PCR is detection of rickettsial organisms during the early or acute phase of disease before the generation of antibody. The targets for primer design have ranged from housekeeping genes (*glt*A) to antigen genes (*omp*A and *omp*B). The sensitivities for detection vary among primer sets. For example, real-time assays utilizing primer set RR.190.547F (CCTGCCGATAATTATACAGGTTTA) and RR.190.701R (GTTCCGTTAATGGCAGCAT), which generates a product of 154 bp, can detect five copies of rickettsial DNA (12). The primer set CS-5 and CS-6 detects 1 copy of *R. rickettsii* DNA and 10 copies of *R. bellii* DNA. Perhaps the best potential demonstration of real-time PCR as a diagnostic assay was observed in the primers targeting *ompB* and an *ompB* probe (70). For *O. tsutsugamushi*, the 56-kDa protein gene is the usual target of diagnostic PCR (68). The nested 56-kDa gene PCR assay is highly sensitive for the diagnosis of *O. tsutsugamushi* infections during the acute phase of the disease (61). PCR amplification of DNA from blood samples, using primers p34 (TCAAGCTTATTGCTAGTGCAATGTCTGC) and p55 (AGGGATCCCTGCTGCTGTGCTTGCTGCG), which generate a 1,003-bp product, followed by nested PCR with primers p10 (GATCAAGCTTC-

CTCAGCCTACTATAATGCC) and p11 (CTAGGGATCC-CGACAGATGCACTATTAGGC), amplifies a 483-bp product. This assay detects 10 prototype strains of *O. tsutsugamushi* and does not amplify *R. typhi* or *R. honei*. A duplex PCR assay using the *groEL* gene identifies both SFG rickettsiae and *O. tsutsugamushi* (46). Real-time PCR assays utilizing the *Orientia htrA* gene have been applied to diagnosis with clinical samples (24, 66).

Molecular and immunohistochemical diagnostic testing, the most useful methods for establishing a diagnosis during the acute stage of illness when therapeutic decisions are critical, are available at this time, to the best of our knowledge, in only a few reference laboratories including ours. Individual cases for immunohistochemistry may be referred to the following laboratories after contacting the directors for consultation: David H. Walker, M.D., Department of Pathology, University of Texas Medical Branch, 301 University Blvd., Keiller Building, Room 1.116, Galveston, TX 77555-0609, telephone (409) 772-3989, fax (409) 772-1850, e-mail dwalker@utmb.edu; J. Stephen Dumler, M.D., Division of Medical Microbiology, Department of Pathology, The Johns Hopkins Medical Institutions, Meyer B1-193, 600 North Wolfe Street, Baltimore, MD 21287, telephone (410) 955-5077, fax (410) 614-8087, e-mail sdumler@jhmi.edu; and Sherif Zaki, M.D., Ph.D., Department of Pathology, Centers for Disease Control and Prevention, 1600 Clifton Road Mail Stop G32, CDC, Atlanta, GA 30333, telephone (404) 639-3133, e-mail sxz1@cdc.gov.

SEROLOGIC TESTS

For most clinical microbiology laboratories, assays for antibodies to rickettsiae are the only tests performed. This situation is unfortunate for the patient with a life-threatening, acutely incapacitating rickettsial disease because these assays are useful principally for serologic confirmation of the diagnosis in convalescence and usually do not provide information that is helpful in making critical therapeutic decisions during the acute stages of illness. Patients who died of rickettsioses usually had received many antibiotics, none of which had antirickettsial activity owing in part to the lack of laboratory data providing clinical guidance for a rickettsial diagnosis. The earlier a diagnosis is established, the shorter the course of rickettsial illness after an appropriate antirickettsial antibiotic is administered.

Serologic assays for the diagnosis of rickettsial infections include the "gold standard" indirect immunofluorescence assay (IFA), indirect immunoperoxidase assay, latex agglutination, enzyme immunoassay (EIA), *Proteus vulgaris* OX-19 and OX-2 and *Proteus mirabilis* OX-K strain agglutination, line blot, Western immunoblotting, and rapid flow assays (4, 7, 9–11, 19, 22, 25–30, 52, 73, 81, 83). Only a portion of these assays are available as commercial kits or as assays performed in reference laboratories for some, but not all, rickettsial diseases. Other serologic tests such as indirect hemagglutination, microagglutination, and complement fixation are no longer in general use.

The IFA contains all the rickettsial heat-labile protein antigens and group-shared lipopolysaccharide antigen and, thus, provides group-reactive serology. IFA reagents are available commercially for SFG and TG rickettsiae from Panbio, Inc., Baltimore, Md.; Focus Technologies, Cypress, Calif., and bioMerieux, Durham, N.C.; they are also available for *O. tsutsugamushi* from Panbio, Inc. In cases of RMSF, IFA detects antibodies at a diagnostic titer of ≥64, usually in the second week of illness. Effective antirickettsial treatment of RMSF must be initiated by day 5 of illness to avoid a potentially fatal outcome. For boutonneuse fever, a diagnostic IFA titer of ≥40 occurs in 46% of patients between days 5 and 9 of illness, in 90% of patients between 20 and 29 days, and in 100% of patients thereafter. In murine typhus, diagnostic IFA titers are present in 50% of cases by the end of the first week of illness and in nearly all cases by 15 days after onset (10). In areas where particular rickettsial diseases are endemic, a higher diagnostic cutoff titer is required. For example, for the IFA diagnosis of scrub typhus in patients residing in zones of endemicity, an IFA titer to *O. tsutsugamushi* of ≥400 is 96% specific and 48% sensitive, with sensitivity rising from 29% in the first week to 56% in the second week (4). Lowering the diagnostic cutoff titer to 100 raises the sensitivity only to 84% and reduces the specificity to 78%. These considerations are not as important when testing patients who have visited regions of endemicity for only a short period. Each laboratory performing the test should establish its own cutoff titers for the patient population, the microscope and reagents used, and the laboratorian's judgment of the minimal positive signal.

Indirect immunoperoxidase assays for scrub typhus, murine typhus, boutonneuse fever, and presumably other rickettsioses yield results similar to those of IFA when the immunoglobulin G (IgG) diagnostic titer is set at 128 and that of IgM is set at 32 (29). Advantages include the use of a light microscope rather than a fluorescent microscope and the production of a permanent slide result.

Latex agglutination test reagents are available commercially from Panbio, Inc., only for RMSF in the United States. Latex beads coated with an extracted rickettsial protein-carbohydrate complex containing rickettsial lipopolysaccharide are agglutinated mainly by IgM antibodies, with reports of a sensitivity of 71 to 94% and a specificity of 96 to 99% (19). A diagnostic titer of 128 is often detected early in the second week of illness.

EIAs have been developed in various formats including antigens coating microtiter wells or immobilized on nitrocellulose or other sheets for use in the commercial reference laboratory setting. Dot-EIA kits are commercially available as Dip-S-Ticks from Panbio, Inc., for detecting antibodies against *R. rickettsii, R. conorii, R. typhi*, and *O. tsutsugamushi* (29, 81). No peer-reviewed publications have evaluated the use of the dot-EIA for the diagnosis of RMSF. When compared with an IFA titer of ≥64 for the diagnosis of murine typhus, the dot-EIA showed a sensitivity of 88% and specificity of 91% (29). The dot-EIA for diagnosis of scrub typhus had sensitivities and specificities of only 80 and 77%, respectively, when compared with an IFA cutoff titer of 64, and 89 and 66%, respectively, at an IFA cutoff titer of 128 (81). These SFG rickettsiae and *R. typhi* kits detect cross-reactive antibodies as demonstrated in an outbreak of African tick bite fever by clinical and epidemiologic data. The dot-EIA of *R. conorii* antigen provided early diagnostic evidence of a SFG rickettsiosis. Subsequent analysis revealed poor specificity with a high rate of false-positive results. These assays are diagnostic tools that do not require expensive, specialized equipment, but they suffer from apparent low specificity. Standard EIAs for detecting IgG or IgM antibodies against SFG rickettsiae or *O. tsutsugamushi* are also available from Panbio. The utilization of these tests for paired sera from populations with clinical (fever, headache, or rash) and epidemiologic (tick exposure) features consistent with rickettsiosis would most likely yield useful information. A multitest Dip-S-Ticks EIA for scrub typhus, murine typhus, and leptospirosis was not useful in Thailand, where nearly all patients had

antibodies to more than one agent and others had antibodies against an agent that was not the cause of the illness (79). The diagnosis of scrub typhus by detection of antibodies in clinical samples in an IgM capture enzyme-linked immunosorbent assay can be utilized for single serum samples from early-stage infections (22). The performance values of this assay are a sensitivity of 96.3% and a specificity of 99%. A comparison of serologic methods for scrub typhus revealed that EIA containing recombinant p56 of Karp, Kato, and Gilliam strains was most sensitive (100%) and rapid lateral flow assay of antibodies against recombinant Karp p56 had a sensitivity of 86% (22). The latter, which is especially useful in situations with limited laboratory facilities and a low number of specimens, is available from Panbio Ltd., Brisbane, Australia.

The assays that have been most widely used for the diagnosis of rickettsial diseases are agglutination of the OX-19 and OX-2 strains of *Proteus vulgaris* for rickettsioses and the OX-K strain of *Proteus mirabilis* for *O. tsutsugamushi* infections. These assays have poor sensitivity and specificity (26, 73). They should be replaced by more accurate serologic methods such as IFA or EIA. However, there are situations in developing countries where the choice is between the *Proteus* agglutination tests and none at all for the detection of important public health problems such as outbreaks of louse-borne typhus. In fact, the evidence leading to the discovery of Japanese spotted fever and Flinders Island spotted fever included *Proteus* agglutinating antibodies.

Shared antigens of OmpA, OmpB, and group-specific lipopolysaccharide impede establishment of a species-specific diagnosis by serologic methods. The criterion of a fourfold or greater difference in IFA titers between the two suspected agents distinguished infections by *R. prowazekii* and *R. typhi* in only 34% of cases and infections by *R. africae* from those by *R. conorii* in only 26% (34, 54). Western immunoblotting detection of antibodies against OmpA or OmpB of only one *Rickettsia* species has also been proposed as a criterion for species-specific diagnosis. However, it was effective in distinguishing *R. prowazekii* and *R. typhi* infections or *R. africae* and *R. conorii* infections in only one-half of the cases (23, 34, 54). Cumbersome, expensive cross-absorption of sera prior to IFA or Western immunoblotting is more effective in establishing a species-specific diagnosis. However, interpretation of these results requires careful evaluation of the performance of valid controls, the quality and quantity of each antigen preparation, and the potential for the occurrence of infection by an untested, even as yet unrecognized, agent. In the past, knowledge of the geographic origin of the case sufficed to designate the specific diagnosis. However, the increasing number and geographic overlap of rickettsioses challenge the old assumptions.

ANTIMICROBIAL SUSCEPTIBILITY TESTING

Data supporting the use of doxycycline or another tetracycline antibiotic as the drug of choice for the treatment of infections caused by *Rickettsia* spp. and *O. tsutsugamushi* and the use of chloramphenicol as an alternative drug have been derived principally by empiric experience and retrospective case studies (20). In addition to historic studies of the activity of antimicrobial agents against these obligately intracellular bacteria in infected animals and embryonated eggs, studies of the effects of antimicrobial agents in cell culture have supported the consideration of alternative drugs such as fluoroquinolones, josamycin, azithromycin, and clarithromycin. Indeed, several fluoroquinolones, josamycin, and azithromycin have been used successfully for the treatment of boutonneuse fever under certain circumstances but cannot be recommended for more pathogenic rickettsioses (1, 6, 40, 53, 56). Except for cases of scrub typhus in Thailand which have not responded to doxycycline or chloramphenicol but for which azithromycin may be effective, there is little concern regarding rickettsial development of antimicrobial resistance (53, 78). During pregnancy, chloramphenicol has been used to treat RMSF and josamycin for boutonneuse fever. Antimicrobial susceptibility studies of rickettsiae are not routinely performed clinical laboratory tests.

INTERPRETATION AND REPORTING OF RESULTS

When reporting the results of an assay for antibodies in a single serum sample, the laboratorian seldom knows the duration of illness and whether the serum sample is from the acute or the convalescent phase of the disease. For sera that are nonreactive by dot-EIA, by IFA at a dilution of 1:64, by indirect immunoperoxidase assay at a dilution of 1:128, by latex agglutination at a dilution of 1:64, or by Weil Felix *Proteus* agglutination at a titer of 1:160, the laboratory report should state that no antibodies were detected at the particular cutoff dilution, which may differ among some laboratories and some patient populations, that negative results are expected in the acute stage of rickettsial illness, and that a second sample should be submitted to evaluate the possibility of seroconversion if no alternative diagnosis has been established. If paired acute- and convalescent-phase sera separated by an appropriate interval are available, they should be tested simultaneously. It is wise to test for all the rickettsial and ehrlichial agents to which the patient is likely to have been exposed in the United States. SFG rickettsiae, *Ehrlichia chaffeensis*, *Anaplasma phagocytophilum*, and *R. typhi* are the likely agents unless travel to an area where scrub typhus is endemic has occurred. If the paired sera are negative, the report should state that the results do not support the diagnosis of rickettsial infection, but that occasionally antibody synthesis is delayed, particularly in cases with early antirickettsial therapy. If a single serum sample contains an IFA antibody titer of $\geq$64, an IgM-IFA titer of $\geq$1:32, an indirect immunoperoxidase antibody titer of $\geq$128, a latex agglutination titer of $\geq$64, or a Weil Felix titer of $\geq$1:320, the laboratory report should state that antibodies reactive with the particular rickettsial antigen were detected at the measured titer, that the result provides supportive evidence for the diagnosis of the rickettsial disease, and that a convalescent-phase sample should be submitted to assess the possibility of seroconversion. If paired sera measured simultaneously show a fourfold or greater rise in titer, the interpretation is stated that the results strongly support the rickettsial diagnosis indicated by the tested antigen. If a significant titer was detected in the acute-phase sample, but no rise or only a single doubling dilution rise was measured, it should be stated that an additional later sample should be tested to evaluate a fourfold rise or fall in titer. The concept that recrudescent typhus could be distinguished from primary louse-borne typhus by the absence of IgM antibodies to *R. prowazekii* and of *Proteus* OX-19 agglutinating antibodies has been challenged (11). The manufacturers of the dot-EIA have recommended the interpretation that strongly reactive samples (three or four dots) may indicate the presence of a specific antibody response and that weakly reactive samples (one or two dots) are infrequent but possible in normal populations. Retesting 2 to 3 weeks later would establish the diagnosis if three or four dots develop in the convalescent serology and should always be performed.

Isolation of a rickettsia from blood or tissue may be interpreted as indicating an etiologic role. The level of identification of the isolate should be stated, whether identified only as to a group containing particular organisms or to the species level.

Immunohistologic and immunocytologic diagnostic interpretation states the method, reactivity of the method (e.g., antibody reactive with SFG rickettsiae), and location of the antigen (e.g., in vascular endothelium and frequently adjacent vascular smooth muscle for *R. rickettsii*). Detection of three or more rickettsiae in vascular endothelium in biopsy specimens or four or more rickettsiae in captured circulating endothelial cells is diagnostic of rickettsial infection.

Interpretation of PCR results should state the target gene, the organisms that would be detected, and the presence or absence of a DNA product of a particular size. If a specific oligonucleotide probe or DNA sequencing confirmed the specificity of the identification, this result should be stated. For negative immunohistologic, immunocytologic, and PCR results, it should always be stated that the failure to detect the agent does not exclude the diagnosis along with data regarding the sensitivity and specificity of the assay in the particular laboratory and the effects of antirickettsial treatment on the sensitivity.

Special efforts should be made to establish the diagnosis of fatal cases including rickettsial isolation, immunohistology, PCR, and serology on samples collected at necropsy.

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Ehrlichia, Anaplasma, and Related Intracellular Bacteria*

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67

TAXONOMY

Members of the genus *Ehrlichia* and *Anaplasma* have become important agents of human disease. Together with a number of other pathogens in several genera, they are obligate intracellular bacteria currently placed in the *Proteobacteria* phylum, section alpha, order *Rickettsiales* and family *Anaplasmataceae*. While most closely related to the genera *Rickettsia* and *Orientia,* organisms classically considered ehrlichiae could be divided into four major clades. Taxonomic classification of this group of bacteria (Fig. 1) is largely based upon sequence analysis of 16S rRNA genes and *groESL* operons (50). This classification has been further supported by sequence analysis of the citrate synthase (*gltA*), the β-subunit of RNA polymerase (*rpoB*), FtsZ protein (*ftsZ*), and rRNA genes (73–75, 83, 94, 132). The phylogenetic approach is further supported by serologic cross-reactions, comparisons among major immunodominant surface proteins, and the cellular tropisms of these bacteria (46, 77). All members of the tribes *Ehrlichieae* and *Wolbachieae* are included in the family *Anaplasmataceae* with elimination of the tribe structure of the family *Rickettsiaceae*. Current and former denominations of these bacteria associated with human and veterinary infections are found in Table 1. All tick-borne bacteria of *Anaplasmataceae* are grouped within two closely related genera, *Ehrlichia* and *Anaplasma*. The former members of the *Ehrlichia phagocytophila* group that include the species *Ehrlichia equi* and the bacterium formerly known as human granulocytic ehrlichiosis (HGE) agent were merged into a single species within the genus *Anaplasma* as *A. phagocytophilum*. Although minor sequence differences in the 16S rRNA gene exist between the former members of the *E. phagocytophila* group, it seems that such differences are related to their biological and ecological differences (34, 95, 128). Genetic variants of *A. phagocytophilum* have been reported in ticks and mammals in the Northeast United States and Europe (95, 128). Recently, a new agent closely related to *Ehrlichia ruminantium* has been found in *Ixodes ricinus* ticks removed from asymptomatic individuals in Northern Italy. This agent has been proposed as "*Candidatus* Ehrlichia walkerii" (27, 82, 123).

While *Ehrlichia* species infect predominantly leukocytes of humans and other mammals, *Anaplasma* species infect bone marrow-derived cells of all lineages in different animal hosts. *Neorickettsia* species infect predominantly mononuclear phagocytes and occasionally enterocytes in mammalian hosts. The genus *Aegyptianella* is currently listed as incerta sedis. *Wolbachia* spp. are "endocytosymbionts" of insects and helminths that are transmitted only by transovarial and transstadial (between stages of development) passage.

DESCRIPTION OF THE GENERA

Ehrlichia and *Anaplasma* spp. are obligate intracellular gram-negative bacteria that propagate and reside in cytoplasmic membrane-lined vacuoles in bone marrow-derived cells including granulocytes, monocytes, erythrocytes, and platelets, and for *E. ruminantium*, also within endothelial cells (118, 137, 141). Infection of endothelial cells is not surprising since both hematopoietic and endothelial cells are derived from a common ancestor, the hemangioblast that gives rise to angioblasts (endothelial precursor cells) and the hematopoietic stem cells. In fact, angioblasts share several cell surface markers with the hematopoietic stem cells and some of those markers are retained down to the endothelial differentiation (116).

For the genera *Ehrlichia* and *Anaplasma*, organisms are transmitted by tick bite, whereas organisms in the genera *Wolbachia* and *Neorickettsia* are not. Individual small (0.2- to 0.4-μm) dense forms of bacterial cells resembling chlamydial elementary bodies have been described, as have larger forms (0.8 to 1.5 μm) resembling reticulate bodies (113). Both are capable of binary fission, and a developmental cycle has not been demonstrated. After a few days, the elementary bodies dividing in the phagosome form an initial pleomorphic inclusion that matures into a microscopic colony known as a morula. *Neorickettsia sennetsu* grows as bacterial cells that may maintain their individual vacuolar membranes when they undergo binary fission, and thus an intracellular inclusion may not be identified by light microscopy. Cell lysis is a feature of the infectious process and leads to the release of cell-free bacteria that can infect other competent cells (22). Unlike *Rickettsia,* members of the genera *Ehrlichia* and *Anaplasma* do not show thickening of either leaflet of the outer membrane and the outer membrane appears to be more ruffled in *A. phagocytophilum* than in *N. sennetsu* or *Ehrlichia chaffeensis* (118). By transmission electron microscopy, *Ehrlichia* and *Anaplasma* spp. do not appear to contain significant amounts of peptidoglycan, a fact confirmed by the absence of genes

*This chapter contains information presented in chapter 65 by Maria E. Aguero-Rosenfeld and J. Stephen Dumler in the eighth edition of this Manual.

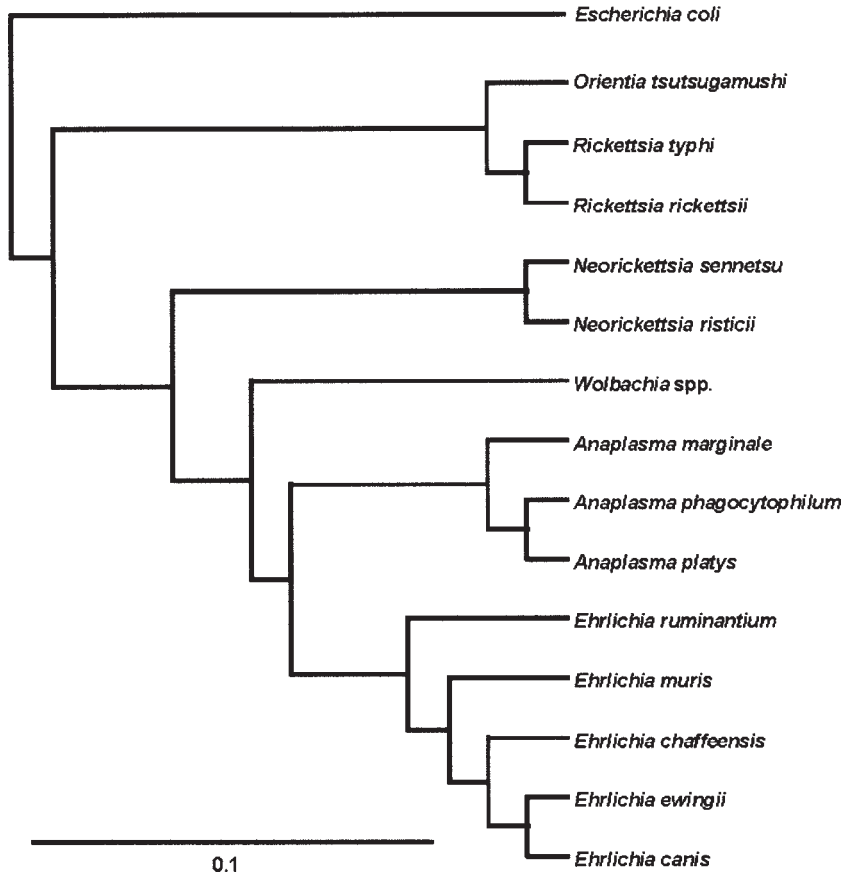


FIGURE 1 Neighbor-joining phylogenetic tree inferred from 16S ribosomal RNA gene sequences of selected *Ehrlichia, Anaplasma, Neorickettsia,* and *Wolbachia* spp. *Escherichia coli* is used as an outgroup. The bar represents the estimated number of substitutions per site.

necessary for the synthesis of this cell wall component on the full genome sequences currently available for *Anaplasma marginale* and *E. ruminantium* (20, 40). The genome size of *N. sennetsu* is approximately 800 kb, whereas those of *E. chaffeensis* and *E. ruminantium* are 1,200 kb, and those of *A. phagocytophilum* and *A. marginale* are 1,500 and 1,200 kb, respectively. With the availability of five full genomic sequences (*A. marginale* and *E. ruminantium, E. chaffeensis, Ehrlichia canis,* and *A. phagocytophilum),* certain themes have emerged including the low G+C content seen in other endosymbionts (*A. marginale* is the exception; it contains more G+C than any other intracellular bacterium) and a large proportion of noncoding sequences that have resulted from both reductive evolution due to their intracellular niche and ongoing sequence duplication events. Many of the genes for the glycolytic pathway are absent (142). Carbon sources are proline and glutamate. All enzymes for Krebs cycle are present, as well as the enzymes necessary for purine and pyrimidine metabolism, in contrast to rickettsiae, which lack such enzymes. ATP synthesis is possible since they possess the ATP synthase complex and other enzymes of the aerobic respiration chain. However, ATP/ADP translocases are not present, in contrast to the *Rickettsia* genus. Genes for type IV secretion systems are present as in other α-proteobacteria and most likely represent virulence genes (100). A surprising feature of the genomes is the presence of a large number of pseudogenes that are the product of sequence duplication events rather than reductive evolution seen in other intracellular bacteria (20). The active duplication of tandemly repeated sequences probably counteracts the reductive evolution to create new genes and therefore increase antigenic diversity as a mechanism of survival.

A. marginale and *Aegyptianella* spp. are intraerythrocytic bacteria of ruminants and birds, amphibians, or reptiles, respectively. These organisms also reside in small membrane-bound inclusions (18) and are not known to be associated with human disease.

EPIDEMIOLOGY AND TRANSMISSION

Ehrlichia and *Anaplasma* spp. are zoonotic agents that are transmitted to animals and humans most frequently after a tick bite or possibly by ingestion of fish infested by *Neorickettsia*-infected flukes. *Wolbachia* spp. are symbionts of a broad range of arthropods and helminths. Thus, most species have the potential for vertebrate and invertebrate life stages (Table 1). Based on a recent publication, transovarial transmission in ticks does not appear to occur, and thus, *Ehrlichia* and *Anaplasma* species depend upon high levels of bacteremia and in some cases persistent infection for enzootic maintenance (86). Transstadial and interstadial transmission in ticks allows ehrlichiae to be acquired from blood meals on naturally infected animals by the immature stages of ticks (larvae and nymphs), which can transmit the ehrlichiae to other mammalian hosts after molting to the next stage (nymph or adult).

TABLE 1 Selected features of *Ehrlichia, Anaplasma, Neorickettsia,* and *Aegyptianella* species of human and veterinary interest

Organism name	Former denomination(s)	Vector	Disease[a]	Hosts developing disease	Infected cells	Reservoir host
Anaplasma phagocytophilum	*Ehrlichia equi*	*Ixodes persulcatus* group	EGE	Horses	Granulocytes	Deer, sheep
	Ehrlichia phagocytophila HGE agent		Tick-borne fever HGA	Ruminants Humans, dogs		White-footed mice
Anaplasma marginale, A. centrale, A. ovis		Several tick species	Ruminant anaplasmosis	Cattle (sheep, goats)	Erythrocytes	Ruminants, wild cervids?
Anaplasma platys	*Ehrlichia platys*	*Rhipicephalus sanguineus?* *Amblyomma* spp.?	Canine cyclic thrombocytopenia	Dogs	Platelets	Ruminants?
Anaplasma bovis	*Ehrlichia bovis*	*Rhipicephalus appendiculatus* *Amblyomma variegatum*	Bovine ehrlichiosis	Cattle	Mononuclear leukocytes	Ruminants? Rabbits?
Ehrlichia chaffeensis		*Amblyomma americanum* *Dermacentor variabilis*	HME	Humans Dogs	Mononuclear leukocytes	White-tailed deer Domestic dogs Others?
Ehrlichia ewingii		*Amblyomma americanum*	CGE Ehrlichiosis ewingii	Dogs Humans	Granulocytes	Canids?
Ehrlichia canis		*Rhipicephalus sanguineus*	CME	Dogs Humans?	Mononuclear leukocytes	Canids
Ehrlichia ruminantium	*Cowdria ruminantium*	*Amblyomma* species	Cowdriosis (heartwater)	Domestic and wild ruminants	Granulocytes Endothelial cells	Ruminants
Ehrlichia muris		*Haemaphysalis flava*	Not named	Laboratory mice	Mononuclear leukocytes	Not known
Neorickettsia sennetsu	*Ehrlichia sennetsu*	Unknown; possibly acquired by ingestion	Sennetsu fever	Humans	Mononuclear leukocytes	Fluke-infested fish?
Neorickettsia risticii	*Ehrlichia risticii*	Ingestion of fluke-infested insects	Potomac horse fever	Horses	Mononuclear leukocytes	*Juga* spp. flukes
Neorickettsia helminthoeca		Ingestion of fluke-infested salmonid fish	Salmon poisoning disease	Dogs, bears	Mononuclear leukocytes	Fluke-infested fish
Aegyptianella spp. (genus and species incertae sedis)		*Amblyomma* spp.	Not known	Birds, amphibians, reptiles	Erythrocytes	Not known

[a] EGE, equine granulocytic ehrlichiosis (anaplasmosis); CGE, canine granulocytic ehrlichiosis; CME, canine monocytic ehrlichiosis.

Humans are only inadvertently infected and represent an end stage host for ehrlichiae. Natural maintenance of tick-borne ehrlichiae depends on the presence of appropriate tick vectors and mammalian hosts in the local environment (6, 87, 117, 135).

Natural reservoirs that are known to exist for *E. chaffeensis* involve both white-tailed deer *(Odocoileus virginianus)* and domestic dogs, and perhaps other animals that host *Ambylomma americanum* ticks (43, 85). Other minor reservoirs include opossums, raccoons, voles, coyotes, and goats (104). White-tailed deer also serve as a reservoir of *Ehrlichia ewingii* and of another ehrlichial agent closely related to *Anaplasma platys,* the white-tailed deer agent, which has not yet been associated with human disease (8). The identities of the major reservoirs for *A. phagocytophilum* are incompletely investigated, although small mammals, especially the white-footed mouse *(Peromyscus leucopus)* in the eastern United States, and other small mammals such as chipmunks *(Tamias striatus)* and voles *(Clethrionomys gapperi)* are naturally infected and are frequent hosts of the immature stages of *Ixodes scapularis* (135, 139). The role of white-footed mice as a reservoir of *A. phagocytophilum* in nature has been questioned, since immunity that develops after infection may render them temporarily host incompetent (84). Persistent or prolonged infection of animal reservoirs is essential for maintenance of zoonotic pathogens. Mice infected with *Borrelia burgdorferi* remain infected for many months, essential for continuous transmission to different stages of developing *I. scapularis* ticks (56). In Europe, red deer, sheep, cattle, and goats are persistently infected and serve as reservoirs of *A. phagocytophilum*. The reservoir for *N. sennetsu* is not known; however, epidemiological data suggest that consumption of raw fish was associated with sennetsu fever. Other species of *Neorickettsia* seem to have a complex transmission process involving trematode stages. *Neorickettsia risticii*, the agent of Potomac horse fever, appears to be transmitted to horses by accidental ingestion of insects carrying *N. risticii*-infected cercariae (89). Similarly, *Neorickettsia helminthoeca* infects dogs through ingestion of trematode-infested fish.

Over 20 species of ticks have been identified in the transmission of *A. marginale* worldwide (81). Other modes of transmission such as accidental inoculation of infected erythrocytes or blood-sucking insects have also been reported for *A. marginale.* Tick vectors also appear to be important in the transmission of members of the genus *Aegyptianella* to birds and amphibians (71).

CLINICAL SIGNIFICANCE

Human Diseases

HME

The causative agent of human monocytotropic ehrlichiosis (HME) is *E. chaffeensis,* a monocytotropic ehrlichia that was first identified as a human pathogen in a patient with a severe febrile illness after tick bites in 1986 (91). More than 1,300 cases of HME have been identified by the CDC between 1999 and 2004 (32, 33, 59); however, serologic data compiled at a major commercial laboratory that performs serologic testing for *E. chaffeensis* comprise more than 722 diagnostic serologic results between 1992 and 1995 alone, suggesting that HME occurs much more frequently than is reported (137). In another study based on a commercial laboratory, between 1997 and 1998, a total of 486 cases were diagnosed serologically (101). Passive and active case identification has revealed that clinically apparent HME occurs as frequently as or more frequently than Rocky Mountain spotted fever in Oklahoma, Georgia, Maryland, Tennessee, and North Carolina (127, 137). In fact, a 3-year prospective study conducted in Cape Girardeau, Mo., revealed an incidence between 2 and 4.7 per 100,000 population, a figure that is almost 1 order of magnitude higher than the one obtained by passive surveillance (102). Most cases are identified in the south central and southeastern United States, but increasingly infections have been identified in the mid-Atlantic region as well. Prospective evaluation of individuals with high rates of tick exposure shows that approximately 75% of seroconversions with antibody to *E. chaffeensis* are subclinical (57). Recent reports from Latin America, Africa, Europe, and Asia seem to indicate that *E. chaffeensis,* or closely related microorganisms, is also found in those localities (24, 39, 60, 120, 143). *E. chaffeensis* nucleotide sequences were found in different tick species from different regions of China, using PCR to amplify ehrlichial 16S rRNA genes (30).

The median incubation period for HME is 9 days, and two of every three patients are males; the median age is 44 years (57, 104). Patients typically present with high fever (97%), headache (81%), malaise (84%), and myalgia (40 to 60%), usually without other specific physical findings. Manifestations of gastrointestinal involvement (nausea, vomiting, and diarrhea), respiratory involvement (cough or pulmonary infiltrates), and joint pain are rare. Central nervous system involvement (stiff neck, confusion) and meningitis have been described (127). Rash patterns are variable in character, distribution, and temporal appearance. It could be petechial, macular, maculopapular, and erythematous (103, 104). Rash frequency is higher in the pediatric population and is present in up to 67% of cases (124). In spite of the nonspecific clinical symptoms and signs, laboratory findings are abnormal in at least 86% of patients, including leukopenia (60 to 74%) with lymphopenia and neutropenia, thrombocytopenia (70 to 90%), and increased serum aspartate transaminase activities (80 to 90%). Severe complications include a toxic shock-like syndrome with multiorgan failure, meningoencephalitis, diffuse alveolar damage associated with adult respiratory distress syndrome, and fulminant infections in patients immunocompromised by human immunodeficiency virus (HIV), high-dose corticosteroids, or organ transplantation (55, 93, 106, 107, 122). The case fatality rate of approximately 2 to 3% would be higher if effective antimicrobial therapy were not available. Fatal disease has been described most frequently in males, older patients, and those immunocompromised and with debilitating illnesses.

HGA, Formerly Known as Human Granulocytic Ehrlichiosis

The causative agent of human granulocytotropic anaplasmosis (HGA) is *A. phagocytophilum,* causing an illness identical to that of the formerly named *E. equi* and *E. phagocytophila* in horses and ruminants, respectively (58, 90). HGA was first identified in 1990 in a patient from Wisconsin after tick bites in an area where *E. chaffeensis* and its tick vector do not exist (11). Although the most frequent mode of transmission is through the bite of an infected *Ixodes* tick, other modalities of infection are likely to exist; perinatal transmission has been reported (68). Transmission by accidental inoculation of infected blood or by transfusion is probably rare but should also be considered.

As for HME, HGA is not a reportable illness in most states; thus, the true incidence and prevalence of the infection are unknown. However, prospective passive case collection in

northwestern Wisconsin during 1990 through 1995 revealed a yearly incidence rate of 16 cases per 100,000 population, with peak rates identified as high as 58 cases per 100,000 population in some counties in 1995 (13). Between 1999 and 2004 over 2,000 cases have been reported nationwide (32, 33, 59). The highest annual incidence rates were in the Northeast and upper Midwest states. Infected patients have been identified in many other states as well as in several European and Asian countries (9, 17, 21, 23, 41, 65, 79, 110, 112, 115, 137). Subclinical infection is probably the norm, as nearly 14 and 11% of the population assessed in northwestern Wisconsin and Westchester County, N.Y., respectively, have antibodies indicative of prior infection (2, 12). The tick vectors for HME and HGA coexist in the mid-Atlantic, southern New England states, and in the southern Midwest. Thus, both diseases can occur in these areas (41). Furthermore, human ehrlichioses, including HME, HGA, and *E. ewingii* infections, are clinically undistinguishable.

HGA has a median incubation period of 5 to 11 days after *Ixodes* spp. tick bites and occurs in patients with a median age of 43 to 60 years (1, 3, 13). Twice as many men as women have the illness (14). Patients present with high fever, myalgias, headache, and malaise, with gastrointestinal, respiratory, musculoskeletal, or central nervous system involvement in fewer patients. Rash is observed in less than 11% of patients. Leukopenia with lymphopenia, thrombocytopenia, and increased serum aspartate transaminase activities are present in most patients during early stages of the disease. The hematological abnormalities may normalize before antimicrobial treatment, and lymphocytosis with atypical lymphocytes has been observed after the first week of infection (14, 70). Severe complications of HGA include a septic shock-like illness with multiorgan failure, adult respiratory distress syndrome, and opportunistic infections (13, 62, 70, 138); meningitis has not been documented with HGA. At least six deaths have been associated with HGA; at least three of those patients had opportunistic infections including *Candida* esophagitis, *Cryptococcus* pneumonia, invasive pulmonary aspergillosis, and herpes esophagitis (13, 62, 138).

Ehrlichiosis Ewingii

The association of *E. ewingii* with human disease was first demonstrated by detection of its DNA in the blood of patients presenting with clinical and routine laboratory findings indistinguishable from HME in Missouri (29). The main vector is also the Lone star tick (*A. americanum*), and therefore, the distribution of the disease is similar to that of HME. Evidence of *E. ewingii* in *Dermacentor variabilis* ticks has also been documented (145, 149). White-tailed deer are also reservoirs for this bacterium, and recent epidemiological studies suggest that dogs might also play a role as such (8, 149). Ehrlichiosis ewingii seems to affect mostly immunosuppressed patients including patients with HIV. Although clinical descriptions exist for only a few patients, it has not been reported as a cause of death and patients coinfected with HIV and *E. ewingii* seem to have a milder disease than those patients coinfected with HIV and *E. chaffeensis* (29, 105). In dogs, *E. ewingii* is responsible for canine granulocytotropic ehrlichiosis.

Sennetsu Ehrlichiosis (Neorickettsiosis)

Named after the Japanese term for glandular fever, *N. sennetsu* was first isolated from patients with suspected infectious mononucleosis in 1953 (97). Rarely identified now, patients develop a self-limited febrile illness with chills, headache, malaise, sore throat, anorexia, and generalized lymphadenopathy. Cases were identified only in Japan and possibly Malaysia. Laboratory findings include early leukopenia and atypical lymphocytes in the peripheral blood during early convalescence. No fatalities or severe complications have been reported.

Other Human Ehrlichioses

In 1996, an *E. canis*-like agent was isolated from the blood of an asymptomatic man from Venezuela who had morulae in peripheral blood monocytes. Serological investigations in children with febrile illness and healthy adults exposed to dogs with canine ehrlichiosis in that geographic area showed antibodies in two healthy individuals reacting with the Venezuelan agent (Venezuelan human ehrlichia), *E. chaffeensis*, *E. canis,* and *Ehrlichia muris*. Comparison of the 16S rRNA gene of the Venezuelan human ehrlichia agent with two strains of *E. canis* showed one or two base differences (111). Two additional cases were identified by real-time PCR in patients from Cape Girardeau, Mo., as part of a prospective study performed between 1997 and 1999. In fact, one of the cases was a dual infection caused by *E. chaffeensis* and *E. canis* (J. P. Olano and J. W. McBride, unpublished data).

While *Wolbachia* spp. are not known to be directly pathogenic for humans or animals, emerging experimental evidence indicates a potential role for the intracellular bacterial components ("symbionts") of such helminths as *Brugia malayi*, *Onchocerca volvulus*, and *Wuchereria bancrofti* as potentiators of the inflammatory reactions that are considered significant parts of the pathogenesis of the parasite infection (66, 134).

Animal Diseases

Members of *Ehrlichia, Anaplasma, Neorickettsia,* and *Aegyptianella* and related genera were known as veterinary pathogens long before they were recognized as human pathogens. Work in this area by dedicated veterinary microbiologists has contributed tremendously to the recognition and understanding of the diseases they cause in humans.

Canine Ehrlichiosis

Several agents produce infections in dogs, as shown in Table 1, which include canine monocytic ehrlichiosis caused by *E. canis* and *E. chaffeensis,* granulocytic ehrlichiosis caused by *E. ewingii,* granulocytic anaplasmosis caused by *A. phagocytophilum,* and canine cyclic thrombocytopenia caused by *A. platys* (53, 54, 118, 129). *Neorickettsia helminthoeca,* agent of salmon poisoning disease, also causes infection in dogs; it infects mononuclear cells and is acquired after ingesting salmonid fish encysted with a fluke harboring the organism (118).

Equine Ehrlichiosis

Potomac horse fever is a febrile gastrointestinal disease of horses caused by *N. risticii,* which infects monocytes (67), and is acquired through a complex aquatic ecosystem where infected insects seem to be implicated (89).

Equine granulocytic ehrlichiosis caused by *A. phagocytophilum,* formerly *E. equi,* is a seasonal disease first described in 1969. Although it has been described as occurring mostly in the foothills of Northern California (88), it also occurs in other regions of the United States and Europe. It causes a self-limiting disease in horses, which is similar to HGA in humans (15) and appears to be transmitted in California through the bite of infected *Ixodes pacificus* ticks. Tick-borne fever of ruminants is caused by the granulocytotropic *A. phagocytophilum,* formerly *E. phagocytophila,* and is transmitted via the bite of infected *I. ricinus* ticks in Europe (58).

Anaplasmosis of Ruminants

A. marginale is the etiologic agent of bovine anaplasmosis, the most prevalent tick-borne disease of cattle worldwide. Mechanical transmission by biting flies and other insects may also occur. Organisms develop as an intraerythrocytic inclusion and can produce severe anemia, weight loss, abortion, and sometimes death. A feature of this infection is the persistent low-level bacteremia even after clinical recovery; therefore, infected animals become a reservoir for transmission to other animals. This feature is shared with the related *A. phagocytophilum* infections of deer and *A. platys* infection of dogs. Antigenic variation has been postulated to play a role in the persistence of *A. marginale* infection and transmission (108).

Aegyptianella Infection of Birds and Amphibians

Members of the genus *Aegyptianella* infect erythrocytes of different animal species throughout the world. The type species is *Aegyptianella pullorum* of birds. Other species such as *A. bacterifera* have been associated with infections in amphibians. *A. botuliformis,* which infects helmeted guineafowls in Africa, appears to be transmitted through the bite of infected *Amblyomma* species (71). Based on 16S rRNA and *groEL* gene sequencing, *A. pullorum* is most closely related to *Anaplasma* spp.

Heartwater (Cowdriosis)

Ehrlichia (Cowdria) ruminantium is an intracellular bacterium that infects neutrophils and endothelial cells of cattle, sheep, goats, and certain wildlife species (118). Heartwater is an acute disease that can cause mortality rates up to 90% in susceptible ruminants. It occurs most frequently in sub-Saharan Africa and in the islands of the Atlantic and Indian Oceans and the Caribbean Sea and is transmitted by *Amblyomma* tick vectors.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

EDTA- or acid-citrate-dextrose-anticoagulated blood or cerebrospinal fluid (CSF) and serum should be obtained for diagnosis or confirmation of HME or HGA. Currently, there are three methods for diagnosis during the acute phase of illness, when ehrlichiae are likely to be present in circulating peripheral blood or CSF leukocytes: PCR amplification of *Ehrlichia* or *Anaplasma* species nucleic acids, detection of morulae in the cytoplasm of infected leukocytes by nonspecific Romanowsky stains (e.g., Giemsa or Wright) or by specific immunocytologic or immunohistologic stains using *E. chaffeensis* or *A. phagocytophilum* antibodies, and culture of *Ehrlichia* or *Anaplasma* species from blood or CSF. EDTA-anticoagulated blood is the most useful specimen for most tests and should be obtained during the acute phase of illness (1, 61, 103).

Samples for PCR should be tested promptly, but if the blood is maintained at 4°C, it may still be possible to amplify nucleic acids several days to a week or more later. If delays beyond several days are anticipated, the blood should be frozen at –20°C until used. There is less experience with collection and storage of CSF for PCR, but it is expected that similar conditions will yield appropriate results. PCR using serum or plasma is far less sensitive than PCR using samples that contain infected leukocytes.

Peripheral blood buffy coat smears or cytocentrifuged preparations of CSF cells should be prepared within several hours of obtaining the samples since leukocytes degenerate rapidly. Once prepared, air-dried blood smears and cytocentrifuged CSF preparations are stable at room temperature for months or years.

The culture conditions for *Ehrlichia* and *Anaplasma* species are still being optimized. Currently the preferred specimen for culture is peripheral blood. Samples should be obtained by sterile venipuncture or lumbar puncture and submitted as soon as possible to the laboratory that is to attempt culture. Usually, this requires overnight courier service since culture is currently performed only in a few public health and research laboratories. Samples to be cultured should be maintained at approximately 4°C during shipping; it is important to avoid freezing, which is likely to reduce the content of viable ehrlichiae. Success rates in culturing *A. phagocytophilum* are far better than those for *E. chaffeensis*. The time elapsed between collection of the blood sample and inoculation in cell lines appears to be especially critical for *E. chaffeensis* isolation. EDTA-anticoagulated blood stored for up to 18 days at 4°C has also been successfully used to culture *A. phagocytophilum* in vitro (78). After cultivation, ehrlichia-infected cells may be stored in a frozen state at −80°C for several months or in liquid nitrogen for longer periods. Storage of infected cells is best accomplished when more than 50 to 90% of the host cells are infected and is achieved by suspension of at least 10^6 cells per ml in tissue culture medium that contains 10% dimethyl sulfoxide (DMSO) and at least 30% fetal bovine serum.

LABORATORY CONFIRMATION OF *EHRLICHIA CHAFFEENSIS*

Direct Examination

Microscopy by Romanowsky Staining of Peripheral Blood

Patients with suspected HME should have Romanowsky-stained (Giemsa or Wright stain) peripheral blood or buffy coat leukocytes examined for the presence of ehrlichial morulae. This method is very insensitive, identifying up to 29% (2 of 7) of culture-confirmed *E. chaffeensis*-infected patients (127). Ordinarily *E. chaffeensis* organisms are detected predominantly in monocytes, and their detection seems to correlate with severity of infection. When present, ehrlichial morulae are small (1- to 3-μm diameter) round-to-oval clusters of bacteria that stain basophilic to amphophilic with Romanowsky stains (Fig. 4). These clusters are present in the cytoplasm and have a stippled appearance owing to individual bacteria within the vacuole. Detection of morulae is more common in immunocompromised patients. Numbers of infected cells range from 0.2 to 10%, suggesting that up to 500 cells should be examined in suspicious cases.

Antigen Detection by Immunohistology

Immunohistologic methods may be used to identify *E. chaffeensis* within human tissues, including bone marrow, liver, and spleen. An immunohistologic study of bone marrow, however, detected *E. chaffeensis* in only 40% of specimens obtained during the active infection (48). Most immunohistologic studies have been performed using polyclonal antibodies that react with other *Ehrlichia* species. A monoclonal antibody may specifically detect *E. chaffeensis* in human tissues (150). Unfortunately, commercial sources for direct immunohistologic detection of *E. chaffeensis* are not currently available.

Nucleic Acid Detection Techniques

The most widely used nucleic acid amplification test is PCR amplification of *E. chaffeensis* nucleic acids from clinical samples by using the HE1/HE3 primer set (7, 52, 126). This primer pair amplifies a 389-bp fragment of the 16S rRNA gene

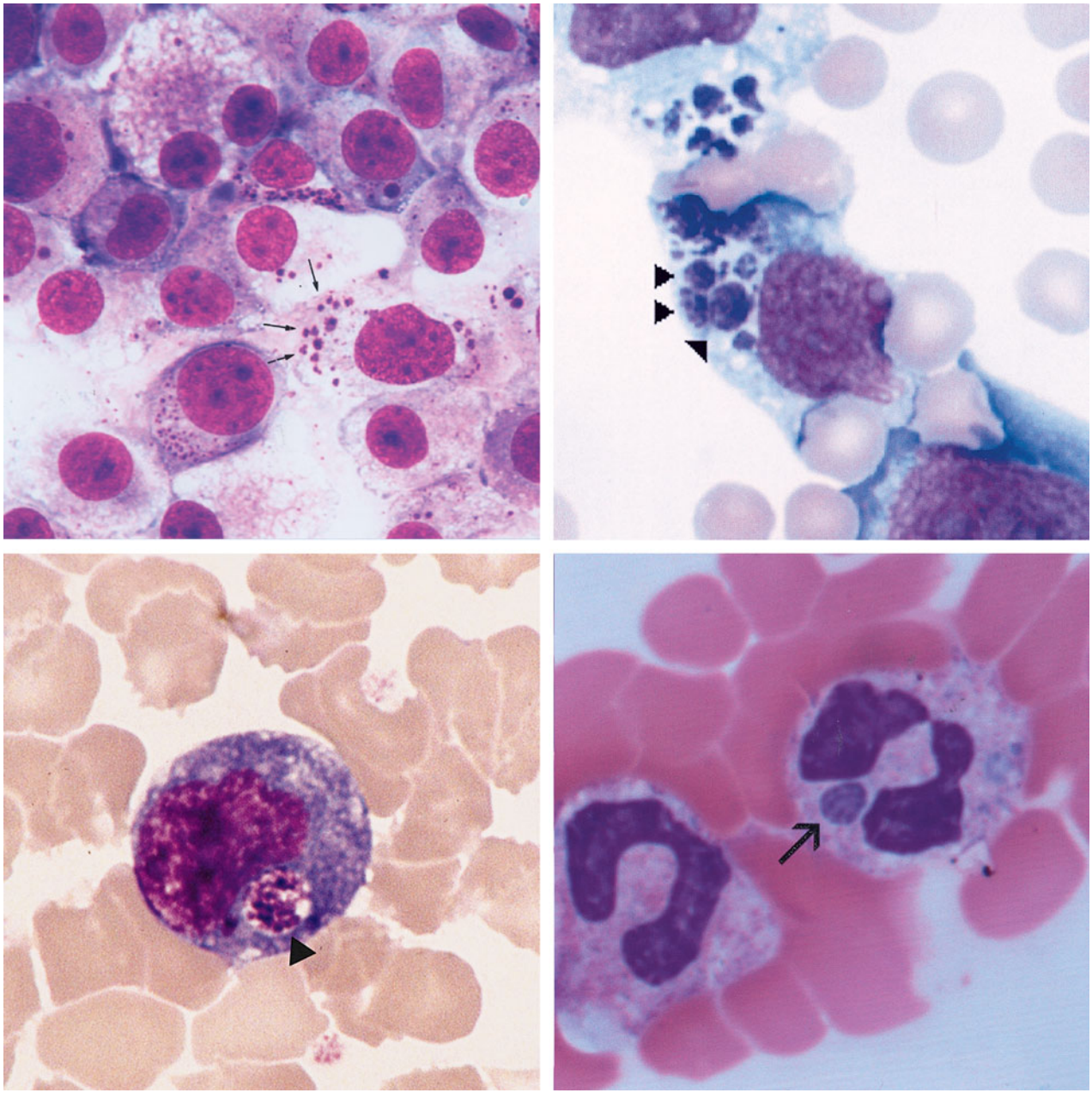


FIGURE 2 (top left) *E. chaffeensis* cultured in the canine histiocyte cell line, DH82. Note the presence of basophilic, stippled, intracytoplasmic inclusions approximately 2 to 3 μm in diameter (arrows). The smaller intracytoplasmic granules may also be ehrlichial morulae (Romanowsky [Leukostat] stain; original magnification, ×1,000).

FIGURE 3 (top right) *A. phagocytophilum* cultured in the human promyelocytic cell line HL-60 from the blood of an infected patient. Note the presence of multiple basophilic, stippled, intracytoplasmic inclusions (arrowheads) in an HL-60 cell (Wright stain; original magnification, ×1,000).

FIGURE 4 (bottom) *E. chaffeensis* (A [left]) and *A. phagocytophilum* (B [right]) in peripheral blood leukocytes. Note that the *E. chaffeensis* morula (arrowhead) is present in a monocyte (A) and that *A. phagocytophilum* morula (arrow) is present in a neutrophil (B) (Wright stain; original magnification, ×1,000).

sequences. The product may be detected by simple nucleic acid staining (e.g., ethidium bromide) after agarose gel electrophoresis or by Southern hybridization of the amplified products, using an internal probe that is reported to increase analytical sensitivity (38). A clinical evaluation of *E. chaffeensis* PCR using the HE1/HE3 system showed a sensitivity of 79 to 100% compared with detection of *E. chaffeensis* or *E. canis* antibodies in convalescence; however, *E. chaffeensis* nucleic acids were frequently detected in patients who never developed antibodies, a finding of uncertain significance given the high degree of analytical specificity demonstrated with this system (7, 126). Similar results occur when using a nested PCR that employs broad-range "*Ehrlichia* genus" primers in an initial step followed by PCR with the HE1/HE3 primer pair (80). A nested PCR assay with broad-range 16S rRNA primers (8F and 1448R) followed by a second (nested) reaction with primers 15F and 208R on whole blood yielded similar results to culture in another study (127). Other targets for PCR that have not been fully evaluated for clinical sensitivity or specificity include the *groESL* operon, a variable-length PCR target of unknown significance that is present in *E. chaffeensis* (105, 127, 131), the 120-kDa antigen gene that encodes an immunodominant antigen with tandemly repeated subunits that may vary among *E. chaffeensis* isolates, the quinolinate synthase A gene, *nadA,* and the p28 multigene family (105, 136, 152, 153). In a prospective study, the overall PCR sensitivity and specificity were 56 and 100%, respectively, using the 16S rRNA subunit, *nadA,* and 120-kDa protein genes (102). However, in this study several blood samples had high titers of antiehrlichial antibodies by indirect fluorescent-antibody (IFA) and were in the late phases of the disease, suggesting that the sensitivity was decreased due to lower numbers of circulating ehrlichiae. When sensitivity was calculated based on seroconversion rates, it increased to 84%. Posttest probabilities for a positive and negative PCR result were 96 and 11.1%, respectively. Posttest probabilities take into account a clinical case definition and therefore are most useful when used in the appropriate clinical setting (102).

Real-time multicolor PCR and real-time multiplex reverse transcriptase PCR assays have been developed recently with extremely high analytical sensitivity and specificity comparable to that of nested PCR assays (45, 125). Advantages of this technique include a lower risk of contamination, greater speed, lower cost, and a multicolor and multiplexing format allowing detection of multiple ehrlichial pathogens at the same time.

Isolation Procedures

E. chaffeensis has been isolated from peripheral blood of more than 30 patients with HME, and an *E. canis*-like organism was isolated only once from an asymptomatic human (42, 107, 111, 127). The most frequently used cell for primary isolation is the canine histiocytic cell line, DH82; however, *E. chaffeensis* has been successfully cultivated in other cells including the human macrophage-like THP-1 cells, the fibroblast-like HEL-22 cells, Vero cells, and HL-60 cells (human promyelocytic cell line differentiated to monocytic pathway), among others (63). Isolation may be successful even when infected leukocytes are not observed on examination of peripheral blood (127). The usual format for isolation involves direct inoculation of leukocyte fractions or, less optimally, whole blood into flasks with a confluent layer of adherent cells or into flasks that contain approximately 2×10^5 to 1×10^6 nonadherent cells per ml of tissue culture medium. Macrophage-like cells that are highly phagocytic may be adversely affected by the presence of numerous erythrocytes; thus, it is recommended that either (i) leukocytes be fractionated from erythrocytes by density gradient centrifugation (e.g., Ficoll-Paque), (ii) leukocytes be harvested after erythrocyte lysis (hypotonic lysis, $NHCl_4$ lysis, etc.), or (iii) cell confluency be reestablished after cultivation with erythrocyte-containing samples by addition of uninfected host cells. Since *E. chaffeensis* may be present in a very small proportion of peripheral blood leukocytes, it is usually advisable to inoculate cultures with as many peripheral blood leukocytes as possible. This may be difficult owing to leukopenia that often accompanies HME. A method using 2 to 3 ml of EDTA-anticoagulated blood, diluted in 2 volumes of sterile Hanks' balanced salt solution, followed by Histopaque (Sigma, St. Louis, Mo.) gradient separation of leukocytes gave a high yield of isolation in a study of the use of culture to diagnose HME (127).

The blood mononuclear cells are resuspended in a 2-ml volume of tissue culture medium supplemented with 5% fetal bovine serum and allowed to interact with adherent host cells in a 25-cm^2 flask for 3 h, usually enhanced by incubation with rocking at 37°C in 5% CO_2. The inoculum is removed if significant erythrocyte contamination is present and the monolayer is replenished with 5 ml of fresh tissue culture medium. Since *Ehrlichia* species are bacteria, antibiotics in the medium must be avoided. The generation time of *E. chaffeensis* is approximately 19 h (19), and thus, cultures must be maintained to allow a slow logarithmic or stable growth phase to avoid the host cells outgrowing the ehrlichiae.

Identification

The presence of infected cells is determined by sampling the medium (DH82 cells and THP-1 cells) or by lightly scraping part of the monolayer. Aliquots of the culture are cytocentrifuged, followed by Romanowsky or immunofluorescent stains, and cells are examined for the presence of intracytoplasmic morulae or *E. chaffeensis* antigen (Fig. 2). Culture may require 1 month or more but has been achieved in as short a time as a few days (42, 47, 127). Confirmation of the infectious agent is currently best achieved by PCR amplification using species-specific primers (5).

Serologic Tests

The majority of cases of HME are diagnosed by retrospective serologic confirmation, and serology is the most sensitive method for diagnostic confirmation. The most widely used test is the IFA test, which utilizes ehrlichia-infected cells fixed to glass slides. Ehrlichial antigens may be difficult to prepare and are available mostly through public health and research laboratories, although commercial production and distribution are now available. Commercial sources of IFA serodiagnostic kits include Focus Technologies (Cypress, Calif.) and PanBio Diagnostics (Columbia, Md.). Immunoblot procedures are becoming increasingly popular because of the perception of increased specificity (25, 35, 146).

Currently, there is little standardization for any method of ehrlichial serology, and cutoff titers are dependent on validation in individual laboratories that perform these assays. The algorithm for serologic testing by IFA includes an initial screen at a dilution of 1:64 or 1:80 for antibodies to *E. chaffeensis*. Reactive samples are then titrated.

Ehrlichia chaffeensis IFA

E. chaffeensis antibodies are detected by IFA using *E. chaffeensis* Arkansas strain-infected DH82 canine macrophage-like cells. Sera are serially diluted starting at a dilution of 1:64. The presence of antibodies is detected after incubation with fluorescein isothiocyanate-conjugated anti-human immunoglobulins. A positive reaction is detected by the fluorescent

demonstration of typical intracytoplasmic ehrlichial morula morphology. It is important to identify the appropriate proportion of infected cells as determined by a positive control serum and the appropriate morphology for each antigen preparation to preclude false-positive interpretations. Prescreening for autoantibodies or routine removal of rheumatoid factors lessens the risk of misinterpretation due to antibodies reactive with cellular components including nuclear or cytoplasmic antigens. A fourfold increase or decrease in antibody titer, with a peak titer or a single convalescent titer of ≥64 for a patient with a consistent clinical history, supports the diagnosis of HME. Antibody titers may be detected in a small proportion of subjects without HME owing to the presence of antigens that are highly conserved among bacterial species, including heat shock proteins (44, 146). Acute-phase sera should be obtained at the time of presentation with acute illness, and convalescent sera are best obtained 3 to 6 weeks later (44).

The sensitivity and specificity of the IFA for *E. chaffeensis* infections in the convalescent phase are not known but are assumed to be high because of a high degree of correlation of the presence of *E. chaffeensis* antibodies and characteristic clinical findings (57). In the early phases of the disease, IFA testing is not sensitive when compared to PCR (37). A systematic evaluation of the usefulness of immunoglobulin M (IgM) testing is not available, although a preliminary evaluation based on nine culture-confirmed cases suggests that it might be slightly more sensitive than IgG for the diagnosis of HME during the acute phase (37). Previously, the serologically cross-reactive *E. canis* was used as a surrogate antigen; however, this serodiagnostic assay has a lower sensitivity than that obtained using *E. chaffeensis,* and its use should be discouraged (7). The role of immunoblots in diagnosis is not well established; however, many patients with *E. chaffeensis* infection can be differentiated from patients with HGA by the demonstration of antibodies reactive with one or more of the 22-, 28-, 29-, 46-, 54-, or 120-kDa antigens of *E. chaffeensis* (25, 35). A recombinant 120-kDa protein that has been applied in a dot blot method appears to offer a sensitive and specific serologic confirmation tool (151). Antibodies to *E. chaffeensis* have also been detected in patients diagnosed with Rocky Mountain spotted fever, Q fever, brucellosis, Lyme disease, and Epstein-Barr virus infections, suggesting that false-positive reactions do occur (44, 146). Antigenic diversity among isolates of *E. chaffeensis* is well described (36) but may not affect the detection of the polyclonal antibody response that is generated with human infection. Several reports have characterized patients with HME without antibody responses, even long after onset (52, 121, 127). However, in the few cases in which *E. chaffeensis* infection has been most clearly proven by cultivation of the agent, most patients who survived developed clear serologic reactions detected by IFA methods in convalescence (36, 42, 47, 105, 127). Hypothetical reasons for false-negative results include infection by antigenically diverse strains (unproven) and abrogation of antibody response by early therapy (127).

LABORATORY CONFIRMATION OF *ANAPLASMA PHAGOCYTOPHILUM*

Direct Examination

Microscopy by Romanowsky Staining of Peripheral Blood

Examination of Romanowsky-stained (Giemsa or Wright stain) peripheral blood or buffy coat leukocytes for the presence of ehrlichial morulae is highly valuable in the diagnosis of HGA. Usually, 800 to 1,000 granulocytes are examined under ×500 to ×1,000 magnification for the presence of morulae (1, 3, 13). Since most patients presenting with positive smears have less than 1% of infected granulocytes and usually have leukopenia, buffy coat preparations have a higher yield than peripheral smears. Infection rates as high as 40% of peripheral granulocytes have been described (11). As for HME, the presence of detectable infected granulocytes in peripheral blood seems to correlate with the severity of infection (3, 11, 13). The sensitivity of the buffy coat smear examination in the acute phase of HGA is approximately 60% (14). When present, ehrlichial morulae are small (1- to 3-μm diameter) round-to-oval clusters of bacteria that stain basophilic to amphophilic with Romanowsky stains (Fig. 4). These clusters are present in the cytoplasm of neutrophils or eosinophils and have a stippled appearance owing to individual bacteria within the vacuole.

Immunohistology for Antigen Detection

Immunohistologic methods may also be used to identify *A. phagocytophilum* within human tissues, including bone marrow, liver, and spleen.

Nucleic Acid Detection Techniques

Multiple PCR assays for detection of *A. phagocytophilum* nucleic acids have been published (51, 96, 130). Most utilize regions of the 16S rRNA gene that are relatively specific as targets for amplification. The most frequently applied and evaluated method employs the primer set ge9f and ge10r, which amplifies a 919-bp fragment, most often used as a single stage reaction, with or without a hybridization probe to enhance sensitivity (34, 51). A popular alternative is the use of nested PCR with an outer set of primers that anneal to and amplify eubacterial 16S rRNA genes, followed by an internal PCR with *A. phagocytophilum*-specific primers (96, 126). PCR amplification of the *groESL* region by use of a nested reaction has also been useful to detect ehrlichial DNA in blood during the acute phase. Primers HS1 and HS6 are used in the primary reaction followed by primers HS43 and HS45. The size of the amplified product distinguishes *E. chaffeensis* from *A. phagocytophilum* (528 versus 480 bp, respectively) (130). The analytical sensitivity and specificity of several published primer sets were evaluated by Massung and Slater (96) by using DNA extracted from serial dilutions of *A. phagocytophilum*-infected HL-60 cells. Specificity was evaluated using DNA extracted from cultures of *E. chaffeensis, Rickettsia rickettsii,* and *Bartonella henselae*. The primer sets with the greatest sensitivity and specificity were those used in a nested reaction to amplify the 16S RNA gene, ge3a-ge10 and ge9-ge2, and those amplifying the *msp2* gene, msp2-3f-msp2-3r (96). Both PCR assays detected as few as 0.25 infected HL-60 cell. Most recently a multiplex assay to detect *Ehrlichia* and *Anaplasma* spp. by real-time reverse transcriptase-PCR was developed and evaluated in peripheral blood of dogs suspected of ehrlichiosis (125). The assay has a sensitivity of 100 16S rRNA transcripts, which corresponds to about one infected cell in a test sample, can detect single or multiple infections, and has the potential for automation.

Isolation Procedures

A. phagocytophilum has been successfully cultivated more often from human patients than has *E. chaffeensis, E. canis,* or *N. sennetsu* owing to the higher quantity of organisms that are present in peripheral blood in many infected patients (1). More than 65 isolates have been cultured from infected patients at a single medical center in the United States. Although HGA has

been described in Europe and Asia, no human isolates of *A. phagocytophilum* have been reported yet in any locality outside the United States. Few animal isolates, however, have been cultured in Europe (16, 144). Isolation is best achieved in the human promyelocytic cell line HL-60 (61) and has also been accomplished when ehrlichiae are not observed in peripheral blood smears. The optimal conditions for recovery of these bacteria have not been conclusively determined, but cultivation in HL-60 cells has been achieved with or without the presence of granulocyte-differentiating chemicals (e.g., DMSO or retinoic acid) (64). Because erythrocytes do not adversely affect the HL-60 cells, direct inoculation of EDTA-anticoagulated blood is effective. Fractionation of blood into leukocytes by preparation of buffy coat or by isolation of granulocyte fractions by density gradient centrifugation is also effective (119). Ordinarily, approximately 100 to 500 μl of EDTA-anticoagulated blood containing approximately between 10^2 and 10^4 infected granulocytes is inoculated into a total of approximately 100-fold more uninfected HL-60 cells that are in the exponential growth phase and are subsequently maintained at a concentration between 2×10^5 and 1×10^6 cells per ml of tissue culture medium.

Identification

Cultures are examined every 2 to 3 days by Romanowsky staining of cytocentrifuged preparations of 20 to 50 μl of culture suspensions. Ehrlichial morulae appear as small aggregates of basophilic bacteria in the cytoplasm of the HL-60 cells (Fig. 3). Since HL-60 cells may contain a variety of cytoplasmic granules, immunocytology can be very helpful for the inexperienced laboratorian. Unfortunately, immunohistological reagents are currently not commercially available. Cultures usually require between 5 and 10 days of cultivation before morulae are clearly identified, but infected cells may be detected as early as 3 days postinoculation. Time to detection of organisms in culture correlates with the amount of bacteria present in blood at the time of culture inoculation (78). Definitive identification is achieved by PCR amplification using species-specific primers (34, 60, 109) or by sequence analysis of 16S rRNA genes that have been amplified by universal eubacterial PCR (34). The exact length of incubation before cultures are considered negative is still unknown, but they should be kept for at least 14 days, with the cell density being maintained at about 2×10^5/ml.

Serologic Tests

A. phagocytophilum IFA

A. phagocytophilum is a single species that now includes three previously distinct organisms, *E. phagocytophila, E. equi,* and the former HGE agent (15, 34, 61). Thus, IFA serologic testing is performed using any of these antigens (46, 72, 140). Previously, most testing was performed using neutrophils obtained from the blood of animals experimentally infected with *E. phagocytophila* or *E. equi* (11, 46, 90). Currently, the preferred method for testing human sera is the use of a human isolate propagated in the human HL-60 promyelocyte cell line (4, 41, 72, 114). It is now well demonstrated that antigenic diversity exists among *A. phagocytophilum* isolates, but it is unlikely that such diversity affects detection under clinical circumstances (10, 140). Interpretation of immunofluorescent patterns is similar to that for *E. chaffeensis* and requires an experienced microscopist. IFA kits are currently available from Focus Technologies and PanBio Diagnostics.

Sera should be screened at a single dilution (1:64 or 1:80), and the presence of antibodies is determined after incubation with fluorescein isothiocyanate-conjugated anti-human IgG/IgM. If specimens test reactive, they are serially diluted to determine the end point titer. A serologic confirmation of the diagnosis is achieved when a fourfold rise in titer is demonstrated in convalescence with a minimum titer of 80, or when a single antibody titer of ≥80 is demonstrated in a patient with typical clinical features of HGA (3, 13). Approximately 25 to 45% of infected patients have antibodies at the time of presentation (3, 4, 13); however, up to 11 to 14% of the population possesses antibodies in some highly endemic regions, rendering this early serologic information less useful (2, 12). The typical response during acute infection is a rapid rise (within 2 weeks of onset) in antibody levels reaching high titers (≥640) within the first month (4). In a treated cohort of patients whose diagnosis of HGA was confirmed by culture, the antibody titers declined gradually over the next few months and about one-half of the patients had detectable antibodies by IFA 1 year after infection (4). However, many patients have antibodies detectable for months to years after initial infection (14).

The sensitivity and specificity of the HGA serologic tests are both believed to be high because of good correlation between typical clinical cases and serologic reactions to *A. phagocytophilum* group antigens (3, 4, 13). HGA serology had a sensitivity of 91.3% in a group of 24 culture-confirmed patients (4) and a median sensitivity of 95% among a group of 28 patients diagnosed by culture, PCR, or the presence of morulae in blood smears (140). IgM testing appears to be a useful tool for identification of recent infection, although the sensitivity may not be as high as that for IgG testing (140). Although enzyme-linked immunosorbent assays (ELISA) and immunoblots have been described (46, 114), they are not routinely used in the serology of HGA. Immunoblots may be used to differentiate among *A. phagocytophilum* and *E. chaffeensis* infections by demonstration of a major *A. phagocytophilum* antigen of approximately 44 kDa in sera of HGA patients (46, 72, 146). Cross-reactivity between *E. chaffeensis* and *A. phagocytophilum* group has been observed in IFA. Under those circumstances, testing with both antigens often shows higher titers with antigens of the homologous infecting agent (3, 140).

False-positive reactions have been observed in patients with other rickettsial infections, Q fever, and Epstein-Barr virus infections. Many patients with HGA develop antibodies that react with *B. burgdorferi* by ELISA and demonstrate diagnostic IgG or IgM immunoblots (148). Although some patients have been confirmed by culture to have concurrent infection with *A. phagocytophilum* and *B. burgdorferi,* the statistical probability that this mechanism accounts for most of these concurrent positive serologic results is very low (99, 146). Another explanation is the exposure to the various tick-borne agents at different times rather than by a single tick bite. The most frequent scenario is the presence of antibodies to the various agents in individuals living in areas of high endemicity (12, 92). Autoantibodies to platelets and other leukocyte components can cause false-positive IFA tests (147).

Other immunoassays employing recombinant p44 antigens have been developed and show promise as alternative tests to IFA (133, 154). Antigens of 42 to 49 kDa are immunodominant in *A. phagocytophilum* infection and are encoded by a multigene family (31, 98) similar to the major surface proteins *(msp)* of *A. marginale* (108).

ANTIMICROBIAL SUSCEPTIBILITIES

Routine antimicrobial susceptibility testing of *Ehrlichia* or *Anaplasma* species isolates is unnecessary. These bacteria are maintained enzootically by transmission among ticks and

feral mammalian reservoir hosts (6, 85, 109, 135, 139). The level of exposure of such vertebrate and invertebrate hosts to antimicrobial selection factors is very low, and thus antimicrobial resistance is very unlikely. Most patients with either HME or HGA become afebrile and have clinical improvement within 48 h of therapy with a tetracycline antibiotic, including doxycycline, the drug of choice (13, 57). Retrospective analysis of patients with HME has shown a significant degree of efficacy for tetracyclines or chloramphenicol compared with other broad-spectrum antimicrobials (57). With in vitro studies, tetracycline antibiotics are uniformly bactericidal for *Ehrlichia* and *Anaplasma* species, whereas the MICs of chloramphenicol cannot be safely achieved in humans with HME or HGA (26, 69, 80). In contrast, the most frequently used antibiotics for patients who have had recent tick bites, amoxicillin and ceftriaxone, and broad-spectrum antibiotics that might be prescribed for undifferentiated fever such as other cephalosporins, aminoglycosides, and macrolides are not effective for inhibition of ehrlichial growth in vitro. The rifamycins (rifampin and rifabutin) can achieve effective inhibition or killing of *Ehrlichia* and *Anaplasma* species in vitro, and the fluoroquinolones (ofloxacin, levofloxacin, and trovafloxacin) have very low MICs for human isolates of *A. phagocytophilum* (69, 80). Rifampin has been successfully used to treat HGA during pregnancy and appears to be a useful alternative for patients who cannot receive tetracyclines (28). A recent publication describes a method to evaluate in vitro susceptibility testing by real-time PCR, showing *E. chaffeensis* to be susceptible to doxycycline and rifampin and to have partial susceptibility to the fluoroquinolones. Resistance to macrolides, cotrimoxazole, and beta-lactam compounds was confirmed (19).

Whereas persistent infections with *Ehrlichia* and *Anaplasma* species may occur frequently in naturally and experimentally infected animals even after treatment with tetracycline (58, 76), persistence of ehrlichiae in humans has rarely been documented (49, 121). Therapy is usually highly effective at eliminating ehrlichiae from the blood of infected humans.

INTERPRETATION AND REPORTING OF RESULTS

The current algorithm for identification and confirmation of infections by *Ehrlichia* and *Anaplasma* species requires a complete evaluation of the history, physical examination, and laboratory results that will suggest the diagnosis. Before specific tests for etiologic determination are attempted, a decision concerning therapy should be made since delays may lead to increased morbidity and perhaps mortality. At the time of acute illness and prior to institution of antiehrlichial therapy, EDTA-anticoagulated blood should be obtained for PCR amplification of *E. chaffeensis* and *A. phagocytophilum* and considered for the possibility of in vitro cultivation. A peripheral blood or buffy coat smear should be examined for the presence of morulae that would confirm the presumptive diagnosis. Serum should be obtained and tested for antibodies to *E. chaffeensis* or *A. phagocytophilum*, with an aliquot saved for repeat testing when a convalescent serum is obtained 2 to 4 weeks later.

The presence of intracytoplasmic inclusions within a leukocyte in peripheral blood might be due to the presence of overlying platelets, Döhle bodies, toxic granulations, nuclear fragments, Auer rods in blasts, other bacteria, yeasts, inorganic materials, or granules of normal large granular lymphocytes or granulocytes. If the typical morphology of an *Ehrlichia* or *Anaplasma* spp. morula is observed, an assessment as to the hematopoietic lineage and the percentage of cells that contain morulae should be made and reported.

A positive PCR result should be reported as such, indicating the presence of *E. chaffeensis* or *A. phagocytophilum* DNA present, and it should be made clear that a positive PCR is not equivalent to the culture of ehrlichiae from blood. Laboratories that use a broad-range PCR to identify *Ehrlichia* or *Anaplasma* spp. DNA in blood may also detect *E. ewingii* infection that may mimic either HME or HGA (29).

IFA serologic results should be reported as the titer of antibodies determined to be reactive with *E. chaffeensis* or *A. phagocytophilum,* including the positive cutoff values determined in the laboratory. An interpretation should indicate whether the titers are considered "significant" or "positive" based upon a fourfold increase or decrease or as single high-titered serum. It should be remembered that infections with *E. ewingii* would yield serologic patterns considered diagnostic of *E. chaffeensis* that are difficult to differentiate even with immunoblot methods. Immunoblot analyses should provide information about antibodies that react with specific antigens considered unique or diagnostic of infection with a single species of *Ehrlichia* or *Anaplasma*.

Human ehrlichioses became nationally notifiable in 1999, but not all states report these diseases. For the purpose of surveillance, the Council of State and Territorial Epidemiologists (CSTE) and the CDC developed a case definition that was amended in 2000 to include ehrlichioses other than HME and HGA (http://www.cste.org/ps/2000/2000-id-03.htm). According to this definition, a case presenting with compatible clinical and routine laboratory abnormalities described above could be classified as confirmed or probable based on specific laboratory findings. A confirmed HME or HGA case is supported by (i) a fourfold change in antibody titer to *E. chaffeensis* or *A. phagocytophilum* antigen, respectively, by IFA in paired serum samples; or (ii) positive PCR and confirmation of *E. chaffeensis* or *A. phagocytophilum* DNA, respectively; or (iii) identification of morulae in leukocytes, and a positive IFA titer to *E. chaffeensis* or *A. phagocytophilum* antigen, respectively (based on a cutoff established by the laboratory performing the assay); or (iv) immunostaining of *E. chaffeensis* or *A. phagocytophilum* antigen, respectively, in a biopsy or autopsy sample; or (v) culture of *E. chaffeensis* or *A. phagocytophilum,* respectively, from a clinical sample. The diagnosis of other human ehrlichiosis or ehrlichiosis caused by an unspecified agent is supported by (i) demonstration of a fourfold change in antibody titer to more than one *Ehrlichia* or *Anaplasma* sp. by IFA in paired serum samples, in which the dominant reactivity cannot be determined; or (ii) identification of an *Ehrlichia* or *Anaplasma* sp. other than *E. chaffeensis* or *A. phagocytophilum* by PCR, immunostaining, or culture. According to the CSTE case definition, a probable HME or HGA case is that with a clinically compatible illness with either a single positive IFA titer (based on the cutoff established by the laboratory performing the assay) or the visualization of morulae in leukocytes.

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Coxiella

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TAXONOMY

Coxiella burnetii is the only species of the genus and has been placed in the γ subdivision of the class *Proteobacteria,* close to *Rickettsiella grylli, Legionella* spp., and *Francisella* spp., on the basis of comparison of the 16S rRNA-encoding gene sequences.

DESCRIPTION OF THE GENUS

C. burnetii, the organism responsible for Q fever, is a pleomorphic coccobacillus with a gram-negative cell wall (4). It is an obligate intracellular microorganism measuring 0.2 by 0.7 μm. It does not stain with Gram stain but does stain with Gimenez stain. *C. burnetii* undergoes a developmental cycle in which there is a large-cell and a small-cell variant (59). The small-cell variant attaches to the host cell (usually a macrophage) and is ingested. *C. burnetii* develops within the phagolysosome, where the acid pH activates its metabolic enzymes. Following maturation to the large-cell variant, sporogenesis begins (59). Spore formation explains why *C. burnetii* is so successful as a pathogen. It can survive for up to 10 months at 15 to 20°C, for more than 1 month on meat in cold storage, and for more than 40 months in skim milk at room temperature (12). *C. burnetii* was shown to survive in free-living amoebae, suggesting a possible mechanism to explain the survival and resistance of the pathogen in the environment (Fig. 1) (47).

C. burnetii undergoes phase variation akin to the smooth-to-rough transition of lipopolysaccharides (LPS) of gram-negative bacteria (91). In nature and in laboratory animals, it exists in the phase I state, in which the organisms react with late-convalescent-phase (45 days) guinea pig sera and only slightly with early-convalescent-phase (21 days) sera (91). Apparently phase II LPS is a deletion mutant of phase I LPS (96), of which the fitness, in vitro, is superior to that of phase I. After numerous passages in cell culture or embryonated eggs, truncation of the LPS occurs, yielding the antigenic form, phase II.

EPIDEMIOLOGY AND TRANSMISSION

Q fever is a worldwide zoonosis. *C. burnetii* has been identified in arthropods, fish, birds, rodents, marsupials, and livestock (3). Indeed, it naturally infects more than 40 species (including 12 genera) of ticks on five continents (4). Lice, mites, and parasitic flukes are also infected (64). Bandicoots, rats, rabbits, mice, porcupines, hedgehogs, tortoises, cattle, sheep, goats, dogs, swine, cats, camels, buffaloes, baboons, leopards, hyenas, chickens, ducks, geese, turkeys, pigeons, bats, and shrews have all been infected with this microorganism (43, 64).

The extreme infectivity of *C. burnetii* results in large outbreaks and makes it a potential bioweapon. The major route of transmission of Q fever to humans is by inhalation of dust particles contaminated with parturient fluids. The organism can stick on wool and dust and can be spread by the wind to distant places. Cases of Q fever have occurred miles away from the lambing site in the direction in which the winds blew (8, 93, 95). Ingestion of milk and milk products is a risk factor for transmission of Q fever (25). Raw milk from cows was considered a risk factor for Q fever in California, and the decrease in the prevalence of Q fever in this state could be partly related to cessation of the practice of drinking raw milk. However, the consumption of goat cheese made from raw as well as pasteurized milk was reported as a risk factor (25, 35). In experimental animal models, the route of infection (aerosol or intraperitoneal) has been shown to determine the specific organs that are involved (lung and liver, respectively) (45). Sexually transmitted Q fever has been suggested, but this route needs to be confirmed (61).

The current understanding of the natural history of Q fever is that a nonimmune patient comes into contact with *C. burnetii*, which causes a primary infection that could be asymptomatic or symptomatic (76). The symptomatic primary infection is acute Q fever. The spontaneous evolution of acute infection is usually a complete recovery in the normal host. In immunocompromised hosts, *C. burnetii* can multiply despite an antibody response following primary infection (symptomatic or not). In these cases, because the immune system is unable to control the infection, chronic infection develops. This hypothesis is supported by all available data from human beings as well as from animal models (75). Preliminary data support that the inoculum size, the route of infection, host factors, and the specific strain all contribute to the clinical expression (e.g., pneumonia versus hepatitis) of acute infection (75, 88).

The immune control of Q fever is T-cell dependent, but it does not lead to *C. burnetii* eradication, and immunosuppression can induce relapse of infection in apparently cured patients or laboratory animals (85, 86). *C. burnetii* DNA can be found in apparently cured people as well as in the dental pulp of experimentally infected and cured guinea pigs (1).

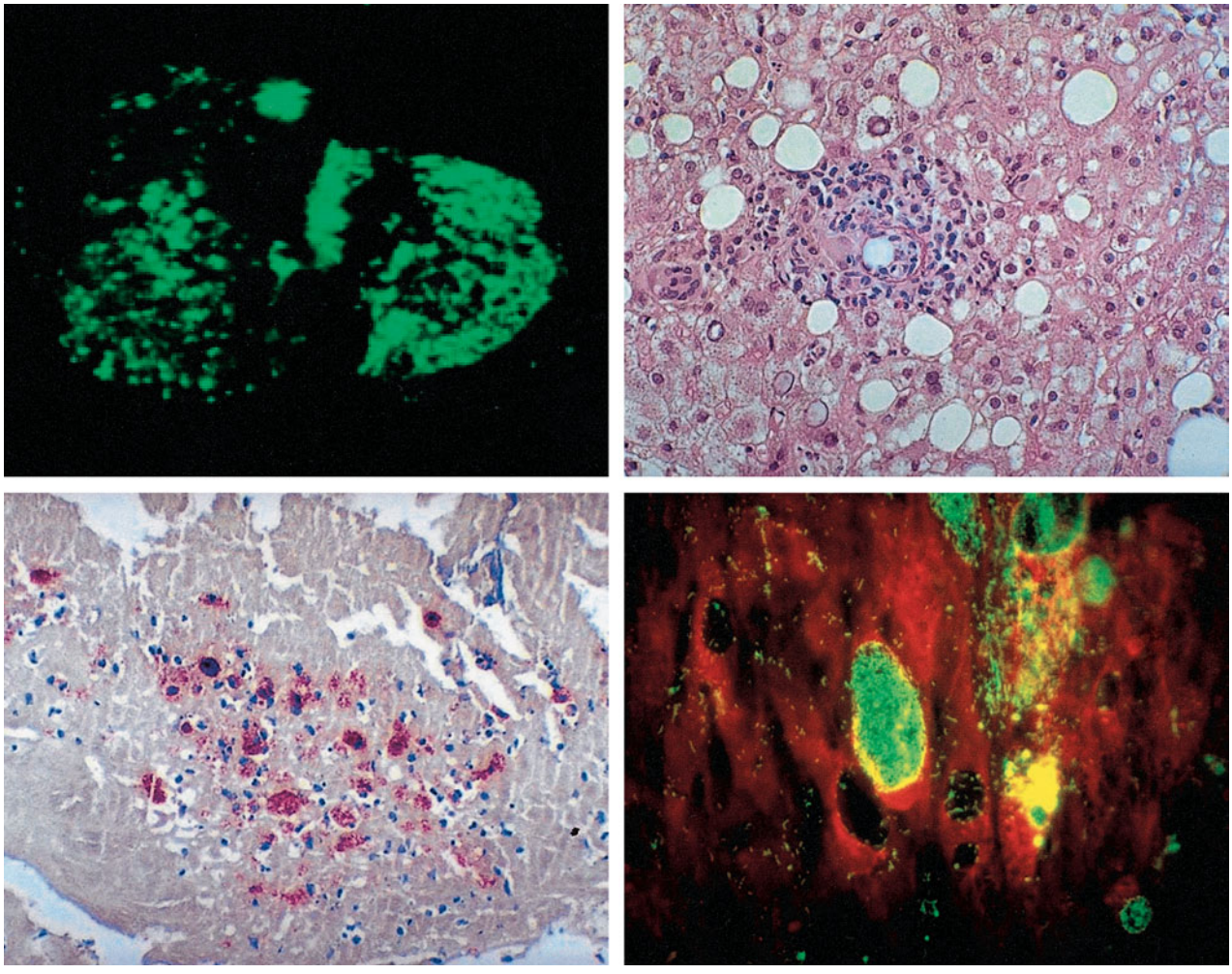


FIGURE 1 (top left) *Coxiella burnetii* in amoebae. Direct fluorescent-antibody stain with anti-C. *burnetii* monoclonal antibodies. Confocal microscopy. Magnification, ×1,000. (Courtesy of B. La Scola, Marseille, France.)

FIGURE 2 (top right) Hematoxylin and eosin stain of liver biopsy in a patient with acute Q fever. Doughnut ring granuloma. Magnification, ×400. (Courtesy of H. Lepidi, Marseille, France.)

FIGURE 3 (bottom left) Alkaline phosphatase immunohistochemistry on the heart valve from a patient with chronic Q fever endocarditis. C. *burnetii* microorganisms are stained pink within mononuclear cells. Magnification, ×400.

FIGURE 4 (bottom right) Identification of C. *burnetii* in shell vial culture at day 6 by the use of specific monoclonal antibody-based direct fluorescent-antibody test. Magnification, ×1,000.

Therefore, patients with acute Q fever who become immunocompromised or who have cardiac valve lesions are particularly at risk to develop chronic infection and especially endocarditis (19, 77).

Following primary infection, 60% of patients seroconvert without clinical manifestations and only 2% need to be hospitalized. A chronic infection develops in less than 1% of acutely infected patients and is often associated with a subtle and unique immune defect (58). Interleukin 10 (IL-10) seems to play a key role in the evolution of chronic infection (36).

Among children with Q fever, boys and girls are equally represented whereas men are 2.45 times more frequently infected than women (54). This has been suggested to be related to occupational exposure, although the protective role of 17β estradiol has been clearly demonstrated in a mouse model (48).

Since 30 to 50% of patients with Q fever and a valve lesion may develop Q fever endocarditis in the 2 years postinfection (19), echocardiography is recommended to detect such lesions as bicuspid aortic valve and minimal valvular leaks in all patients with acute Q fever (18).

CLINICAL SIGNIFICANCE

Acute Q Fever

The onset is usually abrupt, and patients present with high fever (91%), headaches (51%), myalgias (37%), arthralgias

(29%), and cough (34%), less frequently a rash (11%) or a meningeal syndrome (4%). Laboratory investigations show thrombocytopenia (35%), elevated liver enzymes (62%), and an elevated erythrocyte sedimentation rate (55%). The chest radiograph is abnormal in 27% of patients (77). The clinical presentation varies from country to country without clear explanation as to which one of the three major manifestations, i.e., isolated fever, hepatitis, or pneumonia, dominates.

Clinically isolated fever, without pneumonia or hepatitis, is usually associated with severe headache, and in some patients it persists long enough to meet the criteria for fever of unknown etiology. Pneumonia is the major clinical presentation of Q fever in Nova Scotia (Canada), the Basque country (Spain), and in the United Kingdom. Hepatitis is the most common form worldwide including France and Australia. Usually, increased liver enzymes suggest hepatitis; however, a few patients present with jaundice and/or hepatomegaly. Liver biopsy shows inflammatory granulomas, typically organized in the form of a "doughnut"; i.e., the granuloma contains a central lipid vacuole surrounded by a fibrinoid ring (58) (Fig. 2). Neurological manifestations of Q fever occur in 2% or more of cases and include meningitis, meningoencephalitis, peripheral neuropathy, and myelitis (5). Cardiac involvement occurs in 2% of acutely ill patients. This includes myocarditis (29), which led to death in two of the eight patients reported to date (29), and pericarditis, which is frequent but nonspecific (50). Among 204 patients with pericardial effusions, 10 (5%) were diagnosed as having Q fever by systematic testing (49). Other atypical presentations of acute Q fever have been reported such as acute acalculous cholecystitis (81) and isolated lymphadenitis from which *C. burnetii* has been cultured (26). Moreover, as *C. burnetii* occurs worldwide, it has been reported as a cause of fever in travelers returning from tropical countries (65) and may be associated with concurrent malaria (10).

Chronic fatigue following acute Q fever has been described in Australia and the United Kingdom (2, 66). It was reported that 20% of patients with post-Q-fever fatigue syndrome have moderate cytokine dysregulation (66). Q fever in immunocompromised hosts has been reported in patients with cancer (68) as well as in those with human immunodeficiency virus infection (52, 74). Q fever, when contracted during pregnancy, can result in abortion or neonatal death and in premature birth or low birth weight (71). About 4% of parturients in an area where Q fever is endemic have evidence of previous exposure to *C. burnetii,* and this correlates with adverse pregnancy outcomes (44). Twenty-three cases of Q fever during pregnancy have been reported (90). Most of these patients had complications including fetal or newborn death (11 cases) and premature birth (7 cases); only five patients had a normal pregnancy. One-half of the patients develop a serological profile of chronic Q fever during pregnancy, and, as in other mammals, *C. burnetii* can be isolated from the milk, the placenta, and the vaginal discharge. Multiple premature births have been reported in such cases (90), and these patients are subject to relapses during subsequent pregnancies. Patients with a valve lesion, an arterial aneurysm, or a prosthesis who have an episode of acute Q fever are at very high risk for chronic infection (77). Of acutely infected patients with a preexisting valvulopathy, 38% develop endocarditis within 2 years (19). In Greek children acute Q fever accounts for 3% of fever with unknown etiology (53). Children are less frequently symptomatic than adults and have milder disease, mostly pneumonitis (54). Chronic infection has rarely been reported, and when it occurs it manifests as endocarditis and osteomyelitis (11).

Chronic Q Fever

The major clinical form of chronic Q fever is endocarditis. At least 800 cases have been reported since 1949. In France, Q fever causes 5% of endocarditis cases with an estimated prevalence of $1/10^6$ inhabitants per year, which is close to what has been observed in Israel and Switzerland. In the largest published series of blood-culture-negative endocarditis, Q fever represented one-half (48%) of the etiologies, another 28% being due to *Bartonella* spp. and only 1% due to other fastidious growing microbes (38). The clinical presentation of patients with Q fever endocarditis varies according to the delays in diagnosis (70). A better knowledge of the disease has led to a significant reduction in this delay, explaining the decrease in the prevalence of heart failure, hepatomegaly, and abnormal liver function tests in this group of patients (37). Patients are usually afebrile or have low-grade intermittent fever.

Echocardiography is frequently inconclusive and fails to identify vegetations so that the diagnosis of endocarditis can easily be missed. A clue to the diagnosis is the presence of a known valve lesion and unexplained illness (fever, hepatitis, weakness, digital clubbing, weight loss, stroke, or renal insufficiency), an elevated erythrocyte sedimentation rate, increased serum transaminase levels, or thrombocytopenia. In such cases, positive Q fever serology complements the diagnosis made using modified Duke Criteria (27). Vascular infection is the second most commonly identified site of chronic Q fever. We have diagnosed 25 cases in our laboratory (28, 77), and 6 more were reported from other centers including England, the United States, Switzerland, and Australia (16, 17, 21, 60). Other manifestations of chronic Q fever include osteomyelitis (13), chronic hepatitis diagnosed in patients with alcoholism (77), pseudotumor of the spleen, pseudotumor of the lung, and infection of a ventriculoperitoneal drain (51). Recently, a case of vasculitis and pulmonary amyloidosis (41) following acute Q fever and resulting in the death of the patient was reported.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Biosafety level 2 practice and facilities are recommended for nonpropagative laboratory procedures including serological examination and staining of impression smears. Biosafety level 3 practice and facilities are recommended for activities involving inoculation, incubation, and harvesting of cell cultures, the necropsy of infected animals, and the manipulation of infected tissues (14). Although *C. burnetii* can be isolated from blood and a variety of tissues, this biosafety level is not feasible in most laboratories. Since the organism can withstand very harsh environmental conditions, it is unlikely that it will die during transport to a suitable laboratory. Blood should be collected in tubes containing EDTA or sodium citrate (heparin interferes with PCR), and the leukocyte layer should be saved for PCR. Solid specimens should be frozen at −80°C until they are cultured. Most laboratories depend on serological techniques to diagnose *C. burnetii* infection. For the diagnosis of acute Q fever, it is best that acute- and convalescent-phase serum samples collected 2 to 4 weeks apart be tested. Serum samples from patients with Q fever present no hazard to laboratory workers when handled using standard biosafety level 2 precautions.

LABORATORY DIAGNOSIS

Direct Examination

Antigen Detection

C. burnetii can be identified in tissues by a direct immunofluorescence technique. This is of limited utility for the routine diagnosis of C. *burnetii* since, with the exception of heart valve tissue from patients with Q fever endocarditis (9), tissue specimens from patients with acute Q fever are not generally submitted to the laboratory. The main reason for this is that the illness is mild to moderate in severity and death is extremely unusual. However, patients with Q fever endocarditis do have large numbers of C. *burnetii* organisms in the affected valvular tissue. These can be demonstrated by direct immunofluorescence (73) or electron microscopy. Other techniques that can be used to detect C. *burnetii* in tissues include immunohistology (9) (Fig. 3) and capture enzyme-linked immunosorbent assays (ELISAs) or enzyme-linked immunofluorescence assays (92).

Nucleic Acid Detection Techniques

PCR has successfully been used to detect DNA in cell cultures and clinical samples (89). Several genes have then been used to generate specific primers including 16S rRNA, 23S rRNA genes, superoxide dismutase, plasmid-based sequences, and the transposase encoding IS*111* multicopy insertion sequence gene (30). LightCycler nested PCR (LCN-PCR) using the IS*111* multicopy gene has been reported to be very sensitive. Because this is a closed glass capillary system, it does not pose more contamination risk than standard real-time PCR. It allows molecular detection of C. *burnetii* in sera from patients with Q fever endocarditis or vascular infection as well as in acute-phase sera of patients with acute Q fever (10, 20). LCN-PCR is particularly useful for early seronegative acute Q fever patients, but lacks sensitivity after 4 weeks of disease evolution. Thus, it is suggested that this test be added to serology in the first 2 weeks after the onset of symptoms to improve early diagnosis and in the 2 weeks following the onset of symptoms in the remaining seronegative patients only if suspicion of Q fever is high (31). Consequently, a positive PCR in a late-convalescent-phase serum should be carefully interpreted with the help of other conventional diagnostic methods and clinical data (84). LCN-PCR is also valuable for patients with phase I antibody titers between 800 and 6,400. In this range a positive PCR is correlated with chronic infection. For patients with chronic infection who have a higher level of antibody, the PCR is usually negative. Identification of C. *burnetii* at the strain level has been made possible with the use of a multispacer typing method allowing definition of three monophyletic groups among 158 isolates from all over the world (33). More recently a LightCycler PCR assay using hydrolysis probes has been used to detect C. *burnetii* in sera from patients with malaria (10).

Isolation Procedures and Identification

Isolation of C. *burnetii* must be done only in a biosafety level 3 containment facility due to its extreme infectivity. This microorganism can be isolated by inoculation of specimens into conventional cell cultures (e.g., Vero cells) (69), embryonated egg yolk sacs (63), or laboratory animals, such as mice or guinea pigs (39). The spleen of the inoculated animal is the most useful organ for the recovery of C. *burnetii.* Ground spleen extracts (0.2 to 0.5 ml of a 10% suspension) should be inoculated into embryonated eggs, which die 7 to 9 days later. These methods are used infrequently, but inoculation of animals remains helpful when the organism must be isolated from tissues contaminated with multiple bacteria or in order to obtain phase I C. *burnetii* antigens from phase II cells. The adaptation of a viral shell vial culture system using a human embryonic lung fibroblast (HEL cells) has improved the isolation of intracellular bacteria, especially C. *burnetii* (55, 69, 78). Plasma and buffy coat layers from heparinized blood are diluted 1:2 with Eagle's minimal essential medium (EMEM). Tissue specimens are homogenized in sterile phosphate-buffered saline (PBS) and diluted 1:2 in EMEM. Shell vials containing 12-mm-diameter round glass coverslips are seeded with 50,000 human embryonic lung cells in 1 ml of EMEM containing 10% fetal calf serum. The cells are incubated for 3 days at 37°C in an atmosphere containing 5% CO_2 until the monolayer is confluent. Then 1 ml of each homogenized specimen is placed into each of three shell vials. A 1-h centrifugation step (700 × *g* at 23°C) enhances the attachment and penetration of the bacteria into the cells. The supernatant is removed, and the monolayer is washed twice with PBS. Then 1 ml of EMEM containing 10% fetal calf serum is added to each vial. After an incubation period of 6 days, C. *burnetii* organisms are visualized as short rods by microscopic examination after staining with the Gimenez stain. Briefly, the infected cell smear is covered with carbol basic fuchsin (100 ml of 10% [wt/vol] basic fuchsin in 95% ethanol added to 250 ml of 4% [vol/vol] aqueous phenol and to 650 ml of distilled water) and allowed to stand 1 to 2 min. The smear is then washed in tap water and covered with malachite green solution (0.8% aqueous malachite green oxalate) for 6 to 9 s, washed again, and air dried (32). Confirmation of the presence of C. *burnetii* within the cells is performed by an indirect immunofluorescence assay with polyclonal or monoclonal anti-C. *burnetii* antibodies (Fig. 4) (69, 78). Positive cultures are passaged in cells several times to establish the isolated strain.

Serologic Tests

The microagglutination test (24, 64), the complement fixation test (22), the indirect immunofluorescent antibody (IFA) test (22, 62), and ELISA (97) have been used for the serological diagnosis of C. *burnetii* infection. With experimentally infected guinea pigs, ELISA is more sensitive than the IFA test. Antibodies against phase I whole cells are detected by day 9 with ELISA, by day 16 with the IFA test, and by day 20 with the complement fixation test. A commercial ELISA test (PanBio *Coxiella burnetii* immunoglobulin M [IgM] ELISA; QFM-200, Brisbane, Australia) has also been used for the serological diagnosis of acute Q fever, especially for the detection of IgM antibodies (23). However, the ELISA for the diagnosis of Q fever has not been standardized, rendering comparison of titers from laboratory to laboratory impossible. There are no accepted criteria for the diagnosis of acute versus chronic Q fever by this test. The complement fixation test is not as sensitive as the IFA test. Serum samples from about 20% of patients with acute Q fever are anticomplementary. A prozone phenomenon may be present in serum samples from some patients with chronic Q fever. This could result in a false-negative test result unless the laboratory is aware of this possibility.

The IFA test is the serological test of choice for the diagnosis of both acute and chronic Q fever. It is carried out by the procedure of Philip et al. (67) with purified whole-cell antigens at a concentration of 200 μg/ml. Both phase I and phase II antigens are used. Each antigen (phase I and II cells) is spotted onto slides, air dried, and fixed in acetone

for 15 min at room temperature (approximately 20°C). Serum dilutions are added to each antigen spot and incubated in a moist chamber for 1 h at room temperature. Slides are then rinsed three times with PBS before being incubated with a fluorescein isothiocyanate-conjugated anti-human polyvalent (α, γ, or μ chain-specific) immunoglobulin or anti-IgG-, anti-IgA-, or anti-IgM-specific immunoglobulin and incubated for 30 min at 37°C in a moist chamber. The slides are then washed in PBS, rinsed in distilled water, and dried. Coverslips are mounted onto the slides with glycerol mounting medium. The slides are immediately read using a microscope with a UV light source at a magnification of ×400. The end point is the highest dilution showing whole-cell fluorescence. This method can be used to determine IgG, IgM, and IgA antibody fractions to phase I and phase II. However, test results can be confounded by the presence of rheumatoid factor. Thus, rheumatoid factor absorbent is used to remove IgG before the determination of IgM and IgA (94). The choice of negative cutoff titer depends upon the source and purity of the antigen and the amount of background antigen stimulation in the population to be studied. We use 1:50 dilution as our first positive dilution (94). Others have used a 1:8 dilution. An IFA diagnostic kit (Q Fever IFA IgG, Focus Diagnostics, Inc., Cypress, Calif.) is commercially available.

The antigenic variation of *C. burnetii* is extremely useful in differentiating acute and chronic illness. In acute Q fever, antibodies to phase II antigens predominate, and their titer is higher than the phase I antibody titer. IgM antibodies appear usually at about the same time as IgG. On the other hand, in chronic forms of the disease, such as endocarditis, high-titer anti-phase I antibodies are detected uniformly. For the diagnosis of acute Q fever, cutoff values of anti-phase II IgG antibody of 200 or greater and of anti-phase II IgM antibody of 50 or greater are mandatory. For the diagnosis of chronic Q fever endocarditis, a titer of anti-phase I IgG of 800 or greater has been shown to raise the posttest probability to 98% (94).

ANTIMICROBIAL SUSCEPTIBILITIES

Antibiotic susceptibility testing of *C. burnetii* is difficult since this organism cannot be grown in axenic medium and methods are not standardized; therefore, susceptibility testing should be conducted only in appropriately experienced laboratories. Three models of infection have been used: animals, chick embryos, and cell culture; but only the last system is used today.

A shell vial assay with HEL cells for assessment of the bacteriostatic effects of antibiotics against *C. burnetii* has been developed (78). With this system, amikacin and amoxicillin were not effective and ceftriaxone and fusidic acid were inconsistently active, while cotrimoxazole, rifampin, doxycycline, tetracycline, minocycline, and clarithromycin (58) as well as sparfloxacin and the quinolones PD 127,391 and PD 131,628 (40) were bacteriostatic. By the same technique, moxifloxacin demonstrated better activity than ofloxacin and pefloxacin (82), and telithromycin showed better activity than erythromycin, with MICs of 1 μg/ml and 8 μg/ml, respectively (83). When $P388D_1$ and L929 cell multiplication was inhibited with cycloheximide during antibiotic challenges, pefloxacin, rifampin, and doxycycline (69) as well as clarithromycin (58) were bacteriostatic.

Lack of bactericidal activity was related to antibiotic inactivation by the low pH of the phagolysosomes in which *C. burnetii* multiplied. It has been subsequently shown that the addition of chloroquine, a lysosomotropic alkalinizing agent, to antibiotics improved the activities of doxycycline and pefloxacin, which then became bactericidal (57). Real-time quantitative PCR has been reported to be a faster and reliable technique for antimicrobial susceptibility testing of *C. burnetii*-infected cells (6, 7).

INTERPRETATION AND REPORTING OF RESULTS

The diagnosis of Q fever is currently based on serology. In acute Q fever, seroconversion usually is detected 7 to 15 days after the onset of clinical symptoms. Approximately 90% of patients have detectable antibodies by the third week. IFA antibody titers reach their maximum levels 4 to 8 weeks after the onset of disease and then decrease gradually during the ensuing 12 months (34). IgM titers declined to undetectable levels after 10 to 17 weeks (15, 22). In blood culture-negative endocarditis, the sensitivity and the posttest probability of having Q fever in a single-step serological assay with an 800-titer cutoff were 100 and 99.5%, respectively (80). Thus, in chronic Q fever a single serum sample is diagnostic. Consequently it has been suggested to modify the Duke criteria for the diagnosis of endocarditis and proposed that a single-serum *C. burnetii* phase I IgG antibody titer of 800 or a single positive blood culture for *C. burnetii* be considered major criteria for endocarditis (27). Serum samples from patients with Q fever endocarditis may cross-react with *Bartonella* spp., but cross-reaction is weak and differences in titers allow easy differentiation (46). For patients with a serological diagnosis of *Bartonella* endocarditis, the patient's sera should also be assayed for *C. burnetii* antibodies. Cross-reaction with other antigens is distinctly unusual.

The persistence of high levels of anti-phase I antibodies despite appropriate treatment, or the reappearance of such antibodies, should raise the suspicion of possible chronic Q fever. Patients with valvular or vascular abnormalities, those who are immunocompromised, and pregnant women should have repeated *C. burnetii* serology tests if they have a medical history of acute Q fever or a prolonged and unexplained febrile episode. In uncertain cases, a PCR performed with blood may help to determine if the patient is convalescent (negative) or infected (positive).

The follow-up of patients treated for chronic Q fever also should be done serologically. During therapy, serologic testing should be performed once monthly for 6 months and every 3 months thereafter. The levels of antibodies decrease very slowly, but this decrease correlates with the serum doxycycline level and a 2-dilution decrease for anti-phase I IgG and IgA is associated with cure. When the antibodies are initially present, IgM antibodies disappear first, and then IgA antibodies disappear; but IgG titers remain positive for years (79). Antimicrobial treatment can be stopped after 18 months to 3 years if the anti-phase I IgG titer by IFA is below 400 and IgA anti-phase I is undetectable (72).

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Tropheryma

DIDIER RAOULT, FLORENCE FENOLLAR, AND DAVID RELMAN

69

Tropheryma whipplei is the bacterial agent of Whipple's disease and was described for the first time in 1907 by an American pathologist, George Hoyt Whipple (44). The clinical manifestations of this disease are diverse, protean, and nonspecific (19, 33). Due to the varied manifestations of the disease, the differential diagnosis is large, sometimes leading to a delayed diagnosis. Without treatment, the natural evolution of the disease is always fatal. The recent isolation of *T. whipplei* has opened new perspectives for the management of the disease (36).

TAXONOMY

In 1991, Wilson et al. sequenced a fragment of 16S rRNA gene from tissue of a patient with Whipple's disease (45). This partial sequence suggested a bacterium related to the *Actinomyces* clade. In 1992, Relman et al. described a nearly complete bacterial 16S rRNA gene sequence that was detected in five patients with Whipple's disease but not in a group of controls (39). The inferred phylogeny of the presumed causative agent suggested that the organism is an actinomycete and a member of the phylum *Actinobacteria*. The bacterium was originally named "*Tropheryma whippelii,*" but this was subsequently revised to *Tropheryma whipplei*, following its cultivation and phenotypic characterization, in order to accommodate the nomenclature rules of the International Society of Systematic Bacteriology (25).

DESCRIPTION OF THE GENOME

The entire genomes of two different strains of *T. whipplei* have been sequenced (2, 38). Sequencing of *T. whipplei* Twist revealed a 927,303-bp genome which carries 808 predicted protein-coding genes and with a GC content of 47% (38). Sequencing of *T. whipplei* TW08/27 revealed a 925,938-bp genome which carries 784 predicted protein-coding genes and with a GC content of 46.3% (2). *T. whipplei* possesses a unique circular chromosome and is the only known reduced-genome species (<1 Mb) within the *Actinobacteria* (2, 38). The genomic sequences of the two strains are nearly (>99%) identical at the nucleotide sequence level, encoding quasi-identical gene complements. This specific genome includes apparent deficiencies in energy metabolism and amino acids synthesis, suggesting a requirement of external nutrients. Alignment of the two genome sequences reveals a large chromosomal inversion, the extremities of which are located within two paralogous genes (2). These genes belong to a large family of predicted cell surface proteins defined by the presence of a common repeat highly conserved at the nucleotide level. The repeats appear to trigger genome fragment rearrangements in *T. whipplei*, potentially resulting in the expression of different subsets of cell surface proteins (2). This phenomenon might represent a new mechanism for evasion of the host's immune response during the course of this chronic disease.

NATURAL HABITAT

The source of *T. whipplei* and its transmission are presently unknown. The bacterium seems to be present in the environment. Studies based on PCR have shown the presence of *T. whipplei* DNA in effluent from sewage plants (30) and in human stools and saliva (10, 11, 23), and it has been speculated that *T. whipplei* is acquired via the oral route (19). Different studies have been performed to establish the presence of *T. whipplei* DNA in people without Whipple's disease in saliva, duodenal biopsy specimens, gastric liquids, and stools (11, 12, 41). However, the data should be analyzed with caution because they are based on "home-brew PCR" and have not been independently confirmed (10, 15, 31). It has been suggested that *T. whipplei* may have a specific geographic and racial distribution, with a predominance in Caucasians and in Central Europe (10).

CLINICAL FEATURES OF WHIPPLE'S DISEASE

The most common clinical presentation of Whipple's disease is an association of diarrhea, arthralgias, and lymphadenopathy, but all organs may be affected (17, 33). Many cases are associated with long-standing, relapsing and remitting arthralgias. As many as 15% of patients lack gastrointestinal symptoms throughout their illness (42), and small-bowel biopsies may be normal (34). It is therefore not surprising that in many of these atypical cases the diagnosis may not be made for some time and may be entirely missed until it is demonstrated at necropsy. The main clinical manifestations of Whipple's disease are summarized in Table 1.

TABLE 1 Clinical manifestations of Whipple's disease

Manifestations
Classic manifestations
Diarrhea
Arthralgias
Weight loss
Abdominal pain
Lymphadenopathy
Cutaneous pigmentation
Fever of unknown origin
CNS involvements
Cognitive impairment
Ophthalmoplegia
Myoclonic signs
Hypothalamic involvement
Cerebellar involvement
Abnormal ocular movements
Ophthalmologic manifestations
Uveitis
Cardiovascular manifestations
Blood culture-negative endocarditis
Pericarditis
Myocarditis

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

When Whipple's disease is suspected, saliva and stools for PCR and blood for PCR and immunohistochemistry are easy to sample and test. Saliva and stools are not considered useful samples by some authors (11, 12, 41). However, we have never obtained a positive PCR assay from these samples from people without a final diagnosis of Whipple's disease, except in one case (our unpublished data) (15). Small-bowel endoscopic biopsies should also be sampled systematically in suspected cases of Whipple's disease, even in the absence of intestinal symptoms. Several biopsies must be sampled from the duodenum and jejunum because the lesions can be focal and sparse (19). In cases of suspected Whipple's endocarditis, the excised cardiac valve should be tested by periodic acid-Schiff (PAS) staining, PCR, and immunohistochemistry. In cases of suspected neurologic Whipple's disease, cerebrospinal fluid (CSF) should be sampled as well as saliva, stools, blood, and small-bowel mucosa. If these investigations are not diagnostic, another disease site should be sampled based on associated signs and symptoms (lymph node, synovial biopsy, liver, cardiac valve, or vitreous fluid). If all these examinations are negative and accessible lesions are observed on brain magnetic resonance imaging, a stereotaxic biopsy should be performed for PCR and immunohistochemistry. Finally, if a diagnosis of Whipple's disease is established, even in the absence of neurologic signs, CSF should be systematically sampled and tested by PCR due to the common, subclinical involvement of the central nervous system (CNS) in this disease (6, 43). For PCR and culture, fresh samples should be frozen at −80°C and transported in dry ice. For PAS staining and immunohistochemistry, all the samples should be fixed in 10% formalin and transported at room temperature.

DIRECT EXAMINATION

Electronic Microscopy

Electron microscopy has been reported to demonstrate typical bacteria in macrophages from all kinds of tissues (Table 2) (19). Electron microscopy is not a practical tool for the diagnosis of the disease.

PAS Staining

The histological study of small-bowel biopsy specimens by use of PAS staining was the first tool for the diagnosis of Whipple's disease and is still considered an important and sometimes definitive diagnostic criterion (Table 2). The criterion is based on the observation of (i) diastase-resistant, magenta-stained inclusions within macrophages of the lamina propria and (ii) lymphatic dilatation. This PAS staining pattern, which has long been considered pathognomonic of Whipple's disease, is found in other diseases (19, 33). The presence of noncaseous granulomas composed of epithelioid cells may be observed in lymphatic tissues, the gastrointestinal tract, liver, or lung in Whipple's disease. These granulomas are often PAS negative (10, 19, 33). Depending on clinical manifestations, other tissues may be appropriate for PAS staining to establish the diagnosis of Whipple's disease, such as lymph node, synovium, cardiac valve, brain, or CSF (10, 19, 33).

Immunohistochemistry

The culture of *T. whipplei* has made possible the generation of polyclonal antibodies directed against the bacterium (27, 29) and an immunohistochemical diagnosis of Whipple's

TABLE 2 Diagnostic tools for the diagnosis of Whipple's disease

Technique	Sample(s)	Advantage	Disadvantage
Electron microscopy	Small-bowel tissue; all other kinds of tissues; body fluids	None	Not available in all laboratories; laborious
PAS staining	Small-bowel tissue; all other kinds of tissues; body fluids	Available in all laboratories; can be used on archival samples	Low specificity
Nucleic acid amplification	Blood; saliva; stools; small-bowel tissue; all other kinds of tissues; body fluids	Available in some laboratories	Specificity variable depending on sample type and laboratory
Immunodetection	Blood; small-bowel tissue; all other kinds of tissues; body fluids	Good specificity; can be used on archival samples	Noncommercialized antibodies
Culture	Blood; small-bowel tissue; all other kinds of tissues; body fluids	New strain isolation	Specific technology; long time required for the primary isolation
Serology	Serum	Simple procedure	Presently experimental

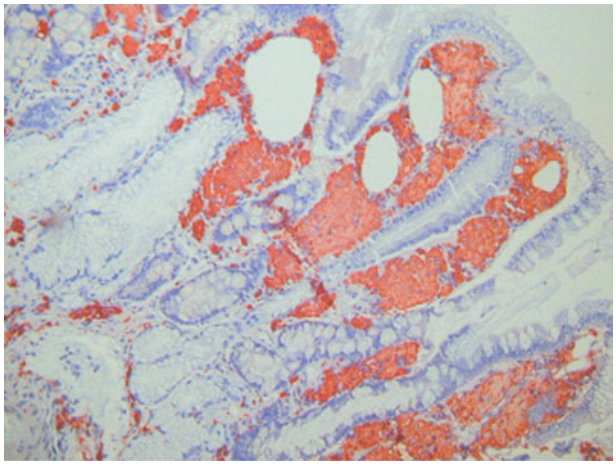


FIGURE 1 Immunohistochemistry performed on duodenal biopsies using rabbit polyclonal antibodies specifically directed against *Tropheryma whipplei*. Original magnification, ×100. Courtesy of Hubert Lepidi.

disease (26–28, 37). This technique has been performed on various kinds of tissues (duodenum, lymph node, brain, and cardiac valve), on body fluids such as aqueous humor, and on blood monocytes (1, 8, 9, 26, 27, 36, 37). This technique provides direct visualization of the bacilli in macrophages (Fig. 1) and offers increased sensitivity and specificity over the traditional PAS staining method (Table 2). It can also be used retrospectively on fixed samples (9).

Molecular Detection

PCR assays can be performed with a variety of tissue types (e.g., intestinal mucosa, lymph nodes, cardiac valves, and brain) and body fluids (CSF, blood, and saliva [Table 2]) (18). DNA extraction is a key step: different protocols have been proposed, involving phenol-chloroform-ethanol purification, chaotropic lysis, Chelex suspension, or a QIAamp DNA binding column (18). Some authors have also used digestion buffer before DNA extraction (18). These protocols can be applied to paraffin-embedded samples, as well as fresh samples. PCR amplification of the 16S rRNA gene was the first molecular tool for the diagnosis of Whipple's disease (39). As with all PCR assays, it is critical to include positive and negative controls (including water and DNA extracted from controls tissues). For each assay, the positive and negative controls must be, respectively, positive and negative to validate the results. Recently, a PCR assay targeting the 16S-23S intergenic region by using a real-time PCR apparatus has been successful for the diagnosis of Whipple's disease (15). This technique offers the advantages of reduced time for PCR analysis and lowered risk of contamination. The availability of the genome has also offered the possibility of selecting DNA targets in a more rational and informed manner. A real-time PCR assay targeting repeated sequences of *T. whipplei* significantly enhanced PCR assay sensitivity without altering its specificity, when compared to regular PCR (16). When amplified product is detected, the identification of the *T. whipplei* sequence should be confirmed by sequencing or hybridization. A problem with nonstandardized PCR assays is DNA contamination. In addition to general recommendations for avoiding contamination, we believe that when positive results from PAS staining or immunohistochemistry are not obtained in parallel with a positive PCR result (especially from blood, CSF, saliva, and stools), it is imperative to obtain confirmation of the diagnosis by using a different sample type tested with a different procedure (e.g., using a different DNA target to reduce the likelihood of false-positive PCR results).

Isolation Procedure

The cultivation of *T. whipplei* from various samples can be achieved with cell culture techniques and human fibroblasts (Table 2) (8, 14, 25, 32, 36, 37). Based on this technique, isolates have been obtained from cardiac valve, duodenal biopsy tissue, blood, aqueous humor, CSF, adenopathy, and synovial fluid (14, 32). Among them, 17 isolates (1 from a duodenal biopsy, 2 from cardiac valves, 7 from CSF, 4 from a blood sample, 2 from lymph node, and 1 from synovial fluid) have been established in serial cultures (2, 14, 32) (our unpublished data). In our experience, the delay before primary detection of *T. whipplei* from clinical samples is approximately 30 days (14), but it may be shorter (32). The cultivation of *T. whipplei* from contaminated specimen types such as intestinal biopsy specimens requires the use of drugs such as colistin, amphotericin B, cephalothin, or ciprofloxacin. *T. whipplei* genome analysis indicates deficiencies in amino acid biosynthetic pathways. As a consequence, the missing amino acids must be obtained from the environment. This information has allowed the design of a medium supplemented with amino acids, in which *T. whipplei* grows axenically (40). By use of this technique, two strains of *T. whipplei* from CSF, two strains from blood, one strain from synovial fluid, one strain from adenopathy, and one strain from cardiac valve have been isolated and established in parallel to cell culture (our unpublished data).

Identification

Every 15 days for 180 days of culture, the culture medium is changed, and before the medium is replaced 100 μl of the supernatant is obtained for centrifugation and stained by immunofluorescence using anti-*T. whipplei* polyclonal rabbit antibodies to detect bacterial growth. In parallel, a PCR assay is performed to confirm the growth and the identification. Confirmation of isolates as *T. whipplei* must be systematically obtained by specific PCR assay and amplicon sequencing or hybridization.

SEROLOGIC TESTING

The culture of *T. whipplei* has led to the development of serologic tests for this disease. However, serologic testing for Whipple's disease is not currently available (Table 2) (33, 35, 36).

ANTIMICROBIAL SUSCEPTIBILITIES

A study of the susceptibilities of three strains of *T. whipplei* to antibiotics in cell culture has indicated that doxycycline, macrolides, ketolides, aminoglycosides, penicillin, rifampin, teicoplanin, chloramphenicol, and trimethoprim-sulfamethoxazole are active against this organism, with MICs ranging from 0.25 to 2 μg/ml (4). Vancomycin was somewhat active with a MIC of 10 μg/ml. Cephalosporins (including ceftriaxone), colimycin, aztreonam, and fluoroquinolones are not active. Variability in the susceptibility of imipenem is also observed. A combination of doxycycline and hydroxychloroquine has been shown to be bactericidal in vitro because *T. whipplei* resides in acidic vacuoles of host cells (4, 22). The antibiotic susceptibilities of these three

TABLE 3 Strategy for interpretation of results depending on sample type and diagnostic technique[a]

Sample(s)	PAS	IHC	PCR	Confirmed diagnosis
Small bowel	+	− or NA	− or NA	No
Lymph nodes,	+	NA	+	Yes
cardiac valve, brain,	− or NA	+	+	Yes
other biopsy	− or NA	− or NA	+	Yes (?)
specimens	− or NA	+	− or NA	Yes (?)
CSF, blood	+	− or NA	− or NA	No
	+	− or NA	+	Yes
	− or NA	+	+	Yes
	− or NA	− or NA	+	?[b]
Saliva, stools	NA	NA	+	Predictive value depending on the laboratory

[a] Abbreviations and symbols: IHC, immunohistochemistry; NA, not available; −, negative; +, positive.
[b] Needs to be confirmed on a second sample, using a second DNA target if possible.

strains grown in axenic medium have also been studied (3). The active compounds on axenic medium are sulfamethoxazole, doxycycline, macrolides, penicillin G, streptomycin, rifampin, chloramphenicol, thiamphenicol, teicoplanin, vancomycin, amoxicillin, gentamicin, aztreonam, levofloxacin, and ceftriaxone, with MICs ranging from 0.06 to 1 μg/ml. Trimethoprim is not effective in vitro as predicted from genomic analysis (5).

INTERPRETATION OF RESULTS

A strategy for interpretation of diagnostic test results based on sample type and technique is summarized in Table 3. When a patient presents with "typical" features of Whipple's disease, including diarrhea, malabsorption, lymphadenopathy, and/or arthralgias, the diagnosis should be suspected. For cases of Whipple's disease without intestinal symptoms, such as Whipple's disease endocarditis and isolated neurologic disease, the diagnosis is more difficult. One of the main problems is the interpretation of an isolated positive PCR. These results must always be confirmed with a second specimen, such as blood or CSF, ideally using a different procedure and/or different DNA target. In these cases, the presence of a positive PCR from saliva or stool may be useful in confirming the diagnosis of Whipple's disease.

TREATMENT

Presently, the recommended treatment is an oral regimen of trimethoprim and sulfamethoxazole administered from 1 to 2 years. Many recommend that this regimen be preceded with treatment for 14 days consisting of intramuscular streptomycin and intravenous benzyl penicillin (penicillin G) (7, 13, 20, 21, 24). Based on recent data from in vitro assays, a therapeutic regimen that includes a combination of doxycycline and hydroxychloroquine to eradicate intracellular organisms is now suggested. Moreover, high doses of sulfamethoxazole in association with doxycycline and hydroxychloroquine may improve the treatment of CNS disease and reduce the likelihood of relapse. Clinical trials are now needed to confirm this strategy.

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ANTIBACTERIAL AGENTS AND SUSCEPTIBILITY TEST METHODS

V

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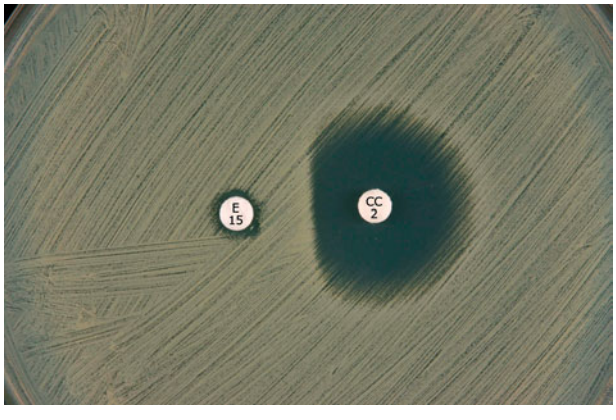
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D-zone test (J. Jorgensen, University of Texas).

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Antibacterial Agents

JOSEPH D. C. YAO AND ROBERT C. MOELLERING, JR.

70

Antimicrobial chemotherapy has played a vital role in the treatment of human infectious diseases in the 20th century. Since the discovery of penicillin in the 1920s, literally hundreds of antimicrobial agents have been developed or synthesized, and dozens of these are currently available for clinical use. While the broad number and variety of agents available provide a great deal of flexibility for the clinician in the use of these agents, the sheer numbers available and the continuing development of agents make it difficult for clinicians to keep up with progress in the field. Similarly, this variety presents significant challenges for the clinical microbiologist, who must decide which agents are appropriate for inclusion in routine and specialized susceptibility testing.

This chapter provides an overview of the antibacterial agents currently marketed in the United States, with major emphasis on their mechanisms of action, spectra of activity, important pharmacological parameters, and toxicities. Antibiotics that have fallen into disuse or remained investigational are mentioned only briefly.

PENICILLINS

The penicillins (Table 1) are a group of natural and semisynthetic antibiotics containing the chemical nucleus 6-aminopenicillanic acid, which consists of a β-lactam ring fused to a thiazolidine ring (Fig. 1a). The naturally occurring compounds are produced by a number of *Penicillium* spp. The penicillins differ from one another in the substitution at position 6, where changes in the side chain may modify the pharmacokinetic and antibacterial properties of the drug.

Mechanism of Action

The major antibacterial action of penicillins is derived from their ability to inhibit a number of bacterial enzymes, namely, penicillin-binding proteins (PBPs), that are essential for peptidoglycan synthesis (358). This ability to inhibit bacterial cell wall enzymes such as the transpeptidases usually confers on the penicillins bactericidal activity against gram-positive bacteria. The bactericidal activity of the penicillins is often related to their ability to trigger membrane-associated autolytic enzymes that destroy the cell wall. Other minor mechanisms of action include inhibition of bacterial endopeptidase and glycosidase, enzymes involved in bacterial cell growth. There is also recent evidence suggesting that penicillins may inhibit RNA synthesis in some bacteria, causing death without cell lysis, but the significance of these observations remains to be proven (223).

Pharmacology

Oral absorption differs markedly among the penicillins. As a natural congener of penicillin G, penicillin V resists gastric acid inactivation and is better absorbed from the gastrointestinal tract than is penicillin G. Amoxicillin is a semisynthetic analog of ampicillin and has greater gastrointestinal absorption than ampicillin (95 versus 40% absorption). Bacampicillin is an ampicillin ester that is absorbed considerably better from the gastrointestinal tract than is ampicillin or amoxicillin. This ester is inactive until naturally occurring esterases in the intestinal mucosa and serum hydrolyze them to release the parent compound, ampicillin, into the serum. The isoxazolyl penicillins, such as oxacillin, cloxacillin, dicloxacillin, and nafcillin, are acid stable and are also absorbed from the gastrointestinal tract (140), in contradistinction to certain other antistaphylococcal penicillins, such as methicillin, which are not acid resistant and cannot be given via the oral route.

Repository forms of penicillin G, available in procaine or benzathine, delay absorption from an intramuscular depot. Procaine penicillin G provides detectable levels for 12 to 24 h, suitable for treatment of uncomplicated pneumococcal pneumonia and gonorrhea due to fully susceptible organisms. Benzathine penicillin G achieves very low levels in blood for prolonged periods (3 to 4 weeks) and is useful for the therapy of syphilis and for prophylaxis of streptococcal pharyngitis and rheumatic fever.

Penicillins are well distributed to many body compartments, including the lungs, liver, kidneys, muscle, bone, and placenta. Penetration into the eye, brain, cerebrospinal fluid (CSF), and prostate is poor in the absence of inflammation. These drugs are metabolized to a small degree and are rapidly excreted, essentially unchanged, via the kidneys. With average half-lives of 0.5 to 1.5 h, they are usually administered every 4 to 6 h to maintain effective levels in blood. The renal tubular excretion of penicillins can be blocked by probenecid, thus prolonging their half-lives in serum.

Dosage reduction of most penicillins is necessary only in severe renal insufficiency (creatinine clearance of ≤10 ml/min). Dosages of all penicillins except nafcillin and the isoxazolyl penicillins are adjusted for hemodialysis. Peritoneal dialysis requires dosage reduction for carbenicillin and ticarcillin.

TABLE 1 Penicillins

- Natural
  - Benzylpenicillin (penicillin G)
  - Phenoxymethyl penicillin (penicillin V)
- Semisynthetic
  - Penicillinase resistant
    - Methicillin
    - Nafcillin
    - Cloxacillin
    - Dicloxacillin
    - Oxacillin
  - Extended spectrum
    - Aminopenicillins
      - Ampicillin
      - Amoxicillin
      - Bacampicillin
      - Pivampicillin
    - Carboxypenicillins
      - Carbenicillin
      - Ticarcillin
    - Ureidopenicillins
      - Azlocillin
      - Mezlocillin
      - Piperacillin
- Penicillin + β-lactamase inhibitor combinations
  - Ampicillin-sulbactam
  - Ticarcillin-clavulanate
  - Amoxicillin-clavulanate
  - Piperacillin-tazobactam

Spectrum of Activity

The penicillins have activity against most gram-positive and many gram-negative and anaerobic organisms. Penicillin G is very effective against penicillin-susceptible *Staphylococcus aureus, Streptococcus pneumoniae, Streptococcus pyogenes,* viridans group streptococci, *Streptococcus bovis, Neisseria gonorrhoeae, Neisseria meningitidis, Pasteurella multocida,* anaerobic cocci, *Clostridium* spp., *Fusobacterium* spp., *Prevotella* spp., and *Porphyromonas* spp. However, the occurrence of penicillin-resistant pneumococci has recently been increasing worldwide (190). Penicillin is the drug of choice for syphilis and *Actinomyces* infections. Penicillin V has a spectrum of activity similar to that of penicillin G except that it is less active against *N. gonorrhoeae.* Penicillinase-resistant penicillins, of which methicillin is the prototype, are primarily effective against penicillinase-producing staphylococci (115). The agents are at least 25 times more active than other penicillins against penicillinase-positive staphylococci. Although they are also active against *S. pneumoniae* and *S. pyogenes,* their MICs for these organisms are higher than those of penicillin G. They are not active against enterococci, members of the family *Enterobacteriaceae, Pseudomonas* spp., or members of the *Bacteroides fragilis* group.

Ampicillin and amoxicillin have spectra of activity similar to that of penicillin G, but they are more active against enterococci and *Listeria monocytogenes.* Although they are also more active against *Haemophilus influenzae* and *Haemophilus parainfluenzae,* up to 35% of *H. influenzae* isolates are resistant, usually because of β-lactamase production. *Salmonella* and *Shigella* spp., including *Salmonella enterica* serovar Typhi, and many strains of *Escherichia coli* and *Proteus mirabilis* are susceptible to these agents. Ampicillin is more effective against shigellae, whereas amoxicillin is more effective against salmonellae. Both of these agents are degraded by β-lactamase and are inactive against many *Enterobacteriaceae* and *Pseudomonas* spp.

The carboxypenicillins and ureidopenicillins have increased activity against gram-negative bacteria that are resistant to ampicillin. Although these drugs are susceptible to staphylococcal penicillinase, they are more stable against hydrolysis by the β-lactamases of *Enterobacteriaceae* and *Pseudomonas aeruginosa.* Carbenicillin and ticarcillin are relatively active against streptococci as well as against *Haemophilus* spp., *Neisseria* spp., and a variety of anaerobes (124). They inhibit *Enterobacteriaceae* but are inactive against *Klebsiella* spp. Although carboxypenicillins are not particularly active against the enterococci, they may act synergistically with aminoglycosides against these organisms.

The ureidopenicillins have greater in vitro activity against streptococci and enterococci than do the carboxypenicillins, and they inhibit more than 75% of *Klebsiella* spp. (87, 343). They have excellent activity against many *Enterobacteriaceae* and anaerobic bacteria, including members of the *B. fragilis* group. On a weight basis, their activities in decreasing order of potency against *P. aeruginosa* are as follows: piperacillin, azlocillin > mezlocillin, ticarcillin > carbenicillin (66). These agents also act synergistically with aminoglycosides against *P. aeruginosa.*

Adverse Effects

Common reactions to penicillins include allergic skin rashes, diarrhea, and drug fever. Severe anaphylactic reactions, which can be fatal, may occur in previously sensitized patients rechallenged with penicillins, but fortunately, such reactions are quite rare. At high doses (usually $>30 \times 10^6$ U/day), penicillin G can cause myoclonic twitching and seizures due to central nervous system toxicity. All of the penicillins may cause interstitial nephritis on an allergic basis, but methicillin is more likely than the other penicillins to cause this complication. Hepatitis has been associated with prolonged use of oxacillin. High-dose carbenicillin can result in sodium overload and hypokalemia. Neutropenia may occur with any of the penicillins. Thrombocytopenia and Coombs-positive hemolytic anemia are rare complications of penicillin therapy. Bleeding tendencies due to interference with platelet function can occur with the use of carboxypenicillins and ureidopenicillins (105). Although pseudomembranous colitis has been associated with all the penicillins, it occurs more frequently with ampicillin (19).

CEPHALOSPORINS

Cephalosporins are derivatives of the fermentation products of *Cephalosporium acremonium* (also designated *Acremonium chrysogenum*). They contain a 7-aminocephalosporanic acid nucleus, which consists of a β-lactam ring fused to a dihydrothiazine ring (Fig. lb). Various substitutions at positions 3 and 7 alter their antibacterial activities and pharmacokinetic properties. The addition of a methoxy group at position 7 of the β-lactam ring results in a new group of compounds called cephamycins, which are highly resistant to a variety of β-lactamases.

Mechanism of Action

Similar to the penicillins, cephalosporins act by binding to PBPs of susceptible organisms, thereby interfering with

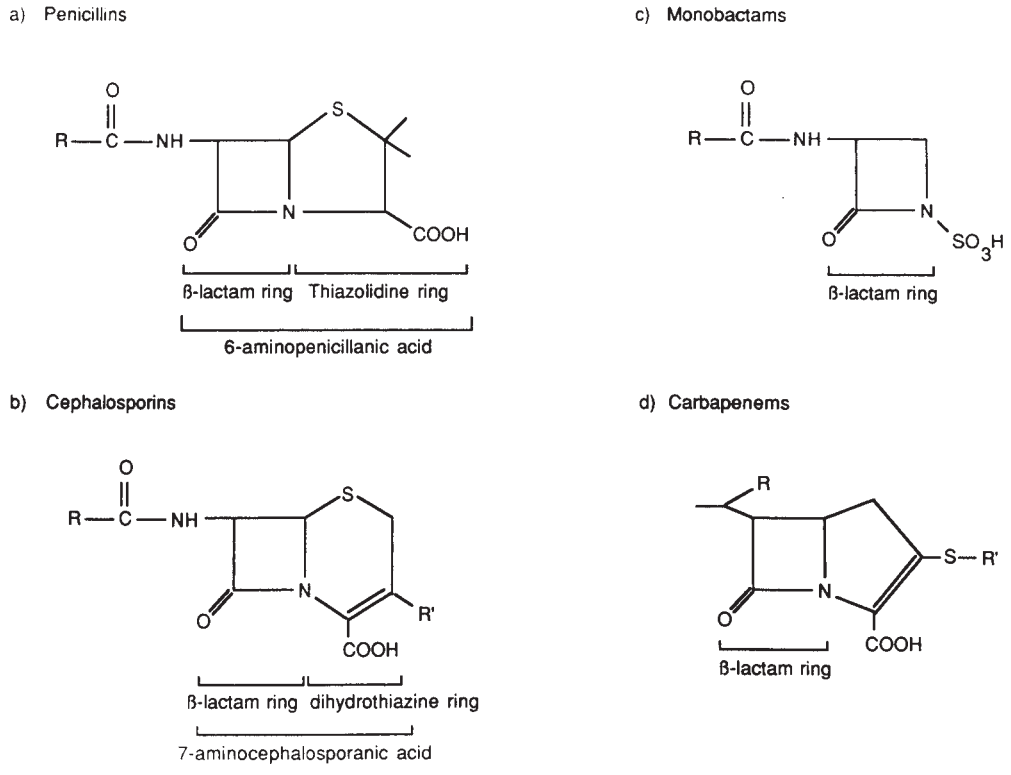


FIGURE 1 Chemical structures of β-lactam antibiotics.

synthesis of peptidoglycan of the bacterial cell wall. In addition, these β-lactam agents may produce bactericidal effects by triggering autolytic enzymes in the cell envelope (358).

Pharmacology

Most cephalosporins require parenteral administration, but a growing number are available in oral form. Cephalexin, cephradine, cefadroxil, cefaclor, cefuroxime axetil, cefprozil, loracarbef, cefdinir, cefditoren pivoxil, cefixime, cefpodoxime proxetil, and ceftibuten are given orally and have good gastrointestinal absorption (60 to 90% of the oral dose). Cefuroxime axetil is an acetoxyethyl ester of cefuroxime, and it is deesterified at the intestinal mucosa and absorbed into the bloodstream as cefuroxime. Cefditoren pivoxil and cefpodoxime proxetil are prodrugs that are absorbed and hydrolyzed by esterases in vivo to release the active drugs, cefditoren and cefpodoxime, respectively. Relatively high concentrations of these agents are attained across the placenta and in synovial, pleural, pericardial, and peritoneal fluids. Levels in bile are usually high, especially with cefoperazone, which is excreted mainly in the bile. Ceftizoxime, cefotaxime, ceftriaxone, cefoperazone, moxalactam, and cefepime enter well into the CSF and are useful for the treatment of meningitis. Cefuroxime penetrates inflamed meninges, but levels in CSF are inadequate in providing bactericidal activity against susceptible bacteria.

Cephalothin, cephapirin, and cefotaxime are converted to the desacetyl forms before excretion. All cephalosporins except cefoperazone are excreted primarily by the kidneys, and for these drugs, dosage adjustments are necessary in patients with renal insufficiency (creatinine clearance of <50 ml/min). Like that of the penicillins, the renal excretion of cephalosporins, except for ceftriaxone, is impeded by probenecid. In general, these agents are removed by hemodialysis but not by peritoneal dialysis. Of the cephalosporins, cefonicid and ceftriaxone have the longest elimination half-lives, at 4.5 and 8 h, respectively, permitting once- or twice-daily drug administration in the treatment of serious infections.

Spectrum of Activity

Cephalosporins are classified by a well-accepted but somewhat arbitrary scheme of grouping by generations based on general features of their antibacterial activity (Table 2). The first-generation (narrow-spectrum) drugs, exemplified by cephalothin and cefazolin, have good activity against gram-positive organisms and relatively modest activity against gram-negative organisms. They are active against penicillin-susceptible and -resistant *S. aureus* as well as *S. pneumoniae, S. pyogenes,* and other aerobic and anaerobic streptococci. Methicillin-resistant *S. aureus, Staphylococcus epidermidis,* and enterococci are resistant. Some *Enterobacteriaceae,* including many strains of *E. coli, Klebsiella* spp., and *P. mirabilis,* are susceptible. *Pseudomonas* spp. (including *P. aeruginosa),* many *Proteus* spp., and *Serratia* and *Enterobacter* spp. are resistant. These agents are active against penicillin-susceptible anaerobes except members of the *B. fragilis* group. They have only modest activity against *H. influenzae.*

The second-generation (expanded-spectrum) cephalosporins are stable against certain β-lactamases found in gram-negative bacteria and, as a result, have increased activity against gram-negative organisms. The agents are more active than narrow-spectrum drugs against *E. coli, Klebsiella* spp., and *Proteus* spp. Their activity also extends to cover some *Enterobacter* and *Serratia* strains, and they have good activity against *Haemophilus* spp., *Neisseria* spp., and many anaerobes.

TABLE 2 Cephalosporins

Narrow spectrum (first generation)
Cefadroxil
Cefazolin
Cephalexin
Cephaloridine
Cephalothin
Cephapirin
Cephradine
Expanded spectrum (second generation)
Cefaclor
Cefamandole
Cefonicid
Ceforanide
Cefuroxime
Cefprozil
Loracarbef
Cefmetazole
Cefotetan
Cefoxitin
Broad spectrum (third generation)
Cefdinir
Cefditoren
Cefixime
Cefoperazone
Cefotaxime
Cefpodoxime
Ceftazidime
Ceftibuten
Ceftizoxime
Ceftriaxone
Extended spectrum (fourth generation)
Cefepime
Cefpirome

Cefaclor, cefuroxime, cefamandole, cefonicid, and cefprozil are active against ampicillin-resistant *H. influenzae* and *Moraxella catarrhalis* (334). However, cefamandole exhibits a significant inoculum effect and is not suitable for treating life-threatening infections due to *H. influenzae.* Ceforanide and cefonicid have spectra of antibacterial activities similar to that of cefamandole, but they are less active than cefamandole against gram-positive cocci. Loracarbef belongs to a new class of cephalosporin derivatives known as carbacephems in which the sulfur atom of the dihydrothiazine ring is replaced by a methylene group to form a tetrahydropyridine ring (67). Since this structural modification of the cephalosporin nucleus is minor, loracarbef is considered a cephalosporin. Its spectrum of antibacterial activity is very similar to those of cefaclor, cefuroxime, and cefprozil. None of the expanded-spectrum agents is active against *Pseudomonas* spp.

Cefoxitin, cefotetan, and cefmetazole belong to a unique group of expanded-spectrum cephalosporins that have marked activity against anaerobes, including members of the *B. fragilis* group (175, 364). Cefotetan is two to four times less active than cefoxitin and cefmetazole against gram-positive cocci, but it is more potent than these two drugs against susceptible *Enterobacteriaceae.* The three drugs are equally active against *H. influenzae, M. catarrhalis,* and *N. gonorrhoeae,* including penicillin-resistant strains. While these drugs are comparable in their activities against the *B. fragilis* group, cefoxitin is the most active against *Prevotella* spp., *Porphyromonas* spp., and gram-positive anaerobic cocci. Cefotetan and cefmetazole have the advantage of more prolonged half-lives in serum.

Third-generation (broad-spectrum) cephalosporins are generally less active than the narrow-spectrum agents against gram-positive cocci, but they are much more active against the *Enterobacteriaceae* and *P. aeruginosa.* Their potent broad spectra of activity against gram-negative organisms are due to their stability against β-lactamases and their ability to pass through the outer cell envelopes of gram-negative rods (104, 254). There are two subgroups among these agents: those with potent activity against *P. aeruginosa* (ceftazidime and cefoperazone) and those without such activity (ceftizoxime, cefotaxime, and ceftriaxone).

Cefotaxime inhibits more than 90% of strains of *Enterobacteriaceae,* including those resistant to aminoglycosides. The MIC_{90}s for strains of *E. coli, Proteus* spp., and *Klebsiella* spp. tested are <0.5 μg/ml. Its activity against strains of *Serratia marcescens, Enterobacter cloacae,* and *Acinetobacter* spp. is variable, and it is inactive against *P. aeruginosa.* It has moderate activity against anaerobes but is inferior to cefoxitin and cefotetan against most of these isolates.

Ceftizoxime and ceftriaxone have spectra of activity similar to that of cefotaxime, with a few exceptions. Ceftriaxone is the most active agent against penicillinase-positive or -negative strains of *N. gonorrhoeae* (108). It is effective as single-dose therapy for infections caused by these organisms (51). Because of its long half-life in serum (the longest of the currently available cephalosporins), ceftriaxone is used frequently in outpatient antibiotic therapy of serious infections, including Lyme disease (229).

Cefoperazone is less active than cefotaxime against many *Enterobacteriaceae* and gram-positive cocci. However, it has activity against *P. aeruginosa,* with an MIC_{50} of ≤ 16 μg/ml. Its activity against anaerobes is similar to that of cefotaxime (177). Ceftazidime has potent activity against *P. aeruginosa,* with an MIC_{90} of <8 μg/ml (248). It is more active than the ureidopenicillins against these strains. This agent has activity similar to that of cefotaxime against the *Enterobacteriaceae* but is not as active against gram-positive cocci. It has little activity against gram-negative anaerobes.

Cefdinir (63), cefditoren (173, 178), cefixime (15), cefpodoxime (116, 298), and ceftibuten (177, 376) are extended-spectrum oral cephalosporins that are more stable than the narrow- and expanded-spectrum oral cephalosporins against gram-negative bacterial β-lactamases. Compared with the earlier cephalosporins, the newer drugs are equally active against streptococci (MIC_{90}s $\leq$ 0.06 μg/ml) but less active against methicillin-susceptible staphylococci (MIC_{90}s $=$ 2 μg/ml). While they have potent activities similar to that of ceftizoxime against many *Enterobacteriaceae, H. influenzae, M. catarrhalis,* and *N. gonorrhoeae* (including β-lactamase-producing strains), they are inactive against *Pseudomonas, Enterobacter, Serratia,* and *Morganella* spp. and anaerobes. None of the currently available cephalosporins is clinically useful against enterococci.

Cefepime is a so-called fourth-generation (extended-spectrum) cephalosporin approved for clinical use in the United States. Cefepime and cefpirome (formerly HR 810), which is currently undergoing clinical evaluations, have the unique features of reduced affinity for and increased stability against the Bush class I β-lactamases. Therefore, these agents are active against stably derepressed class I β-lactamase mutants of *Enterobacteriaceae* and *P. aeruginosa.* In addition, cefepime and cefpirome penetrate well through the outer membrane of gram-negative bacteria, due to a quaternary

nitrogen substitution that makes them zwitterions (net neutral charge). They are more active in vitro than cefotaxime and ceftriaxone against some *Enterobacteriaceae, Proteus, Providencia, Morganella,* and *Citrobacter* (MIC_{90}s ≤ 0.1 μg/ml) (126, 180, 301). Cefepime has activity comparable to that of ceftazidime against *P. aeruginosa,* with MIC_{90}s of ≤4 μg/ml, and it is active against some ceftazidime-resistant strains (279). Against staphylococci (MIC_{90}s ≤ 2 μg/ml) and streptococci (MIC_{90}s ≤ 0.12 μg/ml), the activities of this group of drugs are comparable to those of the narrow-spectrum cephalosporins (126, 180). However, they are not active clinically against enterococci or anaerobes.

Adverse Effects

Cephalosporins are generally very well tolerated. The most common side effects are diarrhea and hypersensitivity reactions such as rash, drug fever, and serum sickness. Cross-reactions with these drugs occur in only 3 to 7% of penicillin-allergic patients (186). Other infrequent side effects include pseudomembranous colitis, elevated serum creatinine and transaminase levels, leukopenia, thrombocytopenia, and Coombs-positive hemolytic anemia. These abnormalities are usually mild and reversible. Prolonged use of ceftriaxone has been associated with formation of gallbladder sludge, which usually resolves after the drug is discontinued (304), and, rarely, cholecystitis.

Disulfiram-like reactions have been described to occur in patients receiving cefamandole, cefotetan, and cefoperazone. This reaction is attributed to the *N*-methylthiotetrazole side chains of these antibiotics, which are similar to the chemical structure of disulfiram. Hypoprothrombinemia and bleeding tendencies have been observed with these cephalosporins. Causes of the coagulopathy included (i) alteration to healthy gut flora by the antibiotics, thus inhibiting the synthesis of vitamin K and its precursors, and (ii) the *N*-methylthiotetrazole side chain, which inhibits the vitamin K-dependent carboxylase enzyme responsible for converting clotting factors II, VII, IX, and X to their active forms and also prevents regeneration of active vitamin K from its inactive form (302).

OTHER β-LACTAM ANTIBIOTICS

■ Monobactams

Aztreonam is the only monobactam antibiotic currently in clinical use. The monobactams are β-lactams with various side chains affixed to a monocyclic nucleus (Fig. 1c).

Mechanism of Action

Aztreonam binds primarily to PBP 3 of gram-negative aerobes, including *P. aeruginosa,* thereby disrupting bacterial cell wall synthesis. It is not hydrolyzed by most commonly occurring plasmid-mediated and chromosomally mediated β-lactamases, and it does not induce the production of these enzymes (42).

Pharmacology

Given intravenously, aztreonam is widely distributed to body tissues and fluids. Average serum drug concentrations exceed the MIC_{90}s for most *Enterobacteriaceae* four to eight times for 8 h and are inhibitory to *P. aeruginosa* for 4 h. Aztreonam crosses inflamed meninges in sufficient amount to be potentially therapeutic for meningitis caused by susceptible organisms. Its half-life in serum is about 1.7 h, and it is excreted mainly unchanged by the kidney. Dosage modification is necessary for patients with renal failure. The drug is removed by both hemodialysis and peritoneal dialysis.

Spectrum of Activity

The antibacterial activity of aztreonam is limited to aerobic gram-negative rods, inhibiting most *Enterobacteriaceae, Neisseria* spp., and *Haemophilus* spp., with MIC_{90}s of ≤0.5 μg/ml (17, 332). It has significant activity against *Enterobacter* spp. and *S. marcescens,* with most strains being inhibited at ≤16 μg/ml. However, many *Acinetobacter* spp., *Burkholderia cepacia,* and *Stenotrophomonas maltophilia* are resistant. It shows in vitro synergism when combined with aminoglycosides against 30 to 60% of aztreonam-susceptible organisms, including *P. aeruginosa* and aminoglycoside-resistant gram-negative rods (41). Bacterial tolerance and inoculum effect are generally not seen with this agent. Aztreonam is not active against gram-positive bacteria or anaerobes.

Adverse Effects

Aztreonam is generally a safe agent, with a toxicity profile similar to those of other β-lactam drugs. Nausea, diarrhea, skin rash, eosinophilia, mild elevation of serum transaminase levels, and transiently elevated serum creatinine levels have occurred. It has minimal cross-reactivity with other β-lactams and can be used safely in patients allergic to penicillins or cephalosporins (303). Hematologic abnormalities have not been reported.

■ Carbapenems

Carbapenems are a unique class of β-lactam agents with the widest spectrum of antibacterial activity of the currently available antibiotics. Structurally, they differ from other β-lactams in having a hydroxyethyl side chain in *trans* configuration at position 6 and lacking a sulfur or oxygen atom in the bicyclic nucleus (Fig. 1d). The unique stereochemistry of the hydroxyethyl side chain confers stability against β-lactamases. Imipenem (*N*-formimidoyl thienamycin), a semisynthetic derivative of thienamycin produced by *Streptomyces* spp., meropenem, and ertapenem are the carbapenems currently available for clinical use (20, 259). Other members of this class currently undergoing preclinical evaluation or clinical trials include biapenem, doripenem, faropenem, and panipenem.

Mechanism of Action

Carbapenems bind to PBP 1 and PBP 2 of gram-negative and gram-positive bacteria, causing cell elongation and lysis (318). They are stable against most plasmid-mediated or chromosomally mediated β-lactamases except those produced by *S. maltophilia* and some strains of *B. fragilis* (249). Bacterial resistance arises from production of carbapenemases (metallo-β-lactamases) capable of hydrolyzing the carbapenem nucleus and from alteration of the porin channels in the bacterial cell wall, reducing the permeability of the drugs.

Pharmacology

After intravenous administration, the carbapenems distribute widely in the body but undergo no significant biliary excretion. Imipenem is metabolized and inactivated in the kidneys by a dehydropeptidase I (DHP-I) enzyme found in the brush border of proximal renal tubular cells. To achieve adequate concentrations in serum and urine, a DHP inhibitor, cilastatin, was developed; it is combined with imipenem in a 1:1 dosage ratio for clinical use. Cilastatin has no antibacterial activity, nor does it alter the activity of imipenem. It has a renal protective effect by preventing excessive accumulation of potentially toxic imipenem metabolites in the renal tubular cells. Meropenem, ertapenem, faropenem, and biapenem contain a β-methyl group substitution at position C-1 of the bicyclic nucleus

resulting in increased stability against inactivation by human renal DHP-I. These agents do not require concomitant administration of a DHP-I inhibitor.

The pharmacokinetics of imipenem and meropenem are very similar, with elimination half-lives in serum of about 1 h. Peak concentrations of the drugs in serum are about 25 to 35 μg/ml and 55 to 70 μg/ml following 0.5- and 1-g doses, respectively. These drugs penetrate inflamed meninges well, with drug levels of 0.5 to 6 μg/ml in the CSF (68, 259). Ertapenem is highly (>95%) bound to human plasma proteins, with poor penetration into the CSF. Its relatively long plasma half-life of 4 h allows for once-daily dosing frequency. The peak concentration in serum of 155 μg/ml is reached following a single intravenous dose of 1 g (256). Dosage adjustment of these carbapenem drugs is necessary for creatinine clearance of ≤30 ml/min. These agents, including cilastatin, are effectively removed by hemodialysis.

Spectrum of Activity

In general, all the carbapenems have similar antibacterial potencies, with minor differences. They have excellent in vitro activity against aerobic gram-positive species: staphylococci (penicillin-susceptible and -resistant isolates); viridans group streptococci; group A, B, C, and G streptococci; *Bacillus* spp.; and *L. monocytogenes.* Imipenem is two to four times more active than meropenem and ertapenem against streptococci and staphylococci, but methicillin-resistant staphylococci are usually resistant to all carbapenems. Although the MICs of carbapenems for penicillin-resistant pneumococci are elevated (MIC_{90}s of 0.25 to 2 μg/ml), many strains remain susceptible to these drugs, with imipenem being most potent (265). Ertapenem has poor activity against *Enterococcus faecalis,* but these isolates are inhibited by other carbapenems at ≤4 μg/ml. However, *Enterococcus faecium* strains are usually resistant.

More than 90% of *Enterobacteriaceae,* including those resistant to other β-lactams and aminoglycosides (254), are susceptible to carbapenems, with the following decreasing order of activity: doripenem, ertapenem, meropenem > biapenem > faropenem, imipenem (89, 97, 124, 157, 179, 215, 259, 362). These agents are highly active against clinical isolates of extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* and *E. coli,* with MIC_{90}s of 0.015 to 0.125 μg/ml (193, 205). Most *Enterobacter* spp., *Citrobacter* spp., and *Serratia* spp. are inhibited by ≤2 μg/ml. Although ertapenem is inactive against *Acinetobacter* and *Pseudomonas,* it is 5- to 10-fold more active than other carbapenems against fastidious gram-negative bacteria such as *Haemophilus, Moraxella, Neisseria,* and *Pasteurella.* Most strains of *P. aeruginosa* are inhibited by other carbapenems at 4 to 8 μg/ml, with meropenem as the most potent agent, including against imipenem-resistant strains (123, 157, 204, 275). While they inhibit *B. cepacia* and *Pseudomonas stutzeri,* carbapenems are inactive against *S. maltophilia* (83). Emergence of resistant *Pseudomonas* spp. has been observed during therapy with imipenem (373). The drug may show in vitro antagonism when combined with broad-spectrum cephalosporins or extended-spectrum penicillins as a result of its ability to induce class I β-lactamase production (249).

Carbapenems are the most potent β-lactams against anaerobes, with activities comparable to those of clindamycin and metronidazole. The MIC_{90}s for anaerobic gram-positive cocci, *Clostridium,* the *B. fragilis* group, *Fusobacterium, Porphyromonas,* and *Prevotella* are ≤4 μg/ml (133, 214, 275, 363, 366, 368). This class of drugs is also active in vitro against *Actinomyces, Nocardia,* and atypical mycobacteria (25, 72, 84, 97, 133).

Adverse Effects

The side effects of carbapenems are similar to those of other β-lactam antibiotics. Nausea, vomiting, and diarrhea occur in up to 5% of patients, usually associated with parenteral administration of ertapenem and imipenem. Pseudomembranous colitis can occur with carbapenems. Allergic reactions such as drug fever, skin rashes, and urticaria are seen in about 3% of patients. Cross-reactivity with other β-lactam agents is possible but has not been fully studied. Seizures of unclear etiology have occurred in up to 1% of patients receiving ertapenem and imipenem, particularly in the elderly age group and in patients with renal insufficiency or underlying neurologic disorders. Meropenem has not been associated with seizures. Reversible elevation of serum transaminases, leukopenia, and thrombocytopenia have been described for carbapenems, but no coagulopathy has been reported.

β-LACTAMASE INHIBITORS

Clavulanic Acid

Clavulanic acid is a naturally occurring, weak antimicrobial agent found initially in cultures of *Streptomyces clavuligerus* (252). It inhibits β-lactamases from staphylococci and many gram-negative bacteria. This agent acts primarily as a "suicide inhibitor" by forming an irreversible acyl enzyme complex with the β-lactamase, leading to loss of activity of the enzyme.

Clavulanic acid acts synergistically with various penicillins and cephalosporins against β-lactamase-producing staphylococci, klebsiellae, *H. influenzae, M. catarrhalis, N. gonorrhoeae, E. coli, Proteus* spp., the *B. fragilis* group, *Prevotella* spp., and *Porphyromonas* spp. (12, 114, 124). Plasmid-mediated TEM β-lactamases present in ceftazidime-resistant strains of *K. pneumoniae* and *E. coli* are inactivated by this drug (169). However, the inducible β-lactamases (chromosomal class I) of *Enterobacter, Citrobacter, Proteus, Acinetobacter, Serratia,* and *Pseudomonas* spp. are not inhibited by clavulanic acid (191). The combination of clavulanic acid with ampicillin, amoxicillin, or ticarcillin is active in vitro against *Mycobacterium tuberculosis,* which is known to produce β-lactamases (73, 380).

In the United States, clavulanic acid is available for clinical use in combination with oral amoxicillin at dosage ratios of 1:2, 1:4, 1:7, and 1:16 and in a 1:15 or 1:30 parenteral combination with ticarcillin. Intravenous combinations of clavulanic acid and amoxicillin at ratios of 1:5 and 1:10 are also used outside of North America. The pharmacological parameters of amoxicillin and ticarcillin are not significantly altered when either drug is combined with clavulanic acid. Amoxicillin-clavulanate is moderately well absorbed from the gastrointestinal tract, with a half-life in serum of about 1 h for each component. One-third of a dose is metabolized, while the remainder is excreted unchanged in the urine. The drug is widely distributed to various body tissues and fluids, but it penetrates uninflamed meninges very poorly.

Adverse reactions are similar to those reported for amoxicillin or ticarcillin used alone. Nausea, vomiting, abdominal cramps, and diarrhea occur in 5 to 10% of patients taking amoxicillin-clavulanate. The incidence of allergic skin reactions is similar to that with ampicillin alone.

Sulbactam

Sulbactam is a semisynthetic 6-desaminopenicillin sulfone with weak antibacterial activity (5). It functions as an effective

inhibitor of certain plasmid-mediated and chromosomally mediated β-lactamases of *S. aureus,* many *Enterobacteriaceae, H. influenzae, M. catarrhalis, Neisseria* spp., *Legionella* spp., the *B. fragilis* group, *Prevotella* spp., *Porphyromonas* spp., and *Mycobacterium* spp. (122, 237). Sulbactam alone is active against *N. gonorrhoeae, N. meningitidis,* some *Acinetobacter* spp., and *B. cepacia* (170, 239). It acts synergistically with penicillins and cephalosporins against organisms that are otherwise resistant to the β-lactam drugs because of the production of β-lactamases. A combination of sulbactam (8 μg/ml) and ampicillin (16 μg/ml) inhibits most strains of staphylococci, *Klebsiella* spp., *E. coli, H. influenzae, M. catarrhalis, Neisseria* spp., the *B. fragilis* group, *Prevotella* spp., and *Porphyromonas* spp. that are ampicillin resistant (284, 365). Like clavulanic acid, sulbactam does not inhibit the β-lactamases of *Enterobacter, Citrobacter, Providencia,* indole-positive *Proteus, Pseudomonas* spp., or *S. maltophilia.*

For clinical use, sulbactam is combined with ampicillin as a parenteral preparation in a 1:2 ratio. The pharmacological properties of the drugs are not affected by each other in this combination. Ampicillin-sulbactam penetrates well into body tissues and fluids, including peritoneal and blister fluids. It enters the CSF in the presence of inflamed meninges. Like ampicillin, sulbactam has a half-life in serum of 1 h, and 85% of the drug is excreted unchanged via the kidneys. Since clearances of sulbactam and ampicillin are affected similarly in patients with impaired renal function, dosage adjustments are similar for the two drugs.

The most common side effects of the ampicillin-sulbactam combination have been nausea, diarrhea, and skin rash. Transient eosinophilia and transaminasemia have been reported. Adverse reactions attributed to ampicillin may also occur with the use of ampicillin-sulbactam.

Tazobactam

Tazobactam (formerly YTR 830) is a penicillanic acid sulfone derivative structurally related to sulbactam. Like clavulanic acid and sulbactam, tazobactam acts as a suicidal β-lactamase inhibitor and binds to bacterial PBP 1 or PBP 2 (239). Despite having very poor intrinsic antibacterial activity by itself, it is comparable to clavulanate and sulbactam in lowering the MICs up to 20-fold for many organisms when combined with various β-lactams against β-lactamase-producing organisms. Tazobactam actively inhibits the β-lactamases of staphylococci, *H. influenzae, N. gonorrhoeae, E. coli,* the *B. fragilis* group, *Prevotella* spp., and *Porphyromonas* spp. (3, 145, 194). It also has activity against the class I β-lactamases of *Acinetobacter, Citrobacter, Proteus, Providencia*, and *Morganella* spp., but it remains inactive against those of *Enterobacter* spp., *Pseudomonas* spp., *S. maltophilia,* and some *Klebsiella* spp. (191, 194, 239). Of the penicillin–β-lactamase inhibitor combinations, piperacillin-tazobactam is the one most active (two- to eightfold-lower MICs) against β-lactamase-producing aerobic and anaerobic gram-negative rods (99, 194).

Available as a 1:8 ratio dosage combination with piperacillin, tazobactam is administered parenterally. The two drugs do not affect each other's metabolism or pharmacokinetics. High concentrations of both agents are achieved in the intestinal mucosa, lungs, and skin, with relatively poor distribution to muscle, fat, the prostate, and CSF (in the absence of inflamed meninges). With a half-life in serum of about 1 h, elimination of tazobactam is mainly via the renal route and is not affected by hepatic failure (316). Major adverse effects of the piperacillin-tazobactam combination are similar to those of piperacillin alone, such as diarrhea, skin rash, and allergic reactions. Mild elevation in serum transaminase levels may be encountered in about 10% of patients.

AMINOGLYCOSIDES AND AMINOCYCLITOLS

Since the first aminoglycoside (aminoglycosidic aminocyclitol), streptomycin, was introduced in 1944, this class of antibiotic has played a vital role in the treatment of serious infections with gram-negative organisms. Among the unique features of the aminoglycosides is the bactericidal activity against aerobic gram-negative rods (including *Pseudomonas* spp.), activity against *M. tuberculosis*, and relatively low incidence of bacterial resistance. The currently available aminoglycosides are derived from *Micromonospora* spp. (gentamicin, sisomicin, and netilmicin) or from *Streptomyces* spp. (streptomycin, neomycin, kanamycin, tobramycin, and paromomycin). The difference in origin of these compounds accounts for the differences of their suffixes, "micin" versus "mycin." Streptomycin, neomycin, kanamycin, tobramycin, and gentamicin are naturally occurring aminoglycosides, whereas amikacin and netilmicin are semisynthetic derivatives of kanamycin and sisomicin, respectively. Structurally, each of these aminoglycosides contains two or more amino sugars linked by glycosidic bonds to an aminocyclitol ring nucleus.

Spectinomycin is an aminocyclitol antibiotic isolated from *Streptomyces spectabilis*. Although it contains an aminocyclitol nucleus, it is not strictly an aminoglycoside because it does not contain an amino sugar or a glycosidic bond.

Mechanism of Action

Aminoglycosides are bactericidal agents that inhibit bacterial protein synthesis by binding irreversibly to the bacterial 30S ribosomal subunit. The aminoglycoside-bound bacterial ribosomes then become unavailable for translation of mRNA during protein synthesis, thereby leading to cell death (79). The aminoglycosides also cause misreading of the genetic code, with resultant production of nonsense proteins. To reach the intracellular ribosomal binding targets, an aerobic energy-dependent process is necessary to enable successful penetration of the bacterial inner cell membrane by the aminoglycosides. Bacterial uptake of these agents is facilitated by inhibitors of bacterial cell wall synthesis such as β-lactams and vancomycin. This interaction forms the basis of antibacterial synergism between aminoglycosides and β-lactam antibiotics (81, 130). There are three known mechanisms of bacterial resistance to aminoglycosides: (i) decreased intracellular accumulation of the antibiotic by altering the outer membrane permeability, decreasing inner membrane transport, or active efflux; (ii) modification of the target site by mutation in the ribosomal proteins or 16S RNA; and (iii) enzymatic modification of the drug (the most common mechanism present).

Spectinomycin acts similarly to the aminoglycosides by binding to the 30S ribosomal subunits and inhibiting protein synthesis. However, it does not cause misreading of the mRNA and is not bactericidal.

Pharmacology

All aminoglycosides have similar pharmacological properties. Gastrointestinal absorption of these agents is unpredictable and always low. Because of its severe toxicity with systemic administration, neomycin is available only for oral and topical use. After intravenous administration, aminoglycosides are freely distributed in the extracellular space but

penetrate poorly into the CSF, vitreous fluid of the eye, biliary tract, prostate, and tracheobronchial secretions, even in the presence of inflammation.

In adults with normal renal function, the aminoglycosides have half-lives in serum of about 2 to 3 h. They are primarily excreted, essentially unchanged, via the kidneys. There is considerable variation in the elimination of aminoglycosides among individuals, especially in patients with impaired renal function. Monitoring of serum aminoglycoside levels in these patients is essential for providing adequate therapy and reducing toxicity. With their features of concentration-dependent killing and prolonged postantibiotic effect, aminoglycosides may be administered once daily to achieve maximum bactericidal activity at high concentrations in serum without increased risk of toxicities (21). In renal failure, the drugs accumulate and dosage reductions are necessary. Aminoglycosides are substantially removed by hemodialysis and to a lesser extent by peritoneal dialysis.

Spectrum of Activity

Aminoglycoside antibiotics are active primarily against aerobic gram-negative rods and *S. aureus*. As a group, they are particularly potent against the *Enterobacteriaceae, P. aeruginosa,* and *Acinetobacter* spp. Certain differences in antimicrobial spectra among the various aminoglycosides do exist. Kanamycin is limited in its spectrum because of the common resistance of *P. aeruginosa* and frequent occurrence of plasmid-mediated inactivating enzymes among other gram-negative bacilli (79). It is now used occasionally as a "second-line" drug in combination with other antibiotics for the therapy of mycobacterial infections (350, 351). Similarly, widespread resistance among *Enterobacteriaceae* has limited the usefulness of streptomycin. As a single agent, streptomycin is used in the therapy of infections due to *Francisella tularensis* (tularemia) and *Yersinia pestis* (plague) (219). It is often used in conjunction with tetracycline for the treatment of brucellosis. It has the greatest in vitro activity of the aminoglycosides against *M. tuberculosis*. It may also be used in combination with penicillin or vancomycin for the treatment of infective endocarditis due to viridans group streptococci or enterococci, provided that the organisms do not possess high-level ribosomal or enzymatic resistance to streptomycin (356, 357, 371, 372).

Although gentamicin and tobramycin have very similar antibacterial activity profiles, gentamicin is more active in vitro against *Serratia* spp., whereas tobramycin is more active against *P. aeruginosa* (247). However, these minor differences have not been correlated with greater efficacy of one agent over the other. For the most part, gentamicin and tobramycin are susceptible to inactivation by the same modifying enzymes produced by resistant bacteria, except that in contrast to gentamicin, tobramycin can be inactivated by 6-acetyltransferase and 4′-adenyltransferase and has variable susceptibility to 3-acetyltransferase. Netilmicin and amikacin are resistant to many of these aminoglycoside-modifying enzymes and therefore are active against most *Enterobacteriaceae* that are resistant to gentamicin and tobramycin (242). Netilmicin is intrinsically less active than gentamicin and tobramycin against *P. aeruginosa*, and most gentamicin-resistant *Serratia*, *Proteus, Providencia*, and *Pseudomonas* isolates are also usually resistant to netilmicin (121). Amikacin is often used as the aminoglycoside of choice when gentamicin and tobramycin resistances are prevalent. In addition, amikacin is active against many *Mycobacterium* spp. (350, 351). Aminoglycosides are only moderately active against *Haemophilus* and *Neisseria* spp. Of the agents active against *Bartonella* spp., aminoglycosides are the only drugs consistently bactericidal toward this group of organisms (218, 219).

Although active against staphylococci, aminoglycosides are not recommended as single agents for the treatment of staphylococcal infections. Gentamicin is often combined with a penicillin or vancomycin for synergy in the treatment of serious infections due to staphylococci, enterococci, or viridans group streptococci (356, 357, 371). The aminoglycosides are not active against anaerobes.

Paromomycin is an aminoglycoside notable for its amebicidal and antihelminthic effects, and it is used clinically for the treatment of intestinal amebiasis and tapeworm infections (228). It has modest antibacterial activity against gram-positive cocci and *Enterobacteriaceae*, but *P. aeruginosa* isolates are generally resistant.

Spectinomycin is used primarily for uncomplicated anogenital infections due to *N. gonorrhoeae* (354), including β-lactamase-producing strains, and gonococci are rarely resistant to this drug (108). It is useful in patients with penicillin allergy. Spectinomycin is ineffective for pharyngeal gonococcal infections, syphilis, or chlamydial infections.

Adverse Effects

Considerable intrinsic toxicity, mainly in the form of nephrotoxicity and auditory or vestibular toxicity, is characteristic of all of the aminoglycosides. The nephrotoxic potential varies among the aminoglycosides, with neomycin being the most toxic and streptomycin the least. This effect is usually reversible when the drug is discontinued. The presence of hypotension, prolonged duration of therapy, preexisting renal insufficiency, and possibly excessive trough serum aminoglycoside concentrations increase the risk of nephrotoxicity.

All aminoglycosides are capable of causing damage to the eighth cranial nerve in humans. Vestibular toxicity is more frequently associated with streptomycin, gentamicin, and tobramycin, whereas auditory toxicity is more typical of kanamycin and amikacin. This frequently irreversible side effect may occur even after discontinuation of the drug and is cumulative with repeated courses of the agent. The ototoxicity is a result of selective destruction of the hair cells in the cochlea. Clinically detectable auditory and vestibular dysfunction has been reported to occur in 3 to 5% of patients receiving gentamicin, tobramycin, or amikacin who underwent audiometric testing (106).

Neuromuscular paralysis, which is usually reversible, can occur after rapid intravenous infusion of aminoglycosides. This phenomenon occurs particularly in the setting of myasthenia gravis or concurrent use of succinylcholine during anesthesia. Other minor adverse reactions include local pain and allergic skin rashes. No known serious adverse reactions have been reported for spectinomycin.

QUINOLONES

Quinolones belong to a group of potent antibiotics biochemically related to nalidixic acid, which was developed initially as a urinary antiseptic. Nalidixic acid and its early analogs, oxolinic acid and cinoxacin, have limited clinical applications as a result of the widespread emergence of bacterial resistance. Newer quinolones have been synthesized by modifying the original two-ring quinolone (or naphthyridone) nucleus with different side chain substitutions (10). These new agents, also known as fluoroquinolones, each contain a fluorine atom attached to the nucleus at position 6. Cinoxacin, enoxacin, norfloxacin, lomefloxacin, ciprofloxacin, ofloxacin, levofloxacin, sparfloxacin, trovafloxacin, gatifloxacin, gemifloxacin, and

moxifloxacin are currently available for clinical use in the United States. Temafloxacin and grepafloxacin have been withdrawn from clinical use due to toxicities. Garenoxacin and sitafloxacin are currently undergoing clinical investigation in the United States. Several closely related fluoroquinolones (enrofloxacin and sarafloxacin) are approved for agricultural and veterinary use in the United States and elsewhere.

Mechanism of Action

The primary bacterial target of the quinolones is DNA gyrase, a type II DNA topoisomerase enzyme essential for DNA replication, recombination, and repair (150). Newer fluoroquinolones also inhibit DNA topoisomerase IV. The DNA gyrase A subunit is the main target of quinolones in gram-negative bacteria, whereas topoisomerase IV is the primary target in gram-positive bacteria. Inhibition of these bacterial enzyme targets causes relaxation or decatenation of the supercoiled DNA, leading to termination of chromosomal replication and interference with cell division and gene expression. By inhibiting bacterial DNA synthesis, these agents are bactericidal. However, the antibacterial activity of quinolones is reduced in the presence of low pH, urine, and divalent cations (Mg^{2+} and Ca^{2+}).

Bacterial resistance to quinolones may occur by one or more of the following mechanisms: single-step chromosomal mutations in the structural genes for DNA gyrase and topoisomerase IV, mutations in the regulatory genes governing bacterial outer membrane permeability to the drug, and expression or overexpression of energy-dependent efflux pumps that can actively remove drugs from the bacterial cell (148, 267). Transferable plasmid-mediated resistance to nalidixic acid and fluoroquinolones can also occur in *Enterobacteriaceae* by acquisition of a resistance gene encoding a protein which inhibits the binding of quinolones to bacterial DNA gyrase (168, 234, 258). In addition, a transferable aminoglycoside acetyltransferase [AAC(6′).1B] has been shown to confer low-level resistance to ciprofloxacin (284a).

Pharmacology

Fluoroquinolones are generally well absorbed from the gastrointestinal tract, with the exception of norfloxacin. The oral bioavailability varies from 60 to 95% for the various fluoroquinolones (325, 375). After oral administration, concentrations in serum peak in 1 to 2 h. The presence of food does not significantly alter the absorption of these drugs. However, coadministration with iron- or zinc-containing multivitamins or with antacids containing aluminum, magnesium, or calcium substantially reduces the gastrointestinal absorption and subsequent peak concentrations of quinolones in serum. The degree of serum protein binding is generally low, ranging from 8% for ofloxacin to 75% for trovafloxacin. The prolonged elimination half-lives of fluoroquinolones, ranging from 3.5 h for ciprofloxacin to 20 h for sparfloxacin, allow for twice- or once-daily dosing (139, 237, 338). Ciprofloxacin, ofloxacin, levofloxacin, trovafloxacin, gatifloxacin, and moxifloxacin are also available for intravenous use. The intravenous formulation of trovafloxacin is prepared as alatrofloxacin mesylate, which is a prodrug of trovafloxacin and is rapidly hydrolyzed in vivo to yield the active drug.

Quinolones have good penetration into the lungs, kidneys, muscle, bone, intestinal wall, and extravascular body fluids. Concentrations in the prostate are about twice those in the serum, and concentrations 25 to 100 times above peak concentrations in serum are achieved in the urine. In the presence of meningeal inflammation, only ofloxacin, levofloxacin, trovafloxacin, gatifloxacin, and moxifloxacin achieve concentrations of >1 μg/ml in the CSF (212, 222). Quinolones penetrate well into phagocytes, such that concentrations within neutrophils and macrophages are as high as 14 times those in serum (340). This feature accounts for their excellent in vivo activity against such intracellular pathogens as *Brucella, Listeria, Salmonella,* and *Mycobacterium* spp.

Trovafloxacin and pefloxacin are metabolized mainly by the liver to form glucuronide conjugates, and pefloxacin is converted into norfloxacin in vivo. Ofloxacin exhibits little or no in vivo metabolism, and it is excreted mainly (90%) via the kidneys. The other quinolones are cleared by both the hepatic and renal routes in various proportions, with elimination primarily via the kidneys. This renal elimination is blocked by probenecid. Small amounts of these drugs are also excreted in the bile.

Hepatic insufficiency prolongs the elimination half-lives of pefloxacin and trovafloxacin, whereas the clearance of other fluoroquinolones is significantly diminished in the presence of renal failure. All of these drugs are only partially removed by hemodialysis (<15%) and are minimally affected by peritoneal dialysis because of their marked extravascular penetration, as reflected in their very large volumes of distribution.

Spectrum of Activity

Quinolones may be categorized into groups with similar spectra of antibacterial activity (Table 3), analogous to the classification of cephalosporins (10). The narrow-spectrum quinolones are inactive against gram-positive cocci, and their clinical utility is limited by widespread prevalence and rapid emergence of bacterial resistance. Broad-spectrum (second-generation) fluoroquinolones are active against both gram-positive and gram-negative bacteria (237, 335,

TABLE 3 Quinolones

Narrow spectrum (first generation)
Cinoxacin
Nalidixic acid
Oxolinic acid
Broad spectrum (second generation)
Ciprofloxacin
Enoxacin
Fleroxacin[a]
Levofloxacin
Lomefloxacin
Norfloxacin
Ofloxacin
Pefloxacin[a]
Rufloxacin[a]
Expanded spectrum (third generation)
Sparfloxacin
Tosufloxacin[a]
Extended spectrum (fourth generation)
Gatifloxacin
Garenoxacin[a]
Gemifloxacin
Moxifloxacin
Sitafloxacin[a]
Trovafloxacin

[a] Not licensed for clinical use in the United States.

346, 378). Increased activity against gram-positive cocci and favorable pharmacodynamic properties (high ratios of area under the curve from 0 to 24 h to MIC) are major features of the newer fluoroquinolones (third and fourth generations), with potencies two- to eightfold greater than those of broad-spectrum agents (9, 23, 37, 62, 64, 75, 82, 217, 271). MIC_{90}s for methicillin-susceptible and -resistant *S. aureus* and coagulase-negative staphylococci are in the range of 0.03 to 1 μg/ml, while methicillin-resistant staphylococci are becoming increasingly resistant to these agents. Although potency against enterococci is lower, gatifloxacin is two- to fourfold (MIC_{90}s of 0.25 to 0.5 μg/ml) and gemifloxacin and moxifloxacin are four- to eightfold (MIC_{90}s of 0.03 to 0.25 μg/ml) more active than levofloxacin against multidrug-resistant *S. pneumoniae*.

In contrast to earlier drugs of this class, many of the expanded- and extended-spectrum quinolones possess potent activity against anaerobes, including members of the *B. fragilis* group and *Clostridium difficile* (57, 95, 257, 317). The relative activities of these newer drugs against all anaerobes in decreasing order of potency are as follows: sitafloxacin > garenoxacin > gatifloxacin, trovafloxacin > moxifloxacin, tosufloxacin. The more active of these agents inhibit *the B. fragilis* group, *Prevotella, Porphyromonas, Fusobacterium, Clostridium,* and anaerobic gram-positive cocci at concentrations of 0.06 to 2 μg/ml. However, increasing fluoroquinolone resistance has emerged among *Bacteroides* spp. since the introduction of the newer drugs (28, 131, 314).

The fluoroquinolones possess excellent activity in vivo against *Enterobacteriaceae, P. aeruginosa, Citrobacter* spp., *Serratia* spp., *Acinetobacter* spp., *H. influenzae*, and gram-negative cocci such as *N. gonorrhoeae, N. meningitidis*, and *M. catarrhalis* (23, 27, 29, 62, 64, 82, 217, 344). Enteropathogenic gram-negative rods such as *Salmonella*, *Shigella*, *Yersinia enterocolitica, Vibrio* spp., *Aeromonas* spp., *Plesiomonas* spp., *Campylobacter jejuni,* and enteroinvasive and enterotoxigenic *E. coli* are all susceptible to the quinolones (98, 378). Clinical studies have shown these drugs to be effective in the prophylaxis and treatment of infectious diarrheas. However, reduced susceptibility and resistance to quinolones have emerged in clinical isolates of *Salmonella, Shigella,* and *Campylobacter* spp. (100, 163). *Legionella* spp. are susceptible to these agents, with MICs of most fluoroquinolones of 0.12 to 1.0 μg/ml for these organisms (29, 37, 82, 94). Fluoroquinolones are the first class of oral antibiotics with outstanding potency against *P. aeruginosa*. Ciprofloxacin and trovafloxacin are the most active among these drugs against *P. aeruginosa,* with MIC_{90}s of 0.5 to 1 μg/ml. However, *Burkholderia* spp. and *S. maltophilia* are variably resistant to quinolones (344)

The fluoroquinolones, especially ciprofloxacin, levofloxacin, ofloxacin, and sparfloxacin, are active in vitro against *M. tuberculosis,* the *Mycobacterium fortuitum* group, *Mycobacterium chelonae*, *Mycobacterium kansasii*, and *Mycobacterium xenopi* (80, 335, 383). Their activity against *Mycobacterium avium* complex is fair to poor. They also exhibit activity against *Chlamydia trachomatis, Chlamydophila pneumoniae,* and *Mycoplasma hominis*, with MIC_{90}s of 0.1 to 1 μg/ml, but are less potent against *Ureaplasma urealyticum* (98, 187). Ciprofloxacin and pefloxacin have been shown to inhibit *Rickettsia conorii*, *Rickettsia rickettsii*, and *Coxiella burnetii* (281, 282, 382). The broad-spectrum fluoroquinolones also possess potent activity against *Bartonella* spp. (218) and *Brucella melitensis* (101). Although quinolones possess in vitro activity against *Plasmodium falciparum* at achievable concentrations in serum, they are relatively ineffective when used clinically for the treatment of malaria. *Nocardia* spp. are relatively resistant to the quinolones (25, 84).

No significant inoculum effect has been observed among the bacteria susceptible to quinolones. Combinations of quinolones with (β-lactam drugs or aminoglycosides are usually indifferent or additive in their effects against gram-negative and gram-positive bacteria and mycobacteria (378). However, bactericidal activities of quinolones can be antagonized by rifampin or chloramphenicol.

Adverse Effects

Gastrointestinal symptoms, occurring in up to 10% of patients as nausea, vomiting, abdominal discomfort, and diarrhea, are the most common side effects (11, 202, 319). However, *C. difficile* colitis occurs infrequently with the use of quinolones. Headaches, fatigue, insomnia, dizziness, agitation, and, rarely, seizures can occur. These adverse neurologic effects are usually associated with high dosages in elderly patients or concurrent use of nonsteroidal anti-inflammatory drugs.

Allergic reactions are uncommon and often manifest as rash, urticaria, and generalized pruritus. Dose-related photosensitivity occurs most frequently in patients on fleroxacin, sparfloxacin, and lomefloxacin. Prolongation of the QT interval has also occurred with sparfloxacin and grepafloxacin. Rare laboratory abnormalities occurring during fluoroquinolone therapy include elevations in serum transaminases, eosinophilia, leukopenia, and thrombocytopenia. Trovafloxacin is rarely used because it results in rare occurrences of liver dysfunction or failure. Gatifloxacin can cause hypoglycemia and hyperglycemia, and its use is contraindicated in diabetic patients.

Enoxacin and, to a lesser extent, ciprofloxacin and pefloxacin increase the levels of theophylline and caffeine in serum as a result of decreased hepatic clearance (11, 277, 369). Other reported drug interactions include augmentation of the anticoagulant effects of warfarin by ciprofloxacin, norfloxacin, and ofloxacin and an increase in serum cyclosporine levels with ciprofloxacin (277).

Although irreversible cartilage erosions and skeletal abnormalities were observed in studies of quinolone toxicity in animals (11), such effects have not yet been documented clinically. However, quinolones are generally contraindicated for use in patients less than 18 years old and in pregnant or nursing mothers. Tendinitis and tendon rupture, mainly involving the Achilles tendon, have occurred with the use of fluoroquinolones, most frequently with pefloxacin and ciprofloxacin (188).

MACROLIDES

Macrolides have been in use since the early 1950s, with erythromycin as the prototypical antibiotic of this class for over 30 years (355). Their chemical structures consist of a macrocyclic lactone ring attached to two sugar moieties, desosamine and cladinose. They differ from each other in the size (14 to 16 atoms) and substitution pattern of the lactone ring. Erythromycin is a naturally occurring 14-membered-ring macrolide derived from *Streptomyces erythraeus*, and other natural analogs include oleandomycin, spiramycin, and josamycin. Clarithromycin and dirithromycin are 14-membered-ring semisynthetic macrolides, while azithromycin is a 15-membered-ring derivative also known as an azalide, with a nitrogen atom incorporated in its lactone ring. These new macrolides offer significant advantages over

erythromycin because of expanded antimicrobial spectra, improved pharmacokinetic parameters, and less frequent adverse effects and drug interactions. Roxithromycin, flurithromycin, and rokitamycin are new macrolides available for clinical use currently in Europe, Asia, and South America (189).

Mechanism of Action

Macrolides are generally bacteriostatic agents that inhibit bacterial RNA-dependent protein synthesis. They may be bactericidal at high drug concentrations and against a small inoculum of bacteria. They bind reversibly to the 23S rRNA of the 50S ribosomal subunits of susceptible organisms, thereby blocking the translocation reaction of polypeptide chain elongation (342). The presence of *erm* gene-encoded rRNA methylases that modify the 23S rRNA target-binding site is the primary mechanism of macrolide resistance and confers macrolide-lincosamide-streptogramin B (MLS_B) coresistance (93). Other less common mechanisms of resistance to macrolides include production of macrolide-inactivating enzymes (esterases, phosphorylases, and glycosidases), *mef* gene-encoded active efflux of drug, and mutations in 23S rRNA and ribosomal proteins (197).

Pharmacology

Erythromycin is available in various topical, parenteral (lactobionate and gluceptate), and oral (base stearate, ethylsuccinate, and estolate) preparations. While clarithromycin and dirithromycin are available only in oral forms, azithromycin is formulated for oral and intravenous administration. When administered orally, erythromycin base is rapidly inactivated by gastric acid, whereas the newer macrolides are stable against acid degradation. Intestinal absorption of erythromycin (except for the estolate form) and azithromycin is reduced up to 50% in the presence of food. Peak levels in serum of 2 to 3, 1 to 2, 0.2 to 0.6, and 0.4 μg/ml are reached at 3 h after oral doses of erythromycin (500 mg), clarithromycin (250 mg), dirithromycin (500 mg), and azithromycin (500 mg), respectively. Much higher concentrations of erythromycin are achieved with intravenous infusion. Tissue distributions of macrolides are excellent, with concentrations in various tissues 10- to 100-fold higher than that in serum (370). The high concentrations reached rapidly within neutrophils and macrophages account for their potent activity against intracellular pathogens (305). They penetrate poorly into the brain and CSF, but they do cross the placenta and are excreted in breast milk.

Erythromycin, clarithromycin, and dirithromycin are metabolized by the liver and primarily excreted in the bile. Azithromycin is excreted largely unchanged in the bile. Clarithromycin exhibits first-pass metabolism, producing a microbiologically active 14-hydroxy derivative that is two to four times more potent than the parent drug against some organisms. Following gastrointestinal absorption, dirithromycin is rapidly converted by nonenzymatic hydrolysis to erythromycylamine, an active derivative with microbiological activity similar to that of its parent compound. Erythromycin, clarithromycin, 14-hydroxy clarithromycin, azithromycin, and dirithromycin have terminal half-lives in serum of 1.5, 5, 8.5, 41, and 44 h, respectively. Because of its exceptionally high tissue penetration, azithromycin has a half-life in tissue of 2 to 4 days (305). Dosage adjustment of clarithromycin is necessary for patients with moderate to severe renal failure (creatinine clearance of <30 ml/min). Except for clarithromycin, macrolides are removed minimally by hemodialysis or peritoneal dialysis.

Spectrum of Activity

Macrolides are relatively broad-spectrum antibiotics, with activity against gram-positive and some gram-negative bacteria, mycoplasmas, chlamydiae, treponemes, and rickettsiae (148, 305, 370). Erythromycin shows good activity against staphylococci and streptococci, including *S. pneumoniae*, but emergence of resistance among these isolates (especially group A streptococci) is a problem in certain parts of the world (55, 190). Erythromycin and dirithromycin exhibit similar in vitro antibacterial activities (22). Clarithromycin is two- to fourfold more active than the other macrolides, and azithromycin is less active than erythromycin against most staphylococci and streptococci (16). These drugs are bactericidal against susceptible strains of streptococci but bacteriostatic toward staphylococci and enterococci. Erythromycin-resistant strains display cross-resistance to these drugs, and methicillin-resistant staphylococci and many enterococci are resistant to all macrolides. These drugs are also active against *Corynebacterium* spp., *L. monocytogenes*, and *Actinomyces israelii* (16).

The antibacterial activity of macrolides against gram-negative rods is influenced by pH, with increasing potency (lower MICs) as the pH rises to 8.5. *H. influenzae* and *M. catarrhalis* are more susceptible to azithromycin (MIC_{90} of 0.5 μg/ml) than to other macrolides (8- to 16-fold-higher MIC_{90}s) (206, 250, 273). However, additive (and possibly synergistic) activity between clarithromycin and its 14-hydroxy metabolite reduces the MIC of clarithromycin for *H. influenzae* two- to fourfold (251). Clarithromycin is the most active drug in this class against *C. pneumoniae* (MIC_{90} of 0.25 μg/ml) and *Legionella* isolates (MIC_{90} of 0.25 μg/ml) (16). All four macrolides are equally potent against *Bordetella pertussis* and *Mycoplasma pneumoniae*, and erythromycin has long been established as the drug of choice for therapy of infections due to these pathogens and *Legionella* spp. Macrolides are active against *Campylobacter* spp., *Helicobacter pylori, P. multocida, N. meningitidis,* and *Borrelia burgdorferi* (16, 225, 273, 341). Unlike other macrolides, azithromycin is also active in vitro against *E. coli, Shigella* spp., *Salmonella* spp., and *Y. enterocolitica* (206, 250).

Macrolide antibiotics are effective in vitro against many pathogens that cause sexually transmitted diseases. *N. gonorrhoeae, Haemophilus ducreyi, C. trachomatis,* and *U. urealyticum* are all susceptible, but only azithromycin is active against *M. hominis* (16, 206). Erythromycin may be used for the treatment of gonorrhea and syphilis in patients who cannot tolerate penicillin G (51), but data on the new macrolides for these indications are limited. Azithromycin is effective as an alternative to tetracyclines for the treatment of genital chlamydial infections (320). As a group, macrolides are among the most potent agents inhibitory toward *Bartonella* spp. (218).

The macrolides have good activity against anaerobic bacteria such as the *B. fragilis* group, *Fusobacterium* spp., *Prevotella* spp., *Porphyromonas* spp., *Propionibacterium acnes,* and anaerobic gram-positive cocci, with MIC_{90}s of 1 to 4 μg/ml (16). Except for dirithromycin, they are active against most *Clostridium* spp., especially *Clostridium perfringens*, with most strains inhibited at ≤ 1 μg/ml. For this reason, erythromycin is commonly used preoperatively with or without neomycin as oral bowel preparations.

Atypical mycobacteria are more susceptible than *M. tuberculosis* to macrolide antibiotics (206, 251). The MIC_{90}s of clarithromycin and azithromycin for *M. avium* complex are in the range of 2 to 4 μg/ml, allowing additive or synergistic killing activity of these organisms within infected macrophages when these drugs are combined with other

antimycobacterial drugs (16). Erythromycin is used occasionally to treat infections due to *Mycobacterium scrofulaceum, M. kansasii,* and *M. chelonae* (236, 350) and in combination with ampicillin against *Nocardia asteroides* (113).

Spiramycin and the new macrolides offer comparable in vitro activity against *Toxoplasma gondii*, and they are effective in the treatment of toxoplasmosis (228). Although spiramycin has been used to treat cryptosporidiosis, the therapeutic efficacy remains to be proven (77).

Adverse Effects

The incidence of serious side effects related to the use of erythromycin is relatively low. Gastrointestinal irritation, such as abdominal cramps, nausea, vomiting, and diarrhea, is common with oral administration and can occur when the drug is given intravenously. These side effects occur less frequently with dirithromycin, clarithromycin, and azithromycin. Thrombophlebitis is associated with intravenous infusion, but it can be avoided by dilution of the dose in a large volume of fluid and by slow infusion. Hypersensitivity reactions may include skin rash, fever, and eosinophilia. Cholestatic hepatitis occurring in adults has frequently been associated with the estolate form but has also been reported with other forms of erythromycin (329) and azithromycin (7). For this reason, erythromycin estolate is no longer recommended for use in adults.

Reversible hearing loss may occur with the use of large doses and very high concentrations of erythromycin in serum (≥4 g/day), usually in elderly patients with renal insufficiency (40, 152). Ototoxicity has also been reported with high doses of clarithromycin and azithromycin used to treat *M. avium* complex infections. Pseudomembranous colitis and superinfection of the gastrointestinal tract or vagina with *Candida* spp. or gram-negative rods occur rarely. Concurrent erythromycin therapy increases the levels of theophylline, cyclosporine, and digoxin in serum by interfering with their hepatic metabolism (345). It also increases the anticoagulant effect of warfarin. To date, no clinically significant interactions have been observed between these drugs and dirithromycin, clarithromycin, or azithromycin. However, cardiac arrhythmias have occurred during concurrent use of terfenadine with erythromycin or clarithromycin.

KETOLIDES

Ketolides are semisynthetic derivatives of erythromycin A, having a ketone group instead of an L-cladinose moiety at the 3 position on the erythronolide A ring. This modification of the chemical structure results in increased stability in acid media, noninducibility of MLS_B resistance, and enhanced activity against gram-positive cocci. The ketolides currently under clinical development also have a substituted carbamate link between carbon atoms 11 and 12 in the macrolide nucleus. This modification enables them to retain activity against bacteria whose ribosomes have been methylated at position A2058 as a result of acquired methylase genes (1). Telithromycin is the first and only ketolide approved for clinical use in the United States. Other ketolides, including cethromycin (ABT-773), are undergoing preclinical and clinical studies.

Mechanism of Action

Like the macrolide antibiotics, ketolides inhibit the translation function in susceptible organisms at the level of the 50S ribosomal subunit. Specifically, ketolides interact with the bacterial 23S rRNA at domains II and V of the peptidyltransferase site (147). These drugs are also able to inhibit the formation of the 30S ribosomal unit. Although ketolides do not induce MLS_B resistance, staphylococci with constitutively expressed MLS_B resistance encoded by *erm* genes are resistant to telithromycin. Although these drugs do not appear to be affected by efflux, mutations occurring in 23S rRNA and ribosomal proteins L4 and L22 can lead to in vitro resistance to ketolides (154). This class of drugs has a low potential to select for resistance or induce cross-resistance among other MLS_B antimicrobials.

Pharmacology

Telithromycin is administered orally as a once-daily dose of 800 mg, with rapid gastrointestinal absorption yielding a mean peak concentration in plasma of 2 μg/ml in 1 to 2 h and steady state in 2 days. A mean trough concentration in plasma of 0.07 μg/ml is attained at 24 h after dosing (246). The oral bioavailability of 57% is unaffected by food ingestion. With about 70% of the drug protein bound, telithromycin exhibits biphasic elimination from plasma with initial and terminal half-lives of 2 to 3 h and 9 to 10 h, respectively. The drug penetrates well into bronchopulmonary, tonsillar, and sinus tissues and middle ear fluid, and it is accumulated by polymorphonuclear neutrophils with an intracellular/plasma concentration ratio of >500 at 24 h. Hepatic metabolism with elimination via feces (~80%) is the main route of excretion, and <15% of the administered dose is eliminated in the urine. Dosage adjustments are not necessary in patients with renal or hepatic impairment (86).

Spectrum of Activity

Ketolides possess a good spectrum of potent activity against respiratory pathogens as well as intracellular bacteria, and telithromycin is designed specifically for the treatment of community-acquired respiratory tract infections. It is more active than macrolides against *S. pneumoniae* isolates, irrespective of penicillin susceptibility, with an MIC_{90} of ≤1 μg/ml for telithromycin, and 90% of penicillin-resistant strains are inhibited at 0.25 μg/ml (102, 110, 183, 210). Almost all macrolide-resistant strains of pneumococci are inhibited at ≤0.5 μg/ml, regardless of the underlying mechanism of macrolide resistance. Telithromycin is more active than erythromycin and clarithromycin and as potent as azithromycin against *H. influenzae* (MIC_{90}s of 2 to 4 μg/ml) and *M. catarrhalis* (MIC_{90}s of 0.06 to 0.125 μg/ml). The activity of telithromycin is unaffected by β-lactamase production in these strains, but the MICs are increased twofold in the presence of 5% CO_2. A significant postantibiotic effect may be observed for up to 9 h with this drug against the major respiratory pathogens (86).

Telithromycin is also active against staphylococci, with MIC_{90}s of 0.125 to 0.25 μg/ml for *S. aureus* and coagulase-negative staphylococci, regardless of the susceptibility to oxacillin. However, isolates harboring the constitutive MLS_B mechanism of resistance are resistant to ketolides. Enterococci without underlying resistance to macrolides and clindamycin are susceptible to telithromycin, with MIC_{90}s of 0.125 μg/ml for *E. faecalis* and *E. faecium*. Higher MIC_{90}s (4 to 8 μg/ml) are observed with erythromycin- or clindamycin-resistant enterococci (306). Telithromycin displays good in vitro activity against beta-hemolytic streptococci and viridans group streptococci, regardless of their susceptibility to penicillin G, with all isolates inhibited at ≤0.5 μg/ml. While streptococcal isolates with the *mef*A gene-mediated drug efflux mechanism of resistance to erythromycin remain susceptible to telithromycin, MICs are higher (2 to 16 μg/ml) among the strains with

inducible or constitutive *erm* gene-mediated resistance to erythromycin. Other gram-positive cocci, such as *Pediococcus, Leuconostoc, Stomatococcus,* and *Rhodococcus equi*, are susceptible to telithromycin, with MIC_{90}s of 0.03 to 0.25 μg/ml.

This drug is also very active against gram-positive rods, inhibiting *Corynebacterium* (including *C. diphtheriae* and *C. jeikeium*), *Listeria, Lactobacillus, Actinomyces,* and *Erysipelothrix* at concentrations of ≤0.125 μg/ml (306). Telithromycin is inhibitory (MIC_{90}s of 0.125 to 0.5 μg/ml) to *Peptostreptococcus* spp., *Prevotella* spp., *Porphyromonas* spp., *Bilophila* spp., and *C. perfringens,* but it is not active against other *Clostridium* spp., *Fusobacterium* spp., or the *B. fragilis* group (367). This drug has poor activity against other gram-negative rods, including the *Enterobacteriaceae, Acinetobacter* spp., *P. aeruginosa,* and *B. burgdorferi* (166).

Intracellular pathogens, such as *Legionella, Mycoplasma, Chlamydia,* and *Chlamydophila* are highly susceptible to telithromycin, with MIC_{90}s of 0.004 to 0.25 μg/ml (110). *Rickettsia* spp., *Bartonella* spp., *C. burnetii,* and *F. tularensis* are also susceptible to this agent. Telithromycin is comparable to the macrolides in its activity against mycobacteria, with an MIC_{90} of 4 μg/ml for *M. chelonae* and *M. avium* complex, and it is not active against *M. tuberculosis, Mycobacterium bovis,* and other atypical mycobacteria (111).

Adverse Effects

Telithromycin is well tolerated by all patient populations, with gastrointestinal symptoms, such as diarrhea (15%), nausea (9%), vomiting, and dizziness, as the most frequent adverse effects (8). Occurrence of *C. difficile*-associated diarrhea has not been reported in clinical trial studies. While elevation of serum transaminase levels is found in <10% of patients, rare cases of severe hepatotoxicity can occur (60). Since ketolides are substrates and inhibitors of the hepatic cytochrome P450 CYP3A4 isoenzyme pathway, their potential to lengthen the QT interval is augmented by concomitant administration of other CYP3A4 inhibitors, such as ketoconazole and itraconazole (24).

TETRACYCLINES AND GLYCYLCYCLINES

Tetracyclines are broad-spectrum bacteriostatic antibiotics with the hydronaphthacene nucleus, which contains four fused rings. The congeners form three groups based on their duration of action. Chlortetracycline, oxytetracycline, and tetracycline are short-acting drugs, demeclocycline and methacycline are intermediate-acting drugs, and doxycycline and minocycline are long-acting drugs. Glycylcyclines are a group of semisynthetic tetracycline derivatives containing a glycylamido substitution at position 9, and tigecycline (formerly GAR-936, and a 9-*t*-butylglycylamido analog of minocycline) is the first in this class of drugs available for clinical use.

Mechanism of Action

Tetracyclines and glycylcyclines act against susceptible microorganisms by inhibiting protein synthesis. They enter bacteria by an energy-dependent process and bind reversibly to the 30S ribosomal subunits, preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor A-site in the RNA-ribosome complex (56). Resistance to tetracyclines occurs among clinical isolates as a result of active efflux of the drug from the cell, an altered ribosomal target site that prevents binding of the drug (ribosomal protection), or production of modifying enzymes that inactivate the drug. With stearic hindrance from the bulky side group at position 9, glycylcyclines are unaffected by bacterial ribosomal protection proteins and evade the efflux pumps present in tetracycline-resistant strains. These drugs also have higher binding affinity for the bacterial ribosomes than tetracyclines. Reduced susceptibility to tigecycline has been found among clinical and laboratory-derived strains of *P. mirabilis, Morganella morganii, Klebsiella pneumoniae, P. aeruginosa,* and *S. aureus* possessing multicomponent efflux pumps (220, 294) and in some *Bacteroides* spp. with the *tet*(X) gene-mediated monooxygenase enzyme that degrades tetracyclines (238).

Pharmacology

Tetracyclines are incompletely absorbed from the gastrointestinal tract, but their absorption is improved in the fasting state. Ingestion of food, especially dairy products, and other substances such as antacids and iron preparations impairs the absorption of these drugs. Less interference with absorption by foods occurs with doxycycline and minocycline. These long-acting tetracyclines are more readily absorbed, and therefore, lower doses are required. Peak concentrations in serum of 3 to 5 μg/ml are reached in 2 h after standard oral dosages. Intravenous preparations are available, and peak concentrations in serum of 10 to 20 μg/ml are reached in 1 h after intravenous administration. Tetracyclines are usually bacteriostatic at these clinically achievable concentrations in serum.

Tissue penetration of these drugs is excellent, but levels in CSF are low even in the presence of meningeal inflammation. Tetracyclines cross the placenta and are incorporated into fetal bone and teeth. They are excreted in high concentrations in human milk. Therefore, tetracyclines are not advised for pregnant or lactating women. Minocycline, the most lipophilic tetracycline at physiological pH, reaches relatively high concentrations in saliva and tears, making it an ideal antibiotic to eradicate the meningococcal carrier state (146, 158).

Tetracyclines are metabolized by the liver and concentrated in the bile. Biliary concentrations of tetracyclines are three to five times higher than concurrent levels in plasma, with significant drug accumulation in the blood of patients with hepatic insufficiency or biliary obstruction. These drugs are excreted primarily in the urine except for doxycycline, which is excreted primarily (90%) as an inactive conjugate via the biliary tract in the feces. Renal failure prolongs the half-lives of the tetracyclines except doxycycline. Therefore, doxycycline is considered the tetracycline of choice for extrarenal infections in the presence of renal failure.

Tigecycline is administered as an intravenous formulation because of limited oral bioavailability. With multiple doses of 50 mg infused every 12 h, the peak concentration in serum is ~0.8 μg/ml (227). Despite having plasma protein binding of 80%, the drug has a rapid and wide distribution into tissues, with highest levels in bone and bone marrow. Although drug levels of >1 μg/ml can be achieved in CSF in a rabbit model of meningitis, penetration into human CSF with or without meningitis remains unknown (260). It is eliminated primarily by the liver via glucuronidation and biliary excretion of unchanged drug, and the mean elimination half-life is 36 h. With <30% of the drug excreted unchanged in the urine, dosage adjustment is not required for renal insufficiency, hemodialysis, or mild to moderate hepatic dysfunction.

Spectrum of Activity

All tetracyclines have similar antimicrobial spectra, with activity against many gram-positive and gram-negative bacteria, mycoplasmas, chlamydiae, rickettsiae, and some protozoa. Many gram-positive aerobic cocci, including *S. aureus, S. pyogenes,* and *S. pneumoniae,* are susceptible at concentrations

achievable in serum. However, tetracycline-resistant strains of *S. pneumoniae* are common (190). Although many *E. coli* isolates are susceptible to tetracyclines, pseudomonads and many *Enterobacteriaceae* are resistant. *Shigella* and *Salmonella* spp. are increasingly resistant to these agents (54). Tetracyclines are used mainly for the treatment of acute, uncomplicated urinary tract infections due to *E. coli* (161) and as effective prophylactic therapy for traveler's diarrhea caused by enterotoxigenic *E. coli* (117). With activity against *Burkholderia pseudomallei, Brucella* spp., *Vibrio* spp., and *Mycobacterium marinum* (351), they have been used successfully in the treatment of infections due to these bacteria. Their efficacy in the therapy of cholera is diminishing owing to the emergence of resistant *Vibrio cholerae* isolates (381). Minocycline is active against *Nocardia* spp. (84). Many anaerobic bacteria, including members of the *B. fragilis* group and *Actinomyces* spp., are susceptible to tetracyclines (265).

These drugs are useful in the treatment of urethritis and acute pelvic inflammatory diseases caused by *N. gonorrhoeae, C. trachomatis, U. urealyticum,* and *M. hominis*. Resistance to tetracyclines among *N. gonorrhoeae* strains is increasing (108). The drugs are effective for the treatment of other chlamydial infections (psittacosis, lymphogranuloma venereum, and trachoma) (51). Other infections responsive to tetracyclines include granuloma inguinale, chancroid, relapsing fever, and tularemia. Tetracyclines are the drugs of choice for treating rickettsial infections (Rocky Mountain spotted fever, endemic and scrub typhus, and Q fever). Many pathogenic spirochetes, including *Treponema pallidum* and *B. burgdorferi,* are susceptible (51, 229, 243). Protozoa such as *P. falciparum* and *Entamoeba histolytica* are also inhibited by these drugs (228, 263).

Due to its potent and wide-spectrum activity against gram-positive, gram-negative, and anaerobic organisms, tigecycline is useful for treatment of complicated skin and skin structure infections, intra-abdominal infections, and nosocomial infections due to multidrug-resistant pathogens except *P. aeruginosa* (32, 119, 156, 167, 260, 264). All staphylococci, including methicillin-resistant and vancomycin-intermediate strains, are inhibited at ≤1 μg/ml. Vancomycin-susceptible and -resistant enterococci typically show MIC_{90}s of 0.25 to 0.5 μg/ml, and *E. faecium* and *E. faecalis* are inhibited equally well by this drug. However, tigecycline exhibits no bactericidal activity (minimum bactericidal concentration at which 90% of strains tested are killed [MBC_{90}] > 32 μg/ml) against staphylococci and enterococci in time-kill studies. Viridans group streptococci, beta-hemolytic streptococci, and multidrug-resistant pneumococci are highly susceptible, with MIC_{90}s ranging from ≤0.25 to 0.5 μg/ml. The drug is also very active against the *Enterobacteriaceae* and nonfermentative gram-negative rods, including strains producing extended-spectrum β-lactamases, with MIC_{90}s of ≤2 μg/ml. Of note is its potent inhibition of multidrug-resistant *Acinetobacter baumannii* and *S. maltophilia* (MIC_{90}s of 2 μg/ml). Among fastidious respiratory tract pathogens, MIC_{90}s are 0.5 and 1 μg/ml for *H. influenzae* and *M. catarrhalis,* respectively. *Proteus, Morganella,* and *Providencia* spp. and *P. aeruginosa* are generally resistant (MIC_{90}s of >16 μg/ml). Tigecycline is also active against most *B. fragilis* group isolates (MIC_{90} = 8 μg/ml), *Peptostreptococcus* spp. (MIC_{90} = 4 μg/ml), *Clostridium* spp. (MIC_{90} = 0.5 μg/ml), *Prevotella* spp., *Propionibacterium* spp., and *Fusobacterium* spp.

Compared to tetracyclines, tigecycline exhibits more potent activity against *M. pneumoniae, M. hominis, C. pneumoniae,* and *C. trachomatis,* with MIC_{90}s in the range of 0.125 to 0.5 μg/ml. It is less active toward *U. urealyticum* and *Legionella* spp., with MIC_{90}s of 8 μg/ml. Rapidly growing mycobacteria, including *Mycobacterium abscessus, M. chelonae,* and the *M. fortuitum* group, are 4- to 11-fold more susceptible (MIC_{90}s of ≤0.25 μg/ml) to this drug than to tetracyclines (347). However, slowly growing nontuberculous mycobacteria, such as *M. kansasii, M. marinum,* and *M. xenopi,* are less susceptible to tigecycline than to minocycline.

Susceptibility testing with tigecycline should be done using freshly prepared media or media containing a biocatalytic oxygen-reducing reagent (e.g., Oxyrase), because the drug is prone to oxidative degradation. Testing using aged broth media (prepared >12 h prior to inoculation) yielded MIC results that are generally 1 to 2 dilutions higher than those obtained using fresh media (34, 162).

Adverse Effects

Tetracyclines have irritative effects on the upper gastrointestinal tract, producing esophageal ulcerations, nausea, vomiting, and epigastric distress. Alterations in the enteric flora occur with the use of tetracyclines, often resulting in diarrhea, and pseudomembranous colitis can develop with prolonged use. Hypersensitivity reactions are unusual, generally manifesting themselves as urticaria, fixed drug eruptions, morbilliform rashes, and anaphylaxis. Cross-reactivity among tetracyclines is the rule. Photosensitivity reactions consist of an erythematous rash on areas exposed to sunlight and can occur with all analogs, especially demeclocycline (120).

Minocycline has been known to cause vertigo, and benign intracranial hypertension (pseudotumor cerebri) has been described with many of the analogs (352). Tetracycline can aggravate preexisting renal failure by inhibiting protein synthesis, increasing the azotemia from amino acid metabolism. Tetracyclines cause depression of bone growth, permanent discoloration of the teeth, and enamel hypoplasia when given during tooth and skeletal development (143). Therefore, these drugs are usually avoided in childhood (<8 years of age) and during pregnancy.

Tigecycline is generally well tolerated, with nausea, vomiting, headache, and diarrhea reported as the most common side effects. Due to adverse effects similar to those of tetracyclines on bone and tooth development, use of this drug is contraindicated in pregnant women, nursing mothers, and those <18 years of age. It may also show cross-hypersensitivity to tetracyclines (385).

LINCOSAMIDES

The lincosamide antibiotics include lincomycin, which was initially isolated from *Streptomyces lincolnensis,* and clindamycin, which is a chemical modification of lincomycin. The chemical structure of each drug consists of an amino acid linked to an amino sugar. Compared with lincomycin, clindamycin has increased antibacterial activity and improved absorption after oral administration (224). Both drugs are available for parenteral and oral use, but lincomycin is rarely used now in the United States.

Mechanism of Action

Lincosamides bind to the 50S ribosomal subunits of susceptible bacteria and prevent elongation of peptide chains by interfering with peptidyl transfer, thereby suppressing protein synthesis. The ribosomal binding sites are the same as or closely related to those that bind macrolides, streptogramins, and chloramphenicol (197, 342). Lincosamides can be bactericidal or bacteriostatic, depending on the drug concentration, bacterial species, and inoculum of bacteria.

Pharmacology

About 90% of an oral clindamycin dose is absorbed from the gastrointestinal tract, with no interference from the ingestion of food. A single oral dose of 150 mg yields a peak concentration in serum of 2 to 3 μg/ml in 1 h. Peak levels in serum of 10 to 12 μg/ml are obtained at 1 h after a 600-mg intravenous dose. Therapeutic serum drug levels are maintained for 6 to 9 h after these dosages (199, 224).

Clindamycin distributes well into bone, the lungs, pleural fluid, and bile, but it penetrates poorly into CSF, even with meningitis. It readily crosses the placenta and enters fetal tissues. Clindamycin is actively concentrated in neutrophils and macrophages.

The normal half-life of clindamycin is 2.4 h. Most of the drug is metabolized by the liver and excreted in an inactive form in the urine. Its half-life is prolonged by severe liver dysfunction, necessitating dosage reduction in patients with severe liver disease. Although the serum drug levels are increased in patients with severe renal failure, dose modification is not essential. The drug is not removed significantly by hemodialysis or peritoneal dialysis.

Spectrum of Activity

Lincosamides have a broad spectrum of activity against the aerobic gram-positive cocci and anaerobes. Clindamycin is more potent than lincomycin against methicillin-susceptible *Staphylococcus* spp., *S. pneumoniae,* and group A and viridans group streptococci (199, 224). The MIC_{90}s are in the range of 0.01 to 0.1 μg/ml for these strains. However, resistance to clindamycin has emerged in clinical isolates of these bacteria that are also resistant to erythromycin (55). The prevalence of clindamycin-resistant *S. aureus* may be 15 to 20% in some institutions. Enterococci are uniformly resistant to the lincosamides. All of the *Enterobacteriaceae* are resistant to lincosamides.

Clindamycin is one of the most active antibiotics available against anaerobes, including members of the *B. fragilis* group and *C. perfringens,* with MIC_{90}s of ≤2 μg/ml (18, 199). However, clindamycin resistance (which appears to be increasing) is found in 10 to 15% of *B. fragilis* group organisms, 15 to 20% of *Prevotella* and *Porphyromonas* spp., 10 to 20% of clostridial species, 10% of peptococci, and most *Fusobacterium varium* strains (2, 314). Clindamycin has been used successfully as single-agent therapy for actinomycosis (287), babesiosis (228, 377), and malaria (200). It is also effective in combination with pyrimethamine for toxoplasma encephalitis (76, 286) and in combination with primaquine for *Pneumocystis jirovecii* pneumonia (336).

Adverse Effects

Clindamycin-associated diarrhea occurs in up to 20% of patients, and use of this drug has been associated with pseudomembranous colitis caused by toxin-producing *C. difficile* (19). This complication is not dose related and may occur after oral or parenteral therapy. Prompt cessation of the antibiotic in conjunction with oral vancomycin, metronidazole, or bacitracin therapy is effective in reversing this complication.

Other uncommon side effects include skin rashes, fever, and reversible elevation of serum transaminases. Clindamycin can block neuromuscular transmission and may potentiate the action of neuromuscular blocking agents during anesthesia.

GLYCOPEPTIDES AND LIPOPEPTIDES

Vancomycin, a bactericidal antibiotic obtained from *Streptomyces orientalis*, is the only glycopeptide marketed for clinical use in the United States. Initially introduced for its efficacy against penicillin-resistant staphylococci, it has become most useful against methicillin-resistant staphylococci and in patients allergic to penicillins or cephalosporins. Teicoplanin (formerly teichomycin A), a new complex glycopeptide chemically related to vancomycin (315), is currently available for clinical use in most countries of the world except the United States. Dalbavancin, oritavancin, and telavancin are semisynthetic lipoglycopeptides (glycopeptide derivatives with hydrophobic substituents) currently at various stages of clinical development (185, 262). Daptomycin is a unique, naturally occurring cyclic lipopeptide antibiotic found among the fermentation by-products of *Streptomyces roseosporus* and has potent activity against gram-positive bacteria. Ramoplanin is an investigational semisynthetic lipoglycodepsipeptide with a spectrum of activity similar to those of the glycopeptides (103), but systemic toxicity limits its clinical trials only to oral administration and topical application.

Mechanism of Action

Glycopeptides inhibit peptidoglycan synthesis in the bacterial cell wall by complexing with the D-alanyl-D-alanine portion of the cell wall precursor (244). Resistance to vancomycin and teicoplanin can occur by one of two mechanisms: (i) the presence of a complex series of bacterial cytoplasmic enzymes in vancomycin-resistant enterococci synthesizing abnormal peptidoglycan precursors terminating in D-Ala-D-lactate residues (instead of D-Ala-D-Ala), thereby markedly lowering the binding affinity with the glycopeptides (52), or (ii) increased accumulation of peptidoglycan precursors (murein monomers) resulting in a thickened cell wall with "trapping" of drug molecules, thereby preventing further diffusion of drug into the inner part of cell wall layers of vancomycin-intermediate *S. aureus* (69, 70, 153).

Daptomycin binds irreversibly to the cytoplasmic membrane of susceptible bacteria via a calcium ion-dependent insertion of the hydrophobic tail of the molecule and causes membrane depolarization, resulting in release of potassium ions and disruption of cellular ion concentration gradients (44, 311). The end effect is cell death without cell lysis, providing potent bactericidal activity of this drug. It is unable to penetrate the outer membrane of gram-negative bacteria. Resistance to daptomycin has occurred in enterococci (208, 297) and *S. aureus* (151, 312), possibly due to the physical barrier of a thickened bacterial cell wall similar to those found in vancomycin-intermediate *S. aureus* or to other mechanisms, such as alteration of the charge of the outer cell envelope (71).

Ramoplanin acts by binding to a lipid II intermediate present in the cell membrane of gram-positive organisms and inhibiting bacterial transglycosylase (PBP 1b), an enzyme essential for peptidoglycan synthesis. With this unique mechanism of action, this drug is bactericidal against vancomycin-resistant enterococci and staphylococci.

Pharmacology

Vancomycin and teicoplanin can be administered orally or parenterally. After oral administration, the drugs are poorly absorbed, and high concentrations in stools are achieved, accounting for their efficacy in treating pseudomembranous colitis (109). Desirable peak and trough levels in serum of 20 to 50 μg/ml and 5 to 15 μg/ml, respectively, are obtained after a 1-g intravenous dose of vancomycin every 12 h in healthy subjects (296). Similar serum drug concentrations are reached with intravenous teicoplanin, which has the advantage of a longer serum half-life and which can be administered once daily. Therapeutic levels of both drugs are

achieved in synovial, ascitic, pericardial, and pleural fluids, with variable penetration into the CSF only in the presence of inflamed meninges (144, 234).

Vancomycin and teicoplanin have half-lives in serum of 6 and 45 h, respectively, in patients with healthy renal function, and they are eliminated from the body by glomerular filtration. In severe renal insufficiency, their excretion is prolonged to about 9 days, and they are not removed by hemodialysis or peritoneal dialysis.

Intravenous infusion of daptomycin at 6 mg/kg of body weight results in peak and trough concentrations of 82 and 6 μg/ml, respectively, in serum. About 90% of the drug is bound to plasma proteins, with limited metabolism. Despite its wide distribution into various body sites and tissues, daptomycin shows poor penetration into the CSF and alveolar space, where it is bound by surfactant, precluding its use for the treatment of meningitis and pneumonia (323). Its elimination half-life is 9 h, and 80% of the drug excreted via the kidneys, with two-thirds as intact drug (299). The dosing interval is increased to every 48 h when creatinine clearance is ≤30 ml/min.

Without systemic absorption or intestinal degradation of the drug, ramoplanin administered orally at dosages of 200 and 400 mg twice daily reaches high concentrations in the stool, with mean minimum drug levels of ~460 and ~760 μg/g in the feces, respectively. This feature enables the drug to be developed for clinical trials in treatment of *C. difficile*-associated diarrhea and gastrointestinal decolonization to prevent nosocomial bacteremia due to vancomycin-resistant enterococci.

Spectrum of Activity

Glycopeptides and lipopeptides are active mainly against aerobic and anaerobic gram-positive organisms, including methicillin-susceptible and -resistant staphylococci, streptococci, enterococci, *Corynebacterium* spp., *Bacillus* spp., *L. monocytogenes, Clostridium* spp., and *Actinomyces* spp. The MICs of vancomycin for *S. aureus, S. epidermidis,* streptococci, and enterococci are typically in the range of 0.25 to 2 μg/ml (176). The bactericidal activity varies, with MBCs 20-fold higher than MICs for viridans group streptococci. These agents are essentially bacteriostatic against enterococci and staphylococci. Teicoplanin (138, 141, 211), ramoplanin (65), and daptomycin (14, 211, 374) are two- to fourfold more active than vancomycin against these gram-positive cocci. Resistance to vancomycin has emerged among clinical isolates of enterococci (52) and staphylococci (153). Cross-resistance with teicoplanin is variable in these strains, but most are susceptible to daptomycin and ramoplanin (172), with MICs of ≤2 μg/ml. Other naturally vancomycin-resistant gram-positive organisms, such as *Leuconostoc, Lactobacillus*, and *Pediococcus* spp., are susceptible to ramoplanin (65, 172, 293).

Vancomycin is useful in the prevention and treatment of endocarditis due to gram-positive bacteria in patients who are allergic to penicillin (74, 371). It is the drug of choice for treating *C. jeikeium* infections (128) and is useful for *Chryseobacterium meningosepticum* meningitis (144) and antibiotic-associated *C. difficile* colitis (19, 109).

The glycopeptides and lipopeptides are not active against gram-negative organisms or mycobacteria. They show no cross-resistance with other unrelated antibiotics. They act synergistically with aminoglycosides or rifampin against staphylococci, streptococci, and enterococci (240, 337, 356, 357), and they are bactericidal with aminoglycosides against *Listeria* spp.

Daptomycin exhibits concentration-dependent bactericidal activity in vitro against most gram-positive bacteria in their growing and stationary phases (295). The MIC_{90}s for multidrug-resistant isolates of staphylococci (including vancomycin-intermediate *S. aureus*), pneumococci, and streptococci are ≤1 μg/ml (182, 327, 328). Vancomycin-susceptible and -resistant enterococci are inhibited equally at an MIC_{90} of ≤4 μg/ml (182, 327). The drug is active (MIC_{90}s of ≤2 μg/ml) against *Listeria, Corynebacterium* spp., *Propionibacterium* spp., *C. difficile, C. perfringens,* and peptostreptococci (134). While *Leuconostoc, Pediococcus,* and *Lactobacillus* are susceptible, other *Clostridium* spp., *Actinomyces*, and *Eubacterium* showed MIC_{90}s in the range of 4 to 16 μg/ml. Since optimal in vitro activity of daptomycin depends on the calcium ion concentration in the growth medium, media used for susceptibility testing with this drug should contain the recommended calcium concentration of 50 μg/ml. Current E-test strips of daptomycin used in the drug gradient diffusion method are manufactured with added calcium. Inconsistent results have been observed with the disk diffusion method, and the agar dilution method has not been validated for testing this drug.

Adverse Effects

The most frequent side effects of vancomycin are fever, chills, and phlebitis at the site of infusion. Rapid or bolus infusion of vancomycin causes tingling and flushing of the face, neck, and thorax, known as the red man syndrome, as a result of histamine release by basophils and mast cells (278). This phenomenon is not due to allergic hypersensitivity. Allergic maculopapular or diffuse erythematous rashes can occur in up to 5% of patients. Reversible leukopenia or eosinophilia can rarely develop with glycopeptide use.

Hearing loss due to ototoxicity has been described occasionally for patients in whom serum vancomycin concentrations exceed 50 μg/ml, but it is hard to find unequivocal evidence of vancomycin ototoxicity in humans or animals (40). Vancomycin-induced nephrotoxicity has been rare since the recent availability of highly purified vancomycin preparations. However, the risk of nephrotoxicity increases during combination therapy with vancomycin and aminoglycosides.

Teicoplanin is generally well tolerated and does not produce the red man syndrome or nephrotoxicity. It does cause irritation at the site of intravenous infusion, and ototoxicity has been reported (78).

Common adverse reactions of daptomycin include diarrhea, rash, dizziness, and dyspnea. Elevated serum creatinine phosphokinase levels, myalgias, and reversible myopathy can occur (4, 45), but these adverse effects are significantly reduced with lower dosages and once-daily dosing.

STREPTOGRAMINS

Streptogramins are natural cyclic peptides produced by *Streptomyces* spp. They are a unique class of antibiotics in which each member of the class is a combination of at least two structurally unrelated components, group A and B streptogramins, acting synergistically against susceptible bacteria (269). Group A streptogramins are polyunsaturated macrolactones consisting of lactam and lactone linkages with an oxazole ring, and the main compounds in this group are pristinamycin II_A and pristinamycin II_B. Group B streptogramins are cyclic hexadepsipeptides, with pristinamycin I_A and pristinamycin I_C as the principal compounds. Quinupristin-dalfopristin is the first injectable streptogramin antibiotic combination developed for clinical use in the United States. It is a 30:70 mixture of the semisynthetic streptogramins quinupristin and dalfopristin, which are water-soluble derivatives of pristinamycin I_A and pristinamycin II_A, respectively.

Mechanism of Action

The streptogramins exert a synergistic bactericidal effect on susceptible organisms by inhibiting bacterial protein synthesis. They enter bacterial cells via passive diffusion and then bind specifically and irreversibly to the 50S subunits of the 70S bacterial ribosomes. Binding of group A streptogramins to the ribosome induces a conformational change in the ribosome which increases its affinity for group B compounds. Group A streptogramins prevent peptide bond formation during the chain elongation step, while group B components cause release of the incomplete peptide chains from the 50S ribosomal subunit (342).

Acquired bacterial resistance to the streptogramins, which may be chromosomal or plasmid mediated, is mainly due to modification of the drug target by methylation of the bacterial 23S rRNA, resulting in resistance to all macrolides, lincosamides, and group B streptogramins (MLS_B resistance phenotype), but not to group A streptogramins. Mutations in the L22 ribosomal protein gene (*rplV*), active efflux of group A and B streptogramins, and drug inactivation by streptogramin A acetylase and streptogramin B hydrolase have been described.

Pharmacokinetics

Quinupristin-dalfopristin is administered intravenously, with distribution into most tissues. Both components are highly protein bound (70 to 90%) and rapidly cleared from plasma via biliary excretion by hepatic conjugation processes (209). Less than 20% of the administered drug combination is excreted in the urine. Following intravenous doses of 7.5 mg/kg, peak concentrations in serum of quinupristin and dalfopristin reach 2.7 and 7.2 μg/ml, respectively, with elimination half-lives of 1 and 0.75 h. The two components penetrate and accumulate in macrophages, and the ratio of peak in vitro cellular and extracellular concentrations is 50:35. The drug combination does not cross the noninflamed blood-brain barrier or placenta to any significant degree. Dosage adjustment is needed for patients with renal insufficiency (creatinine clearance of <30 min/min), and the drug combination is removed in modest amounts by dialysis.

Spectrum of Activity

Streptogramins are active mainly against gram-positive bacteria, with modest activities against selected gram-negative and anaerobic pathogens. Quinupristin-dalfopristin has potent bactericidal activity against methicillin-susceptible and -resistant *S. aureus,* coagulase-negative staphylococci, and streptococci, with MIC_{90}s of ≤ 1 μg/ml and MBCs within two- to fourfold of the MICs (31, 112). Staphylococci and streptococci, including *S. pneumoniae,* that are resistant to β-lactam drugs, macrolides, and fluoroquinolones usually remain susceptible to quinupristin-dalfopristin. While most *E. faecium* strains (MIC_{90}s of ≤ 4 μg/ml) are susceptible, *E. faecalis* is intrinsically resistant (MIC_{90}s ≥ 32 μg/ml) to the drug combination because of active efflux of dalfopristin. Although it is not bactericidal against enterococci, quinupristin-dalfopristin inhibits vancomycin-resistant *E. faecium* (VanA or VanB phenotype), including multidrug-resistant strains, at MIC_{90}s of ≤ 2 μg/ml (209). This drug combination has been a significant addition to the current therapeutic options for serious multidrug-resistant gram-positive bacterial infections (195, 201, 235, 270). *N. meningitidis, N. gonorrhoeae, M. pneumoniae, C. pneumoniae,* and *Legionella pneumophila* are all highly susceptible to the drug (MIC_{90}s of ≤ 2 μg/ml). Quinupristin-dalfopristin is also active against *M. catarrhalis* and *H. influenzae*, with MIC_{90}s of ≤ 4 μg/ml. *Enterobacteriaceae* and other nonfermenting gram-negative rods are resistant.

Among the anaerobes, *C. perfringens* and *C. difficile* are the most susceptible (MIC_{90}s of 0.25 μg/ml). Quinupristin-dalfopristin is active against the *B. fragilis* group (MIC_{90} of 4 μg/ml) as well as other anaerobic bacteria, including *Prevotella, Porphyromonas, Fusobacterium, P. acnes, Lactobacillus,* and peptostreptococci, with MIC_{90}s of 2 to 4 μg/ml.

Adverse Effects

Phlebitis at the site of intravenous infusion is the major local adverse reaction, and the incidence and severity are dose and concentration related (209, 292). The most common systemic side effects that may lead to discontinuation of therapy are arthralgias and myalgia, both of which are reversible on discontinuation of the combination (235, 280). Elevated levels of serum transaminases and cutaneous reactions such as itching, burning, and erythema of the face, neck, or upper body have also been reported.

OXAZOLIDINONES

Oxazolidinones are a unique group of synthetic antibiotics originally discovered in the 1970s (216). Linezolid is currently the only oxazolidinone available for clinical use (233), while other analogs are undergoing preclinical development.

Mechanism of Action

The oxazolidinones inhibit bacterial protein synthesis by preventing the formation of a functional initiation complex consisting of $tRNA^{fMet}$, mRNA, initiation factors, and the ribosome (330). Linezolid binds to the domain V region of 23S rRNA in the 50S ribosomal subunit, thereby distorting the binding site for $tRNA^{fMet}$ and inhibiting formation of a functional 70S initiation complex, thus preventing initiation of mRNA translation. In this regard, this class of antibiotics is unique, without cross-resistance with other antibiotics that also inhibit ribosomal protein synthesis. Resistance to linezolid has occurred in clinical isolates of methicillin-resistant *S. aureus,* vancomycin-resistant enterococci, and pneumococci as a result of point mutations in genes encoding the 23S rRNA (30, 231) or L4 ribosomal protein (379). Oxazolidinones are generally inactive against gram-negative bacteria because of endogenous efflux pumps present in these organisms (330).

Pharmacology

Linezolid is available in oral and parenteral forms. Rapid and extensive absorption occurs after oral administration (>95% bioavailability), reaching maximum concentrations in serum of 15 to 20 μg/ml within 2 h after an oral dose of 600 mg. The drug is metabolized primarily in the liver, and the elimination half-life is about 5 h. With 30% of the drug being protein bound, it is well distributed in all body tissues, including the CSF. The drug is eliminated via the kidneys, with 30% being excreted unchanged in the urine. No dose adjustment is necessary in patients with renal insufficiency or mild to moderate hepatic impairment, while 20% of a dose is removed by hemodialysis (213).

Spectrum of Activity

As a group, oxazolidinones have various activities against most gram-positive bacteria and mycobacteria. Linezolid has excellent activity against staphylococci (including methicillin-resistant strains), streptococci, and multidrug-resistant enterococci, with MIC_{90}s ranging from 1 to 4 μg/ml (61, 203,

386). The MIC_{90}s are in the range of 0.5 to 2 μg/ml for pneumococci. Although the antibacterial effect of linezolid is generally bacteriostatic, the drug is bactericidal against most strains of staphylococci and pneumococci. Other bacteria that are inhibited by linezolid include *Actinomyces* spp., *Bacillus cereus, Corynebacterium* spp., *Leuconostoc* spp., *Pediococcus* spp., *R. equi, L. monocytogenes, Clostridium* spp., and gram-positive anaerobic cocci (134). Both slowly and rapidly growing *Mycobacterium* spp. and *Nocardia* spp. are also susceptible to linezolid, with MIC_{90}s of ≤8 μg/ml (38, 39, 348). Linezolid is an important therapeutic option for skin and soft tissue infections (149, 360), respiratory tract infections (290), and infections due to methicillin-resistant staphylococci (309, 326) and vancomycin-resistant enterococci (272, 276).

Adverse Effects

The most common drug-related adverse events (≤5% incidence) are diarrhea, headache, and nausea (118, 291). Prolonged use of linezolid (usually for >28 days) has led to rare optic neuropathy (198), peripheral neuropathy (36), and myelosuppression, including anemia, leukopenia, thrombocytopenia, and pancytopenia that are reversible upon discontinuation of therapy (6, 129). As a mild nonselective inhibitor of monoamine oxidase, linezolid can interact with adrenergic or serotonergic drugs, and rare cases of serotonin syndrome have been reported with concomitant use of selective serotonin reuptake inhibitor drugs (26, 181).

SULFONAMIDES AND TRIMETHOPRIM (TMP)

Sulfonamides were the first effective systemic antimicrobial agents used in the United States during the 1930s. They are derived from sulfanilamide, which shares chemical similarities with *para*-aminobenzoic acid, a factor essential for bacterial folic acid synthesis. Various substitutions at the sulfonyl radical attached to the benzene ring nucleus enhance the antibacterial activity and also determine the pharmacological properties of the drug.

TMP is a pyrimidine analog that inhibits the enzyme dihydrofolate reductase, interfering with folic acid metabolism, subsequent pyrimidine synthesis, and one-carbon fragment metabolism in the bacteria. Since TMP and sulfonamides block the bacterial folic acid metabolic pathway at different sites, they potentiate the antibacterial activity of one another and act synergistically against a wide variety of organisms. Such a combination, TMP-sulfamethoxazole (TMP-SMX), also called co-trimoxazole, was introduced clinically in 1968 and has proven to be very effective in the treatment of many infections (289, 300).

Mechanism of Action

Sulfonamides competitively inhibit bacterial modification of *para*-aminobenzoic acid into dihydrofolate, whereas TMP inhibits bacterial dihydrofolate reductase. This sequential inhibition of folate metabolism ultimately prevents the synthesis of bacterial DNA (155). Since mammalian cells do not synthesize folic acid, human purine synthesis is not affected significantly by sulfonamides or TMP. The antibacterial effect of these agents may be reduced in patients receiving high doses of folinic acid.

Pharmacology

Sulfonamides are usually administered in the oral and topical forms; the intravenous preparations (sulfadiazine and sulfisoxazole) are rarely used. The sulfonamides vary in their durations of action. Thus, sulfamethizole and sulfisoxazole are short-acting compounds, sulfadiazine and SMX are intermediate-acting compounds, and sulfadoxine is a long-acting compound. Mafenide acetate (Sulfamylon cream) and silver sulfadiazine are applied topically to burn patients and have significant percutaneous absorption. Sulfacetamide is available as an ophthalmic preparation, and various combinations of other sulfonamides are available orally (triple sulfa, or trisulfapyrimidine) or as vaginal creams or suppositories.

The orally administered sulfonamides are absorbed rapidly and completely from the gastrointestinal tract. They are metabolized in the liver by acetylation and glucuronidation and are excreted by the kidneys as free drug and inactive metabolites. Sulfonamides compete for bilirubin-binding sites on plasma albumin and increase levels of unconjugated bilirubin in blood. For this reason they should not be given to neonates, in whom increased serum bilirubin levels may cause kernicterus.

Sulfonamides are well distributed throughout the body, with levels in the CSF and synovial, pleural, and peritoneal fluids about 80% of concentrations in serum. They readily cross the placenta and enter the fetal circulation. Sulfonamides may be used in renal failure, but the drugs may accumulate during prolonged therapy as a result of reduced renal excretion.

TMP is available only for oral use and is absorbed almost completely from the gastrointestinal tract. After the usual 100-mg dose, peak levels in serum reach 1 μg/ml in 1 to 4 h. This drug distributes widely in body tissues, including the kidneys, lungs, and prostate, and in body fluids (267). Concentrations in CSF are about 40% of levels in serum. Its half-life in serum is about 10 h in healthy subjects and is prolonged in those with renal insufficiency. Up to 80% of a dose is excreted unchanged in the urine by tubular secretion; the remaining fraction is excreted as inactive metabolites by the kidneys or in the bile.

A fixed combination of TMP-SMX in a dose ratio of 1:5 is available for oral and intravenous use. An intravenous dose of 160 mg of TMP with 800 mg of SMX produces average peak levels in serum of 3.4 and 47.3 μg/ml, respectively, in 1 h. Similar peak levels are reached at 2 to 4 h after the same dose is taken orally. Widely distributed in the body, both drugs reach therapeutic levels in the CSF (40% of levels in serum). Excretion is primarily by the kidneys; dosage reduction is necessary in patients with creatinine clearances of ≤30 ml/min. Both TMP and SMX are removed by hemodialysis and partially by peritoneal dialysis.

Spectrum of Activity

Sulfonamides are inhibitory to a variety of gram-positive and gram-negative bacteria, actinomycetes, chlamydiae, toxoplasmas, and plasmodia. Their in vitro antimicrobial activities are irregular, being strongly influenced by inoculum size and composition of the test media. Susceptibility testing endpoints are often difficult to determine because of the presence of hazy growth within zones of inhibition in disk diffusion tests and because of the phenomenon of "trailing" in dilution tests. Sulfadiazine and sulfisoxazole are effective for rheumatic fever prophylaxis, but they are not useful in treating established group A beta-hemolytic streptococcal pharyngitis. These drugs may be used for prophylaxis of close contacts of patients with meningitis due to sulfonamide-susceptible *N. meningitidis*. Sulfisoxazole can be used to treat chlamydial urethritis, and sulfacetamide ophthalmic solution is effective for trachoma and inclusion conjunctivitis.

Sulfadiazine in combination with pyrimethamine has been used successfully to treat toxoplasmosis, and sulfadoxine combined with pyrimethamine (Fansidar) is effective in the prophylaxis and therapy of *P. falciparum* malaria (228, 230). Sulfonamides are active against *N. asteroides* (84), and they show moderate activity against *M. kansasii,* the *M. fortuitum* group, *M. marinum,* and *M. scrofulaceum* (285). Other uses of sulfonamides include therapy of melioidosis, dermatitis herpetiformis, lymphogranuloma venereum, and chancroid.

Among the gram-negative rods, *E. coli* strains were initially susceptible to the sulfonamides, especially at levels achievable in the urine. Therefore, these drugs have been used primarily in the treatment of first-episode acute urinary tract infections due to *E. coli*. However, increasing bacterial resistance has limited their efficacy in recent years. *S. marcescens, P. aeruginosa,* enterococci, and anaerobes are usually resistant to the sulfonamides.

TMP is active in vitro against many gram-positive cocci and most gram-negative rods. *P. aeruginosa,* most anaerobes, *M. pneumoniae,* and mycobacteria are resistant. The MIC varies considerably with the test media used. Like the sulfonamides, TMP is used primarily in the therapy of uncomplicated and recurrent urinary tract infections due to susceptible organisms (161). However, the prevalence of TMP-resistant *Enterobacteriaceae* is increasing (135).

Combinations of TMP with other agents, such as rifampin, polymyxins, and aminoglycosides, have demonstrated in vitro synergistic activity against various gram-negative rods. TMP combined with dapsone is effective in the treatment of *P. jirovecii* pneumonia in immunocompromised patients.

Many gram-positive cocci, including staphylococci and streptococci, and most gram-negative rods except *P. aeruginosa* are susceptible to TMP-SMX (43). However, 10 to 50% of strains of *S. pneumoniae* are resistant in many parts of the world (190). The drug combination has variable bactericidal effects on enterococci in vitro, depending on the test media used for susceptibility testing (245). Unlike many bacteria that can utilize only thymidine for growth, enterococci can use thymidine, thymine, exogenous folinic acid, dihydrofolate, and tetrahydrofolate, resulting in higher MICs (25- to 50-fold increase) on media containing these compounds (241). This fact also explains the ineffectiveness of TMP-SMX against enterococci in vivo.

With good activity against *M. catarrhalis* and *H. influenzae,* including β-lactamase-producing strains, TMP-SMX is useful for the therapy of acute otitis media, sinusitis, acute bronchitis, and pneumonia. It has shown excellent results in the prophylaxis and therapy of acute and chronic urinary tract infections (161, 321). It is an effective alternative therapy for uncomplicated urogenital gonorrhea, including cases caused by penicillinase-producing *N. gonorrhoeae* (51). It can also be used for the treatment of chancroid, but resistance to TMP-SMX in *H. ducreyi* is increasing. The drug combination is also useful in treating infections due to salmonellae, shigellae, enteropathogenic *E. coli,* and *Y. enterocolitica* (243). It has been used successfully for prophylaxis and treatment of traveler's diarrhea (90), but resistance to TMP-SMX in *Shigella* spp. and *E. coli* now severely limits its usefulness in many parts of the world.

Other microorganisms susceptible to TMP-SMX include *Brucella* spp., *B. pseudomallei, B. cepacia, S. maltophilia, M. kansasii, M. marinum,* and *M. scrofulaceum. M. tuberculosis* and *M. chelonae* are generally resistant. It is a valuable antibiotic for the treatment of *N. asteroides* infections (349), *B. cepacia* and *S. maltophilia* bacteremia, *L. monocytogenes* meningitis, gastroenteritis due to *Isospora belli* and *Cyclospora* spp. (228, 266), and Whipple's disease. In immunocompromised hosts (e.g., those with leukemia or AIDS and organ transplant recipients), TMP-SMX is effective for the prophylaxis and treatment of *P. jirovecii* pneumonia (49, 50, 384).

Adverse Effects

Sulfonamides are known to cause nausea, vomiting, headache, and fever. Hypersensitivity reactions can occur as rashes, vasculitis, erythema nodosum, erythema multiforme, and Stevens-Johnson syndrome (46). Very high doses of less-water-soluble sulfonamides such as sulfadiazine may result in crystalluria, with renal tubular deposits of sulfonamide crystals. Bone marrow toxicity with anemia, leukopenia, or thrombocytopenia can occur. Sulfonamides should be avoided in patients with glucose-6-phosphate dehydrogenase deficiency because of associated hemolytic anemia. Sulfonamides also potentiate the effects of warfarin, phenytoin, and oral hypoglycemic agents.

In general, TMP is well tolerated. With prolonged use, megaloblastic anemia, neutropenia, and thrombocytopenia can develop, especially in folate-deficient patients. Adverse reactions to TMP-SMX due to either the TMP or, more commonly, the SMX component can occur. Mild gastrointestinal symptoms and allergic skin rashes occur in about 3% of patients (196). Megaloblastic bone marrow changes with leukopenia, thrombocytopenia, or granulocytopenia may develop, usually in patients with preexisting folate deficiency. Nephrotoxicity usually occurs in patients with underlying renal dysfunction. Patients with AIDS have a much higher frequency of adverse reactions (as much as 70%) (137).

POLYPEPTIDES

■ Polymyxins

Polymyxins are a group of related cyclic basic polypeptides originally derived from *Bacillus polymyxa.* They have limited spectra of antimicrobial activity and significant toxicity. Only polymyxins B and E (colistin) are available for therapeutic use in humans.

Mechanism of Action

Acting like detergents or surfactants, members of this group of antibiotics interact with the phospholipids of the bacterial cell membrane, thereby increasing cell permeability and disrupting osmotic integrity. This process results in leakage of intracellular constituents, leading to cell death. The bactericidal action is reduced in the presence of calcium, which interferes with the attachment of drugs to the cell membrane.

Pharmacology

The polymyxins are usually administered via the parenteral, oral, or topical route. They are not significantly absorbed when given orally or topically, and intramuscular injections can be painful. Peak concentrations in serum of 5 μg/ml are obtained with a total daily dose of intravenous polymyxin B at 2.5 mg (25,000 U)/kg of body weight. Colistin sulfate is given orally for local antibacterial effect in the gut, while colistimethate sodium, a sulfomethyl derivative of colistin, is used for intravenous or intramuscular injections. The half-life of polymyxin B in serum is about 6 to 7 h, and that of colistin is 2 to 4 h. They do not penetrate well into pleural fluid, synovial fluid, or CSF, even in the presence of inflammation. Excretion is mostly via the kidneys by glomerular

filtration. Levels in serum and toxicity are increased in states of renal insufficiency. These drugs are not removed by hemodialysis, but small amounts can be removed by peritoneal dialysis.

Polymyxin is often used topically as 0.1% polymyxin in combination with bacitracin or neomycin for treatment of skin, mucous membrane, eye, and ear infections. It is poorly absorbed from these surfaces. When the drug is used for irrigation of serous or wound cavities, systemic absorption can be significant enough to produce toxicity.

Spectrum of Activity

Polymyxins are active only against gram-negative rods, especially *Pseudomonas* spp. The MIC_{90}s for *Pseudomonas* spp., including *P. aeruginosa,* are <8 μg/ml. *Proteus, Providencia, Serratia,* and *Neisseria* isolates are usually resistant. Emergence of resistance during therapy is rare, and there is no cross-resistance with other antibiotics. Polymyxins B and E have identical antimicrobial spectra and show complete cross-resistance to one another.

The combination of polymyxins with TMP-SMX may be synergistic in the treatment of serious infection due to multiply resistant *Serratia* spp., *P. aeruginosa, B. cepacia,* and *S. maltophilia* (83, 288). The polymyxins are usually reserved for serious, life-threatening *Pseudomonas* or gram-negative rod infections caused by organisms resistant to all other antibiotics. Aerosolized polymyxins have been used successfully to treat *P. aeruginosa* colonization or respiratory infections in patients with cystic fibrosis or bronchiectasis (107).

Adverse Effects

Neurotoxicity and nephrotoxicity are the two major side effects of polymyxins. Paresthesia with flushing, dizziness, vertigo, ataxia, slurred speech, drowsiness, or mental confusion occurs when levels in serum exceed 1 to 2 μg/ml. Polymyxins also have a curare-like effect that can block neuromuscular transmission. Dose-related renal dysfunction occurs in about 20% of patients receiving appropriate therapeutic dosages. Allergic reactions such as fever and skin rashes are rare, but urticaria and shock after rapid intravenous infusion have occurred.

■ Bacitracin

Originally isolated from *Bacillus licheniformis* (formerly *Bacillus subtilis*), bacitracin is a peptide antibiotic consisting of peptide-linked amino acids. Although it was introduced initially for the systemic treatment of severe staphylococcal infections, it is now mainly restricted to topical use because of its systemic toxicity.

Mechanism of Action

Bacitracin inhibits dephosphorylation of a lipid pyrophosphate, a step essential for bacterial cell wall synthesis. It also disrupts the bacterial cytoplastic membrane.

Pharmacology

Bacitracin is often used in various topical preparations, such as creams, ointments, antibiotic sprays and powders, and solutions for wound irrigation or bladder instillation. When used as a topical antibiotic, no significant amount of bacitracin is absorbed systemically. Large doses used to irrigate serous cavities may be associated with systemic toxicity.

Spectrum of Activity

The drug is active mainly against gram-positive bacteria, especially staphylococci and group A beta-hemolytic streptococci. However, group C and G streptococci are less susceptible, and group B streptococci are resistant (113). *Neisseria* spp. are also susceptible, but gram-negative rods are resistant. Bacitracin is often combined with neomycin, polymyxin B, or both in topical preparations to provide broad-spectrum antibacterial coverage. Orally administered bacitracin is effective in treating antibiotic-associated *C. difficile* colitis (88).

Adverse Effects

Systemic administration of bacitracin results in significant nephrotoxicity. Side effects are rare when the drug is given orally or applied topically. The drug is nonirritating to skin and mucous membranes. Allergic skin sensitization is rare.

CHLORAMPHENICOL

Chloramphenicol is a unique antibiotic originally derived from *Streptomyces venezuelae*. It contains a nitrobenzene ring. It is a highly effective, broad-spectrum antimicrobial agent with specific indications for use in seriously ill patients. Thiamphenicol is an analog of chloramphenicol with a similar spectrum of antimicrobial activity (253). Only chloramphenicol is available for clinical use in the United States.

Mechanism of Action

The drug is a bacteriostatic agent that inhibits protein synthesis by binding reversibly to the peptidyltransferase component of the 50S ribosomal subunit and preventing the transpeptidation process of peptide chain elongation. At therapeutic concentrations achievable in serum, it can be bactericidal against common meningeal pathogens such as *S. pneumoniae, N. meningitidis,* and *H. influenzae* (212, 253). Bacterial resistance occurs with plasmid-mediated, *cat* gene-encoded production of chloramphenicol acetyltransferase enzyme, which inactivates the drug (308).

Pharmacology

Chloramphenicol is available for topical, oral, or parenteral use. It is not absorbed in any significant amount when applied topically, but it is rapidly and completely absorbed from the gastrointestinal tract. After an oral or intravenous dose of 1 g, peak concentrations in serum at 2 h can reach 10 to 15 μg/ml. It diffuses well into many tissues and body fluids, including CSF, where levels are generally 30 to 50% of concentrations in serum even without meningeal inflammation (313). The antibiotic readily crosses the placental barrier and is present in human milk.

Chloramphenicol is metabolized and inactivated by glucuronidation in the liver, with a half-life of 4 h in adults. The active drug (5 to 10%) and its inactive metabolites are excreted by the kidneys. Careful monitoring of serum chloramphenicol levels, maintaining peak concentrations in serum in the therapeutic range of 10 to 20 μg/ml, is useful for ensuring therapeutic efficacy and reduced toxicity. Patients with hepatic failure have high levels of active drug in serum owing to a prolonged half-life. Dosage modification is not necessary in the presence of renal insufficiency, since the metabolites are not as toxic as the active drug. Levels in serum are not affected by hemodialysis or peritoneal dialysis.

Spectrum of Activity

Chloramphenicol is very active against many gram-positive and gram-negative bacteria, chlamydiae, mycoplasmas, and rickettsiae. MIC_{90}s for most gram-positive aerobic and anaerobic cocci are ≤12.5 μg/ml (253). However, the drug is usually inactive against methicillin-resistant *S. aureus* and *S. epidermidis* and is variably active against enterococci. *N.*

meningitidis, H. influenzae (ampicillin-resistant and -susceptible strains), and most *Enterobacteriaceae* are susceptible. Its activity against *Serratia* and *Enterobacter* isolates is variable, and *Pseudomonas* spp. are usually resistant. Salmonellae, including *S. enterica* serovar Typhi, are also susceptible, but resistant isolates are being encountered with increasing frequency (54).

Chloramphenicol has excellent activity against anaerobic bacteria, including members of the *B. fragilis* group. Almost all of these isolates are inhibited at concentrations of ≤10 μg/ml (2, 314). It is also active against *Rickettsia* spp. and *C. burnetii*.

Adverse Effects

Bone marrow toxicity is the major complication of chloramphenicol use. This side effect may occur as either dose-related bone marrow suppression or idiosyncratic aplastic anemia. Reversible bone marrow depression with anemia, leukopenia, and thrombocytopenia occurs as a result of a direct pharmacological effect of the drug on hematopoiesis. High doses (>4 g/day), prolonged therapy, and excessively high levels in serum (>20 μg/ml) predispose patients to develop this type of complication. The second form of bone marrow toxicity is a rare but usually fatal complication that manifests as aplastic anemia. This response is not dose related, and the precise mechanism is unknown. It can occur weeks to months after the use of chloramphenicol, and it can develop after the use of oral, intravenous, or topical preparations.

Gray baby syndrome, characterized by vomiting, abdominal distention, cyanosis, hypothermia, and circulatory collapse, may occur in premature infants and neonates. This toxicity results from the immature hepatic function of neonates, which impairs hepatic inactivation of the drug. Reversible optic neuritis causing decreased visual acuity has been reported for patients receiving prolonged therapy. Chloramphenicol can occasionally cause hypersensitivity reactions, including skin rashes, drug fevers, and anaphylaxis. It potentiates the action of warfarin, phenytoin, and oral hypoglycemic agents by competitive inhibition of hepatic microsomal enzymes.

METRONIDAZOLE

Metronidazole is a 5-nitroimidazole derivative that was first introduced in 1959 for the treatment of *Trichomonas vaginalis* infections. It now has an important therapeutic role in the treatment of infections due to anaerobic bacteria and certain protozoan parasites. Tinidazole, a second-generation 5-nitroimidazole compound, is approved in the United States for treatment of trichomoniasis, giardiasis, and amebiasis.

Mechanism of Action

Metronidazole owes its bactericidal activity to the nitro group of its chemical structure. After the drug gains entry into the cells of susceptible organisms, the nitro group is reduced by a nitroreductase enzyme in the cytoplasm, generating certain short-lived, highly cytotoxic intermediate compounds, free radicals, that disrupt host DNA (96). Resistance to nitroimidazoles may be due to decreased uptake of the drug or inducible production of 5-nitroimidazole reductase enzyme that can scavenge the free-radical intermediates (207).

Pharmacology

Metronidazole can be administered via the topical, oral, or intravenous route. It is absorbed rapidly and almost completely when given orally. Peak levels in serum of 6 μg/ml are obtained 1 h after an oral dose of 250 mg. Intravenous doses of 7.5 mg/kg result in peak concentrations in serum of 20 to 25 μg/ml. The drug has a half-life in serum of 8 h. Therapeutic levels are achieved in all body tissues and fluids, including abscess cavities and CSF, even without meningeal inflammation. The drug crosses the placenta and is secreted in breast milk. It is metabolized mainly in the liver, and 60 to 80% is excreted by the kidneys. With impaired hepatic function, plasma clearance of metronidazole is delayed and dosage adjustments are necessary. The pharmacokinetics are minimally affected by renal insufficiency. Metronidazole and its metabolites are removed completely by dialysis.

Spectrum of Activity

Metronidazole exhibits potent activity against almost all anaerobic bacteria, including the *B. fragilis* group and *Fusobacterium* and *Clostridium* spp. (18). It is the only antimicrobial agent with consistent bactericidal activity against members of the *B. fragilis* group. However, the susceptibility of gram-positive anaerobic cocci is somewhat variable, with MIC_{90}s of 16 μg/ml for these organisms. Most strains of the genera *Actinomyces, Arachnia,* and *Propionibacterium* are resistant. Frequencies of metronidazole-resistant *B. fragilis* group isolates (MICs of >16 μg/ml) in the range of 2 to 5% have been reported from various institutions (2, 314). Tinidazole is somewhat more potent than metronidazole in its antianaerobe activities (58, 174). Nitroimidazoles have no activity against aerobic bacteria, including the *Enterobacteriaceae*.

The drug is effective in the treatment of antibiotic-associated colitis caused by *C. difficile* (19, 53), with efficacy equivalent to that of oral vancomycin for this indication (333). It is also useful in combination with an aminoglycoside for treating polymicrobial soft tissue infections and mixed aerobic-anaerobic intra-abdominal and pelvic infections.

Metronidazole and tinidazole are active against the protozoa *T. vaginalis, Giardia lamblia,* and *E. histolytica*. It is the drug of choice for the treatment of trichomoniasis, giardiasis, and intestinal and invasive amebiasis, including amebic liver abscess (127, 228, 331).

Adverse Effects

Metronidazole is generally well tolerated, and adverse side effects are uncommon. It can cause mild gastrointestinal symptoms such as nausea, abdominal cramps, and diarrhea. An unpleasant, metallic taste may be experienced with oral therapy. Metronidazole can potentiate the effect of warfarin and prolong the prothrombin time.

Although metronidazole is carcinogenic in mice and rats, there is no evidence for carcinogenicity in humans. However, use of this agent in pregnancy, especially during the first trimester, and in nursing mothers should be avoided.

RIFAMYCINS

Rifamycins are a group of macrocyclic compounds produced by the mold *Streptomyces mediterranei.* Rifampin, also known as rifampicin and a semisynthetic antibiotic derived from rifamycin B, was the first of this class of drugs introduced for clinical use in 1968 as an effective antituberculous drug. A closely related compound, rifabutin, a derivative of rifamycin S, is another potent antimycobacterial agent, especially against *M. avium* complex (261). Rifaximin, a derivative of rifampin, possesses an additional pyridoimidazole ring and is a nonabsorbed oral drug used for therapy of uncomplicated traveler's diarrhea.

Mechanism of Action

Rifamycins exert their bactericidal effects by forming a stable complex with bacterial DNA-dependent RNA polymerase, preventing the chain initiation process of DNA transcription (359). Mammalian RNA synthesis is not affected, because the mammalian enzyme is much less sensitive to the drug. Rifampin-resistant isolates possess an altered RNA polymerase enzyme that arises easily from single-step mutations during monotherapy with rifampin.

Pharmacology

Rifampin is well absorbed after oral administration, reaching peak concentrations in serum of 5 to 10 μg/ml in 2 to 4 h following a 600-mg dose. A parenteral preparation is also available. Rifampin is deacetylated in the liver to an active metabolite and excreted in the bile, and it undergoes enterohepatic circulation. The normal half-life in serum varies from 1.5 to 5 h. The drug distributes well to almost all body tissues and fluids, reaching concentrations equal to or exceeding that in serum. Levels in CSF are highest in the presence of inflamed meninges. It is able to enter phagocytes and kill living intracellular organisms (215), and it crosses the placenta. About 30 to 40% of the drug is excreted in the urine, and it does not accumulate in patients with impaired renal function. Hemodialysis and peritoneal dialysis do not eliminate the drug. Dosage adjustments are necessary for patients with severe hepatic dysfunction.

Due to an additional pyridoimidazole ring in its chemical structure, rifaximin is largely unabsorbed after oral administration, with >99% of the drug present in the stool and <1% of an oral dose detectable in plasma of both healthy volunteers and persons with damaged intestinal mucosa (164). Average fecal concentrations of drug reach 8,000 μg/g on the third day of therapy at a dosage of 400 mg orally twice daily (171).

Spectrum of Activity

In addition to its well-known antimycobacterial effects (59), rifampin has a wide spectrum of antimicrobial activity. It is bactericidal against gram-positive cocci such as staphylococci (including methicillin-resistant strains), streptococci, and anaerobic cocci, with MICs in the range of 0.01 to 0.5 μg/ml. It remains an important adjunct in combination therapy of serious and chronic staphylococcal infections (340). However, it is bacteriostatic against enterococci, with usual MICs of <16 μg/ml (240).

N. gonorrhoeae, N. meningitidis, and *H. influenzae,* including β-lactamase-producing strains, are susceptible to rifampin, which is used frequently in the prophylaxis of meningococcal and *H. influenzae* type b meningitis (146). MICs for *Enterobacteriaceae* are ≤12 μg/ml, while MICs for *S. marcescens* and *P. aeruginosa* are higher (240). Besides fluoroquinolones, rifampin is one of the most active agents against *L. pneumophila* and other *Legionella* spp., with MICs of ≤0.03 μg/ml. Because of its ability to enter phagocytes, rifampin inhibits the growth of *Brucella* spp. and *C. burnetii* intracellularly (282), and it is used frequently in combination therapy of infections due to these organisms. Although *Chlamydia* spp. are very susceptible to rifampin in vitro, resistance emerges rapidly when rifampin is used alone.

Rifaximin has broad-spectrum inhibitory activity against enteric bacterial pathogens, including enterotoxigenic and enteroaggregative strains of *E. coli, Aeromonas, Shigella,* and *Salmonella,* with MIC_{90}s ranging from 4 to 16 μg/ml (136, 164, 310). Although it is less active against *C. jejuni* (MIC_{90} of 512 μg/ml), *Y. enterocolitica* (MIC_{90} of 128 μg/ml), *C. difficile* (MIC_{90} of 128 μg/ml), and *H. pylori* (MIC_{50} of 4 μg/ml), concentrations of rifaximin achieved in the intestinal lumen are >10-fold higher than the MICs of the drug for these pathogens. This drug has been used successfully to treat and prevent uncomplicated traveler's diarrhea (91, 92, 324).

Adverse Effects

Rifampin has many side effects, including gastrointestinal discomfort and hypersensitivity reactions, such as drug fever, skin rashes, and eosinophilia. It produces a harmless, orange-red coloration of saliva, tears, urine, and sweat. In up to 20% of patients, an influenza-like syndrome with fever, chills, arthralgias, and myalgias may develop after several months of intermittent therapy (142). This immunologic reaction may be associated with hemolytic anemia, thrombocytopenia, and renal failure. Rifampin-induced hepatitis occurs in <1% of patients and is more frequent during concurrent isoniazid therapy for tuberculosis. As a result of its ability to induce human hepatic cytochrome P450 enzyme, rifampin has clinically significant interactions with many drugs, such as antagonizing the effect of oral contraceptives and diminishing the anticoagulant activity of warfarin.

Headache, nausea, abdominal pain, and fatigue are the most frequent side effects reported for rifaximin. Rare hypersensitivity reactions, including allergic dermatitis, rash, angioneurotic edema, urticaria, and pruritus, can occur with rifaximin, which is contraindicated in those who are allergic to rifampin (164). However, unlike rifampin, rifaximin does not cause clinically relevant interactions with drugs because of the lack of systemic absorption of this drug.

NITROFURANTOIN

Nitrofurantoin belongs to a class of compounds consisting of a primary nitro group joined to a heterocyclic ring. Its role in human therapeutics is limited to treatment of urinary tract infections (161).

Mechanism of Action

The mechanism of action of nitrofurantoin is not fully understood, but highly reactive electrophilic intermediates of the drug activated by bacterial reductases were found to bind nonspecifically to bacterial ribosomal proteins and inhibit inducible synthesis of essential bacterial enzymes, causing complete cessation of protein synthesis and damage to bacterial DNA and RNA (221). However, inhibition of bacterial protein synthesis can occur in the absence of reductive activation of the drug (226). Despite over 30 years of clinical use, there is no emergence of clinically significant bacterial resistance to this drug.

Pharmacology

The drug is available in microcrystalline (Furadantin) and macrocrystalline (Macrodantin) forms. It is administered orally and is well absorbed from the gastrointestinal tract. Very low levels of the drug are achieved in serum and most body tissues after usual oral doses. With a half-life in serum of about 20 min, two-thirds of the drug is rapidly metabolized and inactivated in various tissues. The remaining one-third is excreted unchanged into the urine. An average dose of nitrofurantoin yields a concentration in urine of 50 to 250 μg/ml in patients with healthy renal function. In alkaline urine, more of the drug is dissociated into the ionized form, with lowered antibacterial activity. Nitrofurantoin accumulates in the sera of patients with creatinine clearances of <60 ml/min. The drug is removed by

hemodialysis. The risk of systemic toxicity increases in the presence of severe uremia. It is contraindicated in patients with significant renal impairment and hepatic failure.

Spectrum of Activity

Nitrofurantoin has a broad spectrum of activity against gram-positive and gram-negative bacteria, particularly the common urinary tract pathogens. It is active against gram-positive cocci, such as *S. aureus, S. epidermidis, Staphylococcus saprophyticus,* and *E. faecalis*, with MICs in the range of 4 to 25 μg/ml (159). *S. pneumoniae, S. pyogenes,* and *Corynebacterium* spp. are also susceptible, but they rarely cause urinary tract infections. Over 90% of *E. coli* isolates and many coliform bacteria are susceptible to nitrofurantoin at MICs of <32 μg/ml. However, only one-third of *Enterobacter* and *Klebsiella* isolates are susceptible. *Pseudomonas* and most *Proteus* spp. are resistant. Susceptible organisms rarely become resistant to this drug during therapy.

Adverse Effects

Gastrointestinal irritation, with anorexia, nausea, and vomiting, is the most common side effect. Diarrhea and abdominal cramps may occur. Hypersensitivity reactions, such as drug fever, chills, arthralgia, skin rashes, and a lupus-like syndrome, have been observed (160). Pulmonary reactions are the most common serious side effects associated with nitrofurantoin use. Acute pneumonitis with fever, cough, dyspnea, eosinophilia, and pulmonary infiltrates present on chest radiographs can occur after a few days of therapy (160). This immunologically mediated reaction is more common in elderly patients and is rapidly reversible after cessation of therapy. Chronic pulmonary reactions with interstitial pneumonitis leading to irreversible pulmonary fibrosis can occur in patients on continuous therapy for 6 months or more (232).

Peripheral polyneuropathy is a serious side effect that occurs more often in patients with renal failure. Hemolytic anemia, megaloblastic anemia, and bone marrow suppression with leukopenia can occur. Rare hepatotoxic reactions, such as cholestatic jaundice and chronic active hepatitis, have been reported (307).

FOSFOMYCIN

Fosfomycin, first isolated from cultures of *Streptomyces* spp. in 1969, is a phosphonic acid derivative originally named phosphonomycin (322). In the United States, it is used as single-dose therapy for uncomplicated urinary tract infections due to susceptible organisms (161).

Mechanism of Action

Fosfomycin is bactericidal by inhibiting pyruvyl transferase, a bacterial cytoplasmic enzyme that catalyzes the formation of uridine diphosphate-*N*-acetylmuramic acid during the first step of peptidoglycan synthesis (184). There is little cross-resistance between fosfomycin and other antibacterial agents, most likely because it differs from other agents in its chemical structure and site of action. Rare resistance to fosfomycin can occur by two mechanisms: (i) mutations of the structural or regulatory genes for the bacterial proteins that transport the drug into the cell or (ii) plasmid-mediated production of a drug-inactivating enzyme (255).

Pharmacology

Originally formulated as sodium and calcium salts for oral and intravenous use, fosfomycin is available in the United States as an oral, water-soluble tromethamine salt. Following oral administration, it is rapidly absorbed and converted to the free acid, fosfomycin. With markedly improved oral bioavailability (35 to 40%), fosfomycin has a mean elimination half-life of 5.5 h, and it is primarily excreted unchanged in the urine (268). Following a single oral dose of 3 g, peak concentrations in serum (range, 22 to 32 μg/ml) are achieved within 2 h after administration, with peak urinary concentrations (1,000 to 4,400 μg/ml) occurring within 4 h and remaining high (>128 μg/ml) for 24 to 48 h, sufficient to inhibit most urinary tract pathogens. Peak urinary concentrations are reached later and are lowered when the drug is administered with food or antiperistaltic agents. In patients with renal impairment (creatinine clearance of <30 ml/min), peak serum fosfomycin concentrations are increased, with decreased urinary elimination and reduced urinary concentrations of the drug.

While not bound to plasma protein, it is widely distributed in various body fluids and tissues, including the kidneys, prostate, and seminal vesicles, from which it is cleared slowly. Although it crosses the placental barrier, the drug can be used safely during pregnancy if clearly needed.

Spectrum of Activity

Fosfomycin has a broad spectrum of activity against most gram-positive and gram-negative bacteria isolated from patients with lower urinary tract infections. *E. coli, Serratia, Klebsiella, Citrobacter, Enterobacter, S. aureus,* and enterococci are generally inhibited by fosfomycin at concentrations of <64 μg/ml (13, 268, 322). Fosfomycin is bactericidal at concentrations that are similar to the MICs, at ≤2-fold differences. It is more active than TMP and nalidixic acid, while similar to norfloxacin and co-trimoxazole, in its activity against these organisms (13). At a breakpoint concentration of ≤128 mg/ml, 60, 20, and 80% of isolates of *Pseudomonas* spp., *M. morganii,* and *S. saprophyticus,* respectively, are susceptible to fosfomycin (274). In multiple-dose use, bacterial resistance to fosfomycin emerges rapidly, and it can be chromosomal or, more rarely, plasmid mediated. However, cross-resistance with other antimicrobials has been uncommon (283).

The in vitro activity of fosfomycin is affected by test medium and conditions (268, 274). Fosfomycin has much greater in vitro activity, and closer correlation with in vivo activity, when the test medium is supplemented with glucose-6-phosphate at 25 μg/ml, which is recommended for susceptibility testing with the agar and broth dilution methods. The disk diffusion testing method utilizes disks containing 200 μg of fosfomycin tromethamine and 50 or 100 μg of glucose-6-phosphate.

Adverse Effects

Mild, self-limiting gastrointestinal disturbances, mainly diarrhea, are the most frequent side effects (3 to 5%). Other minor adverse events include headaches, dizziness, rash, and vaginitis.

METHENAMINE

Methenamine is a tertiary amine with properties of a monoacidic base; it is used as a urinary antiseptic. To be activated, it is combined chemically with a poorly metabolized acid and administered as the mandelate (Mandelamine) or hippurate (Hiprex or Urex) salt.

Mechanism of Action

Methenamine has no antibacterial action by itself, but it is converted at acid pH to ammonia and formaldehyde, which provides the antiseptic action. This hydrolytic process occurs

in the urine, and an effective bacteriostatic concentration of formaldehyde is reached at a urine pH of <5.5. Since the serum is at physiological pH, formaldehyde is not released while methenamine circulates in the body.

Pharmacology

The agent is well absorbed from the gastrointestinal tract and is rapidly excreted in the urine. The elimination half-life of methenamine is about 4 h. At a urinary pH of 5.0, about 20% of methenamine excreted in the urine is hydrolyzed to formaldehyde and ammonia. Bactericidal levels (>20 mg/ml) of formaldehyde are generated in the bladder urine at 2 h after oral administration and may be maintained for at least 6 h or until the patient voids (192). The mandelate and hippurate moieties are also rapidly excreted in the urine in active, unchanged forms by glomerular filtration and tubular secretion. The agent is contraindicated in patients with hepatic insufficiency because of the ammonia produced.

Spectrum of Activity

With the liberation of enough formaldehyde into the urine, methenamine is essentially active against all gram-positive and gram-negative bacteria and also against fungi (192). However, it is not effective for treating urinary tract infections due to urea-splitting organisms such as *Proteus* and *Morganella*, which can convert urea to ammonium hydroxide, thereby preventing the hydrolysis of methenamine to formaldehyde. Combination with acetohydroxamic acid, a urease inhibitor, has been suggested for treating these infections by *Proteus* and *Morganella*. Since bacteria and fungi do not become resistant to formaldehyde, emergence of resistance to methenamine is not a problem.

Methenamine is not useful for acute urinary tract infections. It has been used successfully as prophylactic therapy for recurrent bacteriuria, particularly infections caused by highly resistant gram-negative rods or yeasts. It is also effective as prolonged suppressive therapy for chronic bacteriuria in the absence of structural abnormalities of the urinary tract.

Adverse Effects

Methenamine and its acid salts are generally well tolerated. Some patients may develop nausea, vomiting, abdominal cramps, and diarrhea. High doses or prolonged administration of the drug can cause urinary tract irritation by the free formaldehyde, resulting in urinary frequency, dysuria, albuminuria, and hematuria. Skin rashes may also occur. To avoid precipitation of urate crystals in the urine, methenamine salts should not be used in patients with gout or hyperuricemia.

MUPIROCIN

Mupirocin, formerly pseudomonic acid A, is a topical antibacterial agent derived from the fermentation products of *Pseudomonas fluorescens* (125). It contains a unique 9-hydroxynonanoic acid moiety in its chemical structure, and it inhibits isoleucyl-tRNA synthetase, resulting in cessation of bacterial protein synthesis (165). Low-level resistance (MICs of 4 to 256 μg/ml) results from an altered synthetase enzyme due to mutations in the bacterial chromosomal *ileS* gene, whereas high-level, transferable resistance (MIC of ≥512 μg/ml) is mediated by the *mupA* gene, which encodes an entirely different synthetase.

Originally developed for the topical treatment of superficial soft tissue infections, particularly those due to staphylococci, mupirocin is available as a 2% ointment or cream in the United States. After topical application, <1% of the drug is absorbed systemically, with no detectable levels in the urine or feces. Penetration into deeper dermal layers of the skin is increased with traumatized skin or use of occlusive dressings. The drug is highly protein bound (95%), and its activity is lowered in the presence of serum. It is most active at moderately acid pH, with no inoculum effect (353). Mupirocin is slowly metabolized in the skin to the inactive monic acid.

It has excellent in vitro activity, primarily against the gram-positive cocci. *S. aureus*, including methicillin-resistant strains, and coagulase-negative staphylococci are uniformly very susceptible, with MIC_{90}s of <0.5 μg/ml (47). Emergence of resistant strains of staphylococci can occur with widespread use of mupirocin (33, 35, 339). Most streptococci (including *S. pneumoniae*, beta-hemolytic streptococci of groups A, B, C, and G, and viridans group streptococci) are inhibited by concentrations of ≤1 μg/ml. Resistant bacteria include enterococci, *Corynebacterium* spp., *Erysipelothrix* spp., *P. acnes*, gram-positive anaerobes, and most gram-negative bacteria. However, *H. influenzae, N. gonorrhoeae, N. meningitidis, M. catarrhalis, B. pertussis*, and *P. multocida* are quite susceptible, with MICs in the range of 0.02 to 0.025 μg/ml. There is no cross-resistance between mupirocin and other major groups of antibiotics. Clinically, mupirocin is efficacious in the therapy of superficial skin infections, such as impetigo, folliculitis, and burn wound infections, that are caused by staphylococci or streptococci (132). Although this drug has been used successfully to eradicate nasal carriage of *S. aureus*, including methicillin-resistant strains (48, 85), it may not prevent nosocomial *S. aureus* infections (361).

No systemic toxic effects have been reported with mupirocin. Local irritation, such as burning, stinging, itch, and rash, which may be due to the polyethylene glycol base in the vehicle ointment, may occur.

APPENDIX

Approximate Concentrations of Antibacterial Agents in Serum

The concentrations of antimicrobial agents listed below are approximations taken from various reports and publications. Several factors can influence the level of antimicrobial agent in individual patients, including inherent differences in the patients themselves, their physical condition, the dosages, and the routes of administration. The values can also be influenced by the assay methods used to obtain them. Therefore, these concentrations should be used only as approximate values, and clinicians should use their knowledge of the patient and the drugs, the recommendations from U.S. Food and Drug Administration-approved package inserts, or other reputable sources in planning their therapeutic regimens.

Antimicrobial agent	Serum half-life (h)	Unit dose	Average peak level in serum (μg/ml)[a]		
			p.o.	i.m.	i.v.[b]
Amikacin	2–2.5	7.5 mg/kg		15–20	20–40
Amoxicillin	1	500 mg	6–8		
Amoxicillin-clavulanate	1.3 / 1.0	250/125 mg	3.3 (Amox) 1.5 (Clav)		
		500/125 mg	6.5 (Amox) 1.8 (Clav)		
		875/125 mg	11.6 (Amox) 2.2 (Clav)		
		1,000/62.5 mg	17 (Amox) 2.1 (Clav)		
Ampicillin	1.1	500 mg	2.5–5	8–10	
		1 g			40
Ampicillin-sulbactam	1.1/1.0	3 g			120 (Amp) 60 (Sulb)
		1.5 g			18 (Amp) 13 (Sulb)
Azithromycin	48	500 mg	0.4		3.5
Azlocillin	1	2 g			130
Aztreonam	1.7	1 g		45	90–160
Bacampicillin	1.1	800 mg	13		
Carbenicillin	1.1	1 g		20–30	150
Carbenicillin indanyl sodium	1.1	764 mg	10		
Cefaclor	0.6	500 mg	16		
Cefadroxil	1.5	500 mg	10		
Cefamandole	0.5–1	1 g		20–36	90–140
Cefazolin	1.8	1 g		65	185
Cefepime	2	1 g		30	82
Cefdinir	1.7	300 mg	1.6		
Cefditoren	1.6	200 mg	3.1		
		400 mg	4.4		
Cefixime	3–4	400 mg	3.5		
Cefmetazole	1.5	1 g			70
Cefonicid	4	1 g		98	220
Cefoperazone	2	1 g		65–75	153
Ceforanide	3	1 g		70	125
Cefotaxime	1	1 g		20	40–45
Cefotetan	3–4.5	1 g		50–80	160
Cefoxitin	1	1 g		20–25	55–110
Cefpirome	2	1 g		45	85
Cefpodoxime	2.5	200 mg	2.3		
Cefprozil	1.5	500 mg	10.5		
Ceftazidime	2	1 g		40	70
Ceftibuten	2.5	400 mg	15		
Ceftizoxime	1.5	1 g		39	80–90
Ceftriaxone	6–9	500 mg		40–45	
		1 g			150
Cefuroxime	1.5	750 mg		27	50
Cefuroxime axetil	1.5	500 mg	9		
Cephalexin	0.9	500 mg	18		
Cephalothin	0.6	1 g			30–60
Cephapirin	0.6	1 g			40–70
Cephradine	0.8	500 mg	16		
Chloramphenicol	4	1 g	10–18		10–15
Chlortetracycline	6–9	500 mg	2–4	12	
Cinoxacin	1–1.5	500 mg	15		
Ciprofloxacin	5–6	400 mg			4.5
		500 mg	3.0		
		500 mg XR[c]	1.6		
		750 mg	4.0		
		1,000 mg XR	3.1		

(Continued on next page)

Antimicrobial agent	Serum half-life (h)	Unit dose	Average peak level in serum (μg/ml)[a]		
			p.o.	i.m.	i.v.[b]
Clarithromycin	5–7	250 mg	1–2		
		500 mg	3–4		
		1,000 mg XR	2–3		
Clinafloxacin	5.2	200 mg	1.5		
Clindamycin	2.5	300 mg	3	6	
		600 mg			10–12
Cloxacillin	0.5	500 mg	10		
Colistimethate sodium	2–4.5	150 mg		5–6	
Daptomycin	9	4 mg/kg			70
		6 mg/kg			82
Demeclocycline	12	300 mg	1–2		
Dicloxacillin	0.5–0.7	500 mg	15		
Dirithromycin	40	500 mg	0.5		
Doxycycline	18–22	100 mg	2.5		4
Enoxacin	4–6	400 mg	3–5		
Ertapenem	4	1 g		70	155
Erythromycin	1.5	500 mg	2–3		
		1 g			10
Fleroxacin	12	400 mg	5		7–8
Fosfomycin	5.7	3 g	25		
		50 mg/kg			275
Fusidic acid	13–19	500 mg	25–30		50
Gatifloxacin	7	400 mg	4		4.5
Gemifloxacin	7–8	320 mg	1.8		
Gentamicin	2–3	1.5 mg/kg	4–6		4–8
Imipenem	1	500 mg			25–35
Kanamycin	2.2–3	7.5 mg/kg		20–25	
Levofloxacin	6–8	500 mg	5.5		6.5
		750 mg	8.5		12
Lincomycin	5	500 mg	3.5		
		600 mg		10	16–21
Linezolid	5	600 mg	15		15
Lomefloxacin	6.5	400 mg	3		
Loracarbef	1	400 mg	14		
Meropenem	1	500 mg			25–35
Methicillin	0.5	1 g	15		60
Metronidazole	8	500 mg	12		20–25
Mezlocillin	1	1 g			15
		3 g			260
Minocycline	14–16	100 mg	1		
Moxifloxacin	12	400 mg	4.5		4.5
Nafcillin	0.5	500 mg			5–8
		1 g			20–40
Nalidixic acid	1.5	1 g	20–50		
Netilmicin	2.5	2 mg/kg		5–7	6–8
Nitrofurantoin	0.3	100 mg	<2		
Norfloxacin	3.3	400 mg	1.5		
Ofloxacin	5	400 mg	4		
Ornidazole	13	500 mg	10		20
Oxacillin	0.5	500 mg	4–6	14–16	
		1 g			40
Oxytetracycline	9	500 mg	1–2		
Pefloxacin	10	400 mg	3		5.5
Penicillin G	0.5	500 mg		1.5–2.5	
Aqueous		1×10^6 U		8–10	10
Benzathine		1.2×10^6 U		0.1–0.15	
Procaine		1.2×10^6 U	3		
Penicillin V	0.5	500 mg	3–5		
Piperacillin	1.1	2 g			36
		4 g			240

(Continued on next page)

Antimicrobial agent	Serum half-life (h)	Unit dose	Average peak level in serum (μg/ml)[a]		
			p.o.	i.m.	i.v.[b]
Piperacillin-tazobactam	1.1/1.0	3.375 g			242 (Pip) 24 (Tazo)
		4.5 g			298 (Pip) 34 (Tazo)
Pivampicillin	0.5–1	350 mg	2		
Polymyxin B	6–7	2.5 mg/kg		5	
Quinupristin-dalfopristin	1/0.75	7.5 mg/kg			3 (Q) 7.5 (D)
Rifampin	2–5	600 mg	7–9		10
Sparfloxacin	20	200 mg	1.1		
Spectinomycin	1–2	2 g		100	
Spiramycin	3.8	2 g	3		
Streptomycin	2–3	1 g		25–50	
Sulfadiazine	17	2 g	100–150		
Sulfadoxine	150–200	1 g	50–75		
Sulfamethizole	4–7	2 g	60		
SMX	10–12	1 g	40		
Sulfisoxazole	5–7	2 g	170		
Teicoplanin	45	200 mg		7	
		400 mg			20–40
Telithromycin	9–10	800 mg	2		
Tetracycline	8	500 mg	4		8
Ticarcillin	1.2	1 g		20–30	
		3 g			190
Ticarcillin-clavulanate	1.2/1.0	3.1 g			330 (Ticar) 8 (Clav)
Tigecycline	36	50 mg			0.8
Tinidazole	12–14	2 g	48		
Tobramycin	2–2.8	1.5 mg/kg		4–6	4–8
TMP	10–12	100 mg	1		
TMP-SMX		160/800 mg	3 (TMP) 46 (SMX)		9 (TMP) 106 (SMX)
Trovafloxacin (alatrofloxacin i.v.)	11	300 mg			4.5
Vancomycin	6	500 mg			20–40

[a] p.o., oral; i.m., intramuscular; i.v., intravenous.
[b] At 30 min following intravenous infusion.
[c] XR, extended-release formulation.

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Mechanisms of Resistance to Antibacterial Agents*

LOUIS B. RICE AND ROBERT A. BONOMO

71

GENERAL CONCEPTS, INOCULUM EFFECTS, AND TOLERANCE

In considering the growing problem of antimicrobial resistance in bacteria, it is worth remembering that resistance is neither a new phenomenon nor unexpected in an environment in which potent antimicrobial agents are used. The diversity of the microbial world and the relatively specific activities of our antimicrobial agents virtually ensure widespread resistance among bacteria. In many cases, this resistance is recognized when an antibiotic is first tested for development. For example, it is not considered a problem or threat that *Escherichia coli* is resistant to vancomycin. We simply understand that *E. coli* is not among vancomycin's spectrum of activity and avoid using vancomycin when *E. coli* infection is known or highly suspected (i.e. *E. coli,* has natural or primary resistance to vancomycin). Conversely, the increasing resistance of *E. coli* to ciprofloxacin represents an important problem, since we frequently use the fluoroquinolone class of antimicrobial agents to treat infections in which *E. coli* is likely to be involved. So when we speak of the problem of resistance, we must recognize that most problems result from expression of resistance by bacteria that are intrinsically susceptible to the antibiotic in question (i.e, acquired resistance).

It is also important to recognize that resistance as a clinical entity is essentially a relative phenomenon, in many ways a problem only indirectly related to the microbiologic techniques often used to detect it. For example, it is possible to incorporate enough ticarcillin into an agar plate to inhibit an ampicillin-resistant strain of *Enterococcus faecium* (in many cases this requires about 10,000 μg/ml). So in one sense, ampicillin-resistant *E. faecium* isolates are susceptible to high concentrations of ticarcillin. However, such concentrations cannot be achieved at the site of infection, so ampicillin-resistant *E. faecium* strains are not considered susceptible to ticarcillin. It is the responsibility of the Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards), the U.S. Food and Drug Administration, and other standard-setting bodies in different countries to make determinations on susceptible, intermediate, and resistant breakpoints for new antimicrobial agents, considering factors of in vitro susceptibility, pharmacokinetics, and pharmacodynamics. These issues become very important in treating meningitis, in which relatively minor increases in the MIC of penicillin for *Streptococcus pneumoniae* can foil treatment, but in many cases are less relevant in the treatment of simple urinary tract infections, given the tendency of many antibiotics to concentrate in the urine.

The relativity of resistance is no better exemplified than by considerations of susceptibility in bacterial strains that exhibit significant inoculum effects, such as those resistant by virtue of producing β-lactamase. β-Lactamase-mediated resistance results from a chemical interaction in which the β-lactamase molecule binds to the β-lactam antibiotic in a manner that ultimately results in the hydrolysis of the critical β-lactam ring structure (Fig. 1). The rapidity and efficiency with which binding and hydrolysis proceed are dependent upon the affinity with which the β-lactamase molecule binds the antibiotic and the efficiency of the subsequent hydrolysis. High affinity and rapid hydrolysis mean that the cell wall synthesis machinery (penicillin binding proteins, or PBPs) can be defended with few β-lactamase molecules compared to the number of β-lactam molecules likely to be present in the vicinity of the PBPs. Low affinity and slow hydrolysis mean that more β-lactamase molecules are necessary for effective resistance but also that resistance can be more easily overcome by adding more antibiotic molecules. Increasing the inoculum of organisms in a solution with a fixed concentration of β-lactam antibiotic has the effect of increasing the number of β-lactamase molecules and can in some instances result in clinically important levels of resistance. The MIC for a *Klebsiella pneumoniae* strain that produces extended-spectrum β-lactamase TEM-26, for example, may be 1 μg of cefotaxime/ml with a standard inoculum (ca. 10^5 CFU/ml). However, when the inoculum is increased to 10^7 CFU/ml, the MIC increases to greater than 256 μg/ml (210). This type of resistance is likely to be important in treating a large-inoculum infection such as pneumonia. The existence of inoculum effects has led to the common practice of considering all *K. pneumoniae* strains that are resistant to ceftazidime to be resistant to all cephalosporins (excepting the cephamycins), regardless of in vitro susceptibility test results using the standard inoculum.

A second, more amorphous and difficult-to-evaluate concept in considering antimicrobial resistance is that of

* This chapter contains information presented in chapter 68, by Louis B. Rice, Daniel Sahm, and Robert A. Bonomo in the eighth edition of this Manual.

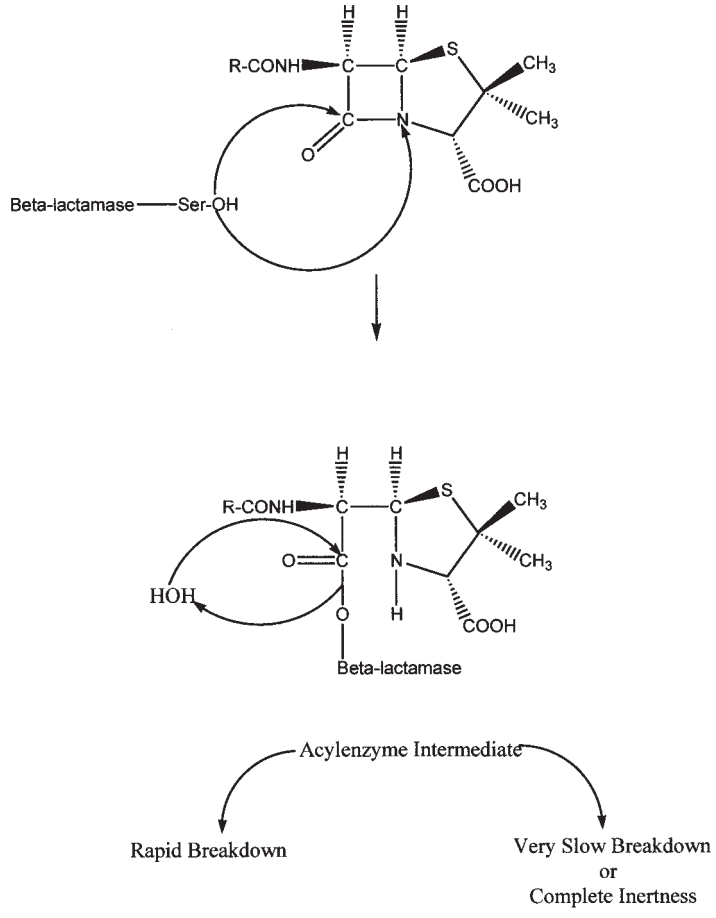


FIGURE 1 Penicillin-interactive, active-site serine peptidases and their reactions with β-lactam carbonyl donors. Modified from reference 87 with permission of Annual Reviews (http://www.annualreviews.org).

tolerance, or resistance to killing at antimicrobial concentrations sufficient to inhibit further growth. Tolerance is an unimportant concept for the treatment of most infections, since for therapeutic success it is usually sufficient to inhibit further growth of bacteria (bacteriostatic activity), allowing the patient's immune defenses to kill the growth-inhibited organisms and clean up the debris. In some instances, however, antimicrobial killing of the bacteria (bactericidal activity) is required to yield a high percentage of treatment success. Instances in which bactericidal activity is preferred include cases of endocarditis, meningitis, and osteomyelitis, in which the immune system has limited access to the infection site. They also include circumstances in which the immune system is severely compromised, such as it is in patients undergoing high-dose chemotherapy for hematologic malignancies. In these instances, antibiotics that are primarily bacteriostatic, such as the tetracyclines and the macrolides, are considered poor choices for therapy whereas β-lactam antibiotics, which are primarily bactericidal, are preferred. Some bacteria are naturally tolerant to β-lactam antibiotics. Bactericidal activity against enterococci, for example, requires two agents, one active against cell wall synthesis and an aminoglycoside (154). Recognition of this bactericidal synergism has raised cure rates for enterococcal endocarditis from about 40 to 70% or greater (207). Unfortunately, expression of aminoglycoside-modifying enzymes (AMEs) negates the synergism and appears to decrease the cure rates for enterococcal endocarditis. Although some level of tolerance to β-lactam antibiotics can be demonstrated for several different bacterial species, the impact on the treatment of clinical infections appears to be less dramatic than for enterococci.

Emergence and Spread of Antibiotic Resistance

The emergence of antimicrobial resistance phenotypes is inevitably linked to the clinical (or other) use of the antimicrobial agent against which resistance is directed. One reason for this association is trivial—we do not generally test for resistance to antibiotics that are not in clinical use. The second reason is that nature abhors a vacuum, so when an effective antibiotic eliminates susceptible flora, resistant varieties soon fill the niche. Once a resistance phenotype has emerged within a previously susceptible species, the rapidity and efficiency with which it spreads are impacted by a host of different factors, including the degree of resistance expressed, the ability of the organism to tolerate the resistance mechanism, linkage to other genes, the site of primary colonization, and others. The rapidity and completeness of resistance gene spread are often unpredictable. For example, the staphylococcal β-lactamase gene (conferring resistance to penicillin) was first described shortly after the introduction of penicillin into clinical use and is now almost universally present within

staphylococci in the hospital and the community. It was not until the early 1980s that this gene was described in *Enterococcus*, and it has never spread widely in this genus. The reverse appears to be true with the vancomycin resistance genes, which are found widely in *E. faecium* but remain exceedingly rare in *Staphylococcus aureus.*

An important cause of the spread of antimicrobial resistance is the failure to adhere to appropriate infection control techniques, both within and outside the hospital. It is well established that strains of methicillin-resistant *S. aureus* (MRSA) within individual hospitals, and even within entire cities, are often clonally related, as determined by genetic techniques such as pulsed-field gel electrophoresis and staphylococcal protein A typing (211). The spread of these problematic pathogens has been attributed to transmission from patient to patient, presumably by transiently or persistently colonized health care workers (228). The primary site of *S. aureus* colonization is the anterior nares. Colonization of the nares facilitates aerosol transmission of the resistant bacteria, particularly during periods of viral upper respiratory infection in the colonized worker. It also facilitates direct transmission, given the frequent contact between hands and noses in many people and the frequently poor hand-washing practices of health care workers. The clinical consequences of patient colonization can be significant. Studies have shown a correlation between patient colonization with MRSA and subsequent infection during periods of high risk, such as the postoperative period (184).

Although antibiotic resistance is predominantly a nosocomial problem, resistant bacteria are also spread in the community setting. Sites in which resistant bacteria have been known to spread include day care centers and nursing homes (1, 274). Penicillin-resistant pneumococci have been found to colonize as many as 25% of a day care center's population. Transmission probably reaches its peak in the winter months, when viral upper respiratory infections are prevalent. The prevalence of viral upper respiratory tract infections works in two ways to increase transmission: (i) it probably increases the inoculum of resistant organisms being spread by those already colonized (see above-mentioned references for *S. aureus* colonization), and (ii) it makes those who are not colonized more likely to become colonized because of the increased likelihood that they will be receiving antimicrobial therapy. Nursing homes are predisposed to resistance for a variety of reasons, including the debilitated state of much of their populations, frequent movement back and forth to tertiary care hospitals, and frequent use of antimicrobial agents in an effort to ward off infections that will necessitate hospital admissions.

A final important source for the emergence and spread of antibiotic-resistant bacteria is nonhuman niches in which antibiotics are used. It is now well established that antimicrobial use in food animals is associated with both resistance in bacterial species that contaminate food and infect humans, primarily *Salmonella* and *Campylobacter* species, and the transfer of resistance determinants to human counterparts, such as *Enterococcus* (69). Compelling evidence also exists that high rates of ciprofloxacin resistance in *E. coli* can be associated with the use of fluoroquinolones in poultry (64). Finally, the European outbreak of vancomycin-resistant enterococci with the *van*A determinant was almost certainly fueled by the use of avoparcin (a glycopeptide antibiotic) as a growth promoter in food animals (266). Data are also emerging that the use of antibiotics to promote growth of animals is often expensive and unnecessary, which has prompted many stakeholders in this issue to outline specific instances in which such antimicrobial use will be permitted.

Genetic Bases of Resistance

Acquired antimicrobial resistance results from biochemical processes that are encoded by bacterial genes. A general list of mechanisms of resistance is presented in Table 1. In order to understand the biochemical processes, it is useful to first discuss the genetic underpinnings of resistance and its evolution. Antimicrobial resistance arises by (i) mutation of cellular genes, (ii) acquisition of exogenous resistance genes, or (iii) mutation of acquired genes.

Mutation of Cellular Genes

All antibiotics have targets, which are often (but not always) proteins with important functional responsibilities in cell growth or maintenance. Cellular genes encode these proteins. Interactions between antibiotics and target proteins are often quite specific, and changing a single amino acid, frequently as a result of a single base change in the gene, can sometimes alter these interactions. Perhaps the most familiar example of this mechanism is resistance to rifampin. Rifampin targets the cellular RNA polymerase (encoded by *rpoB*), and a single point mutation in the RNA polymerase gene may confer complete resistance. These mutations occur in most bacterial species at a relatively high frequency (ca. 10^{-8}/CFU). Incubating enough cells with inhibitory concentrations of rifampin will eliminate susceptible cells and allow the resistant mutants to proliferate. The rifampin in the medium is not actually causing resistance but rather selecting mutants that occur naturally but which have no selective advantage for survival in the absence of rifampin in the environment. Other examples of mutational resistance include resistance to streptomycin through ribosomal mutation (63), resistance to fluoroquinolones through mutations of cellular topoisomerases (144), and resistance to linezolid through mutations in the rRNA (197), among others.

Resistance mutations may also be found in genes that regulate cellular processes. Perhaps the most completely studied example of regulatory mutation resulting in resistance is the derepression of the chromosomal β-lactamase of *Enterobacter* spp. (111). Mutations in a cellular amidase gene (designated *ampD*) result in the buildup of a cell wall breakdown product that has the effect of dramatically increasing expression of a chromosomal β-lactamase gene (*ampC)*. Other examples of regulatory changes include the downregulation of expression of the porin OmpD2 in *Pseudomonas aeruginosa* associated with resistance to imipenem (134) and the insertion of an insertion sequence (IS element) upstream of a chromosomal carbapenemase gene conferring imipenem resistance on *Bacteroides fragilis* (188).

Whether mutational resistance is likely to persist will depend in some measure on whether the resistance mutation is tolerable to the cell. For example, although decreased expression of OMPD2 appears to be readily achievable for *P. aeruginosa,* the fact that the resistant strains have not spread widely in the nearly 20 years of carbapenem use probably reflects the fact that this porin has functions that are beneficial to the bacterium, favoring reexpression of the porin once the imipenem threat has been dissipated. Similarly, intermediate levels of susceptibility to vancomycin in *S. aureus* have thus far been attributed to marked changes in the composition of the cell wall (231). These changes are unlikely to be favored in an environment free of vancomycin, since *S. aureus* likely "decided" a long time ago the optimal size and composition of its cell wall. The deleterious effects of acquiring resistance are often referred to as the fitness cost.

Disadvantageous resistance mutations do not always disappear. Although initial point mutations in the *rpoB* gene

TABLE 1 Common associations of resistance mechanisms

Antibiotic class	Resistance type	Resistance mechanism	Common example
Aminoglycosides	Decreased uptake	Changes in outer membrane permeability	*P. aeruginosa*
	Enzymatic modification (AMEs)	Phosphotransferase	Wide range of enteric gram-negative bacteria
		Adenyltransferase	Wide range of enteric gram-negative bacteria
		Acetyltransferase	Wide range of enteric gram-negative bacteria
		Bifunctional enzyme	*aac(6′)-aph(2″)* in *S. aureus, E. faecium,* and *E. faecalis*
ß-Lactams	Altered PBP(s)	PBP2a (additional PBP)	*mecA* in *S. aureus* and coagulase-negative staphylococci
		PBP2x, PBP2b, PBP1a (acquired from other streptococci by transformation)	*S. pneumoniae*
		PBP5 (point mutation)	*E. faecium*
	Enzymatic degradation (β-lactamases)	Ambler class A	TEM-1 in *E. coli, H. influenzae,* and *N. gonorrhoeae* SHV-1 in *K. pneumoniae* K1 (OXY-1) in *Klebsiella oxytoca* ESBLs (TEM-3+, SHV-2+, and CTX-M types) in *K. pneumoniae* and *E. coli* BRO-1 in *Moraxella catarrhalis* PC1 in *S. aureus* PSE-1 in *P. aeruginosa* ß-lactamases of *C. koseri* and *P. vulgaris*
		Ambler class B	L-1 in *Stenotrophomonas maltophilia* CcrA in *Bacteroides fragilis*
		Ambler class C	AmpC in *E. cloacae* and *Citrobacter freundii,* and similar enzymes in *Serratia marcescens, Morganella morganii, Providencia stuartii,* and *P. rettgeri*
		Ambler class D	OXA-1 in *E. coli*
Chloramphenicol	Enzymatic degradation	CATs	CAT in *S. pneumoniae*
	Efflux	Membrane transporters	*cmlA* and *flo*-encoded efflux in *E. coli* and *Salmonella* spp.
Glycopeptides	Altered target	Altered peptidoglycan cross-link target (D-Ala–D-Ala to D-Ala–D-Lac or D-Ala–D-Ser) encoded by complex gene cluster	VanA gene cluster in *E. faecium, E. faecalis,* and *S. aureus;* VanB gene cluster in *E. faecium* and *E. faecalis*
	Target overproduction	Excess peptidoglycan	Glycopeptide-intermediate strains of *S. aureus* and *Staphylococcus haemolyticus*
Oxazolidinones	Altered target	Mutation leading to reduced binding to active site	G2576U mutation in rRNA in *E. faecium* and *S. aureus*
Macrolides-lincosamides-streptogramin B	Altered target	Ribosomal active-site methylation with reduced binding	*erm*-encoded methylases in *S. aureus*, *S. pneumoniae*, and *Streptococcus pyogenes*
Macrolides	Efflux	Mef efflux pump	*mef*-encoded efflux in *S. pneumoniae* and *Streptococcus pyogenes; msrA* in *S. aureus*
Streptogramin A	Enzymatic degradation	Acetyltransferases	*vat*(A)-, *vat*(B)-, and *vat*(C)-encoded enzymes in *S. aureus* *vat*(D)- and *vat*(E)-encoded enzymes in *E. faecium*
Quinolones	Altered target	Mutation leading to reduced binding to active site(s) (quinolone resistance-determining region)	Mutations in *gyrA* in enteric gram-negative bacteria and *S. aureus* Mutations in *gyrA* and *parC* in *S. pneumoniae*

(Continued on next page)

TABLE 1 Common associations of resistance mechanisms *(Continued)*

Antibiotic class	Resistance type	Resistance mechanism	Common example
	Efflux	New membrane transporters	NorA in *S. aureus*
	Protection from DNA binding	Acquired protection protein	*qnr* gene and variants
	Enzymatic modification	Mutated aminoglycoside acetyltransferase	*aac(6′)-Ib* variant in *E. coli*
Rifampin	Altered target	Mutations leading to reduced binding to RNA polymerase	Mutations in *rpoB* in *S. aureus* and *Mycobacterium tuberculosis*
Tetracyclines	Efflux	New membrane transporters	*tet* genes encoding efflux proteins in gram-positive bacteria (mainly group 2) and gram-negative bacteria (mainly group 1)
	Altered target	Production of proteins that bind to the ribosome and alter the conformation of the active site (ribosomal protection proteins)	*tet*(M) in a variety of gram-positive bacteria
Sulfonamides	Altered target	Mutation or recombination of genes encoding DHPS	Found in a wide range of species, e.g., *E. coli, S. aureus,* and *S. pneumoniae*
		Acquisition of new, low-affinity DHPS genes	*sul*I and *sul*II in enteric gram-negative bacteria
Trimethoprim	Altered target	Mutations in gene encoding DHFR	*S. aureus, S. pneumoniae,* and *H. influenzae*
		Acquisition of new, low-affinity DHFR genes	*dhfr*I and *dhfr*II encoded, found in a wide range of species
	Overproduction of target	Promoter mutation leading to overproduction of DHFR	*E. coli*

that confer rifampin resistance on *Salmonella enterica* serovar Enteritidis appear to decrease the fitness of the organism for survival in vivo, persistence in a live host is frequently associated with compensatory mutations that at least partially restore fitness to the strain while retaining the resistance (rather than mutating back to susceptibility) (22). Similarly, transfer of mutated *pbp5* into *E. faecium* strains is often associated with decreases in the expression of ampicillin resistance, but growth on increased concentrations of ampicillin easily yields colonies that grow well at higher concentrations (209). Similar findings have been reported for *S. aureus* strains transformed with the *mec*A gene encoding methicillin resistance (156). In summary, although mutational resistance often confers a fitness cost, subsequent adaptations may make the expression of resistance less costly.

Acquisition of Resistance Genes

If resistance is not achievable through mutation, threatened bacterial species have little choice but to look elsewhere for resistance determinants. They are aided in this effort by the fact that most antimicrobial agents are natural products or derivatives of natural products. Therefore, resistance genes for most antibiotics must exist in the microbial world, either in the species that produce the antibiotic or within species that live in the same ecological niche as the antibiotic producers (54). The challenge for susceptible human pathogens is to find and acquire these resistance determinants. To assist in this acquisition, bacteria have evolved a range of mechanisms that promote gene exchange. Perhaps the simplest of these techniques is natural transformation, referring to the ability of some bacterial species to absorb naked DNA molecules from the environment under the appropriate circumstances (92). Once taken up by the susceptible bacterium, these foreign pieces of DNA enter the bacterial chromosome by recombining across regions of sufficient homology. In some cases, functional genes result from this recombination. If the acquired gene encodes a protein that is less susceptible to inhibition than the native protein, a reduction in susceptibility may result. Perhaps the best-studied example of the formation of these "mosaic" genes to confer resistance is penicillin and cephalosporin resistance in *Streptococcus pneumoniae* (92). A variety of mosaic PBP *(pbp)* genes in resistant strains have been described previously, with the level and degree of resistance determined by the number and nature of gene recombinations. Mosaic topoisomerase genes in fluoroquinolone-resistant transformable bacteria have also been described previously (55).

Most bacteria are incapable of natural transformation and so have developed other mechanisms for acquiring useful genetic determinants. A commonly employed mechanism for genetic exchange is the transfer of conjugative plasmids. These extrachromosomal replicative DNA forms may carry a variety of important genes. Some plasmids are relatively narrow in their host range, and others transfer into and replicate within several different species. Transfer frequencies can be very high, as in the F factor of *E. coli* (virtual complete transfer in 1 h) and the pheromone-responsive plasmids found in *Enterococcus faecalis* (ca. 10^{-1}

transconjugant/recipient CFU in 24 h), or more modest, as observed with the broad-host-range enterococcal plasmids such as pAMß1 one (10^{-7} to 10^{-6} transconjugants/recipient CFU in 24 h) (32). Having entered into a new genus on broad-host-range plasmids, resistance determinants can readily transfer onto more frequently transferable plasmids to increase their movement through the new genus. In the first case of true vancomycin-resistant *S. aureus* described in the literature, it appears that vancomycin resistance transposon Tn*1546* entered into *S. aureus* from *E. faecalis* on a broad-host-range plasmid and then transposed into a conjugative plasmid native to the staphylococcus (79, 268). Plasmids may also integrate into the chromosomes of the recipient strains, potentially increasing the stability of the genetic information they carry.

Bacteria also take advantage of bacterial viruses (bacteriophages) for genetic exchange. These discreet packages deliver to uninfected cells quantities of DNA approximating the sizes of their genomes (in most cases roughly 40 kb). Designed to incorporate their own genomes into the manufactured phage head, they sometimes incorporate bits of chromosomal DNA adjacent to the phage integration site (specialized transduction) and other times incorporate an appropriately sized plasmid or chromosomal DNA segment unrelated to the integrated phage genome (generalized transduction). Since the staphylococcal ß-lactamase gene is frequently identified on nonconjugative plasmids of approximately 35 to 40 kb and since bacteriophages in staphylococci have been well described for decades, it has been speculated that the high prevalence of β-lactamase production in staphylococci has resulted from the bacteriophage-mediated transfer of these plasmids. Bacteriophages have also been implicated in the transfer of virulence determinants (71).

Nonreplicative mobile elements known as transposons have also been implicated in the transfer of resistance genes (204). Transposons encode their own ability to transfer between replicons (autonomously replicating DNA segments). In some cases, the transposons themselves encode conjugation functions, which allow them to transfer from bacterial chromosome to bacterial chromosome. The best characterized of these conjugative transposons is Tn*916,* an 18-kb element originally identified in *E. faecalis* but which has a very broad host range (205). Tn*916* encodes resistance to tetracycline and minocycline through the *tet*(M) resistance gene. Many different Tn*916*-like transposons have now been identified in enterococci and beyond, and some of them, such as Tn*1545* from *S. pneumoniae,* possess additional resistance genes (conferring resistance to erythromycin and kanamycin) (205). Some investigators have suggested that the conjugation events associated with Tn*916*-like transposons are akin to cell fusion events, in which portions of the genome distinct from those adjacent to the inserted transposon can exchange via homologous recombination (249). Transposons with structural similarity to Tn*916* have been implicated in the transfer of vancomycin resistance between *E. faecium* strains (37).

Transposons lacking conjugative functions may also transfer between strains. The most common mechanism through which this transfer is presumed to occur is either transient or more permanent integration into transferable plasmids. Among the more common classes of nonconjugative transposons are the Tn*3* family of elements (including Tn*917* conferring erythromycin resistance, and Tn*1546,* conferring VanA-type vancomycin resistance) (11, 223) and the composite elements formed by mobile IS elements flanking resistance genes (including Tn*4001*, conferring high-level gentamicin resistance in many gram-positive species) (81).

The precise origin of resistance genes is often difficult to discern, but in some cases it is at least possible to determine that acquired resistance determinants originated in other genera. The VanB-type vancomycin resistance gene in enterococci, for example, has a G+C content of nearly 50% (67). The enterococcal genome, in contrast, has an approximately 35 to 38% G+C content. These differences virtually confirm the origin of the *vanB* determinant in a genus other than *Enterococcus.* The likely origin appears to be streptomycetes, probably species that manufacture glycopeptide antibiotics, with entry into enterococci facilitated by the incorporation of these resistance operons into transposons.

It is worth noting at this point that any concept of the bacterial genome as a fixed entity is untenable. Comparisons of *E. coli* genomes reveal striking differences between enteropathogenic and uropathogenic strains, with the different regions constituting "pathogenicity islands" that confer specific virulence traits that give the strains their clinical profiles (95, 270). Data emanating from a comparative study of 36 *S. aureus* genomes indicate that 22% of the genome is dispensable, with many of the variable regions constituting presumed pathogenicity islands and regions of antimicrobial resistance (78). Finally, it is estimated that 25% of the genome of *E. faecalis* strain V583 was acquired from outside the genus (183).

Mutation of Acquired Genes

As bacteria have responded to the challenge of antimicrobial agents, so have we responded to the challenge of antibiotic resistance. Our typical response to the appearance of antimicrobial resistance has been a concerted effort to develop novel antimicrobial agents that will be active against resistant strains. The emergence of β-lactamase-mediated resistance to antibiotics is an instructive example of this interplay. Ampicillin was developed as the first penicillin with clinically significant activity against gram-negative rods, primarily *E. coli.* Within a few years of the clinical introduction of ampicillin, strains of *E. coli* were described that were resistant to this antibiotic by virtue of production of a plasmid-mediated β-lactamase designated TEM (named after the patient from whom the resistant strain was isolated). *S. aureus* expressed a similar β-lactamase, prompting a concerted effort on the part of the pharmaceutical industry to develop β-lactam antibiotics resistant to hydrolysis. Among the more successful compounds that were developed were methicillin, with activity against β-lactamase-producing *S. aureus;* the cephalosporins and carbapenems, with widespread activity against many β-lactamase-producing species; and the β-lactamase inhibitors, which restored the activity of β-lactams susceptible to hydrolysis.

The most successful and widely developed class of β-lactamase-resistant β-lactam antibiotics is the cephalosporins. So many of these agents have been developed for clinical use that they were frequently lumped into "generations" to facilitate remembrance of their spectra of activity. The extended-spectrum (previously referred to as third-generation), cephalosporins cefotaxime, ceftizoxime, ceftriaxone, and ceftazidime are particularly potent antibiotics that are resistant to hydrolysis by the original TEM enzyme. Unfortunately, increasing clinical use of these agents, particularly ceftazidime, has been associated with the emergence of resistant gram-negative rods, particularly *K. pneumoniae* (114). Molecular analysis of these resistant strains revealed that the resistance is mediated by β-lactamases and that

many of these β-lactamases are derived from the native TEM enzyme through one or more point mutations in the bla_{TEM} gene.

Biochemical Mechanisms of Resistance

Modification of the Antibiotic

Many antibiotic-modifying enzymes have been described previously, including the β-lactamases, the AMEs, and chloramphenicol acetyltransferases (CATs). Although these enzymes are in many cases acquired, some are intrinsic to certain genera. For example, chromosomally encoded β-lactamases are intrinsic to almost all gram-negative rods. Expression of these enzymes is often only at very low levels, conferring resistance to only very susceptible β-lactams, as in the case of *K. pneumoniae* resistance to ampicillin through expression of the chromosomally expressed SHV-1 enzyme (208), or to no β-lactams at all, as in the case of wild-type *E. coli* strains. In some bacterial genera (notably *Enterobacter* and *Pseudomonas*), chromosomally expressed β-lactamases are under regulatory control, with derangements in these regulatory mechanisms resulting in high-level, broad-spectrum β-lactam resistance (112). In some instances, AMEs are intrinsic to bacterial species as well, as in the cases of the chromosomally expressed acetyltransferases of *Providencia stuartii* and *Serratia marcescens* (203, 224).

Modifying enzymes in general confer high levels of resistance to the antibiotics against which they have activity. Expression of the TEM-1 β-lactamase by *E. coli,* for example, can increase the MIC of ampicillin from 8 to >10,000 μg/ml. Similarly, expression of the bifunctional aminoglycoside resistance enzyme in *E. faecalis* raises the MIC of gentamicin from 32 to 64 μg/ml to >2,000 μg/ml. As effective as these mechanisms are, however, some antibiotics appear to be immune to inactivating enzymes. Vancomycin has been in clinical use since 1958, yet there are still no examples of vancomycin-modifying enzymes in bacteria.

Modification of the Target Molecule

Since antibiotic interaction with target molecules is generally quite specific, minor alterations of the target molecule can have important effects on antibiotic binding. Numerous examples exist of antibiotic target modification as a mechanism of resistance, including the many erythromycin ribosomal methylases that confer resistance to the macrolide-lincosamide-streptogramin B classes of antibiotics (269). Modifications of PBPs can affect the affinities of these molecules for β-lactam antibiotics, as noted above for *S. pneumoniae,* and especially for ampicillin-resistant *E. faecium* through mutations in PBP5 (92, 209). Modification of PBPs seems to be a favored mechanism of β-lactam resistance in gram-positive bacteria, whereas β-lactamase production is favored in gram-negative rods. Although the reason for this difference is unknown, it is interesting that β-lactamases produced by gram-positive bacteria diffuse into the external medium once produced whereas those produced by gram-negative rods are kept within the periplasmic space by the outer membrane. The ability to concentrate β-lactamases enhances their efficacy and may help explain the preference for this mechanism among gram-negative rods.

Other important examples of target modifications include the altered cell wall precursors that confer resistance to glycopeptide antibiotics; mutated DNA gyrase and topoisomerase IV, conferring resistance to fluoroquinolone antimicrobial agents; ribosomal protection mechanisms conferring resistance to tetracyclines; and RNA polymerase mutations conferring resistance to rifampin. The degree of resistance conferred by target modifications is variable and may be dependent upon the ability of the mutated target to perform its normal function. Mutations in PBPs of *S. pneumoniae,* for example, confer a relatively low level of resistance (although one that is significant in the treatment of meningitis) (92), whereas VanA-type vancomycin resistance mutations confer a very high level of resistance to vancomycin on enterococci (12).

Restricted Access to the Target

It is axiomatic that an antibiotic must reach its target in order to be effective. Therefore, for targets for which barriers must be crossed by the antibiotic, strengthening of these barriers can be a highly effective mechanism of resistance. All gram-negative bacteria have an outer membrane that must be traversed before the cytoplasmic membrane can be reached. Reductions in the quantities of known or presumed porins (channels for movement of materials across the outer membrane) have been documented as important contributors to resistance to imipenem in *P. aeruginosa*, cefepime in *Enterobacter cloacae*, and cefoxitin and ceftazidime in *K. pneumoniae* (129, 134, 145). In most instances, this restricted entry must be in combination with the production of an at least moderately active β-lactamase to confer high-level resistance. Barriers to entry can also exist in the cytoplasmic membrane. Movement of aminoglycosides across the cytoplasmic membrane is an oxygen-dependent process, so these antibiotics are inactive in anaerobic environments (and hence against strictly anaerobic species) (127).

Efflux Pumps

Among the most active areas of research in antimicrobial resistance is the identification and characterization of pumps that efflux one or more antibiotic classes from the bacterial cell. Several classes of pumps in gram-positive and/or gram-negative bacteria have been described previously. The pumps may be quite selective, or they may have broad substrate specificities. The majority of these pumps are located in the cytoplasmic membrane and use proton motive force to drive drug efflux. The major families of efflux transporters are (i) the major facilitator superfamily, which includes QacA and NorA/Bmr of gram-positive bacteria and EmrB of *E. coli;* (ii) the small multidrug-resistance family, including Smr of *S. aureus* and EmrE of *E. coli;* and (iii) the resistance-nodulation-cell division (RND) family, including AcrAB-TolC of *E. coli* and MexAB-OprM of *P. aeruginosa.* The structure of the AcrAB-TolC RND-type efflux pump is shown in Fig. 2. Deciphering the crystal structure of this pump was a major achievement (157). Among other things, it revealed that there is a periplasmic opening in the pump that can allow passage of molecules, explaining the previously confusing observation that RND pumps include β-lactam antibiotics (which do not enter the cytoplasm) among their substrates. In some instances, combinations of different types of pumps can result in higher levels of resistance than are achieved by the activity of a single pump alone (128).

Attribution of resistance to a specific mechanism may be difficult when more than one mechanism is involved. For example, resistance to imipenem in *P. aeruginosa* is contributed to by reduced access (through down-regulation of OmpD2) and production of AmpC β-lactamase (134). Neither mechanism alone is sufficient to yield clinically significant levels of resistance, yet both are required for high levels of resistance to result.

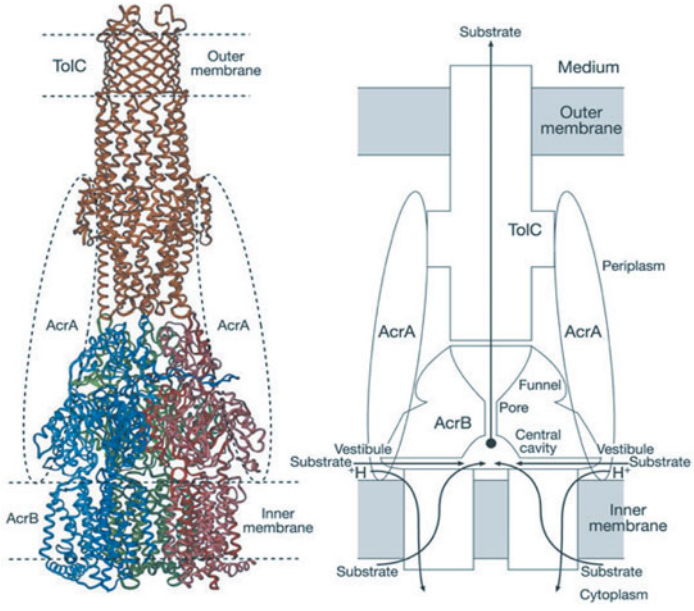


FIGURE 2 Representation of the crystal structure of the AcrAB-TolC three-component resistance nodulation-cell division-multidrug efflux pump. On the left are the three components of the pumps as they link the cytoplasmic (inner) membrane to the outer membrane. The periplasmic linker protein (AcrA) is shown only in outline to allow visualization of the linkage between AcrB and TolC. On the right, an outline of the pump shown at the left is presented, detailing the functional regions of the pump. Reprinted from reference 157 with permission of the publisher.

RESISTANCE MECHANISMS FOR DIFFERENT ANTIMICROBIAL CLASSES

Aminoglycoside Resistance

In explaining resistance to aminoglycosides (amikacin, gentamicin, kanamycin, neomycin, netilmicin, paromomycin, streptomycin, and tobramycin), we will first explain how aminoglycosides reach their targets in bacterial cells and then review their mechanism of action. The clinical indications for aminoglycoside therapy will also be summarized.

The aminoglycosides contain an aminocyclitol ring (streptidine or 2-deoxystrepamine) and two or more amino sugars linked by glycosidic bonds. They are hydrophilic antibiotics whose antimicrobial activity is concentration dependent. They are particularly active against aerobic, gram-negative rods. As a class, these agents have other highly desirable qualities: they are rapidly bactericidal, demonstrate a "postantibiotic" effect, and exhibit predictable pharmacodynamics.

Mechanism of Action of Aminoglycosides

Aminoglycosides have unique effects on protein translation in prokaryotes. These effects may explain why aminoglycosides are bactericidal whereas most other antibiotics that target the ribosome (chloramphenicol, macrolides, and tetracyclines) are bacteriostatic.

First, aminoglycosides penetrate bacteria by three main steps. The positively charged aminoglycosides bind to the negatively charged bacterial cell. Next, in an energy-dependent process driven by the membrane-bound electron transport system present only in aerobic bacteria, a small fraction of the aminoglycosides that are attached to the bacterium are taken into the cell.

Once inside the cell, the principal target of aminoglycoside action is the 30S subunit of the ribosome. The molecular details of aminoglycoside binding to the ribosome are unique. Gentamicin attaches to the highly conserved tRNA acceptor A site of the *E. coli* 16S rRNA (152). This binding prevents the elongation of the growing peptide chain by causing misreading or premature termination of peptide synthesis. By interfering with the translation of mRNA, protein production is altered, aberrant proteins are synthesized and inserted into the cell membrane, cell permeability is increased, more aminoglycosides are taken up into the cell, and cell death ensues.

Mistranslated proteins may also affect the bacterial cell. Paromomycin increases the error rate of protein translation by allowing the incorporation of the wrong tRNAs (the missense substitution rate of amino acids at specified positions in proteins is 1 in 3,000). Spectinomycin, a nonaminoglycoside aminocyclitol, inhibits translocation of the peptidyl-tRNA from the A site to the P site. Streptomycin also makes the ribosome error prone by allowing the binding of different tRNAs (39). Resistance to aminoglycosides can occur through four mechanisms: (i) loss of cell permeability (decreased uptake); (ii) alterations in the ribosome that prevent binding; (iii) expulsion by efflux pumps; and (iv) enzymatic inactivation by AMEs.

Resistance Due to Decreased Uptake and Altered Electrical Potential, Divalent Cations, and Efflux

It is well known that bacterial respiration generates an electrical potential across the membrane. Hence, anaerobes and facultative anaerobes (e.g., enterococci and certain small-colony variants of staphylococci) do not allow movement of aminoglycosides across their cell membranes. This

intrinsic resistance confers low-level cross-resistance to all aminoglycosides.

Studies of *P. aeruginosa* and *E. coli* indicate that movement across the outer membrane may be due to a self-promoted uptake mechanism. The cationic aminoglycosides displace divalent cations (e.g., Mg^{2+}) that bridge adjacent lipopolysaccharide molecules, thereby permeabilizing the outer membrane and allowing the entry of aminoglycosides (118). Consistent with this model, divalent cations have long been known to antagonize the activity of aminoglycosides against gram-negative bacteria (35).

Recent data indicate that *P. aeruginosa* possesses an inducible RND-type pump (MexXY-OprM) that effluxes aminoglycosides (115, 141). In *E. coli*, aminoglycosides are captured from both the periplasm and the cytoplasm by the AcrD multidrug efflux transporter (2). Evaluating a multidrug-resistant strain, *Acinetobacter baumanii* BM4454, that was resistant to aminoglycosides, Magnet et al. found an RND-type efflux pump (139), and Marchand et al. showed that the pump was under stringent control by a two-component regulatory and sensor system, *ade*RS (142).

Modification of the Ribosome

Bacterial cells have multiple copies of the rRNA genes. Mutations that affect aminoglycoside binding to the ribosome include alterations in ribosomal proteins and 16S rRNA and enzymatic methylation of the rRNA. Ribosomal mutations have been demonstrated to confer resistance to spectinomycin and streptomycin (258) and more recently to other aminoglycosides as well (148). The newly identified 16S rRNA methylases (RmtA, RmtB, and ArmA) in gram-negative rods have been noted as emerging problems (280). The first such novel enzyme was found in *K. pneumoniae* BM4536 and designated *armA* ("aminoglycoside resistance methylase A") (82, 83).

Aminoglycoside-Modifying Enzymes

As that of β-lactamases (see below), inactivation of aminoglycosides by AMEs is the most important mechanism in terms of frequency and level of resistance (15). AMEs are believed to originate from *Actinomycetes* that synthesize these antibiotics (*Streptomyces* and *Micromonospora* spp.). It is also possible that AMEs originated from enzymes involved in normal cellular respiration (housekeeping functions) (202). These enzymes are phosphotransferases (APHs), nucleotidyltransferases or adenyltransferases (ANTs), and acetyltransferases (AACs). AMEs covalently modify specific amino or hydroxyl groups, resulting in aminoglycosides that bind poorly (higher K_ms, lower affinities) to the ribosome. AMEs can be passed from one bacterium to another by mobile genetic elements. These resistance determinants are frequently carried on specialized genetic elements called integrons (225, 273).

Sites of AME modification of aminoglycosides are identified by a standard numbering system. The streptidine or 2-deoxystreptamine nucleus forms the center for the numbering scheme (from 2-deoxystreptamine derive all aminoglycosides except streptomycin and spectinomycin). The first sugar moiety at the 4 position has positions numbered with a single prime (1′ to 6′); the second sugar moiety at the 6 position gets positions numbered with a double prime (1″ to 6″). Hence, AAC(6′) acetylates an amino group at the 6′ position (via an acetyltransferase) on the amino sugar attached to the 4 position (Fig. 3). Each AME class consists of numerous enzymes that can modify different OH or NH_2 groups. These classes are divided into subclasses. In each subclass, there are different enzyme types that are designated by a roman numeral, e.g., AAC(3)-I. Isoenzymes are also described and are designated by a lowercase letter "a" or "b," etc. These isoenzymes are functionally identical and confer identical resistance phenotypes.

Currently, there are seven major phosphotransferases [APH(3′), APH(2″), APH(3″), APH(6), APH(9), APH(4), and APH(7″)], four nucleotidyltransferases [ANT(6), ANT(3″), ANT(4′), and ANT(2″)], and four acetyltransferases [AAC(2′), AAC(6), AAC(1), and AAC(3)]. A bifunctional AME also exists that is able to acetylate and phosphorylate AAC(6)-APH(2″). This enzyme is found in *Staphylococcus*, *Streptococcus,* and *Enterococcus* species and is responsible for high-level resistance to aminoglycosides (70). The gene *aac(6′)-aph(2″)* that encodes the synthesis of this enzyme is present on Tn*4001*-like transposons which are inserted both into plasmids and into the chromosomes of aminoglycoside-resistant isolates. Most health care-associated MRSA strains express this enzyme.

Resistance to β-Lactam Antibiotics

Penicillin-Binding Protein-Mediated Resistance

β-Lactam antibiotics act by inhibiting the PBPs, the transpeptidases that manufacture peptidoglycan. The specific functions of different PBPs have been identified for some bacteria, but the precise ways in which they interact with one another and with cell wall precursors remain largely a mystery. Some are clearly essential for cell viability (generally the high-molecular-weight transpeptidases and their partner transglycosylases), whereas others appear to be dispensable, with

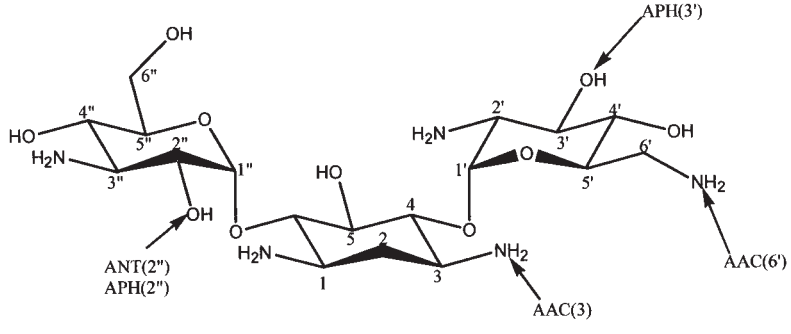


FIGURE 3 Sites of modification on kanamycin B by various AMEs. The arrows point to the sites of modification by the specific enzymes, namely, acetyltransferases, phosphotransferases, and nucleotidyltransferases. Reprinted from reference 125 with permission.

no apparent deleterious effects on cellular structure or function resulting from their absence (most commonly the low-molecular-weight carboxypeptidases). It has long been suspected that some PBPs are redundant and can serve the functions of others. For example, *E. faecium* strains in which all of the PBPs except low-affinity PBP5 are saturated grow normally, implying that PBP5 can perform all of the penicillin-inhibiting functions required for cell wall synthesis (276). On the other hand, *E. faecium* strains in which *pbp5* has been deleted grow normally as well, implying that the other PBPs can provide all of these functions (209).

PBPs are all members of a larger family of serine peptidases that includes most of the β-lactamases. PBPs and β-lactamases interact with β-lactam molecules (which themselves are structural analogues of the peptidyl-D-alanyl-D-alanine termini of peptidoglycan precursors) by catalytically disrupting the β-lactam bond, resulting in a serine-ester-linked acyl-enzyme derivative (Fig. 1). In the case of β-lactamases, a water molecule then hydrolyzes the ester linkage of the acyl-enzyme intermediate, releasing the irreversibly damaged penicilloyl (or cephalosporyl) moiety and regenerating the active enzyme. PBP-β-lactam acyl-enzyme derivatives are in general less accommodating of nucleophilic attack by the water molecule, resulting in persistence of the covalent bond and inactivation of the PBP. The stability of this interaction allows identification of these proteins by binding to radiolabeled penicillin and is the genesis of their designation as "penicillin binding proteins."

Inhibition of PBPs interrupts cell wall synthesis, which by itself should inhibit cell growth rather than kill the cell. However, the interaction of β-lactam molecules with PBPs triggers the activity of cell wall-degrading molecules known as autolysins, which rupture the cell, leading to cell death (248). The extent to which these autolytic enzymes are activated correlates in most cases with the killing activity of a β-lactam against a particular bacterial strain.

Some bacterial species are intrinsically resistant to some β-lactam antibiotics by virtue of decreased PBP affinity. For example, enterococci are resistant to clinically achievable levels of cephalosporin antibiotics because of the presence of low-affinity PBP5. Similar low affinities are demonstrated for the semisynthetic antistaphylococcal penicillins nafcillin and oxacillin, as well as for the antipseudomonal penicillins carbenicillin and ticarcillin. Ampicillin, mezlocillin, and piperacillin bind enterococcal PBP5 with diminished affinity, resulting in MICs that are higher than those for streptococci. These concentrations are within the achievable range in human serum.

PBP-mediated resistance for normally susceptible bacteria takes several forms, including (i) overproduction of a PBP, (ii) acquisition of a foreign PBP with low affinity, (iii) recombination of a susceptible PBP with more resistant varieties, and (iv) generation of point mutations within PBPs that lower affinities for the β-lactam antibiotic.

PBP Overexpression

Increased expression of a PBP as a mechanism of conferring resistance is relatively uncommon. Clear examples of settings in which increased quantities of a PBP are associated with resistance include the increased levels of methicillin resistance found in *S. aureus* strains overexpressing PBP4 (99) and increased levels of penicillin resistance in *E. faecium* strains that overexpress PBP5 (209). The existence of this mechanism serves as a reminder that, like that of β-lactamases, susceptibility or resistance depends on the number of β-lactam molecules relative to the number of targets. Increasing the number of target molecules can, under the correct circumstances, result in resistance. Imipenem's effectiveness against *E. coli* has been partially attributed to the fact that its primary target is PBP2, which is present in roughly 200 copies, in contrast to PBP3, estimated to be present in 2,000 copies.

Acquisition of Foreign PBPs

Acquisition of a foreign PBP as a mechanism of resistance is best exemplified by the expression of methicillin resistance in *S. aureus* strains by virtue of the expression of PBP2a, a low-affinity PBP not native to *S. aureus* (42). Structural analysis of PBP2a suggests that it possesses both transglycosylase and transpeptidase domains. However, it is not clear that the transglycosylase domain is functional. In fact, the transglycosylase domain from PBP2 is required for proper functioning of PBP2a (187). PBP2a confers resistance to all β-lactam antibiotics, although it is bound relatively well by ampicillin (43). The fact that virtually all MRSA strains express β-lactamase limits the utility of ampicillin, but in vitro and animal studies suggest that combinations of ampicillin with β-lactamase inhibitors may be effective (43). To date, there are no clinical data in humans to support using these combinations to treat MRSA infections.

The origin of the *mecA* gene (which encodes PBP2a) is unknown. A *mecA* homologue has been identified in *Staphylococcus sciuri*, a staphylococcal species associated with rodents and primitive mammals (50). The deduced amino acid sequences of the two product enzymes exhibit 88% similarity across the entire proteins and 91% identity within the transpeptidase domain. *Staphylococcus sciuri* strains are not methicillin resistant, however, which may owe to the lack of an effective promoter upstream of the gene (279).

Expression of methicillin resistance in *S. aureus* is commonly under regulatory control, either by the product of the upstream *mecI* gene or *in trans* by the homologous *blaI* gene that regulates the expression of β-lactamase (the promoter regions of *mecA* and the *blaZ* β-lactamase genes are similar) (91). The *mecI* and *blaI* repressors are in turn controlled by the *mecR1* and *blaR1* sensor-transducers, although the precise mechanism of this interaction is incompletely understood. The efficiency of induction varies with the mechanism (*blaR1-blaI*-mediated induction is faster), leading to difficulties in detecting the methicillin resistance phenotype. In fact, only a small minority of a resistant population may express high levels of resistance. Several techniques are used in the laboratory to bring out the resistance (e.g., prolonged incubation or an increase of salt in the media), and more recently techniques have been developed to bypass phenotypic expression in favor of directly identifying the *mecA* gene or directly detecting the PBP2a protein.

Expression of PBP2a-mediated resistance to β-lactams in *S. aureus* is also influenced by the expression of other genetic loci called *fem* ("factors essential for methicillin resistance") or *aux* ("auxiliary") genes (42). *fem* and *aux* genes were first identified by transposon mutagenesis of MRSA to look for insertions that would reduce the expression of resistance. Many *fem* and *aux* factors have now been identified, and all are involved in the formation of the staphylococcal cell wall (42). These data indicate that minor perturbations of the normal processes prevent PBP2a from functioning, suggesting that it has a restricted substrate specificity.

The *mecA* gene is located within a larger (ca. 21 to 67 kb) region of the chromosome known as the staphylococcal cassette chromosome *mec* (SCC*mec*) region (110). It has now been conclusively demonstrated that SCC*mec* is a mobile

element, with mobility conferred by the presence of the *ccrA* and *ccrB* genes. The basic elements of SCC*mec* are the *mecR1-mecI-pbp2a* region and *ccrA* (Fig. 4). In recent community-acquired *S. aureus* isolates, little else is included in the SCC*mec* complex (173). Hence, the SCC*mec* regions in these isolates are relatively small (20 to 30 kb) and the isolates themselves tend to be resistant only to β-lactams. Nosocomial isolates have larger SCC*mec* regions, owing to the accumulation over time of integrated plasmids or transposons that contribute to the multiresistance of these isolates (110). Although the SCC*mec* element has never been shown to be transferable in vitro, compelling data have emerged from the study of clinical isolates indicating that the type IV SCC*mec* element has been recently acquired (72). It is intriguing that these data are being found now that the smaller SCC*mec* elements are more prevalent. Since they fall below the 40 kb typical of the staphylococcal bacteriophage, it is conceivable that SCC*mec* type IV is transferable by transduction. The large sizes of SCC*mec* types I to III preclude this sort of transfer, providing a rationale for the prominence of person-to-person spread of MRSA in the hospital rather than the spread of the resistance determinant from strain to strain. Transfer of the *mec* region between staphylococcal strains has never been conclusively documented. The spread of MRSA within institutions is therefore largely due to the transmission of resistant organisms from patient to patient, probably on the hands of transiently colonized health care workers (228). Single strains have spread through entire hospitals and even cities (211). Recent data indicate that *mecA* stabilities differ among different *S. aureus* clones (119), suggesting one potential explanation for the limited lineages within which the resistance determinant has been found.

Resistance Mutations by Recombination with Foreign DNA

Resistance through recombination between PBPs of native, susceptible species and those of less susceptible species is a phenomenon largely restricted to species that are capable of natural transformation or of taking up of naked DNA from the environment. Prominent among these species are *S. pneumoniae,* viridans group streptococci, *Neisseria gonorrhoeae,* and *Neisseria meningitidis* (92). *S. pneumoniae* contains six PBPs (1a, 1b, 2a, 2b, 2x, and 3), all of which are subject to recombination with foreign PBPs taken up by transformation. In most cases, resistant *pbp*

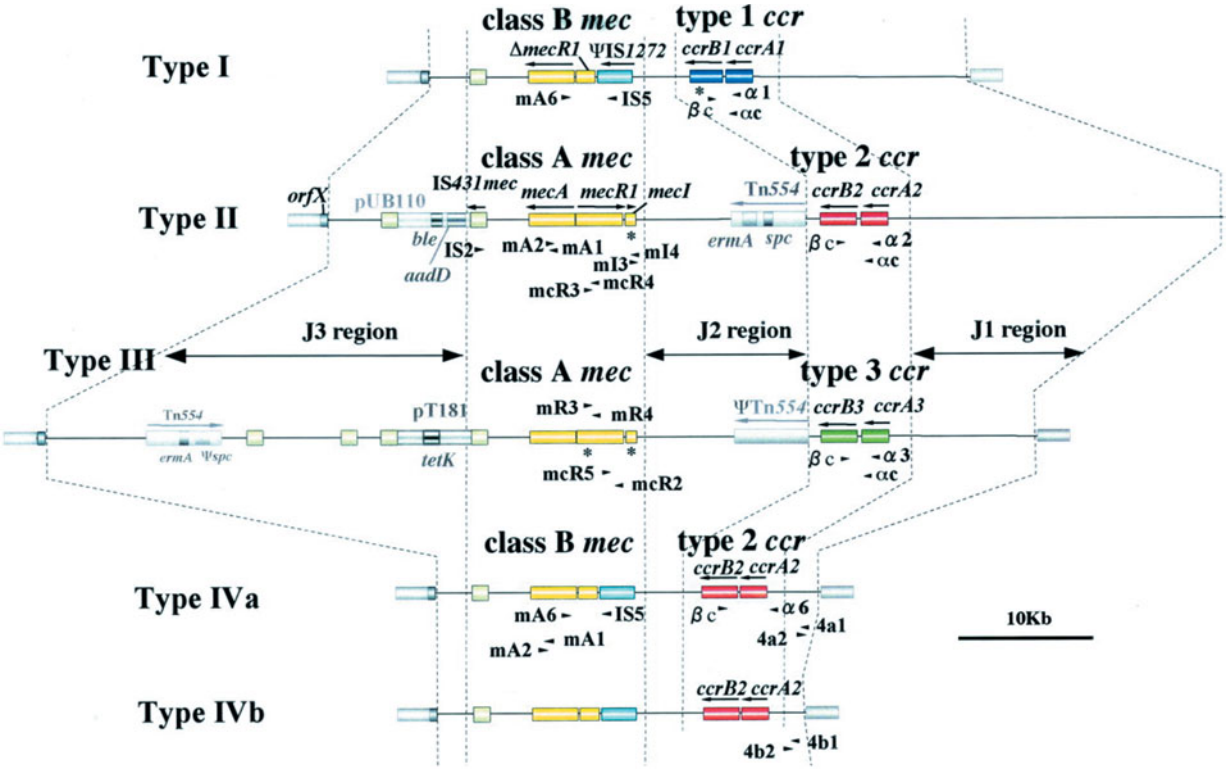


FIGURE 4 Four (of five currently described) SCC*mec* types. Types I, II, and III are found predominantly in health care-associated MRSA, whereas type IV is commonly found in the more susceptible community-associated MRSA around the world. The individual classes of *mec* regions are indicated, as are the variants of the *ccrA* and *ccrB* genes that confer mobility. IS elements and integrated transposons are also indicated. J, or junkyard, regions indicate areas that are not components of the basic SCC*mec*. Antimicrobial resistance genes found within the integrated transposons or plasmids are indicated below the lines, whereas the names of the integrated transposons or plasmids are indicated above the lines. Type V SCC*mec*, recently described, is slightly larger than the type IV elements, reflecting the inclusion of three genes of a putative restriction-modification system. Reprinted from reference 173.

genes demonstrate mosaic patterns (individual segments of foreign *pbp* genes integrated with the native *pbp* gene) with the foreign DNA from the less-penicillin-susceptible viridans group streptococci (92). In fact, genetic exchange appears to be common among these closely related species, with mosaic patterns demonstrable even in PBPs from susceptible *Streptococcus mitis* strains (7). Penicillin resistance in *S. pneumoniae* can be established by alterations in PBP2x, PBP2b, or PBP1a, whereas alterations in PBP2x or PBP1a are required for cephalosporin resistance (17). Penicillin resistance in *S. pneumoniae* has been reported for some time, with cephalosporin resistance emerging more recently, most commonly in strains already resistant to penicillin. However, strains resistant to cephalosporins but susceptible to penicillin have also been reported (234). High-level resistance (≥2 μg/ml) usually implies modification of more than one PBP, sometimes with several mosaic regions in each one (17).

Like that of PBP2a-mediated resistance to methicillin in *S. aureus,* expression of penicillin and cephalosporin resistance in *S. pneumoniae* is dependent on the proper functioning of auxiliary genes. The *fib* locus of *S. pneumoniae (fibA* and *fibB)* is analogous to *femA* and *femB* in *S. aureus*. It is involved in the formation of interpeptide bridges, and inactivation of the *fib* locus is associated with reduction of cross-linked muropeptides and loss of penicillin resistance even in the presence of low-affinity mosaic PBPs (265). The *murM-murN* operon encodes enzymes involved in the biosynthesis of branched, structured cell wall muropeptides commonly found in penicillin-resistant pneumococci (74). Inactivation of *murM-murN* results in the loss of branched-chain muropeptides as well as the loss of penicillin resistance.

Among gram-negative species, *Neisseria* species are well known to be naturally transformable. It is therefore not entirely surprising that strains of both *N. gonorrhoeae* and *N. meningitidis* have been described in which mosaic PBP genes are associated with decreased susceptibility to penicillin (92). Similar to those in *S. pneumoniae*, the resistant portions of the PBP genes have been acquired from closely related commensal *Neisseria* species that are more resistant to penicillin (239). Although penicillin resistance in *N. meningitidis* remains extremely rare, both β-lactamase-mediated resistance and PBP-mediated resistance to β-lactams in *N. gonorrhoeae* are quite common.

Generation of Point Mutations

The final mechanism of PBP-mediated β-lactam resistance is the generation of point mutations within the *pbp* genes that result in lower affinity for the β-lactam in question. This form of mutational resistance is seen most commonly in PBP5 of *E. faecium* strains, raising MICs of penicillin from 4 to 16 μg/ml to as high as >1,000 μg/ml. Strains with high-level penicillin resistance now represent the majority of clinical *E. faecium* isolates in the United States (218). These mutations further reduce the affinities for cephalosporins and other β-lactams with lower affinities for the nonmutated version, leading to an increase in resistance that may have implications for the likelihood that antibiotic use will promote colonization with multiresistant *E. faecium* (57). Most of the mutations occur in the vicinity of one or more of several conserved boxes important for β-lactam binding (216). A systematic study of several common mutations of PBP5 has revealed that resistance increases with an increasing number of mutations, with some loci conferring higher levels of resistance than others (206). Among those in gram-negative species, point mutations of the *ftsI* gene of *Haemophilus influenzae* (encoding the transpeptidase domain[s] of PBP3a and/or PBP3b) have been associated with non-β-lactamase-mediated resistance to ampicillin and cephalosporins (255). Although mutations within PBP4 have also been noted in this species, it is not clear that these have a significant impact on β-lactam susceptibility.

β-Lactamase-Mediated Resistance

Classification of β-Lactamases

Two schemes are currently used to classify β-lactamases: the Ambler classification system and the Bush-Medeiros-Jacoby classification system (6, 33, 34). The Ambler classification separates β-lactamases into four distinct classes based on similarities in amino acid sequences. Classes A, C, and D are serine β-lactamases, whereas class B enzymes are metallo-β-lactamases that require zinc for activity (see above). The Bush-Medeiros-Jacoby scheme classifies β-lactamases according to functional similarities (substrate and inhibitor profiles). There are four categories and multiple subgroups in the Bush-Medeiros-Jacoby system (groups 1, 2, 2a, 2b, 2br, 2d, 2be, 2c, 2f, 3, and 3a, etc.) (34). A comparison of the two classification systems is summarized in Table 2. In the discussions that ensue, we will refer to both classification systems.

TABLE 2 ß-lactamase classification

Bush-Jacoby-Medeiros system	Major subgroups	Ambler system	Main attributes
Group 1 cephalosporinases		C (cephalosporinases)	Usually chromosomal; resistance to all ß-lactams except carbapenems; not inhibited by clavulanate
Group 2 penicillinases (clavulanic acid susceptible)	2a	A (serine ß-lactamases)	Staphylococcal penicillinases
	2b	A	Broad-spectrum: TEM-1, TEM-2, SHV-1
	2be	A	Extended-spectrum: TEM and SHV variants, predominantly
	2br	A	Inhibitor-resistant TEM
	2c	A	Carbenicillin hydrolyzing
	2e	A	Cephalosporinases inhibited by clavulanate
	2f	A	Carbapenemases inhibited by clavulanate
	2d	D (oxacillin hydrolyzing)	Oxacillin-hydrolyzing (OXA)
Group 3 metallo-β-lactamase	3a	B (metalloenzymes)	Zinc-dependent carbapenemases
	3b	B	
	3c	B	
Group 4		Not classified	Miscellaneous enzymes, most not yet sequenced

At the time of this writing, 533 β-lactamases have been reported (Karen Bush, personal communication). These include 64 group 1 chromosomal cephalosporinases and 415 group 2 β-lactamases (these are penicillinases that are usually inhibited by clavulanic acid). The group 2be β-lactamases consist of 213 extended-spectrum β-lactamases (ESBLs). As will be discussed below, ESBLs in gram-negative bacteria confer resistance to ceftazidime and other extended-spectrum cephalosporins and are susceptible to inhibition by clavulanic acid. Twenty-nine inhibitor-resistant β-lactamases (resistant to clavulanic acid inactivation; group 2br) have been found, and 12 carbapenemases have been described previously. There are 45 group 3 enzymes (metallo-β-lactamases), and fewer than a dozen β-lactamases that are still unclassified (group 4).

β-Lactamase Mechanism

β-Lactamases are members of a superfamily of active-site serine proteases (147). The mechanism of hydrolysis of β-lactams by β-lactamases has been best studied with TEM-1. TEM-1 β-lactamase disrupts the amide bond of a β-lactam in a two-step reaction. First, the negatively charged carboxylate group of the β-lactam antibiotic is attracted to the active site by the enzyme's positively charged residues. There, the β-lactam is properly positioned, participating in key hydrogen bonding interactions with the enzyme (122). Next, the β-lactam is acylated (Fig. 1). A conserved serine, Ser70, in the active site of TEM-1 serves as the reactive nucleophile in this acylation reaction. Recent ultrahigh-resolution X-ray crystallography studies of TEM-1 (0.85 Å) indicate that Glu166, acting through the catalytic water, activates Ser70 for nucleophilic attack of the β-lactam ring. Then, a strategically positioned water molecule is activated by a general base (e.g., again Glu166). This water molecule deacylates the β-lactam and regenerates the active β-lactamase. This symmetric mechanism is also supported by the ultrahigh-resolution structure (0.90 Å) of another common class A β-lactamase, SHV-2, found in *K. pneumoniae* (170).

β-Lactamase Processing

Generally speaking, β-lactamases are secreted into the periplasmic space in gram-negative bacteria and into the surrounding medium by gram-positive bacteria. Membrane-associated enzymes (in *Bacillus licheniformis, Bacillus cereus,* and *Bacteroides vulgatus*) have been reported rarely. β-Lactamases are synthesized as precursor proteins. As that in other bacteria, the export of proteins into the periplasmic space is mediated by an amino-terminal signal peptide. After transport, the signal peptide of the β-lactamase is cleaved by a processing enzyme, signal peptidase I.

Genetic Environment of β-Lactamases

β-Lactamases can be chromosome-, plasmid-, or transposon- expressed enzymes that are produced in a constitutive or inducible manner. An increasing number of β-lactamases have been found that are carried on integrons. Integrons are genetic elements of variable lengths that contain a 5′ conserved integrase gene (*int*), mobile antibiotic resistance genes (called cassettes), and an integration site for the gene cassette, *attI* (att, "attachment"). To date, five distinct integron classes have been found to be associated with cassettes that contain antibiotic resistance genes. Three main classes of integrons (classes 1, 2, and 3) have been identified in gram-negative bacteria. Integrons capture antibiotic resistance gene cassettes by using a site-specific recombination mechanism. In the class 1 integrons, the 3′ conserved segment includes two open reading frames of known function: *qacEΔ1,* a deletion derivative of the antiseptic resistance gene *qacE,* and *sul*I, a sulfonamide resistance gene (Fig. 5). As integrons carry multiple resistance determinants and can be readily mobilized, their impact on antibiotic resistance is significant. In the words of Hall and Collis, "integrons thus act both as natural cloning systems and as expression vectors" (93). The capture and spread of antibiotic resistance determinants by integrons underlie the rapid evolution of multiple-antibiotic resistance among diverse gram-negative clinical isolates (213). It should be noted that gene cassettes are mobile and can also exist in free circular form. However, these cassettes do not include all functions required for their mobility. Cassettes are formally part of the integron only when they are integrated at the integron-receptor site. The genes within the cassette do not have promoters.

Integrons are an important source of the spread of *bla* genes. Integrons carrying β-lactamases encompassing Ambler classes A, B, and D have been found in *Acinetobacter baumannii, P. aeruginosa,* and other species of gram-negative bacteria (271). The β-lactamases carried on integrons are VEB-1, VEB-2, GES-1, GES-2, IBI-1, CTX-M-2, CTX-M-9, PSE-1, and numerous OXA β-lactamases (163, 190, 191). OXA and metallo-β-lactamases that confer resistance to carbapenems (IMP-1 to IMP-4 and IMP-8 to IMP-12, VIM-1, VIM-2, and GIM-1) are also included among integron-carried β-lactamases (41, 271).

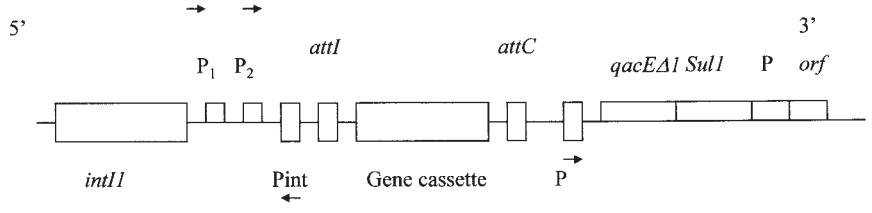


FIGURE 5 Simplified representation of a class 1 integron. P_1, promoter for the gene cassette; P_2, second promoter; Pint, promoter for the integrase; *attI,* integration site; *att*C, sequence in the gene cassette recognized by the integrase; *qacEΔ1,* partially deleted gene that encodes quaternary ammonium compound resistance; *Sul1*, gene for sulfonamide resistance; P, promoter; *orf,* open reading frame. As pictured, integrons contain components of a site-specific recombination system that recognizes and captures mobile gene cassettes. Gene cassettes can be antibiotic resistance genes followed by a repeat sequence called a 59-bp element (59be) or *att*C. Cassette-free integrons exist in which the *int* and *sul* regions are contiguous.

Class A β-Lactamases (Bush Group 2b Penicillinases)

Class A β-lactamases possess four important structural motifs that create a complex hydrogen bonding network to fix the β-lactam in the substrate binding pocket. Residues Ser70-Xaa-Xaa-Lys73, Ser130-Asp131-Asn132 (SDN loop), and Lys/Arg234-Thr/Ser235-Gly236 define the conserved residues critical for β-lactam binding and hydrolysis (122). The Ω loop (amino acids Arg164 to Asn179) is unique in class A β-lactamases. A highly conserved Glu166 residue that functions as a general base (electron donor) in the catalytic process is located in the Ω loop. The salt bridge formed between Arg164 and Asn179 defines the limits, or neck, of the Ω loop.

The two commonly encountered class A β-lactamases found in *Enterobacteriaceae* are designated TEM-1 and SHV-1. TEM-1 and SHV-1 β-lactamases are primarily penicillinases with diminished activity against cephalosporin substrates. These two families of β-lactamases have received considerable attention over the past two decades since they are the progenitors of the ESBLs and the inhibitor-resistant TEM β-lactamases now common in many hospitals.

Bush group 2be. ESBLs are generally class A β-lactamases that have expanded or changed substrate profiles as a result of amino acid substitutions. Normally, extended-spectrum cephalosporins are very poor substrates for hydrolysis by Bush group 2b enzymes (high K_ms and low k_{cat}s). Extended-spectrum cephalosporins are extremely potent β-lactams. Mutations at critical amino acids expand the spectra of these enzymes and allow the hydrolysis of extended-spectrum cephalosporins (185). In most cases, ESBL mutations render the enzymes more susceptible to inhibition by mechanism-based inactivators (clavulanic acid, sulbactam, and tazobactam). It is unclear why this increased susceptibility occurs. The impact of enteric gram-negative rods possessing ESBLs on the choice of empiric and definitive antimicrobial therapy has been substantial (123, 135, 178, 180, 181).

Among the TEM family enzymes, five amino acid residues appear to be most important for conferring the ESBL phenotype: Gly238 and Ala237 (located on the b3 β-pleated sheet), Arg164 and Asp179 (located on the neck of the Ω loop), and Asp104 (located directly across from G238 and A237 at the opening of the active-site cavity (122, 200). Of note, the replacement of Gly to Ser, Ala, or Asp at the Ambler position 238 is a common mutation in both TEM and SHV ESBLs (http://www.lahey.org/studies/webt.html).

Non-TEM, non-SHV ESBLs. Among the non-TEM, non-SHV ESBLs, the CTX-M β-lactamases are the most prevalent. They can be divided into distinct clusters (see http://www.lahey.org/studies/webt.html). Unlike most (but not all) TEM- and SHV-derived ESBLs, CTX-M β-lactamases hydrolyze cefotaxime and cetriaxone better than they do ceftazidime. It appears that CTX-M enzymes are more readily inhibited by tazobactam than they are by clavulanic acid. The first CTX-M type β-lactamase (MEN-1) was described nearly a decade ago. There are now nearly 60 members of this family.

CTX-M β-lactamases are commonly found in *K. pneumoniae, E. coli,* typhoidal and nontyphoidal *Salmonella, Shigella* spp., *Citrobacter freundii, Enterobacter* spp., and *Serratia marcescens* (121, 126, 179). The chromosome-carried β-lactamase of *Kluyvera ascorbata* is a probable progenitor of some plasmid-carried CTX-M enzymes (121). Other CTX-M β-lactamases may derive from *Kluyvera georgiana* (174). A recent comprehensive review delineates the lineage of many CTX-M enzymes resulting from *Kluyvera* spp. (25). Of note, different genetic elements may be involved in the mobilization of bla_{CTX-M} genes.

Many other clinically important non-TEM, non-SHV ESBLs have been described previously (K1, GES-1, PER-1, PER-2, VEB-1, BES-1, IBI-1, IBI-2, and OXA type) (28, 29, 193).

Structural biology of ESBLs. Important insights have emerged from the study of a number of atomic structures of class A ESBL enzymes. The common theme that emerges is that the active site is selectively remodeled and expanded to accommodate the bulky R1 side chain of extended-spectrum cephalosporin. Although the details of this modification are different for many of the ESBLs, the remodeling comes at a price. Many of these ESBLs are not as catalytically efficient as are the wild-type progenitors against certain substrates. With the expanded substrate spectrum, one uniformly observes decreases in MICs of penicillin and in k_{cat}/K_m ratios. In addition, these enzymes are less stable under proteolysis and heat. The structures of Toho-1, TEM-52, TEM-64, the Gly238Ala ESBL in TEM, SHV-2, and K1 β-lactamases all reveal insights into why expanded-spectrum cephalosporins fit into the active site (45, 108, 109, 170, 175, 229, 251, 264).

Serine carbapenemases of Bush group 2f or class A type. In the past, β-lactamases able to hydrolyze carbapenems were rare. It is regrettable that this is no longer the case. Representatives of these carbapenemases are Sme-1, Sme-2, NMC-A, IMI-1, GES-2, and KPC-1, KPC-2, and KPC-3 (191, 281). Class A carbapenemases hydrolyze imipenem but are not resistant to clavulanic acid inhibition. bla_{NMC-A} and bla_{IMI-1} are chromosomally located genes in *E. cloacae* and are induced by cefoxitin and imipenem (168, 201). bla_{NMC-A} is regulated by a LysR-type regulatory protein (160). It is felt that bla_{IMI-1} is regulated in the same manner (201). bla_{Sme-1} is a chromosomally located gene encoding serine carbapenemase of class A in *E. cloacae* and *Serratia marcescens.*

A notable increase in bacteria expressing class A carbapenamases has occurred in the United States. Numerous studies are revealing that KPC ("*Klebsiella pneumonia* carbapenemase") β-lactamases are becoming endemic in East Coast cities. First found in *K. pneumoniae,* these β-lactamases have been detected in *Salmonella enterica* serotype Cubana, *Klebsiella oxytoca,* and *Enterobacter* spp. strain MS 412 (106, 153, 281, 282). bla_{KPC-1} is located on a nonconjugative plasmid (281), and bla_{KPC-2} in *Salmonella* and *Klebsiella oxytoca* was isolated from conjugative plasmids (153, 282). The plasmid carrying bla_{KPC-3} from New York was also transferable by conjugation (278).

Detection of KPC β-lactamases may be a problem for clinical laboratories because of the positive ESBL confirmation tests (clavulanate-potentiated activities of ceftriaxone, ceftazidime, cefepime, and aztreonam). To improve detection of KPC-expressing *K. pneumoniae*, careful inoculum preparation for broth-based susceptibility methods must be carried out to avoid underinoculation. Investigators in this area have found that resistant isolates are readily detected by agar-based methods but not by broth methods (31). Bratu et al. have also advised that using ertapenem or meropenem will improve detection (31).

Among these serine carbapenemases exists a notable curiosity. bla_{GES-2} is a plasmid-carried β-lactamase gene

found in *P. aeruginosa* (194). bla_{GES-2} is a point mutation mutant of the clavulanic acid-inhibited ESBL gene bla_{GES-1} (a non-TEM, non-SHV ESBL gene). It is curious that GES-1 is an ESBL and that a single point mutation (Gly → Asp at position 170 in the Ω loop) can add an imipenemase activity to GES-2. The OXA carbapenemases will be discussed subsequently.

Inhibitor-resistant class A β-lactamases: Bush group 2br. Amino acid mutations within TEM and SHV that confer resistance to inhibition by β-lactamase inhibitors have also been characterized at the following amino acid positions: Met69, Ser130, Arg244, Arg275, and Asn276 (26, 56, 68, 196, 220, 242, 247, 257, 260). Most inhibitor-resistant β-lactamases are variants of the TEM-1 enzyme, with only two descriptions of clinical isolates expressing SHV β-lactamases that are resistant to inhibitors (SHV-10 and SHV-49) known at the time of this writing (59, 196). It is possible that the frequency of inhibitor-resistant β-lactamases in TEM and SHV family enzymes is underestimated, since many laboratories in the United States do not identify the corresponding strains routinely (120). Cephalosporins or high-dose piperacillin-tazobactam may be effective for the treatment of infection with *E. coli* strains expressing some inhibitor-resistant β-lactamases.

Mutants of TEM β-lactamases are being recovered that maintain the ESBL phenotype but also demonstrate inhibitor resistance. These are referred to as complex mutants of TEM (CMTs). There are now four identified CMTs (73, 164, 192, 232). It is still unknown how these enzymes will impact empirical treatment. At this time, clinical microbiology laboratories do not have the resources to detect such complex phenotypes.

Class B β-Lactamases (Bush Group 3 Enzymes)

In contrast to the serine-dependent β-lactamases (those of classes A, C, and D), class B β-lactamases are metalloenzymes. These enzymes contain an αββα motif with a central β sandwich and two α helices on each side. As such, class B β-lactamases require zinc or another heavy metal for catalysis and their activities are inhibited by chelating agents (EDTA). The zinc atom is held in place by three histidines and a water molecule. Some metallo-β-lactamases contain a second Zn binding site. These two sites function separately, with the primary Zn binding site assisted by the secondary site. A coordinated water molecule also serves a critical role in catalysis (263).

With few exceptions (see below), class B β-lactamases confer resistance to a wide range of β-lactam compounds, including cephamycins and carbapenems. The class B β-lactamases are resistant to inactivation by clavulanate, sulbactam, and tazobactam. Aztreonam, a monobactam, may act as an inhibitor, but there are metallo-β-lactamases that hydrolyze aztreonam. Of note, isolates expressing VIM-2 can be susceptible to aztreonam (189).

Class B β-lactamases can be grouped into three different subclasses (B1, B2, and B3). Amino acid positions can be assigned according to a specific numbering system, designated BBL, that is based upon structural alignment (84). Although the genes encoding metallo-β-lactamases show very little primary structure sequence identity (17 to 37%), the three-dimensional structures of the known metallo-β-lactamases appear similar (36, 48, 238, 256).

Because of the metal ion, the catalytic pathway for metallo-β-lactamases does not involve an acyl-enzyme intermediate as it does for class A and C β-lactamases. The catalytic pathway for class B also incorporates a hydrolytic water molecule (the bridging water molecule) that possesses enhanced nucleophilicity due to the proximity to the metal ion. The zinc ions coordinate two water molecules. The addition of the hydroxide to the carbonyl carbon of the β-lactam leads to the formation of a transient, noncovalent reaction intermediate.

The majority of metallo-β-lactamases are chromosomally encoded, and their expression may be constitutive or inducible. The metallo-β-lactamases of *Bacillus cereus, Stenotrophomonas maltophilia, Aeromonas hydrophilia,* and *Aeromonas jandaei* are inducible. In *Aeromonas jandaei,* regulation of the metallo-β-lactamase appears to involve two-component-signal transduction systems (4).

The metallo-β-lactamases of the VIM and IMP types are becoming increasingly important as threats to our antimicrobial armamentarium. These metallo-β-lactamases are broad-spectrum enzymes, are active against most β-lactams, including carbapenems, and have been found in various gram-negative clinical isolates mostly in the Far East and the Mediterranean regions. bla_{VIM} is an integron-borne metallo-β-lactamase gene that is usually found in *P. aeruginosa* isolates. bla_{VIM-2} has now spread to more than 20 countries. The majority of metallo-β-lactamase genes are mobilized on integrons, transposons, and mobile common regions (263). At the time of this writing, there are 13 VIM and 18 IMP types identified. Like VIM metallo-β-lactamase genes, IMP metallo-β-lactamase genes are widespread. IMP metallo-β-lactamase genes have been found as parts of integrons in the following bacteria: *P. aeruginosa, Pseudomonas putida, Serratia marcescens, Pseudomonas stutzeri, Acinetobacter baumannii, Pseudomonas fluorescens, K. pneumoniae, Klebsiella oxytoca, Enterobacter aerogenes, Achromobacter xylosoxidans,* and *E. coli* (263).

As stated above, the atomic structures of a number of class B β-lactamases have been solved (36, 48, 84, 85). The structures are being used to design novel inhibitors of class B β-lactamases.

Class C β-Lactamases

Ambler class C (Bush-Jacoby-Medeiros group 1) chromosomally encoded β-lactamases are produced to a greater or lesser degree by almost all gram-negative bacteria (*Salmonella* and *Klebsiella* being the only known exceptions). Chromosomally encoded (and inducible) enzymes are particularly important in clinical isolates of *Citrobacter freundii, Enterobacter aerogenes, E. cloacae, Morganella morganii, P. aeruginosa,* and *Serratia marcescens.* Although class C β-lactamases hydrolyze cephalosporins (including extended-spectrum cephalosporins) more effectively than they do penicillins, it should be kept in mind that these enzymes have great efficiencies for the hydrolysis of penicillins (the K_ms are very low). Most class C enzymes are resistant to inhibition by clavulanate, sulbactam, and tazobactam.

Class C β-lactamases have larger active-site cavities than class A enzymes, which may allow them to bind the bulky extended-spectrum cephalosporins (oxyimino-β-lactams) (51, 136). It is claimed that this conformational expansion and flexibility facilitate hydrolysis of oxyimino-β-lactams by making the acyl-enzyme intermediate more open to attack by water (51, 136, 151).

The important structural elements described for class A enzymes are also present in class C β-lactamases. The active-site serine (Ser64) is located near the N terminus of a long helix and is followed on the next helix turn by a lysine (Ser64-Xaa-Xaa-Lys67). The second element contains a Tyr-Xaa-Asn (Tyr150) or Ser-Xaa-Asn pattern corresponding to the Ser130-Asp131-Asn132 loop of class A β-lactamases.

The opposite side of the active site is marked by Lys315/Arg/His-Thr/Ser-Gly corresponding to the KTG motif of class A enzymes.

Class C cephalosporinases acylate β-lactams in the same manner that class A enzymes do. First, the catalytic Ser64 attacks the β-lactam carbonyl carbon and the acyl-enzyme forms. The approaches of the activated water molecule are different between AmpC and class A enzymes (for class C, the approach is from the β-face). This difference implies that the deacylation mechanism is distinct. Unlike that of class A enzymes, the ring amine of the acyl-enzyme of class C facilitates deacylation. This substrate-assisted catalysis distinguishes class C from class A. In examination of the catalytic mechanism, a Glu166 equivalent in class C β-lactamases is not readily apparent; this role may be filled by the tyrosine (Tyr150) in the Tyr-Xaa-Asn motif.

The first class C β-lactamase structure determined was that of the AmpC cephalosporinase of *Citrobacter freundii* by Oefner et al. (171). Determination of the structures of the P99 β-lactamase of *E. cloacae,* AmpC β-lactamase from *E. coli,* and *E. cloacae* GC1 and *E. cloacae* 908R β-lactamases has ensued (51, 137; http://www.rcsb.org). The GC1 β-lactamase of *E. cloacae* has improved hydrolytic activity for oxyimino-β-lactam antibiotics because of a tripeptide insertion in the Ω loop (tandem repeat Ala211-Val212-Arg213). In a strict sense this is a class C ESBL. As a result of this addition, the opening of the active-site binding cavity is wider and the substrate spectrum has expanded (51).

In the clinically important class C enzyme-producing gram-negative rods, β-lactamase production is normally repressed. The details of the repression have been mostly elucidated for *Enterobacter* spp. (111). Repression and activation are closely linked to the processes of cell wall synthesis and breakdown. The molecule that serves as both the repressor and the activator of *amp*C transcription is AmpR, a transcriptional regulator of the LysR family. AmpR is present as a repressor by virtue of its interaction with UDP-MurNac-pentapeptide, a peptidoglycan precursor molecule. In this form AmpR is incapable of activating *amp*C and in fact serves as a repressor of *ampR* expression. In the setting of high concentrations of cell wall breakdown product anhydro-MurNAc-tripeptide (or anhydro-MurNac-pentapeptide), however, UDP-MurNAc-pentapeptide is displaced from its site in AmpR, resulting in the conversion of AmpR into an activator of *amp*C transcription.

Increases in *amp*C expression may result from the action of β-lactam antibiotics, certain of which cause the release of significant quantities of anhydro-MurNAc-tripeptide and/or -pentapeptide from the peptidoglycan. This anhydro-UDP-MurNAc-tripeptide enters the cell through a channel (AmpG) and overwhelms the recycling ability of the cytosolic amidase (AmpD) specific for recycling of muropeptides. Under these circumstances (induction), β-lactamase is produced only as long as the antibiotic is present in the medium.

Constitutive high-level production of AmpC β-lactamase most commonly results from a mutation in the *ampD* gene, reducing the quantity of (or eliminating) AmpD from the cytoplasm. Under these circumstances, a constant high level of anhydro-MurNAc-tripeptide is present in the cytoplasm, and AmpR serves as a constitutive activator of *amp*C transcription. Constitutive production can also result from deletion of *ampR,* but in this circumstance β-lactamase production is generally at a low level.

The widespread dissemination of *amp*C-type β-lactamase genes on transferable plasmids is a continuing challenge. These plasmid-carried AmpC cephalosporinases are separated into four general groups. Group 1 plasmid-carried AmpC cephalosporinases consist of those that originated from the chromosomally encoded AmpC of *Citrobacter freundii* (BIL-1, CMY-2, LAT-1, and LAT-2). Members of group 2 are related to the chromosomally encoded cephalosporinase of *E. cloacae* (MIR-1 and ACT-1); group 3 β-lactamases belong to the AmpC group of *P. aeruginosa* (CMY-1, FOX-1, and MOX-1), and group 4 enzymes belong to the CMY-1 β-lactamases (CMY-1 cluster). Plasmid-mediated class C β-lactamases have been identified in many gram-negative bacteria from all parts of the world. Host strains harboring these enzymes include *K. pneumoniae, Enterobacter aerogenes, S. enterica* serotypes Enteritidis and Senftenberg, *E. coli, Proteus mirabilis, Morganella morganii,* and *Klebsiella oxytoca*. The loss of porin proteins in clinical isolates with plasmid-carried AmpC enzymes may result in resistance to carbapenems (30, 240).

Class D β-Lactamases

The OXA-type (oxacillin-hydrolyzing) β-lactamases have been most commonly identified in *Enterobacteriaceae, Acinetobacter* spp., and *P. aeruginosa* (161). OXA enzymes confer resistance to a wide variety of penicillins. They are only weakly inhibited by clavulanic acid but are inhibited by sodium chloride (NaCl). Overall, the amino acid identities between class D and class A or class C β-lactamases are less than 20%. The frequent location of class D genes on mobile genetic elements (plasmids or integrons) facilitates spread (162, 190, 261).

Several OXA β-lactamases (OXA-11 and OXA-14 to OXA-20) are associated with an ESBL phenotype. These OXA types have been found exclusively in *P. aeruginosa*. A comparison of the crystal structure of the OXA-10 β-lactamase with that of the class C enzyme from *E. cloacae* P99 and that of the class A TEM-1 enzyme from *E. coli* shows that the class D and class A enzymes share a common fold pattern (α helices and β-pleated sheets), although the distributions of secondary-structure elements are different. A remarkable feature is the nearly perfect symmetry of all atoms that constitute the catalytic machinery for acylation (Ser67, Lys70, Ser115, and Lys205 in OXA-10 and Ser70, Lys73, Ser130, and Lys234 in TEM-1). There seems to be an extension of the substrate binding site (tripeptide strand) in OXA-10. The role of the peptide extension is not known. The oxyanion hole is provided by the main-chain nitrogen atoms of Ser67 and Phe208. In class D the same residue (Lys70) is involved in acylation and deacylation (149, 176).

OXA enzymes are assuming greater importance due to the ability of members of this class to hydrolyze carbapenems. The first description of a serine carbapenemase in *Acinetebacter baumannii* was that of ARI-1 (OXA-23) in 1985 (182). Although OXA carbapenemases hydrolyze imipenem inefficiently, their presence in an organism with an active efflux pump or a porin mutation may confer clinically significant levels of resistance (101). It is notable that *Acinetobacter baumannii* isolates possess a chromosomally encoded oxacillinase, OXA-69, that confers very low level imipenem resistance. The OXA-69 gene is ubiquitous in *Acinetobacter baumannii* and is referred to as a housekeeping gene (100).

Resistance to Chloramphenicol

Acetyltransferases

Chloramphenicol is a broad-spectrum antimicrobial agent whose use has waned in recent years due to well-characterized hematologic toxicity and a wealth of less toxic therapeutic options. The most common mechanism of resistance to

chloramphenicol is the elaboration of CATs. A large number of CAT genes have been reported, and these determinants generally confer extremely high levels of resistance on the organisms expressing them. Substantial structural similarities exist among the different CAT variants, although their nucleotide sequences may be quite divergent (159). Relationships among the different CATs have been described in detail in a recent review by Schwarz and colleagues (222). Chloramphenicol contains two hydroxyl groups that are acetylated in a reaction catalyzed by CAT in which acetyl coenzyme A serves as the acyl donor. Initial acetylation occurs at the C-3 hydroxyl group to give 3-acetoxy-chloramphenicol (226). Following nonenzymatic rearrangement into 1-acetoxy-chloramphenicol and reacetylation, the 1,3-diacetoxy-chloramphenicol product is formed. Neither the mono- nor the diacetoxy derivatives are able to bind to the 50S ribosomal subunit and inhibit prokaryotic peptidyltransferase (226).

CATs are generally divided into two types: type A (classical) CATs and type B (xenobiotic) CATs (222). Five structurally similar type A CATs (A, B, C, D, and that expressed by the prototypic plasmid pC194) in *S. aureus* have been described previously (77). The *cat* genes encoding these enzymes are commonly located on small, multicopy plasmids, and expression is inducible through a translational attenuation mechanism (222). *E. faecalis* and *S. pneumoniae* also express inducible CAT genes that are similar to the type D gene of *S. aureus*. Two CAT genes encoding constitutive CAT expression in *Clostridium perfringens* have been described previously. *catP* is generally found within transposon Tn*4451*, whereas *catQ* (nearly identical to *catD* of *Clostridium difficile*) is chromosomal (222).

Three types of type A CATs (I, II, and III) have been identified in gram-negative bacteria. The widely prevalent type I enzymes are distinguished by their ability to bind and inhibit (without acetylation) the activity of fusidic acid (19). These enzymes are frequently found associated with transposon Tn9 or related elements. Type II CATs are notable for their sensitivity to inhibition by thiol-reactive agents and for their association with *H. influenzae* (158). Most knowledge of the structural features of the type A CAT enzymes comes from the study of the type III enzyme, for which the tertiary structure is known at high resolution (159). The structural determinants of binding for each substrate are also known for this enzyme.

Type B (xenobiotic) acetyltransferases (159) are structurally unrelated to classic CATs, and those that have been demonstrated to acetylate chloramphenicol confer only low levels of chloramphenicol resistance even when the genes are present in high copy numbers. Their natural substrate is likely something other than chloramphenicol, explaining their limited ability to acetylate this antibiotic. First described in *Agrobacter tumefaciens,* they have now been identified in a wide range of species (222). Included among this class of agents are the virginiamycin acetyltransferases found in *S. aureus* and *E. faecium* (see "Resistance to Macrolides" below). In fact, although they are members of this class, the *vat* gene products do not confer resistance to chloramphenicol nor have they been demonstrated to be able to acetylate chloramphenicol in vitro (159). They are, however, quite adept at acetylating streptogramins. The crystal structures of two trimeric type B CATs have been determined previously (18, 198).

Decreased Accumulation of Chloramphenicol

It is now well recognized that chloramphenicol serves as a substrate for many of the multidrug-resistance efflux pumps that exist in gram-positive and gram-negative bacteria, including those found in *E. coli, P. aeruginosa, Bacillus subtilis,* and *S. aureus* (165). In addition, there are efflux systems that are specific for chloramphenicol. The first chloramphenicol-specific efflux gene to be described was *cmlA* within the In*4* integron of Tn*1696* (21). *cmlA* encodes an efflux mechanism that uses chloramphenicol but not florfenicol (a chloramphenicol derivative licensed for use in animals in 1996 in the United States for the treatment of bovine respiratory pathogens) as a substrate. Gram-negative bacteria also express efflux genes specific for both chloramphenicol and florfenicol (flo_{Pp} and flo_{St}). These resistance genes are being reported with increasing frequency from animal-derived *E. coli* and *Salmonella* isolates (24, 272). In fact, the chloramphenicol resistance expressed by multiresistant *S. enterica* serotype Typhimurium DT104 is most commonly encoded by flo_{St} (24), emphasizing again the potential negative impact of using similar antimicrobial agents in humans and animals.

Resistance to Glycopeptides

Glycopeptide antibiotics (vancomycin and teicoplanin) inhibit cell wall synthesis by binding to the pentapeptide peptidoglycan precursor molecule as it exits the cytoplasmic membrane. This binding prevents the cross-linking (transpeptidation) of peptidoglycan precursors necessary for the formation of normal, stable cell walls. The large size of the glycopeptide molecules also appears to inhibit the other major peptidoglycan linkage reaction (transglycosylation) by steric hindrance. The specific moiety bound by vancomycin is the terminal D-alanyl-D-alanine of the pentapeptide. The vast majority of bacteria that have been studied have peptidoglycan precursors that are pentapeptides terminating in D-Ala-D-Ala and therefore are theoretically susceptible to vancomycin. However, the large size of the vancomycin molecule exceeds the exclusion limits of the porins in gram-negative-bacterium outer membranes, so vancomycin cannot access the target in these species. Hence, vancomycin is active only against bacteria lacking outer membranes, which are predominantly gram positive.

Acquired resistance to vancomycin in gram-positive bacteria comes in three varieties largely defined by the species in association with which they have been described: (i) altered precursor formation in enterococci, (ii) mutational cell wall changes in staphylococci, and (iii) tolerance in pneumococci. The importance of the first type of resistance is characterized more by its prevalence than by the importance of the species as causes of infection, whereas the other two types of resistance are defined more by the importance of the species involved than by their prevalence.

To date, six varieties of enterococcal glycopeptide resistance have been described (corresponding to VanA through VanE and VanG). Of these, the most clinically important are VanA and VanB (12). VanA and VanB are encoded by similar operons in which three genes (*vanH, vanA,* and *vanX* or $vanH_B$, *vanB,* and $vanX_B$) are required for the expression of resistance (12). Two other genes (*vanY* and *vanZ* or $vanY_B$ and *vanW*) serve to amplify resistance but are not required for its expression (8, 9), and two more genes (*vanS* and *vanR* or $vanS_B$ and $vanR_B$) regulate the transcription of the three essential genes (10, 66). The ultimate purpose of these genes is to alter the structure of the pentapeptide precursor from terminating in D-alanyl-D-alanine to terminating in D-alanine-D-lactate and in so doing to reduce the binding affinity of vancomycin for its target (roughly 1,000 fold). Since the terminal amino acid is cleaved off of the pentapeptide in the

transpeptidation reaction, the final composition of the cell wall is indistinguishable from that in strains lacking the resistance determinant.

VanA enterococci are phenotypically resistant to vancomycin and teicoplanin, whereas VanB strains are resistant to vancomycin but appear to be susceptible to teicoplanin. This susceptibility results from the fact that teicoplanin does not induce expression of resistance (66). Once the VanB operon is expressed, however, resistance to teicoplanin results. Consequently, teicoplanin has been disappointing as a therapy for infections caused by VanB enterococci, since mutations in the VanB regulatory apparatus resulting in either inducibility by teicoplanin or constitutive expression occur readily during therapy (13, 96, 117).

Both VanA and VanB operons are carried by transposons. The VanA operon is found exclusively within transposon Tn*1546*, a 10.4-kb Tn*3* family element that is presumed to disseminate among enterococci by integrating into conjugative plasmids (11). The genes of the VanA operon are highly conserved in their sequences among different strains, but the restriction maps of the operons and of Tn*1546* often differ markedly among clinical strains (53). These differences result from insertions of a variety of IS elements with or without subsequent deletions of parts of the mobile element and have been used by some investigators to establish lineages of strains within defined clinical settings. The VanB operon is most commonly carried on transposons designated Tn*5382* or Tn*1549* (37, 86). It is likely that Tn*5382* and Tn*1549* are identical. These transposons exhibit significant homology to prototype enterococcal conjugative transposon Tn*916*. In contrast to the *vanA* gene, *vanB* has three allelic variants (*vanB1*, *vanB2*, and *vanB3*). The *vanB2* gene is associated with Tn*5382*.

The overwhelming majority of clinical vancomycin-resistant strains are *E. faecium*, a predilection that remains unexplained (218). In the United States, the vast majority of *E. faecium* strains that are resistant to vancomycin also express high levels of resistance to ampicillin, thereby negating the two most effective and reliable antienterococcal agents. In some strains, this linkage is actually physical, with insertion of Tn*5382* immediately downstream of the *pbp5* gene resulting in high-level ampicillin resistance (37). These two determinants transfer between enterococcal chromosomes together through mechanisms that are yet to be determined.

The origins of the vancomycin resistance determinants are obscure, but it is clear from sequence comparisons that they did not develop within the enterococci (see above). Their entry into enterococci, markedly delayed considering that vancomycin was introduced into clinical use in 1958, was likely facilitated by the administration of high concentrations of glycopeptides to mammalian gastrointestinal tracts. In Europe, this exposure occurred largely in the gastrointestinal tracts of food animals that were fed the glycopeptide avoparcin as a growth promoter (266). Evidence is now quite convincing that the animal strains transferred to the European human population through the food chain (237). In the United States, where glycopeptides were never used in animal feeds, the exchange event likely occurred in the gastrointestinal tracts of hospital patients who were given vancomycin orally to treat real or presumed *Clostridium difficile*-associated diarrhea. The U.S. *E. faecium* strains were likely highly adapted to the hospital environment through acquisition of antibiotic resistance and virulence determinants. These differences in origin may explain why vancomycin-resistant infections in European hospitals are quite rare, despite sometimes heavy colonization of the community population, whereas vancomycin-resistant infections in the United States now constitute more than 25% of all enterococcal infections acquired in the hospital, despite no compelling evidence of colonization in the community at all. Emerging data indicate that many of the vancomycin-resistant strains isolated from outbreaks of clinical infection belong to a specific clonal group, suggesting that some vancomycin-resistant *E. faecium* strains are particularly likely to disseminate and cause clinical infections (275). Thankfully, the VanA and VanB operons have remained restricted to *E. faecalis* and *E. faecium*, with few exceptions. Despite in vitro transfer of the VanA determinant to *S. aureus* (167), and at least three instances in which VanA-expressing *S. aureus* isolates have been recovered from clinical samples (41a), vancomycin-resistant *S. aureus* remains exceedingly rare. In all cases, resistance has been conferred by the VanA operon and in one well-characterized case transfer appears to have been facilitated by the presence of Tn*1546* on a broad-host-range plasmid in *E. faecalis*, with transposition of Tn*1546* to a staphylococcal plasmid once entry into the staphylococcus occurred (79, 268).

The VanC operon is intrinsic to the cell wall synthesis machinery of minor enterococcal species *Enterococcus casseliflavus* (including the biotype formerly classified as *Enterococcus flavescens*) and *Enterococcus gallinarum* (60, 262). The peptidoglycan precursor in VanC strains terminates in D-alanine-D-serine, reducing vancomycin affinity about sevenfold and resulting in low levels of resistance. Precursors terminate in D-alanine-D-serine in strains of *E. faecalis* with VanE (76), whereas in VanD-expressing *E. faecium* it terminates in D-alanine-D-lactate. The failure to observe dissemination of VanD may be explained in part by the fact that the VanX equivalent enzyme in *E. faecium* BM4339 appears to be ineffective in an enterococcal background. Resistance is expressed in BM4339 because that strain lacks a functional cellular ligase *(ddl)* gene, eliminating the need for VanX activity to express resistance (40).

Mutational resistance to glycopeptides in *S. aureus* is a rare clinical entity that most commonly takes the form of reduced susceptibility rather than frank resistance. For resistant strains, alternately called vancomycin-intermediate *S. aureus* or GISA (glycopeptide-intermediate *S. aureus*), MICs of vancomycin are in the 4- to 8-μg/ml range (133). However, within these cultures are smaller populations of cells that express higher levels of resistance. Animal studies suggest that the level of resistance expressed by GISA strains will reduce the effectiveness of vancomycin therapy (47).

The exact mechanism of glycopeptide resistance in GISA strains remains incompletely understood (27). One consistent finding is a thickened cell wall with increased numbers of unlinked precursors and abnormal septum formation (52). Consistent with this phenotype is the fact that all clinically isolated GISA strains studied have virtually undetectable quantities of PBP4, a low-molecular-weight PBP associated with peptidoglycan cross-linking (75). Restoration of PBP4 on a high-copy-number plasmid restores cross-linking and vancomycin susceptibility to these strains. Preliminary work suggests that a range of genes may be involved in the expression of vancomycin resistance, that the phenotype may be due to different genetic mutations in different strains, and that vancomycin-resistant mutants may evolve from strains that are hypermutators (14). It has been suggested that vancomycin resistance results from the absorption of most of the vancomycin by the unlinked precursors (false vancomycin targets) before it can arrive at its true target, the peptidoglycan precursors attached to lipid II as they emerge from the

cytoplasmic membrane (104). Further work will be required before we understand the details of vancomycin resistance in these rare but troublesome strains.

Glycopeptide resistance in coagulase-negative species of staphylococci has also been reported. In contrast to that in *S. aureus,* resistance in *Staphylococcus haemolyticus* has been associated with changes in the compositions of the cross-links of the peptidoglycan (20). The mechanism by which this would lead to vancomycin resistance is unknown.

Vancomycin is, in general, a less bactericidal antibiotic than are the β-lactams. Evidence for the importance of this observation can be found in several studies. The bacteremia associated with *S. aureus* endocarditis, for example, takes roughly twice as much time to clear with vancomycin treatment as with treatment with β-lactam oxacillin (131). Recent clinical data also suggest that vancomycin treatment is associated with higher rates of failure and relapse than nafcillin treatment of bacteremia due to methicillin-susceptible *S. aureus* (44). Reports of vancomycin tolerance in *S. pneumoniae* first appeared in 1990 (253). *S. pneumoniae* is the most common cause of bacterial meningitis in most patient populations, and bactericidal therapy is optimal for treatment of this condition. At least one case of presumed recrudescence of meningitis after treatment of a case of vancomycin-tolerant pneumococcal meningitis has been reported (98). Tolerance appears to involve mutations within an operon *(vex123)* encoding an ABC transporter, but the mechanism by which this occurs remains undefined (90). Recent work suggests that tolerance may be more common in clinical pneumococcal isolates than was previously believed, that tolerance is associated with antibiotic resistance, and that vancomycin-tolerant strains are more likely to be tolerant to other classes of antimicrobial agents as well (212). Further work will be required before we understand the true importance of pneumoccocal tolerance for the treatment of clinical infections.

Resistance to Linezolid

The oxazolidinone antibiotic linezolid inhibits bacterial protein synthesis by interacting with the *N*-formylmethionyl-tRNA-ribosome-mRNA ternary complex commonly referred to as the initiation complex (230). Linezolid exerts excellent bacteriostatic activity against a wide range of gram-positive pathogens, including methicillin-resistant staphylococci and multiresistant enterococci. Clinical use of this agent has been associated with the emergence of resistant strains, most commonly after prolonged therapy of infections with difficult-to-eradicate bacteria. Resistance in both *E. faecium* and *S. aureus* has now been described, and there are suggestions that the rate of emergence of resistance in *E. faecium* may be significant. Analysis of linezolid-resistant enterococcal laboratory mutants suggests that resistance is associated with a G2576U (*E. coli* numbering scheme) point mutation in the 23S rRNA, although mutations at other positions may also contribute to resistance (197). A G2576U mutation has been described in resistant clinical isolates of *E. faecium* and in the analogous position of the 23S ribosomal subunit of a resistant *S. aureus* isolate (88, 252). Since the 23S subunit genes exist in multiple copies in different bacteria (four in *E. faecalis* and six in *E. faecium*), it has been suggested that more than one copy of the genes must be mutated to confer resistance. This in fact appears to be the case, with strains having a higher percentage of mutated 23S genes expressing greater levels of resistance (143). Multiple mutated gene copies do not arise independently, however. Once the first gene is mutated, subsequent copies can be mutated through recombination with the resistant gene (138). In this fashion, persistent selective pressure exerted by linezolid can lead to rapid development of high-level (MIC, >128 μg/ml) resistance. Despite this potential and the increasing use of linezolid in clinical settings, rates of resistance to this antimicrobial agent remain very low.

Resistance to Macrolides

Erythromycin (the first macrolide) was initially isolated from *Streptomyces erythreus,* a soil organism found in the Philippines. There are currently four macrolides in common use: erythromycin, clarithromycin, azithromycin, and roxithromycin. Macrolides inhibit protein synthesis in susceptible organisms by binding reversibly to the peptidyl-tRNA binding region of the 50S ribosomal subunit, inhibiting translocation of a newly synthesized peptidyl-tRNA molecule from the acceptor site on the ribosome to the peptidyl (or donor site). Erythromycin does not bind to mammalian ribosomes. Most gram-negative organisms are resistant to erythromycin because entry of erythromycin into the cell is restricted.

Resistance to macrolides occurs through several mechanisms. Among the more important of these mechanisms is methylation of the ribosome, preventing erythromycin binding (269). This methylation is most commonly accomplished by different *erm* ("erythromycin ribosomal methylase") genes. Methylated ribosomes confer resistance to macrolides, the related lincosamides (clindamycin and lincomycin), and streptogramin B (MLS_B resistance). Many *erm* genes have been described previously, such as *erm*(A) and the related *erm*(TR) and *erm*(B) and the related *erm*(AM), and resistance is frequently inducible by macrolides but not by clindamycin ($iMLS_B$). In some strains, *erm*-type resistance is expressed constitutively ($cMLS_B$), resulting in resistance to clindamycin as well.

The second major mechanism of resistance to macrolides is through expression of efflux pumps encoded by *mef* genes (Mef in gram-positive bacteria and AcrAB-TolC in *H. influenzae* and *E. coli*) (284). The efflux pumps confer resistance to the macrolides but not to clindamycin, hence the phenotypic description of this resistance type as M type. *mef* genes have been studied most extensively in *S. pneumoniae* [*mef*(E)] and *Streptococcus pyogenes* [*mef*(A)], but similar genes in a variety of gram-positive genera have been described. The prevalence of *mef*-mediated resistance versus that mediated by MLS_B-type mechanisms in *S. pneumoniae* varies in different parts of the world. Minor mechanisms of resistance to macrolides include esterases that hydrolyze the antibiotics and point mutations within the genes for the 50S ribosomal subunit.

Resistance to Ketolides

Ketolides belong to a new class of semisynthetic 14-membered-ring macrolides, which differ from erythromycin by having a 3-keto group instead of the neutral sugar L-cladinose. Ketolides bind to an additional site on the bacterial ribosome, increasing their binding affinities relative to those of other macrolides (58). Telithromycin, a ketolide, is uniformly and highly active against pneumococci (regardless of their susceptibility or resistance to erythromycin and/or penicillin), erythromycin-susceptible *Streptococcus pyogenes,* and erythromycin-resistant *Streptococcus pyogenes* strains of the M phenotype or the $iMLS_B$ or $cMLS_B$ phenotype [in which resistance is mediated by a methylase encoded by the *erm*(TR) gene] (16). Ketolides are less active against erythromycin-resistant *Streptococcus pyogenes* strains with the $cMLS_B$ phenotype or the $iMLS_A$ subtype [in which resistance is mediated by a methylase encoded by the *erm*(AM) gene],

these strains ranging in phenotypes from the upper limits of susceptibility to resistant. Methicillin-resistant staphylococci, which commonly express a $cMLS_B$ phenotype, are not susceptible to telithromycin (16).

Resistance to Quinupristin-Dalfopristin

Quinupristin-dalfopristin is a mixture of semisynthetic streptogramins A and B licensed in Europe and the United States. A related streptogramin A and B combination, virginiamycin, has been used for years as a growth promoter in animal feed. Resistance to these mixtures can result from resistance to streptogramin A alone, was first described in staphylococci, and was conferred by genes encoding streptogramin A acetyltransferases [*vat*(A), *vat*(B), and *vat*(C)] or ATP binding efflux genes [*vga*(A) and *vga*(B)]. Quinupristin-dalfopristin's excellent activities against *E. faecium* and MRSA make it an attractive alternative for the treatment of multiresistant *E. faecium* and health care-associated infections with methicillin-resistant staphylococci, especially since the combination retains in vitro activity against streptogramin B-resistant strains. Two acetyltransferase-encoding resistance genes have now been described that confer resistance to quinupristin-dalfopristin in *E. faecium* strains—*vat*(D) [previously *sat*(A)] and *vat*(E) [previously *sat*(G)]. In most cases, these resistance genes are found along with an *erm* resistance gene (236), suggesting that resistance to both streptogramin A and B may be necessary to confer clinically significant levels of resistance to quinupristin-dalfopristin in *E. faecium*. The presence of these resistance genes on transferable plasmids suggests that the potential for their spread within the genus is significant. Although quinupristin-dalfopristin remains active against the majority of human *E. faecium* strains, the use of virginiamycin in animal feeds has been associated with high percentages of resistance in isolates derived from animals (97). In many cases, the known mechanisms of resistance to quinupristin-dalfopristin are not present in these isolates (97), indicating that there is still much to be learned about resistance to quinupristin-dalfopristin in *E. faecium.*

Metronidazole Resistance

Metronidazole is a member of the nitroimidazole family of bactericidal antimicrobials. The 5-nitroimidazole molecule is a prodrug whose activation depends upon reduction of the nitro group in the absence of oxygen. An exception to this rule occurs in *Helicobacter pylori,* where the RdxA protein reduces metronidazole in a microaerophilic environment (259). The nitro group of metronidazole accepts a single electron from electron transport proteins (ferredoxins) in bacteria, yielding a toxic radical anion. Metronidazole's activity appears to result in DNA damage and cell death (62). Resistance to metronidazole is rare. Decreased uptake and/or a reduced rate of reduction is believed to be responsible for metronidazole resistance in some cases (61). Five *Bacteroides* genes, *nimA* to *nimE,* have been implicated in resistance to 5-metronidazole antibiotics. Analysis of the NimA-susceptible and -resistant *Bacteroides* strains and recent crystal structure analysis suggest that the enzyme utilizes pyruvate for a two-electron reaction resulting in an amine that prevents the formation of the toxic anion radical (38, 130). Expression of *nim* genes varies depending on the positioning of a variety of IS elements that supply active promoters (235).

Resistance to Nitrofurantoin

The antibiotics nitrofurazone and nitrofurantoin are used in the treatment of genitourinary infections and as topical antibacterial agents. Nitrofurazone is used primarily as a topical antiseptic (89). Nitrofurantoin, 1-[(5-nitrofurfurylidene)amino]hydantoin, is a synthetic antibacterial agent used primarily in the treatment of urinary tract infections. The mechanisms of action of nitrofurazone and nitrofurantoin have not been fully elucidated. Investigators have reported that the ability of nitrofurantoin to kill bacteria correlates with the presence of bacterial nitroreductases which convert nitrofurantoin into highly reactive electrophilic intermediates (150). These intermediates are believed to attack bacterial ribosomal proteins nonspecifically, causing complete inhibition of protein synthesis. In *E. coli,* nitroreductases are type I oxygen-insensitive enzymes encoded by the *nfn*A (*nfs*A) and the *nfn*B (*nfs*B) genes. Strains of bacteria that are resistant to nitrofurantoin have been shown to possess diminished nitroreductase activity (199). Resistance to nitrofurantoin from reduced nitroreductase activity seems to be present in other genera as well.

Resistance to Polymyxin B and Polymyxin E (Colistin)

Clinical and scientific interest in the cationic polypeptides colistin (polymyxin E) and polymyxin B is increasing. Although they were first used in the early 1960s, these agents are now viewed as the last-line therapeutic option for multidrug-resistant gram-negative-bacterium infections. As a chemical class, the polymyxins are polycationic peptide antibiotics isolated from *Bacillus polymyxa* (116). These polypeptide antibiotics exert their bactericidal activity by binding to the gram-negative-bacterium cell membrane and disrupting its permeability, resulting in leakage of intracellular components. They also disrupt bacterial biofilm formation. In mechanistic terms, polymyxin binds to phosphorylated head groups of lipid A. Hence, by disrupting cell membranes, these agents become rapidly bactericidal against certain gram-negative bacteria (244, 254).

Not all gram-negative bacteria are susceptible to polymyxins. Organisms that are resistant to polymyxins have cell walls that prevent access of the drug to the cell membrane. In general, polymyxins are bactericidal against *P. aeruginosa, Acinetobacter* spp., some *Proteus mirabilis* strains, and some strains of *Serratia marcescens*. *Proteus* spp., *Providentia* spp., *Neisseria* spp., and gram-positive bacteria are resistant to polymyxins (244, 254).

Polymyxin-resistant mutants and bacteria exhibit a modified lipopolysaccharide. In *E. coli, S. enterica* serotype Typhimurium, and many other pathogenic gram-negative bacteria, modification of the phosphate groups of lipid A confers resistance to polymyxin and cationic antimicrobial peptides. Lipopolysaccharide modifications that include alteration of the fatty acid content of lipid A, phosphoethanolamine addition to the core and lipid A head groups, and 4-amino-4-deoxy-L-arabinose (Ara4N) addition to the core and lipid A regions have been well studied (254). Recent evidence also implicates the presence of the MtrC-MtrD-MtrE efflux pump and lipid A modification as well as the type IV pilin secretion system to modulate levels of polymyxin resistance in *N. meningitidis* (254).

Concerns exist about the reliability and reproducibility of susceptibility testing methods for polymyxins. A recent article addresses the urgent need for quality control ranges for susceptibility determination (116).

Resistance to Quinolones

The fluoroquinolones are among the most widely used antimicrobial agents in both hospital and community settings.

Quinolone antibiotics all act by directly inhibiting DNA synthesis. Their targets include two type II topoisomerases, DNA gyrase and topoisomerase IV. These two enzymes are structurally related in that both exist as tetramers composed of two different subunits (GyrA and GyrB of DNA gyrase; ParC and ParE of topoisomerase IV). DNA gyrase maintains the negative supercoiling of DNA, whereas topoisomerase IV separates interlocked daughter DNA strands formed during replication, facilitating segregation into daughter cells. Fluoroquinolones bind to the topoisomerase-DNA complexes and disrupt various cellular processes involving DNA (such as those involving the replication fork, transcription of RNA, and DNA helicase) (102, 227, 277). The end result is cellular death through unclear mechanisms.

The affinities of fluoroquinolones for the two targets vary, explaining to some degree the differing potencies of the various agents against different bacterial species. The enzyme for which a particular fluoroquinolone exerts the greatest affinity is referred to as the primary target (5, 23, 177). In general, the primary target of fluoroquinolones in gram-negative bacteria is DNA gyrase, whereas in gram-positive bacteria it is topoisomerase IV.

Alterations in Target Enzymes

The most common mechanism of clinically significant levels of fluoroquinolone resistance is through alterations of the topoisomerase enzymes. These alterations are created by spontaneous mutations that occur within the respective genes. In GyrA and ParC, resistance-associated mutations are often localized to a region in the amino termini of the enzymes that contains the active-site tyrosine that is covalently linked to the broken DNA strand. This 130-bp region of *gyrA* has been referred to as the quinolone resistance-determining region. X-ray crystallographic studies of a fragment of the GyrA enzyme suggest that these mutations are clustered in three dimensions, lending support to the hypothesis that the region constitutes a part of the quinolone binding site (155). Particularly frequent sites for resistance-associated mutations are Ser83 and Asp87 of DNA gyrase and Ser79 and Asp83 of ParC (186).

Experimental data suggest that point mutations occur singly in roughly 1 in 10^6 to 10^9 cells. The level of resistance conferred by a single point mutation in the primary target enzyme depends upon the reduction of enzyme affinity created by the mutation, as well as the affinity of the fluoroquinolone for the secondary target. In this scenario, it is expected that fluoroquinolones exhibiting strong affinity for both target enzymes would be less likely to be associated with the emergence of resistant strains, since the retained activity against the secondary target would be enough to inhibit the bacterium even in the presence of a primary target mutation. Fluoroquinolone-species combinations for which single mutations result in significantly higher MICs (such as ciprofloxacin and *S. aureus* or *P. aeruginosa*) would be expected to readily select out resistant mutants in the clinical setting (and have [49]).

Most highly resistant strains exhibit more than one mutation in both the GyrA and ParC enzymes, a phenomenon that can be reproduced in the laboratory by serial passage of strains on progressively higher concentrations of fluoroquinolones. It is noteworthy in this context that fluoroquinolone resistance conferred by enzyme mutations is essentially class resistance. In other words, the activities of all fluoroquinolones are impacted by mutations that result in resistance. Therefore, while single point mutations that confer resistance to one fluoroquinolone may not result in MICs of another that represent clinical resistance, the MICs of the second fluoroquinolone will inevitably be increased. In the setting of such preexisting mutations, the second fluoroquinolone could then select out an additional mutation that would result in clinically significant levels of resistance. This reasoning has led to the recommendation that the most potent and broadly active fluoroquinolone should always be used first, to prevent the emergence of resistance. The wisdom of this recommendation remains to be tested.

Mutations in GyrB and ParE are far less common than those in their companion subunits and tend to cluster in the middle portion of the subunit (105). A clear understanding of the impact these mutations have on enzyme structure or function will await detailed crystallographic studies of enzyme-fluoroquinolone complexes.

Resistance Due to Decreased Intracellular Accumulation

Fluoroquinolones penetrate the outer membranes of gram-negative bacteria through porins, and so the absence of specific porins can impact the level of susceptibility. However, their ability to diffuse through outer and cytoplasmic membranes is sufficient to retain activity against strains lacking solely porins (166). More important in reducing intracellular accumulation of fluoroquinolones is the expression of multidrug-resistance pumps (186). Several such pumps have been identified in human pathogenic bacteria. Resistance results when the expression of pumps is increased due to mutations within the regulatory genes (285). By themselves, pumps generally confer only a low level of resistance to fluoroquinolones. However, their expression may amplify the level of resistance conferred by point mutations within the topoisomerase genes. By so doing, they may increase the risk that a given fluoroquinolone will select out resistant mutants through single point mutations.

In the past few years, plasmid-mediated and transferable resistance to fluoroquinolones has been recognized in a variety of gram-negative-bacterium species. This resistance is conferred by the product of the *qnr* gene and its homologues (113, 169). The mechanism underlying the resistance is protection of the DNA from quinolone binding (250). In general, only low levels of resistance are conferred by this mechanism, but as with other accessory mechanisms, the presence of Qnr can facilitate the clinical emergence of strains resistant by virtue of point mutations in the topoisomerase genes. The prevalence of this mechanism appears to be increasing, which may be explained partly by the frequent presence of *qnr* within complex *sul*I-type integrons (169).

Resistance to Rifampin

Rifampin is particularly active against gram-positive bacteria and mycobacteria. It acts by inhibiting bacterial DNA-dependent RNA polymerase. Point mutations in the chromosomal *rpoB* gene confer resistance to rifampin (267). The frequency with which these point mutations occur precludes using rifampin as a single agent for the treatment of bacterial infections.

Resistance to Tetracyclines

The tetracyclines are a group of bacteriostatic antibiotics that act by inhibiting attachment of aminoacyl-tRNA to the ribosome acceptor site, thereby preventing elongation of the peptide chains of nascent proteins (221). In order to gain access to the bacterial ribosome, tetracyclines need to enter the cell. In *E. coli* and presumably in other gram-negative bacteria, they enter the periplasmic space through outer membrane porins

OmpC and OmpF, probably chelated to magnesium ions (221). Once in the periplasmic space, the weakly lipophilic tetracycline molecule dissociates from the magnesium ion and crosses into the cell by diffusing through the lipid bilayer in an energy-dependent process. Once inside the cell, tetracycline-ion complexes bind to the ribosome at a single, high affinity binding site on the 30S subunit, blocking access of the aminoacyl-tRNA to the ribosome acceptor site. Although high affinity, binding of tetracycline to the ribosome is reversible (46).

Tetracyclines are broad-spectrum and effective antimicrobial agents. Unfortunately, widespread use of tetracyclines to treat clinical infections and to promote growth in livestock has been associated with the emergence and dissemination of a variety of resistance determinants. As a consequence, the number of infections for which tetracyclines are considered recommended first-line therapy has been limited for many years (217). The vast majority of tetracycline resistance determinants fall into one of two classes: (i) efflux or (ii) ribosomal protection. The designations of the different resistance determinants and their classes can be found in detail in an excellent review of tetracyclines by Chopra and Roberts (46). Initial designations of tetracycline resistance determinants used the prefix *tet* or *otr* with letters (A, for example) designating the different determinants. Since the number of resistance determinants now exceeds the number of letters in the alphabet, a system using numbers has been devised (132).

Tetracycline efflux proteins are all membrane associated and members of the major facilitator superfamily of proteins. They expel tetracycline from the cell by exchanging a proton for a tetracycline-cation complex. In general, the efflux proteins confer resistance to tetracyclines but tend to spare minocycline (46). The single exception to this rule is the gram-negative-bacterium Tet(B) protein that confers resistance to both tetracycline and minocycline. The efflux proteins have been divided into six groups based on amino acid identity. Group 1 consists of Tet efflux proteins that are found primarily in gram-negative species [with the exception of Tet(Z)], whereas group 2 [consisting only of Tet(K) and Tet(L)] is found primarily in gram-positive species. Groups 3 through 6 are small groups consisting of one or two efflux proteins each.

Ribosome protection proteins account for the other major mechanism of tetracycline resistance. These proteins exhibit homology to elongation factors EF-Tu and EF-G and exhibit ribosome-dependent GTPase activity (219). They act by binding to the ribosome, thereby changing its conformation and inhibiting binding of tetracycline. Tet(M) and Tet(O) are the best characterized of these proteins. Ribosome protection proteins are widespread in bacteria, in many cases as a result of the incorporation of the corresponding genes into broad-host-range conjugative transposons.

Both efflux proteins and ribosome protection proteins are regulated in ways such that their expression is increased in the presence of tetracyclines. The gram-negative-bacterium efflux proteins are regulated by repressors that are divergently transcribed relative to the efflux proteins (103). Binding of a repressor to tetracycline changes the conformation of the repressor so that it can no longer bind to the operator region, resulting in increased transcription of both the efflux protein and the repressor genes. The gram-positive-bacterium efflux genes are not associated with specific protein repressors, but sequence analysis suggests that these determinants may be regulated by mechanisms similar to translational attenuation. Study of this area has been limited (241). Transcription of ribosome protection genes is augmented by growth in the presence of tetracycline.

Intrinsic mechanisms of tetracycline resistance exist in many if not all gram-negative bacteria. Among the best characterized of these systems is the *mar* ("multiple antibiotic resistance") operon (3). This locus consists of a repressor (MarR) that represses transcription of *mar*A, which encodes a transcriptional activator of a variety of genes. Overexpression of MarA results in decreased expression of OmpF, a porin through which tetracycline enters the periplasmic space, and increased expression of multidrug efflux pump AcrAB, a member of the resistance-nodulation-cell division family of efflux proteins that includes tetracyclines among its substrates. Several similar pump systems have been identified in *P. aeruginosa* and other gram-negative bacteria (195). As our knowledge of the genomes of different bacterial species becomes more complete, we will no doubt discover several other pump systems that impact levels of susceptibility to tetracyclines and other antibiotics.

The remarkable diversity of species within which tetracycline resistance determinants are found owes much to the inclusion of these resistance genes within broad-host-range transferable genetic elements. These include transferable plasmids in gram-negative species, where *tet* genes may be found included within integrons, and conjugative transposons. Among the best studied of the conjugative transposons is the Tn*916* family, originally described in *E. faecalis* (205). The complete sequence of Tn*916* has been determined and is remarkable for its dearth of restriction enzyme digestion sites [except in the region of the *tet*(M) gene, which appears to be a late arrival to the element] (172). This lack of restriction sites likely facilitates the entry of this transposon into a variety of different bacterial species. Transfer of Tn*916*-like elements from enterococci into many other species has been demonstrated in vitro and in animal models, and the remnants of Tn*916*-like sequences in *N. gonorrhoeae* are impressive testimony to the ability of these elements to travel widely (205). Transfer of Tn*916*-like elements, which is increased after exposure to tetracycline, has also been suggested to facilitate transfer of unlinked genes, further amplifying the risks of overexposure to tetracycline in the environment.

Resistance to Tigecycline

The recent licensing of the glycylcycline tigecycline offers a broad-spectrum antimicrobial alternative for treating infections due to resistant pathogens, including MRSA and ESBL-producing *K. pneumoniae.* Tigecycline's broad spectrum of antimicrobial activity is due to its resistance to the common efflux or ribosome protection mechanisms that confer resistance to older tetracyclines. Some bacterial species, notably *P. aeruginosa* and *Proteus* spp., are intrinsically resistant to tigecycline because they express RND-type efflux pumps that effectively extrude the antibiotic (214). Resistance to tigecycline in other gram-negative species has also been reported, generally resulting from activation of normally repressed AcrAB-type RND efflux pumps (215). Determination of the ultimate importance of these pump activations for clinical resistance to tigecycline will await more extensive clinical use.

Resistance to Trimethoprim-Sulfamethoxazole

Biosynthesis of several amino acids and purines depends upon the availability of tetrahydrofolate. With few exceptions, bacteria are unable to absorb preformed folic acid and hence rely upon their ability to synthesize it. Sulfamethoxazole and trimethoprim are inhibitors of two enzymes (dihydropteroic acid synthase [DHPS] and dihydrofolate reductase [DHFR],

respectively) that act sequentially in the manufacture of tetrahydrofolate. It is thought that the two inhibitors act synergistically to inhibit folate synthesis, although the mechanism for possible synergism (since sequential blockage of a fully inhibited pathway should not augment resistance) is not clear.

Intrinsic Resistance

Trimethoprim-sulfamethoxazole is a remarkably broad spectrum antimicrobial agent. Intrinsic resistance is relatively rare and may occur through decreased access to the target enzymes *(P. aeruginosa)* (245), low affinity for DHFR enzymes (*Neisseria* spp., *Clostridium* spp., *Brucella* spp., *Bacteroides* spp., *Moraxella catarrhalis,* and *Nocardia* spp.) (246) or the ability to absorb exogenous folate (*Enterococcus* spp. and *Lactobacillus* spp.) (283) or thymine (*Enterococcus* spp.) (94). The decreased access to the target enzyme in *P. aeruginosa* appears to be the result of both a permeability barrier and active efflux from the cell (124, 146). The percentage contribution of each of these mechanisms to resistance remains unclear.

Acquired Resistance to Trimethoprim

Mutational resistance to trimethoprim has been described for several species and involves promoter mutations leading to overproduction of DHFR (in *E. coli*), point mutations within the *dhfr* gene leading to resistance (in *S. aureus and S. pneumoniae*) or both mechanisms (in *H. influenzae*) (107). More common is the acquisition of low-affinity-associated *dhfr* genes, of which approximately 20 have been described previously (107). Expression of *dhfr*I and variant *dhfr*II genes, which are most commonly found on plasmids in gram-negative bacteria, increases resistance to levels greatly exceeding clinically achievable concentrations of the drug.

Acquired Resistance to Sulfonamides

Point mutations or small insertions of DNA segments within chromosomal *dhps* genes conferring resistance to sulfonamides have been reported for many different species (65, 107). More extensive changes within *dhps* genes resulting in resistance have been reported for *N. meningitidis* and *Streptococcus pyogenes.* In these instances, the extensive changes have suggested acquisition of at least some parts of the *dhps* genes from other species via transformation and recombination (233, 243). Plasmid-mediated, transferable resistance to sulfonamides has been reported for gram-negative bacteria (107). In contrast to the diversity in *dhfr* genes, only two acquired low-affinity *dhps* genes (*sul*I and *sul*II) have been described. Genes conferring resistance to sulfonamides are frequently incorporated into multiresistance integrons, which are themselves frequently integrated into transferable plasmids. The transferability of these resistance plasmids and the frequent association with other resistance genes explain in part the widespread nature and persistence of resistance to sulfonamides. One trimethoprim-sulfamethoxazole-resistant *E. coli* strain was reported to have spread widely in the United States, causing urinary tract infections in young women in at least two states (140), although more recent data suggest that this widespread prevalence may owe more to the parallel emergence of related strains than to the direct spread of an outbreak isolate (80).

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Susceptibility Test Methods: General Considerations

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Determination of the antimicrobial susceptibilities of significant bacterial isolates is one of the principal functions of the clinical microbiology laboratory. From the physician's pragmatic point of view, the results of susceptibility tests are often considered as important as or more important than the identification of the pathogen involved. This is particularly true in an era of increasing antimicrobial resistance in which treatment options are at times limited to newer, more costly antibacterial agents. As a result, the laboratory must give high priority not only to producing technically accurate data but also to reporting those data to physicians in an easily interpretable manner.

The main objective of susceptibility testing is to predict the outcome of treatment with the antimicrobial agents tested. The implication of the result "susceptible" is that there is a high probability that the patient will respond to treatment with the appropriate dosage regimen for that antimicrobial agent. The result "resistant" implies that treatment with the antimicrobial agent is likely to fail. One group has coined the term "90-60 rule," that is, for many infections we can expect treatment success about 90% of the time when the organism tests as "susceptible" to that treatment and success may still occur in around 60% of cases when the organism tests as "resistant" to the agent used (36). The 60% response rate to ineffective antimicrobials is said to reflect the natural response to many bacterial infections in the immunologically normal host (31).

Most test methods also include an "intermediate" category of susceptibility, which can have several meanings. With agents that can be safely administered at higher doses, this category may imply that higher doses may be required to ensure efficacy or that the agent may prove efficacious if it is normally concentrated in an infected body fluid, e.g., urine. Conversely, for body compartments where drug penetration is restricted even in the presence of inflammation (e.g., subarachnoid space containing cerebrospinal fluid), it suggests that extreme caution should be taken in the use of the agent. It may also represent a buffer zone that prevents strains with borderline susceptibilities from being incorrectly categorized as resistant.

A further aim of susceptibility testing is to guide the clinician in the selection of the most appropriate agent for a particular clinical problem. In most clinical settings, susceptibility test results are usually obtained 24 to 48 h or more after the patient has been given empirical treatment. The test results may confirm the susceptibility of the organism to the drug initially prescribed or may indicate resistance, in which case alternative therapy will likely be required. The report describing the susceptibility testing results should provide the clinician with alternative agents to which the organism is susceptible. These alternatives also may be useful if the patient subsequently develops an adverse reaction to the initial antimicrobial agent. There is a growing emphasis from the professional societies and managed care organizations on the use of susceptibility test results to direct therapy toward the most narrow-spectrum, least expensive agent to which the pathogen should respond. This is particularly true for hospitalized patients, in whom the rate of antimicrobial resistance tends to be higher, and it is easier to make therapeutic changes for inpatients than for outpatients. This makes the accuracy of susceptibility testing even more critical for effective patient care.

The clinical microbiology laboratory should perform susceptibility testing only with pathogens for which well-standardized methods are available and pathogens whose resistance is known or suspected to be a clinical problem; susceptibility testing should not be performed on normal flora or colonizing organisms. Currently, routine susceptibility testing methods are best standardized for the common aerobic and facultative bacteria and systemic antibacterial agents. For some uncommon or fastidious bacteria and for topical antibacterial agents, simple routine test methods have not been standardized. Taking into account this limitation, the Clinical and Laboratory Standards Institute (CLSI; formerly the NCCLS) has recently released recommendations on how some of these bacteria may be tested and the results interpreted (10). With some pathogens (e.g., *Mycobacterium tuberculosis* and invasive fungi), routine testing is important for patient management, but testing is best performed by specialized laboratories in which test volumes are sufficient to maintain technical proficiency and where unusual results are likely to be recognized. Susceptibility testing methods for certain other pathogens (e.g., mycoplasmas, chlamydiae, legionellae, spirochetes, viruses, protozoa, and helminths) may not be well established at present and/or are limited to a few specialty laboratories. A number of choices exist in antibacterial susceptibility testing with respect to methodology and selection of agents for routine testing.

SELECTING AN ANTIMICROBIAL SUSCEPTIBILITY TESTING METHOD

Clinical microbiology laboratories can choose from among several conventional or novel methods for performance of routine antibacterial susceptibility testing. These include the broth microdilution, disk diffusion, antimicrobial gradient, and automated instrument methods. In recent years there has been a trend toward the use of commercial broth microdilution and automated instrument methods instead of the disk diffusion procedure. However, there may be renewed interest in the disk diffusion test because of its inherent flexibility in drug selection, ability to respond quickly to changes in interpretive breakpoints, and low cost. The availability of numerous antibacterial agents and the diversity of antimicrobial agent formularies at different institutions have made it difficult for manufacturers of commercial test systems to provide standard test panels that fit everyone's needs. Thus, the inherent flexibility in drug selection that is provided by the disk diffusion test is an undeniable asset of the method. The test is also one of the most established and best proven of all susceptibility tests and continues to be updated and refined through frequent (usually annual) CLSI publications (12, 13). Furthermore, the qualitative interpretive category results of susceptible, intermediate, and resistant provided by the disk test are readily understood by clinicians. Instrumentation is now available for reading and interpreting inhibition zone diameters, as well as storing this information, and may reduce interobserver reading errors (28, 32).

Advantages of the microdilution and agar gradient diffusion methods include the generation of a quantitative result (i.e., an MIC) rather than a category result, the ability to test accurately some anaerobic or fastidious species that may not be tested by the disk diffusion method (6, 8, 11, 22, 25), and the ancillary benefits of computer systems that accompany many of the microdilution and automated systems (22). Indeed, computerized data management systems are very important in laboratories that may have limited or inflexible laboratory information systems. However, an MIC method should not be chosen on the basis that MICs are routinely more useful to physicians. There is no clear evidence that MICs are more relevant than susceptibility category results to the selection of appropriate antibacterial therapy for most infections (17).

A laboratory may choose to perform rapid, automated antibacterial susceptibility testing in order to generate results faster than manual methods can generate them. The provision of susceptibility results 1 day sooner than that provided by conventional methods seems a logical advance in patient care. Two studies have demonstrated both the clinical and economic benefits derived from the use of rapid susceptibility testing and reporting (2, 19), while a further study has not shown such a benefit (4). However, rapid susceptibility testing results may not have substantial impact unless the laboratory uses more aggressive means of communication to make physicians aware of the results (43). This may be because physicians have come to expect antimicrobial susceptibility testing results approximately 48 h after the submission of a specimen or because the results, although generated more rapidly, are still not available soon enough to assist with the initial selection of antimicrobial therapy.

A previously cited shortcoming of rapid susceptibility testing methods was the failure to detect some inducible or subtle resistance mechanisms (21, 28, 41, 42). However, the instruments most notorious for such problems are no longer marketed and the manufacturers of the remaining instruments have made substantial efforts to correct earlier problems (29, 35, 39, 44) or to extend testing to include fastidious organisms (24). It is important to emphasize that accuracy should not be sacrificed in an effort to generate a rapid susceptibility testing result.

SELECTING ANTIBACTERIAL AGENTS FOR ROUTINE TESTING

The laboratory has the responsibility to test and report on the antimicrobial agents that are most appropriate for the organism isolated, the site of infection, and the clinical practice setting in which the laboratory functions. The battery of antimicrobial agents routinely tested and reported on by the laboratory will depend on the characteristics of the patients under care in the institution and the likelihood of encountering highly resistant organisms (23). A laboratory serving a tertiary-care medical center, which specializes in the care of immunosuppressed patients, may need to test routinely agents that are broader in spectrum than those tested by a laboratory that supports a primary-care outpatient practice in which antibiotic-resistant organisms are less commonly encountered.

When a laboratory's routine susceptibility testing batteries are determined, several principles should be followed. First, the antimicrobial agents that are included in the institution's formulary and that physicians prescribe on a daily basis should be tested. Second, the species tested strongly influences the choice of antimicrobial agents for testing. The CLSI publishes tables that list the antimicrobial agents appropriate for testing against various groups of aerobic and fastidious bacteria (13). The guidelines indicate the drugs that are most appropriate for testing against each organism group and for treatment based upon the specimen source (e.g., cerebrospinal fluid, blood, urine, or feces). The lists also include a few agents that may be tested as surrogates for other agents because of the greater ability of a particular agent to detect resistance to closely related drugs (e.g., the use of the cefoxitin disk test to predict overall β-lactam resistance in staphylococci) (13). This initial list of agents must be tailored to an individual institution's specific needs through discussions with infectious disease physicians, pharmacists, and committees concerned with infection control and the institutional formulary (23).

A third important step in defining routine testing batteries is ascertaining the availability of specific antimicrobial agents for testing by the laboratory's routine testing methodology. Certain methods (e.g., the disk diffusion, gradient diffusion, and in-house-prepared broth and agar dilution methods) allow the greatest flexibility in the selection of test batteries. In contrast, some commercial systems may have less flexibility or delays in adding the latest antimicrobial agents approved for clinical use. However, practicality limits the maximum number of drugs that can be tested simultaneously with an isolate by any susceptibility testing method. For example, a maximum of 12 disks can be placed on a 150-mm-diameter Mueller-Hinton agar plate and a similar number can ordinarily be accommodated in a microdilution panel if full concentration ranges of each agent are to be included for routine determination of MICs. Some commercial test panels attempt to resolve this problem by testing a larger array of antimicrobial agents, although in a very limited concentration range (perhaps two to four dilutions for each agent).

ESTABLISHING SUSCEPTIBILITY BREAKPOINTS

There is general agreement that the MIC is the most basic laboratory measurement of the activity of an antimicrobial agent against an organism. It is defined as the lowest concentration that will inhibit the growth of a test organism over a defined interval related to the organism's growth rate, most commonly 18 to 24 h. The MIC is the fundamental measurement that forms the basis of most susceptibility testing methods and against which the levels of drug achieved in human body fluids may be compared to determine breakpoints for defining susceptibility.

The conventional technique for measuring the MIC involves exposing the test organism to a series of twofold dilutions of the antimicrobial agent in a suitable culture system, e.g., broth or agar for bacteria. The twofold dilution scheme was originally used because of the convenience of preparing dilutions from a single starting concentration in broth or agar dilution methods. Subsequently, this system proved to be meaningful because an antibiotic's MICs for a single bacterial species in the absence of resistance mechanisms have a statistically normal distribution when plotted on a logarithmic scale. This provides investigators with the opportunity to examine the distributions of MICs for bacterial populations and distinguish strains for which MICs are abnormally high (potentially resistant strains) from those for which MICs are normal (susceptible strains) (26).

MIC measurements are influenced in vitro by a number of factors, including the composition of the medium, the size of the inoculum, the duration of incubation, and the presence of resistant subpopulations of the organism. The in vitro test conditions also do not encompass other factors that can have an influence on in vivo antimicrobial activity. These include sub-MIC effects, postantibiotic effects, protein binding, effects on organism virulence or toxin production, variations in redox potential at sites of infection, and the pharmacokinetic changes resulting from different drug levels in blood and at the site of infection over time. Nevertheless, if determined under standardized conditions, MIC measurements provide a fixed reference point for the setting of pharmacodynamic breakpoints with the power to predict efficacy in vivo. Pharmacological breakpoints can be applied directly to routine dilution testing methods that generate MICs, such as broth microdilution, agar dilution, gradient methods, and some automated instruments. They also provide reference values for deriving breakpoints for disk diffusion methods. Breakpoints (or interpretative criteria) are the values that determine the categories of susceptible, intermediate, and resistant. The approach to setting breakpoints varies by organization or regulatory body, but with few exceptions it is based on the agent's MICs. Depending on the approach taken, up to four sources of data can be examined in establishing breakpoints.

MIC Distributions

Examination of MIC distributions can indicate the range of MICs for a population of strains that lack any known mechanisms of resistance to the particular drug. These distributions may aid in the recognition of new resistance mechanisms by highlighting strains for which the MICs fall outside the normal distribution. However, the distributions of MICs have limited direct application since they vary between species, and for some strains for which the MICs are outside the normal range, the MICs may be below clinically derived breakpoints. Such strains may or may not respond to treatment. An example of the latter point is the fact that the penicillin MICs for some β-lactamase-producing organisms and the expanded-spectrum cephalosporin MICs for extended-spectrum β-lactamase-producing gram-negative bacilli may be relatively low but do not translate to reliable clinical efficacy. Indeed, knowledge of the presence of specific resistance mechanisms that inactivate compounds of a particular drug class is very useful in deriving microbiological breakpoints.

Pharmacokinetics and Pharmacodynamics

Pharmacokinetics examines the absorption, distribution, accumulation, and elimination (metabolism and excretion) of a drug in the body over time. These parameters are usually determined with healthy volunteers. A drug's MICs can be compared with the concentration of the drug achievable in blood or other body fluids (e.g., cerebrospinal fluid). In the past, breakpoints were chosen generally such that the MICs for susceptible pathogens would be exceeded by the drug level for most or all of the dosing interval. Newer data now considered when establishing breakpoints include pharmacodynamic calculations. Pharmacodynamics is the study of the time course of drug action against the microorganism. For antimicrobial agents, the desired action is pathogen eradication. In vitro pharmacodynamic studies have revealed that agents fall into three classes: those with principally time-dependent antimicrobial action and no or short postantibiotic effects, those with time-dependent action and long postantibiotic effects, and those with prominent concentration-dependent action (17). For drugs with time-dependent action and no or short postantibiotic effects, the critical determinant of bacterial killing in vivo is the percentage of time in a dosing interval that the drug concentration is above the MIC. For the other two classes, the important determinant is the ratio of the area under the concentration-time curve to the MIC and/or the ratio of the peak concentration to the MIC. For β-lactams, short-acting macrolides, and clindamycin, the relevant measure is the percentage of time that the drug concentration is above the MIC, and the ratio of the area under the concentration-time curve or of the peak concentration to the MIC is the relevant parameter for aminoglycosides, long-acting macrolides, tetracyclines, glycopeptides, and fluoroquinolones (18). These values can be used to calculate the maximum MICs or breakpoints that would allow the achievement of optimum efficacy with standard drug dosing schedules.

Clinical and Bacteriological Response Rates

During clinical trials, the clinical and/or bacteriologic eradication response rates of organisms for which the MICs of new antimicrobial agents have been determined give an indication of the relevance of breakpoints selected by using the MIC distributions and the pharmacokinetic-pharmacodynamic properties of the drug. Response rates of at least 80% may be expected for organisms classified as susceptible, although the rates may be lower depending on the site and type of infection. While in some countries breakpoints are determined primarily from clinical and bacteriological response rates, the CLSI evaluates clinical and bacteriological response rates in conjunction with population distributions and pharmacokinetics and pharmacodynamics in establishing the breakpoints in an attempt to provide the best correlation between in vitro test results and clinical outcome (7).

Inhibition Zone Diameter Distributions for Disk Diffusion Methods

Once the MIC breakpoints are selected, disk diffusion breakpoints can be chosen by plotting the inhibition zone diameters against the MICs derived from the testing of a large number of strains of various species. A statistical approach that uses the linear regression formula may be used to calculate the appropriate zone diameter intercepts for the predetermined MIC breakpoints. An alternative, pragmatic approach to deriving disk diffusion breakpoints is the use of the error rate-bounded method, in which the zone diameter criteria are selected on the basis of the minimization of the disk interpretive errors, especially the very major errors (5, 34) (Fig. 1). Newer statistical techniques are being studied to improve correlation with MICs (16). The newest CLSI approach focuses on the rate of interpretive errors near the proposed breakpoint versus those with MICs more than a single $\log_2$ dilution from the MIC breakpoints (7). The concept is that errors that occur with isolates for which MICs are very close to the MIC breakpoints are less of a concern than errors that occur with more highly resistant or susceptible strains.

Breakpoints derived by professional groups or regulatory bodies in various countries are often quite similar. For instance, there are a small number of breakpoint discrepancies between the CLSI and the U.S. Food and Drug Administration, which are under review by both groups. Also, there can be notable differences in the breakpoints used in different countries or regions for the same agents. The reasons for the differences may be that certain countries use different dosages or administration intervals for some drugs. In addition, some countries are more conservative in assessing the susceptibility to antimicrobial agents and place greater emphasis on the detection of emerging resistance, noted primarily by examination of microorganism population distributions. Technical factors such as the inoculum density, atmosphere of incubation, and test medium can also affect MICs and zone diameters, thereby justifying different interpretive criteria in some countries. These technical differences are summarized in chapter 73 of this Manual. Two non-U.S. methods minimize or avoid the use of an intermediate category of susceptibility based on the rationale that such results are of little value to the clinician (3, 31). The lack of a buffer between susceptible and resistant categories can result in higher rates of incorrect categorization. It may be safer for a laboratory to employ a method that uses an intermediate category or, if not, to report intermediate results as resistant.

Information on a range of international susceptibility testing methods and/or breakpoints can be downloaded or purchased from the following websites: the CLSI website at http://www.clsi.org; the European Committee on Antimicrobial Susceptibility Testing website at http://www.eucast.org; the British Society for Antimicrobial Chemotherapy website at http://www.bsac.org.uk; the Société Française de Microbiologie website at http://www.sfm.asso.fr/; the Deutsches Institut für Normung website at http://www.beuth.de/; the Swedish Reference Group for Antibiotics website at http://www.srga.org; a website featuring the Danish commercial disk diffusion method at http://www.rosco.dk; and a website featuring the Australian calibrated dichotomous sensitivity (CDS) disk diffusion method at http://www.med.unsw.edu.au/pathology-cds/.

FUTURE DIRECTIONS AND NEEDS IN ANTIMICROBIAL SUSCEPTIBILITY TESTING

Antimicrobial resistance is becoming widespread among a variety of clinically significant bacterial species (40, 45). Therefore, the microbiology laboratory plays a key role in the patient management process by providing accurate data on which physicians can base therapy decisions. Susceptibility testing results, however, are also used by investigators in surveillance studies and by infection control practitioners to detect and control the spread of antibiotic-resistant organisms (9, 38). Surveillance can be performed at the laboratory, local, regional, national, and international levels through direct interchange of data from laboratory information systems to centralized databases (37). Thus, the

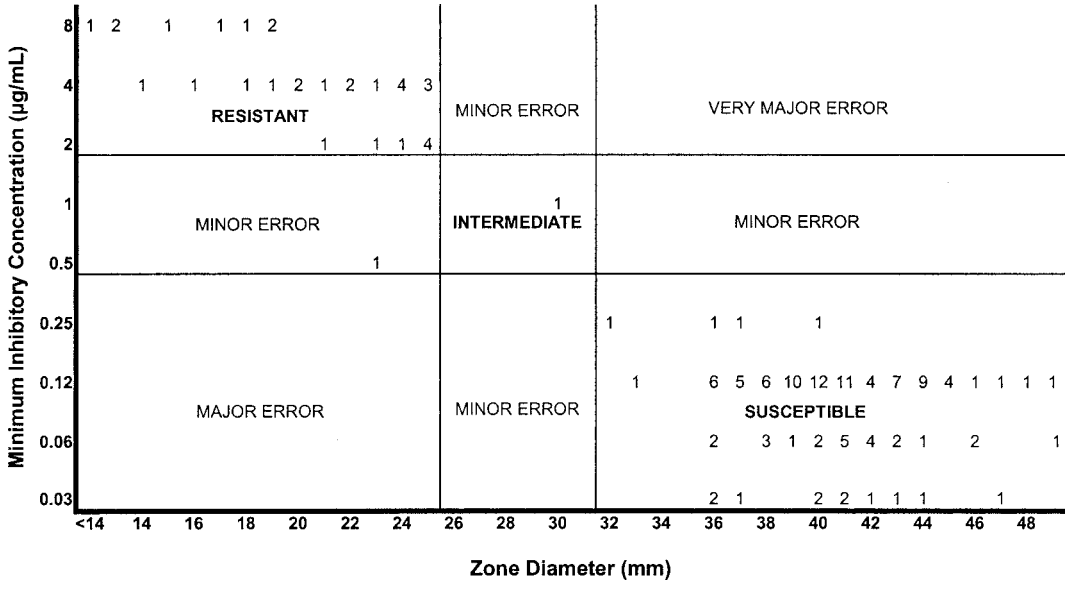


FIGURE 1 Comparison of zone diameters with MICs of a hypothetical antimicrobial agent.

accuracy of stored results becomes almost as important as the accuracy of test performance and interpretation.

To meet these challenges and responsibilities, clinical microbiologists must continuously assess and update their susceptibility testing strategies. The first priority is to use accurate and reliable methods, whether they are conventional or perhaps newer molecular methods. Then, careful monitoring of test performance with well-characterized control strains that challenge the capability of the testing methods becomes essential. Today, laboratories must use a variety of testing methods, each tailored specifically to a particular species or group of organisms. It is not likely that a single method, whether conventional or commercial, will be optimal for all antimicrobial agents, organisms, and resistance mechanisms. This will require increased education and training for clinical microbiologists in the future. Some assistance may be sought from the computer-based "expert" systems that allow a rapid and accurate view of antimicrobial susceptibility profiles and recognition of potentially aberrant results or novel resistance mechanisms (15, 39). Rapid progress is also being made on molecular methods that are starting to have practical application in the routine clinical laboratory (1, 30, 33; chapter 78).

More effective means of conveying critical antimicrobial susceptibility testing information to clinicians in a time frame that allows efficient and effective management of patients and in a format that is unambiguous to clinicians in various practice specialties are still needed. Clinical microbiologists should become more proactive in the reporting of antimicrobial susceptibility results and in cross-linking that information to other databases (e.g., those for pharmacy prescriptions) to ensure that patients receive the most efficacious cost-effective therapy.

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Susceptibility Test Methods: Dilution and Disk Diffusion Methods

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A number of methods can be used to perform antimicrobial susceptibility tests in a clinical laboratory setting or for research purposes, such as assessing the activities of new antimicrobial agents. Susceptibility testing can be performed reliably by using broth or agar dilution tests or agar diffusion methods. The choice of methodology to be used in individual laboratories may be based on factors such as relative ease of performance, cost, flexibility in selection of drugs for testing, use of automated or semiautomated devices to facilitate testing, and the perceived accuracy of the methodology (54, 55, 57). A misconception has existed regarding the clinical relevance of determining and routinely reporting MICs versus the interpretive category results (i.e., susceptible, intermediate, or resistant) that are derived using the disk diffusion method. This misconception may have been based on the assumption that the dilution test is the inherently more accurate of the two methods. Since there is a direct relationship between the sizes of the zones of inhibition and the MICs with most drugs and since MICs and zone diameters for reference strains have similar interlaboratory and intralaboratory reproducibilities, there is little objective evidence that one method is more accurate than the other with the majority of common, rapidly growing bacteria (29, 55). While an MIC may be perceived as more accurate than a result from a disk test, the MIC generated using the standard doubling-dilution series may be a concentration somewhere between the concentration inhibiting the organism and the next lower concentration tested (i.e., usually 1 $\log_2$ dilution lower). For example, an organism may grow in the presence of an antimicrobial agent at a concentration of 4 μg/ml but not at 8 μg/ml. The actual MIC could be anywhere between 4 and 8 μg/ml. Despite our statement that MICs are not inherently more accurate, there are some species with resistance mechanisms that may not be reliably detected by standard disk testing (e.g., vancomycin-intermediate *Staphylococcus aureus*) (47, 80), some species for which the standard disk test is not well calibrated (e.g., *Stenotrophomonas maltophilia* and *Burkholderia cepacia*), some species for which no disk test standards exist (e.g., *Corynebacterium* spp. and *Bacillus* spp.) (24), a few drugs that do not appear to diffuse well in agar (e.g., colistin, polymyxin B, and daptomycin) (43, 44), and some species with mechanisms of diminished susceptibility for which MICs are needed to guide selection of therapy and appropriate dosing (e.g., penicillin- and cephalosporin-intermediate *Streptococcus pneumoniae*) (29, 58). However, there are a few instances in which disk testing may provide superior detection of some resistance mechanisms, e.g., use of the cefoxitin disk test to screen for *mecA*-mediated oxacillin resistance in staphylococci (78) and disk approximation testing to detect inducible clindamycin resistance in staphylococci and streptococci (61).

There may also be the misconception that physicians prefer a quantitative (MIC) result rather than a report of a susceptibility category. To the contrary, few physicians other than those who specialize in infectious diseases have the training or experience to reliably interpret MICs. For that reason, the laboratory is obliged to provide a category interpretation (susceptible, intermediate, or resistant) with each MIC result in order to avoid potential misinterpretation of the data (25). There may be only a limited number of specific clinical indications for determining MICs to assist in patient management. These include primarily testing of isolates from patients with endocarditis or osteomyelitis (29). At this time, the pharmacodynamic principles that relate to the importance of the levels of antibiotic in serum or body fluid in relation to the MIC for an organism are useful in determining interpretive breakpoints but are not generally used to optimize therapy of individual patients (30). For research purposes, MICs are useful for evaluating relative degrees of susceptibility of bacteria to various antimicrobial agents and for ranking the activities of drugs against various species. For clinical laboratory purposes, however, the decision as to whether to perform dilution or disk diffusion testing is usually based on logistical reasons, including ease of performance of a method, its cost, and factors such as selection of a system that can provide identification of common bacteria in addition to susceptibility testing (57). Several commercial systems are now available for performing susceptibility testing; however, a major challenge posed by such systems is the relative inflexibility of standard panels of antimicrobial agents that can be tested. The inability to match precisely an institution's antimicrobial agent formulary with drugs readily available in commercial systems has led some laboratories to adopt or retain the highly flexible disk diffusion test for routine use (56).

The selection of antibacterial agents for testing is complicated by the large number of agents available today and the diversity of institutional formularies. Some of these compounds, however, exhibit similar if not identical activities in vitro, so that in some cases, one compound can be

tested as a surrogate to represent one or more closely related compounds. Such extrapolations, which have generally been agreed upon internationally, are listed in Table 1. Use of these drug surrogates can substantially reduce the number of agents required for testing and in some cases provide necessary flexibility in adapting commercial test systems for routine use in a variety of institutions. For instance, the susceptibility of *Staphylococcus* spp. to oxacillin (or cefoxitin) can be extrapolated to apply to all currently available penicillinase-stable penicillins, cephalosporins, and carbapenems. It is thus unnecessary to test any of the agents in these chemical classes, with the exception of the beta-lactamase-labile compounds (e.g., penicillin, ampicillin, amoxicillin, and piperacillin) represented by penicillin itself (25, 26). Other extrapolations are possible, especially if there is demonstrated susceptibility to a less potent member of the chemical class of antimicrobial agent.

It is important that the microbiologist work with the hospital formulary committee to ensure that the antibacterial agents being tested in the laboratory reflect those in the institution's current formulary (56). Failure to do so can contribute to antibiotic misuse (36). Guidelines for the selection of antibacterial agents to be tested routinely are published annually by the Clinical and Laboratory Standards Institute (CLSI; formerly called the NCCLS) (27) and are summarized in Table 2. While this listing is sometimes regarded rigidly as the standard for selecting the agents that must be tested, it is a list of agents that should be considered for routine testing only, and many variables go into the decision as to which agents should be tested in any particular setting (56). The CLSI also cautions that the decision about testing and reporting of some agents selectively should be made by the clinical microbiologist in conjunction with the infectious disease practitioners, the pharmacy, and/or the infection control committees (25, 26, 27).

DILUTION METHODS

Broth or agar dilution susceptibility testing methods are used to determine the minimal concentration, usually in micrograms per milliliter, of an antimicrobial agent required to inhibit or kill a microorganism. Antimicrobial agents are usually tested at $\log_2$ (twofold) serial dilutions, and the lowest concentration that inhibits visible growth of an organism is regarded as the MIC. The concentration range used may vary with the drug, the organism being tested, and the site of infection. Ranges should encompass the concentrations defining the interpretive categories (i.e., susceptible, intermediate, and resistant) and also the ranges that include the expected MICs for quality control reference strains. Other dilution methods include those that test a single concentration or a selected few concentrations of antimicrobial agents (i.e., breakpoint susceptibility tests and single-drug-concentration screens; see below).

Dilution methods offer flexibility in the sense that the standard medium used to test frequently encountered organisms (e.g., staphylococci, enterococci, members of the family *Enterobacteriaceae,* and *Pseudomonas aeruginosa*) may be supplemented or even replaced with another medium to allow accurate testing of certain fastidious bacterial species that may not be reliably tested by disk diffusion. Dilution methods are also readily adaptable to automated test systems.

DILUTION TESTING: AGAR METHOD

Dilution of Antimicrobial Agents

The solvents and diluents needed to prepare stock solutions of most commonly used antimicrobial agents are listed in the CLSI document on dilution testing (25).

TABLE 1 Antibacterial susceptibility results that may be extrapolated from other test results

Test drug (result)	Organism(s)	Drugs for which result can be extrapolated
Penicillin G	*Staphylococcus* spp., *Neisseria gonorrhoeae*	Phenoxymethylpenicillin, phenethicillin, ampicillin, amoxicillin, bacampicillin, cyclacillin, hetacillin, carbenicillin, mezlocillin, azlocillin, ticarcillin, piperacillin
Ampicillin	All	Amoxicillin, bacampicillin, cyclacillin, hetacillin
Ampicillin	*Enterococcus* spp.	Penicillin
Oxacillin	*Staphylococcus* spp.	All penicillins including antistaphylococcal penicillins, all cephalosporins, all beta-lactamase inhibitor combinations, all carbapenems, loracarbef
Cephalothin	*Enterobacteriaceae*	Cephapirin, cephradine, cephalexin, cefaclor, and cefadroxil but not other cephalosporins
Erythromycin	Gram-positive cocci	Azithromycin, clarithromycin, dirithromycin, roxithromyxin
Clindamycin	All	Lincomycin
Tetracycline	All (except *Staphylococcus, Enterococcus,* and *Acinetobacter* spp.)	Doxycycline, minocycline chlortetracycline, demeclocycline, oxytetracycline, methacycline
Sulfisoxazole	All	All sulfonamides
Cephalothin or cefazolin (susceptible)	*Enterobacteriaceae*	Susceptibility to broad-spectrum cephalosporins

TABLE 2 Antimicrobial agents recommended for routine dilution and disk diffusion susceptibility testing[a]

Antimicrobial agent	Group[b] recommended for testing with:						
	Enterobacteriaceae	*Pseudomonas* spp. and other non-*Enterobacteriaceae*[c]	*Acinetobacter* spp.[c]	*Burkholderia cepacia*[c]	*Stenotrophomonas maltophilia*[c]	Staphylococci	Enterococci
Penicillins							
Penicillin G						A	A[e]
Ampicillin	A						A[d,e]
Oxacillin[f] or methicillin						A	
Ticarcillin[d]	B	A	B				
Mezlocillin[d]	B	A	B				
Piperacillin[d]	B	A	B				
Ampicillin-sulbactam	B		B				
Amoxicillin-clavulanic acid	B						
Piperacillin-tazobactam	B	B	B				
Ticarcillin-clavulanic acid	B	B[g]	B	B	B		
Cephalosporins							
Cephalothin	A[h]						
Cefazolin	A						
Cefamandole	B						
Cefonicid	B						
Cefuroxime	B						
Cefmetazole	B						
Cefoperazone	B	B					
Cefoxitin	B						
Cefotetan	B						
Cefotaxime	B		B				
Ceftriaxone	B		B				
Ceftizoxime	B	U					
Ceftazidime	C	A	A	B	B		
Cefepime	B	B	B				
Other beta-lactams							
Ertapenem	B						
Imipenem	B	B	A				
Meropenem	B	B	A	B			
Aztreonam	C	B					
Aminogylcosides[h]							
Gentamicin	A	A	B			C	C[i]
Netilmicin	C	C					
Tobramycin	C	B	B				
Amikacin	B	B	B				
Streptomycin							C[i]

Macrolides							
Azithromycin						B	
Clarithromycin						B	
Erythromycin						B	C
Quinolones							
Ciprofloxacin	B	B	B			C	U
Gatifloxacin	U		B			C	
Levofloxacin	B	U	B	B	B	C	U
Lomefloxacin	U	U				U	
Moxifloxacin						C	
Norfloxacin	U	U				U	U
Ofloxacin	U	U				C	
Miscellaneous							
Chloramphenicol	C	C		B	B	C	C
Clindamycin						B	
Daptomycin						B	B
Doxycline			B				
Linezolid						B	B
Minocycline			B	B	B		
Nitrofurantoin	U					U	U
Polymyxin			C				
Quinupristin-dalfopristin						C	B
Rifampin						C	C
Sulfisoxazole	U					U	
Tetracycline	C	U[j]	B[j]			C	U
Telithromycin						B	
Trimethoprim-sulfamethoxazole	B	B	B	A	A	B	
Trimethoprim	U					U	
Vancomycin						B	B[e]

[a] Modified from the CLSI informational supplement (27) with permission. Current standards and supplements to them may be obtained from the CLSI, 940 West Valley Rd., Suite 1400, Wayne, PA 19087-1898.

[b] Group A comprises primary drugs to be tested and reported, group B comprises those to be tested as primary drugs but reported selectively, group C comprises supplemental drugs to be reported selectively, and group U comprises drugs to be tested with urinary isolates only.

[c] Some agents should be tested only by using an MIC method and not by using disk diffusion. See the latest CLSI standards for specific examples.

[d] Results of tests with penicillin apply to other penicillins (e.g., ampicillin, amoxicillin, carboxypenicillins, and ureidopenicillins) against beta-lactamase-negative enterococci.

[e] Combination therapy consisting of penicillin, ampicillin, or vancomycin and an aminoglycoside is recommended.

[f] Staphylococci resistant to the penicillinase-resistant penicillins should also be considered resistant to penicillins, beta-lactam-beta-lactamase-inactivating combinations, cephalosporins, and carbapenems.

[g] Ticarcillin-clavulanic acid or piperacillin-tazobactam should not be considered a therapeutic alternative for *P. aeruginosa* isolates resistant to carboxy- or ureidopenicillins.

[h] Cephalothin test results may also be used to represent those for cephapirin, cephradine, cephalexin, cefaclor, cefadroxil, and cefazolin (except against the *Enterobacteriaceae*). Cefuroxime, cefixime, cefpodoxime, cefprozil, ceftibuten, and loracarbef may be tested separately on a supplemental basis because their activities may be greater than those of cephalothin or cefazolin against the *Enterobacteriaceae.*

[i] For use of aminoglycosides to screen enterococci for synergy resistance, see the section "Breakpoint Susceptibility Tests" and "Resistance Screens."

[j] Doxycycline or minocycline may be tested on a supplemental basis because of their greater activities against some nonfermentative gram-negative bacilli and staphylococci.

Preparation, Supplementation, and Storage of Media

Mueller-Hinton agar is the recommended medium for testing most commonly encountered aerobic and facultatively anaerobic bacteria (25). The dehydrated agar base is commercially available and should be prepared as described by the manufacturer. Before sterilization, the molten agar is usually distributed into screw-cap tubes in exact aliquots sufficient to dilute the desired antimicrobial concentrations 10-fold. Tubes of agar, one for each drug concentration to be tested, are sterilized by autoclaving at 121°C for 15 min, and the agar is allowed to equilibrate to 48 to 50°C in a preheated water bath. Once the agar has equilibrated, the appropriate volume of antimicrobial agent is added, the tube contents are mixed by gentle inversion and poured into 100-mm-diameter round or square sterile plastic petri plates set on a level surface, and the agar is allowed to solidify. For growth controls, plates containing drug-free agar are also prepared. All plates should be filled to a depth of 3 to 4 mm (20 to 25 ml of agar per round plate and 30 ml per square plate), and the pH of each batch should be checked to confirm the acceptable pH range of 7.2 to 7.4 (25).

After sterilization and temperature equilibration of the molten agar, any necessary supplements are aseptically added to the Mueller-Hinton agar at the time of addition of the drug solutions. For testing of streptococci, supplementation with 5% defibrinated sheep or horse blood is recommended (25). However, sheep blood supplementation may antagonize the activities of sulfonamide and trimethoprim against some organisms (11). The presence of blood also affects results with novobiocin and nafcillin as well as the in vitro activities of cephalosporins against enterococci (15, 73); therefore, blood supplementation should not be used unless necessary for bacterial growth (see chapter 75 for acceptable methods for testing of fastidious bacterial species). Performance standards for Mueller-Hinton agar have been defined sufficiently such that calcium and magnesium supplementation is unnecessary (22). The agar should be supplemented with 2% NaCl for testing of oxacillin against staphylococci (49).

Once prepared, plates should be sealed in plastic bags and stored at 4 to 8°C. In general, they should be used within 5 days of preparation or as long as the MICs for control strains that are tested routinely are within the acceptable ranges. However, certain agents are sufficiently labile that plates may not be stored prior to use, e.g., carbapenems, cefaclor, and clavulanic acid. Before inoculation, plates that have been stored under refrigeration should be allowed to equilibrate to room temperature and the agar surface should be dry.

Inoculation Procedures

Variations in inoculum size may substantially affect MICs; therefore, careful inoculum standardization is required to obtain accurate results. The recommended final inoculum for agar dilution is 10^4 CFU per spot (25). This may be achieved in either of two ways. Four or five colonies are picked from overnight growth cultures on agar-based medium and inoculated into 4 to 5 ml of suitable broth that will support good growth (usually tryptic soy broth). Broths are incubated at 35°C until visibly turbid, and then the suspension is diluted until it matches the turbidity of a 0.5 McFarland, barium sulfate ($BaSO_4$), or latex particle turbidity standard (ca. 10^8 CFU/ml). The 0.5 McFarland standard may be purchased or the barium sulfate standard may be prepared as described in the CLSI document (25). The accuracy of the standard should be verified by using a spectrophotometer with a 1-cm light path; for the 0.5 McFarland standard, the absorbance at 625 nm should be 0.08 to 0.13 (25). An alternative inoculum standardization method, one that is preferred by many microbiologists, utilizes direct suspension of colonies from overnight growth cultures on a nonselective agar medium in saline or broth to a turbidity that matches the 0.5 McFarland standard. This approach eliminates the time needed for growing the inoculum in broth (25). In either case, normal saline or sterile broth is used to make a 1:10 dilution of the suspension to give an adjusted concentration of 10^7 CFU/ml (25).

Once the adjusted bacterial inoculum suspension is prepared, inoculation of the antimicrobial agent plates should be accomplished within 30 min, since longer delays may lead to changes in inoculum size. By using a pipette, a calibrated loop, or, more commonly, an inoculum-replicating device, 0.001 to 0.002 ml (1 to 2 μl) of the 10^7-CFU/ml suspension is delivered to the agar surface, resulting in the final desired inoculum of approximately 10^4 CFU per spot. For convenience, use of a replicator is preferred, because consistent inoculum volumes for up to 36 different isolates are simultaneously delivered (25, 76). To use this device, an aliquot of the adjusted inoculum for each isolate is pipetted into the appropriate well of an inoculum seed plate and a multiprong inoculator is used to pick up and gently transfer 1 to 2 μl from the wells to the agar surfaces. Replicators are also available that deliver only 0.1 to 0.2 μl per spot and that do not require the 0.5 McFarland standard suspension to be diluted prior to delivery to the agar surface (25). The surfaces of the agar plates must be dry before inoculation, which should begin with the lowest drug concentration. To check for viability of each test isolate and also as an added check for purity, control plates that do not contain drugs are inoculated last. Finally, plates should be clearly marked so that the locations of the different isolates being tested on each plate are known.

Incubation

Inoculated plates are allowed to stand for several minutes until the inoculum drops have been completely absorbed by the medium; then they are inverted and incubated in air at 35°C for 16 to 20 h before results are read. To facilitate detection of vancomycin-resistant enterococci and methicillin-resistant or vancomycin-resistant or -intermediate staphylococci, plates containing vancomycin or oxacillin should be incubated for a full 24 h before results are read (25). Incubation should not be carried out in the presence of an increased CO_2 concentration unless a fastidious organism is being tested (see chapter 75).

Interpretation and Reporting of Results

Before reading and recording the results obtained with clinical isolates, those obtained with applicable quality control strains tested at the same time should be checked to ensure that their values are within the acceptable ranges (see "Quality Control" below) and the drug-free control plates should be examined for isolate viability and purity. Endpoints for each antimicrobial agent are best determined by placing plates on a dark background and examining them for the lowest concentration that inhibits visible growth, which is recorded as the MIC. A single colony or a faint haze left by the initial inoculum should not be regarded as growth. If two or more colonies persist at antimicrobial concentrations beyond an otherwise obvious endpoint or if there is no growth at lower concentrations but there is growth at higher concentrations, the isolate should be subcultured to confirm purity and the test should be repeated. Substances that may antagonize the antibacterial activities of sulfonamides and trimethoprim may be carried over with the inoculum and cause "trailing," or less definite endpoints (11,

15). Therefore, the MICs of these antimicrobial agents should be interpreted as the endpoints at which 80% or more diminution of growth occurs. Although much less pronounced, trailing endpoints may also occur for some organisms with bacteriostatic agents such as chloramphenicol, the tetracyclines, linezolid, and quinupristin-dalfopristin (25).

The MIC of each antimicrobial agent is usually recorded in micrograms per milliliter, although in Europe and in the international standard reference method, the values are expressed as milligrams per liter (39, 40, 50). These quantitative results should be reported with the appropriate corresponding interpretive category (susceptible, intermediate, or resistant), or the interpretive category may be reported alone. The MIC interpretive standards for these susceptibility categories, as currently recommended by the CLSI (25), are provided in Table 3. For detailed instructions concerning the

TABLE 3 Interpretive standards for dilution and disk diffusion susceptibility testing[a]

Antimicrobial agent and organism	MIC (μg/ml)			Zone diam (mm)		
	Susceptible	Intermediate	Resistant	Susceptible	Intermediate	Resistant
Penicillins						
Penicillin G						
Staphylococci[b]	≤0.12		≥0.25	≥29		≤28
Enterococci[c]	≤8		≥16	≥15		≤14
Methicillin[d]	≤8		≥16	≥14	10–13	≤9
Oxacillin[d]						
S. aureus	≤2		≥4	≥13	11–12	≤10
Coagulase-negative staphylococci	≤0.25		≥0.5	≥18		≤17
Ampicillin						
Enterobacteriaceae	≤8	16	≥32	≥17	14–16	≤13
Staphylococci	≤0.25		≥0.5	≥29		≤28
Enterococci[c]	≤8		≥16	≥17		≤16
Amoxicillin-clavulanic acid						
Staphylococci	≤4/2		≥8/4	≥20		≤19
Other organisms	≤8/4	16/8	≥32/16	≥18	14–17	≤13
Ampicillin-sulbactam	≤8/4	16/8	≥32/16	≥15	12–14	≤11
Azlocillin	≤64		≥128	≥18		≤17
P. aeruginosa						
Carbenicillin						
P. aeruginosa	≤128	256	≥512	≥17	14–16	≤13
Other gram-negative bacilli	≤16	32	≥64	≥23	20–22	≤19
Mecillinam	≤8	16	≥32	≥15	12–14	≤11
Mezlocillin						
P. aeruginosa	≤64		≥128	≥16		≤15
Other gram-negative bacilli	≤16	32–64	≥128	≥21	18–20	≤17
Piperacillin						
P. aeruginosa	≤64		≥128	≥18		≤17
Other gram-negative bacilli	≤16	32–64	≥128	≥21	18–20	≤17
Piperacillin-tazobactam						
P. aeruginosa	≤64/4		≥128/4	≥18		≤17
Other gram-negative bacilli	≤16/4	32/4–64/4	≥128/4	≥21	18–20	≤17
Staphylococci	≤8/4		≥16/4	≥18		≤17
Ticarcillin						
P. aeruginosa	≤64		≥128	≥15		≤14
Other gram-negative bacilli	≤16	32–64	≥128	≥20	15–19	≤14
Ticarcillin-clavulanic acid						
P. aeruginosa	≤64/2		≥128/2	≥15		≤14
Other gram-negative bacilli	≤16/2	32/2–64/2	≥128/2	≥20	15–19	≤14
Staphylococci	≤8/2		≥16/2	≥23		≤22

(Continued on next page)

TABLE 3 Interpretive standards for dilution and disk diffusion susceptibility testing[a] *(Continued)*

Antimicrobial agent and organism	MIC (μg/ml)			Zone diam (mm)		
	Susceptible	Intermediate	Resistant	Susceptible	Intermediate	Resistant
Cephalosporins						
Cefaclor	≤8	16	≥32	≥18	15–17	≤14
Cefamandole	≤8	16	≥32	≥18	15–17	≤14
Cefazolin	≤8	16	≥32	≥18	15–17	≤14
Cefepime	≤8	16	≥32	≥18	15–17	≤14
Cefetamet	≤4	8	≥16	≥18	15–17	≤14
Cefixime	≤1	2	≥4	≥19	16–18	≤15
Cefmetazole	≤16	32	≥64	≥16	13–15	≤12
Cefonicid	≤8	16	≥32	≥18	15–17	≤14
Cefoperazone	≤16	32	≥64	≥21	16–20	≤15
Cefotaxime	≤8	16–32	≥64	≥23	15–22	≤14
Cefotetan	≤16	32	≥64	≥16	13–15	≤12
Cefoxitin	≤8	16	≥32	≥18	15–17	≤14
Cefpodoxime	≤2	4	≥8	≥21	18–20	≤17
Cefprozil	≤8	16	≥32	≥18	15–17	≤14
Ceftazidime	≤8	16	≥32	≥18	15–17	<14
Ceftibuten	≤8	16	≥32	≥21	18–20	≤17
Ceftizoxime	≤8	16–32	≥64	≥20	15–19	≤14
Ceftriaxone	≤8	16–32	≥64	≥21	14–20	≤13
Cefuroxime axetil	≤4	8–16	≥32	≥23	15–22	≤14
Cefuroxime sodium	≤8	16	≥32	≥18	15–17	≤14
Cephalothin	≤8	16	≥32	≥18	15–17	≤14
Loracarbef	≤8	16	≥32	≥18	15–17	≤14
Moxalactam	≤8	16–32	≥64	≥23	15–22	≤14
Other beta-lactams						
Aztreonam	≤8	16	≥32	≥22	16–21	≤15
Ertapenem	≤2	4	≥8	≥19	14–18	≤15
Imipenem	≤4	8	≥16	≥16	14–15	≤13
Meropenem	≤4	8	≥16	≥16	14–15	≤13
Aminoglycosides						
Amikacin	≤16	32	≥64	≥17	15–16	≤14
Gentamicin	≤4	8	≥16	≥15	13–14	≤12
Enterococci (high-level resistance)	≤500		>500	≥10	7–9	6
Netilmicin	≤8	16	≥32	≥15	13–14	≤12
Tobramycin	≤4	8	≥16	≥15	13–14	≤12
Streptomycin						
Enterococci (high-level resistance)						
Broth microdilution method	≤1,000		>1,000			
Agar-based method	≤2,000		>2,000	≥10	7–9	6
Glycopeptides						
Teicoplanin	≤8	16	≥32	≥14	11–13	≤10
Vancomycin						
Enterococci	≤4	8–16	≥32	≥17	15–16	≤14
S. aureus	≤2	4–8	≥16	≥15	Determine MIC if ≤14	
Coagulase-negative staphylococci	≤4	8–16	≥32	≥15	Determine MIC if ≤14	
Lipopeptide						
Daptomycin						
Enterococci	≤4					
Staphylococci	≤1					

(Continued on next page)

TABLE 3 *(Continued)*

Antimicrobial agent and organism	MIC (μg/ml)			Zone diam (mm)		
	Susceptible	Intermediate	Resistant	Susceptible	Intermediate	Resistant
Macrolides						
Azithromycin	≤2	4	≥8	≥18	14–17	≤13
Clarithromycin	≤2	4	≥8	≥18	14–17	≤13
Dirithromycin	≤2	4	≥8	≥19	16–18	≤15
Erythromycin	≤0.5	1–4	≥8	≥23	14–22	≤13
Ketolide						
Telithromycin	≤1	2	≥4	≥22	17–21	≤18
Quinolones						
Ciprofloxacin	≤1	2	≥4	≥21	16–20	≤15
Enoxacin	≤2	4	≥8	≥18	15–17	≤14
Fleroxacin	≤2	4	≥8	≥19	16–18	≤15
Gatifloxacin	≤2	4	≥8	≥18	15–17	≥14
Levofloxacin	≤2	4	≥8	≥17	14–16	≤13
Lomefloxacin	≤2	4	≥8	≥22	19–21	≤18
Moxifloxacin	≤0.5	1	≥2	≥24	19–23	≤20
Nalidixic acid[e]	≤8	16	≥32	≥19	14–18	≤13
Norfloxacin[e]	≤4	8	≥16	≥17	13–16	≤12
Ofloxacin	≤2	4	≥8	≥16	13–15	≥12
Sparfloxacin	≤0.5	1	≥2	≥19	16–18	≤15
Tetracyclines						
Doxycycline	≤4	8	≥16	≥16	13–15	≤12
Minocycline	≤4	8	≥16	≥19	15–18	≤14
Tetracycline	≤4	8	≥16	≥19	15–18	≤14
Other						
Chloramphenicol	≤8	16	≥32	≥18	13–17	≤12
Clindamycin	≤0.5	1–2	≥4	≥21	15–20	≤14
Colistin	≤2		≥4			
Fosfomycin	≤64	128	≥256	≥16	13–15	≤12
Linezolid						
Enterococci	≤2	4	≥8	≥23	19–22	≤20
Staphylococci	≤2			≥21		
Nitrofurantoin	≤32	64	≥128	≥17	15–16	≤14
Polymyxin	≤2		≥4			
Quinupristin-dalfopristin	≤1	2	≥4	≥19	16–18	≤15
Rifampin	≤1	2	≥4	≥20	17–19	≤16
Sulfonamide	≤256		≥512	≥17	13–16	≤12
Trimethoprim[e]	≤8		≥16	≥16	11–15	≤10
Trimethoprim-sulfamethoxazole	≤2/38		≥4/76	≥16	11–15	≤10

[a] Adapted from CLSI data (27) with permission. The interpretive data are valid only if the methodologies in documents M2-A8 (26) and M7-A6 (25) are followed. Breakpoints for some agents apply only to certain genera or species. The CLSI frequently updates the interpretive tables through new editions of the standards and supplements to them. Users should refer to the most recent editions. The current standards and supplements to them may be obtained from the CLSI, 940 West Valley Rd., Suite 1400, Wayne, PA 19087-1898.

[b] Penicillin should be used as the class representative for all penicillins (e.g., ampicillin, amoxicillin, mezlocillin, piperacillin, and ticarcillin). Isolates for which MICs are ≤0.03 μg of penicillin per ml generally do not produce beta-lactamase, whereas those for which MICs are ≥0.25 μg/ml do and should be regarded as resistant to penicillins. Isolates for which MICs of penicillin are 0.06 or 0.12 μg/ml should be tested for beta-lactamase.

[c] Therapy for serious enterococcal infections requires high doses of penicillin or ampicillin in combination with an aminoglycoside. Vancomycin may be substituted for the penicillin in instances of penicillin hypersensitivity or of penicillin or ampicillin resistance.

[d] Oxacillin or methicillin may be tested; however, oxacillin is preferred because of its greater stability in vitro. The results from the testing of oxacillin apply also to other penicillinase-resistant penicillins. Oxacillin-resistant staphylococci should be considered resistant to all penicillins, cephalosporins, carbacephems, carbapenems, and beta-lactam–beta-lactamase inhibitor combinations. Disk testing with cefoxitin is the most sensitive and specific method for phenotypic detection of *mec*A-mediated oxacillin resistance in staphylococci (27, 78).

[e] For the treatment of urinary tract infections only.

use of these criteria and categories, the latest CLSI standards for dilution testing methods should be consulted (27). Note that the interpretive standards for most of the penicillin class drugs vary with the organism being tested. In an attempt to minimize confusion regarding different breakpoints that may apply to individual species, the CLSI has divided the interpretive breakpoint tables into a number of different tables for the various organism groups (25).

The three interpretive categories are defined as follows. Susceptible indicates that an infection caused by the tested microorganism may be appropriately treated with the usually recommended dose of antibiotic. Intermediate indicates that the isolate may be inhibited by attainable concentrations of certain drugs (e.g., the beta-lactams) if higher dosages can be used safely or if the infection involves a body site indicating that the drug is physiologically concentrated (e.g., the urinary tract). The intermediate category also serves as a buffer zone that prevents slight technical artifacts from causing major interpretive discrepancies. Resistant isolates are not inhibited by the concentration of antimicrobial agent normally achievable with the recommended dose and/or yield results that fall within a range indicating that specific resistance mechanisms are likely to be present (25).

Advantages and Disadvantages

Dilution testing by the agar method is a well-standardized, reliable susceptibility testing technique that may be used as a reference for evaluating other testing methods. In addition, the simultaneous testing of a large number of isolates with a few drugs is efficient (such as when new agents are evaluated in the pharmaceutical industry), and microbial contamination or population heterogeneity is more readily detected by the agar method than by broth methods. The agar dilution method has been considered the reference test method in many areas of Europe (39), while broth microdilution has been much more widely used for research and clinical purposes in North America (55) and is now considered the international reference method for determining MICs (50). The major disadvantages of the agar method are associated with the time-consuming and labor-intensive tasks of preparing the plates, especially if the number of different antimicrobial agents to be tested for each isolate is high or if only a few isolates are to be tested.

DILUTION TESTING: BROTH METHODS

The general approaches for broth methods include broth macrodilution, in which the broth volume for each antimicrobial concentration is ≥1.0 ml (usually 2 ml) contained in test tubes, and broth microdilution, in which antimicrobial dilutions are most often in 0.1-ml volumes in wells of microdilution trays.

Broth Macrodilution Methods

Dilution of Antimicrobial Agents

Stock solutions are prepared as discussed in the CLSI document on dilution testing (25) and are the same as those used for agar dilution tests. As in the agar method, the actual volumes used for the dilutions would be proportionally increased according to the number of tests being prepared, with a minimum of 1.0 ml needed for each drug concentration. Because addition of the inoculum results in a 1:2 dilution of each concentration, all final drug concentrations must be prepared at twice the actual desired testing concentration (see "Inoculation Procedures" below).

Preparation, Supplementation, and Storage of Media

Cation-adjusted Mueller-Hinton broth (CAMHB) is recommended for routine testing of commonly encountered nonfastidious organisms (25). Adjustment of the cations Ca^{2+} (20 to 25 mg/liter) and Mg^{2+} (10 to 12.5 mg/liter) is required to ensure acceptable results when *P. aeruginosa* isolates are tested with aminoglycosides and when tetracycline is tested with other bacteria (9). However, for convenience and consistency, cation adjustment of Mueller-Hinton broth is now recommended for testing of all species and antimicrobial agents (25). Some manufacturers provide Mueller-Hinton broth that already has appropriate concentrations of divalent cations, so the cation content of commercial dehydrated media must be ascertained and care must be taken to supplement the broth with the appropriate cation levels. If adjustment is necessary, it can be accomplished by the addition of suitable volumes of filter-sterilized, chilled $CaCl_2$ stock (3.68 g of $CaCl_2 \cdot 2H_2O$ dissolved in 100 ml of deionized water for a concentration of 10 mg of Ca^{2+} per ml) and $MgCl_2$ stock (8.36 g of $MgCl_2 \cdot 6H_2O$ in 100 ml of deionized water for a concentration of 10 mg of Mg^{2+} per ml) to the cooled broth (25). Insufficient cation concentrations result in increased aminoglycoside activity (32), and excess cation content results in decreased aminoglycoside activity against *P. aeruginosa* (7, 32). For testing of daptomycin, the calcium level of the test medium must be increased to 50 mg/liter to achieve reliable MICs (25, 43). While the effects of inappropriate calcium and magnesium ion contents are well recognized, other ions, including zinc and manganese, may adversely affect the activities of some drugs, e.g., carbapenems (31). The CLSI has initiated a consensus standard for manufacturers of Mueller-Hinton broth that attempts to specify all known factors that determine performance of the medium (23). Reliable detection of staphylococcal resistance to oxacillin requires that the CAMHB used for testing that agent be supplemented with 2% NaCl (25, 83).

To minimize evaporation and deterioration of antimicrobial agents, tubes should be tightly capped and stored at 4 to 8°C until needed. With most agents, the dilutions should be used within 5 days of preparation or as long as quality control ranges are maintained (see "Quality Control" below). As in agar dilution testing, certain beta-lactam agents are too labile for prolonged storage at final test concentrations.

Inoculation Procedures

The recommended final inoculum is 5×10^5 CFU/ml. Isolates are inoculated into a broth that will support good growth (such as tryptic soy broth) and incubated until turbid. The turbidity is adjusted to match that of a 0.5 McFarland standard (approximately 10^8 CFU/ml). Alternatively, four or five colonies from overnight growth cultures on a nonselective agar plate may be directly suspended in broth to match the turbidity of the McFarland standard (25). This alternative is preferred for testing of oxacillin against staphylococci (25). A portion of the standardized suspension is diluted approximately 1:100 (10^6 CFU/ml) with broth or saline. When 1 ml of this dilution is added to each tube containing 1 ml of the drug diluted in CAMHB, a final inoculum of 5×10^5 CFU/ml is achieved. Broth not containing an antimicrobial agent is inoculated as a control for organism viability (growth control). All tubes should be inoculated within 30 min of inoculum preparation, and an aliquot of the inoculum should be plated to check for purity.

Incubation

Tubes are incubated in air at 35°C for 16 to 20 h before MICs are determined. Incubation should be extended to a full 24 h for the detection of vancomycin-resistant enterococci and oxacillin-resistant or vancomycin-resistant or -intermediate staphylococci (25). An atmosphere with increased CO_2 should not be used.

Interpretation and Reporting of Results

Before MICs for the test strains are read and recorded, the growth controls should be examined for viability, inoculum subcultures should be checked for contamination, and appropriate MICs obtained with the quality control strains should be confirmed (see "Quality Control" below). Growth or lack thereof in the antimicrobial agent-containing tubes is best determined by comparison with the growth control. Generally, growth is indicated by turbidity, a single sedimented button of ≥2 mm in diameter, or several buttons with smaller diameters. As with the agar method, trailing endpoints may be seen when trimethoprim or sulfonamides are tested, and the concentration at which 80% or greater diminution of growth, compared with that of the growth control, occurs should be recorded as the MIC (25). Other interpretation problems include the "skipped tube" phenomenon, in which growth is not observed at one concentration but is observed at lower and higher drug concentrations. Most authorities suggest that when this occurs, the skipped tube should be ignored and the concentration that finally inhibits growth at serially higher concentrations should be recorded as the MIC. If more than one skipped tube occurs or if there is growth at higher antimicrobial concentrations but not at lower ones, the results should not be reported and the test for that drug should be repeated.

The lowest concentration that completely inhibits visible growth of the organism as detected by the unaided eye is recorded as the MIC. The CLSI MIC interpretive standards in effect as of the date of this writing (27) for the susceptibility categories are provided in Table 3. The definitions of and comments concerning these categories that were given for the agar method also pertain to the broth macrodilution method.

Advantages and Disadvantages

The broth macrodilution method is a well-standardized and reliable method that may be useful for research purposes or for testing of one drug with a bacterial isolate. However, because of the laborious nature of the procedure and the availability of more convenient dilution systems (e.g., microdilution), this procedure is generally not useful for routine susceptibility testing in most clinical microbiology laboratories.

Broth Microdilution Method

The convenience afforded by the availability of dilution susceptibility testing in microdilution trays has led to the widespread use of broth microdilution methods. In fact, the broth microdilution method is now considered the international reference susceptibility testing method (50). The disposable plastic trays, containing a panel of several antimicrobial agents to be tested simultaneously, may be prepared in-house or obtained commercially either frozen or freeze-dried. When commercial systems are used, the manufacturers' recommendations concerning storage, inoculation, incubation, and interpretation should be followed. The primary focus of this section will be the in-house preparation and use of broth microdilution panels. However, most of the principles and practices discussed here are pertinent to the broth microdilution method regardless of the source of the antibiotic panels.

Dilution of Antimicrobial Agents

Antimicrobial stock solutions are prepared as outlined in the CLSI document on dilution testing (25). The dilution scheme for preparing broth microdilution panels is the same as that described for agar and broth microdilution methods. Automated dispensing systems for preparation of microdilution panels use tubes that contain from 10 to 200 ml or more of broth containing each antimicrobial concentration. From the master tube dilutions, aliquots of 0.05 or 0.1 ml are simultaneously dispensed into the corresponding wells of each broth microdilution tray by using a mechanized dispenser. If 0.05-ml volumes are dispensed, allowances must be made for the 1:2 dilution of the final drug concentration that will occur when the 0.05 ml of inoculum is added (see "Inoculation Procedures" below). When 0.1-ml aliquots are dispensed, the volume of inoculum normally used is sufficiently small (≤0.005 ml) that adjustments in the antimicrobial dilution scheme are not needed. As a general rule, when the inoculum volume is less than 10% of the broth volume in the well, dilution of the antimicrobial concentration by the inoculum does not have to be taken into account (25).

Preparation, Supplementation, and Storage of Media

CAMHB is the recommended medium for broth microdilution testing of nonfastidious organisms and should be prepared as discussed above for the broth macrodilution method. Also, supplementation of the broth with 2% NaCl is required for detection of oxacillin-resistant staphylococci (25). After the antimicrobial dilutions have been dispensed into the plastic trays, the panels are stacked in groups of 5 to 10, with a tray lid or an empty tray placed on top to minimize contamination and evaporation. Each stack is sealed in a plastic bag and frozen immediately at −20°C or, preferably, at −60°C or colder. At −20°C, preservation is ensured for at least 6 weeks with most drugs, but the shelf life may be extended to months if the trays are stored at −60 to −70°C. Care must be taken in storing highly labile compounds such as cefaclor, clavulanic acid, and carbapenems, which may lose potency during storage. If thawed, panels must be used or discarded but not refrozen, since freeze-thaw cycles cause substantial deterioration of beta-lactam antibiotics. For this reason, −20°C household type freezers with self-defrosting units must not be used.

Inoculation Procedures

As with the macrodilution procedure, the final desired inoculum concentration is 5×10^5 CFU/ml. The isolates may be grown in broth to match the turbidity of a 0.5 McFarland standard (ca. 1×10^8 to 2×10^8 CFU/ml), or a suspension of that density can be made from colonies grown overnight on a nonselective agar medium (8), which is the method preferred for detecting oxacillin-resistant staphylococci (25). For broth microdilution procedures that require 0.001- to 0.005-ml volumes to inoculate wells containing 0.1 ml of broth, a portion of the 0.5 McFarland standard suspension is diluted 1:10 (10^7 CFU/ml) in sterile saline or broth. Multipoint metal or disposable plastic inoculum replicators designed to collect and deliver appropriate volumes are used to transfer the inoculum from the diluted suspension to the wells of the broth microdilution tray, resulting in further dilutions ranging from 1:20 to 1:50. Final inoculum concentrations should be 4×10^5 to 6×10^5 CFU/ml (4×10^4 to 6×10^4 CFU per well). For protocols that use an inoculum volume of 0.05 ml to inoculate 0.05 ml of broth, a 1:100 dilution of a 0.5 McFarland

standard suspension (ca. 10^6 CFU/ml) is used. When the inoculum is added to the wells, the 1:2 dilution of the 10^6-CFU/ml inoculum results in a final inoculum concentration of 5×10^5 CFU/ml (5×10^4 CFU per well) and also halves the antibiotic concentration in each well. Special care should be taken to confirm the inoculum density on a periodic basis to ensure that the appropriate density of inoculum is achieved. Moreover, slight deviations from the initial 1:10 dilution described above may be necessary to provide the target inoculum density with some species or organism groups. Insufficient inoculum can be a significant problem with inducible resistance mechanisms of some organisms and may not be recognized as a problem based on the MICs obtained for the very susceptible quality control strains.

Broth microdilution trays should be inoculated within 30 min of inoculum preparation, and an aliquot should be subcultured to check the purity of the isolates. Finally, one well of each panel not containing an antimicrobial agent should be inoculated and used as a growth control, and a second uninoculated well serves as a sterility control.

Incubation

After inoculation, each tray should be covered with plastic tape, sealed in a plastic bag, or tightly fitted with a lid or an empty tray to prevent evaporation during incubation. Trays are incubated in ambient air at 35°C for 16 to 20 h before results are read and should not be incubated in stacks of more than four trays for uniform temperature distribution. The incubator should be kept sufficiently humid to avoid evaporation but not so humid that condensation results in contamination problems. A full 24 h of incubation is recommended for the detection of vancomycin-resistant enterococci and oxacillin-resistant or vancomycin-resistant or -intermediate staphylococci (25). Incubation in an atmosphere with increased CO_2 should not be used with nonfastidious organisms.

Interpretation and Reporting of Results

Before MICs for the clinical isolates are read and recorded, the growth control wells should be examined for organism viability and the inoculum purity should be checked. The appropriateness of the MICs obtained for the quality control strains should be confirmed if tests of these strains were set up simultaneously with those of clinical isolates (see "Quality Control" below). Various viewing devices are available and should be used to facilitate examination of the broth microdilution wells for growth. The simplest and most reliable method is the use of a parabolic magnifying mirror and tray stand that allow clear visual inspection of the undersides of the microdilution trays. Growth is best determined by comparison with that in the growth control well and generally is indicated by turbidity throughout the well or by buttons, single or multiple, in the well bottom. The occurrence of trailing endpoints with trimethoprim or sulfonamides should be ignored, and the MIC endpoint should be based on ≥80% growth inhibition. Results for drugs with more than one skipped well should not be reported, as with the broth macrodilution test.

The CLSI MIC interpretive criteria (27) for susceptibility categories are given in Table 3. It should be noted that these values are published each year and only the most recent tables should be used for interpretation of results. The definitions of the interpretive categories and the comments concerning the use of these standards for agar and broth macrodilution methods are also applicable to broth microdilution methods.

Advantages and Disadvantages

The broth microdilution method represents a standardized reference method for susceptibility testing. Inoculation and reading procedures allow relatively convenient simultaneous testing of several antimicrobial agents with individual isolates. Because few laboratories have the facilities required for preparation of broth microdilution trays, several sources of commercially prepared antibiotic panels are available today. Such products provide trays with wells containing prepared antimicrobial dilutions either frozen or freeze-dried. Trays with frozen dilutions must be stored frozen in the laboratory, whereas dried panels can be stored at room temperature. Most of these products are accompanied by multipoint inoculating devices, or multichannel pipetters. Results of testing may be determined by visual examination or by use of semiautomated or automated instrumentation.

Breakpoint Susceptibility Tests

Breakpoint susceptibility testing refers to methods by which antimicrobial agents are tested only at the specific concentrations necessary for differentiating between the interpretive categories of susceptible, intermediate, and resistant rather than in a range of five or more doubling-dilution concentrations used to determine MICs. When two drug concentrations are selected adjacent to the breakpoints defining the intermediate and resistant categories, any one of the interpretive categories may be determined. Growth at both concentrations indicates resistance, growth at only the lower concentration signifies an intermediate result, and no growth at either concentration indicates susceptibility.

Like full-range dilution testing, breakpoint methods require the use of appropriately adjusted and supplemented Mueller-Hinton broth or agar. In addition, the standard inoculation, incubation, and interpretation procedures recommended for the full-range dilution methods should be followed.

Considering the limited range of drug concentrations tested, a greater number and variety of antimicrobial agents can be incorporated into a broth microdilution panel for breakpoint testing than into panels designed for full-range dilution testing (37). However, convenient quality control procedures to ensure that appropriate concentrations of each antimicrobial agent are present are lacking for breakpoint panels. One possible approach is to use one organism for which the modal MIC is equal to or no less than one doubling dilution less than the lower or lowest concentration tested and a second organism for which the modal MIC is equal to or no more than one doubling dilution greater than the higher or highest concentration tested (25). One of these two quality control organisms should provide on-scale results (25). Despite the theoretical soundness of this approach, routine quality control of breakpoint panels is difficult and not readily accomplished in the clinical laboratory.

Resistance Screens

In some circumstances, testing a single drug concentration may be a reliable and convenient method for detecting antimicrobial resistance. The most clinically useful resistance screens are those for resistance to oxacillin in *S. aureus,* resistance of *Enterococcus* spp. and *Staphylococcus* spp. to vancomycin, and high-level resistance to gentamicin and streptomycin in enterococci (25). These practical and reliable methods are described in chapter 74 of this Manual.

Gradient Diffusion Method

The E test (AB Biodisk, Solna, Sweden) is a commercial method for quantitative antimicrobial susceptibility testing

that incorporates a preformed antimicrobial gradient applied to one side of a plastic strip to provide drug diffusion into an agar medium. The test is performed in a manner similar to disk diffusion testing, in that a 0.5 McFarland standard suspension of a test isolate is generally swabbed onto the agar surface for inoculation. Following incubation, the MIC is read directly from a scale on the top of the E-test strip at the point where the ellipse of organism growth inhibition intercepts the strip. Several strips, each containing a different antimicrobial agent, can be placed radially on the surface of a large round Mueller-Hinton agar plate, or they can be placed in opposing directions on large rectangular plates. MICs determined by this method generally agree well with MICs generated by standard broth or agar dilution methods (6, 48, 54). The E test combines the simplicity and flexibility of the disk diffusion test with the ability to determine MICs of up to five antimicrobial agents on a single large agar plate. However, E-test strips are much more expensive than the paper disks used for diffusion testing. Strengths of the E-test method include the simplicity of the procedure itself and the ability to determine an MIC of an infrequently tested drug and to test fastidious or anaerobic bacteria by applying the strips onto specialized enriched media (see chapters 75 and 76 of this Manual).

QUALITY CONTROL

Quality control recommendations are designed for evaluation of the precision and accuracy of test procedures, monitoring of reagent performance, and evaluation of the performance of the individuals who are conducting the tests.

Reference Strains

A critical element in accomplishing the goals of quality control is the selection and use of reference bacterial strains that are genetically stable and for which MICs are in the midrange of MICs of each antimicrobial agent tested (25). That is, the dilutions in a series should ideally encompass at least two concentration increments above and below the previously established MIC for the reference strain. If there are four or fewer dilutions in a series or if nonconsecutive dilutions are tested (e.g., in breakpoint susceptibility testing), quality control for the correct interpretive category only rather than actual MIC ranges may be accomplished. *Escherichia coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, and *S. aureus* ATCC 29213 are the recommended reference strains for both agar and broth dilution methods (25). The beta-lactamase-producing strain *E. coli* ATCC 35218 is recommended only for penicillin–beta-lactamase inhibitor combinations (25). These organisms may be obtained from the American Type Culture Collection or other reliable commercial sources. For proper storage and subculture procedures, the recommendations of either the CLSI (25) or the commercial provider should be followed.

MIC Ranges

The acceptable quality control MIC ranges for the various reference strains are given in the CLSI document on dilution testing (25). Updates of these MIC ranges are published annually (27) and should be readily available in each clinical laboratory. An out-of-control result is defined as an MIC not within the acceptable range of values. Certain out-of-control results can be directly related to the medium used for testing. High MICs of gentamicin for *P. aeruginosa* ATCC 27853 suggest an inappropriately high divalent cation content or excessively low pH of the Mueller-Hinton medium, and low MICs indicate an insufficient divalent cation concentration or elevated pH. Although trimethoprim-sulfamethoxazole is not recommended for therapy of *Enterococcus faecalis* infections, results obtained with the ATCC 29212 strain can be useful for detecting excessive amounts of substances such as thymidine that interfere with the in vitro activity of antifolate drugs. Trimethoprim-sulfamethoxazole MICs of >0.5 to 9.5 μg/ml indicate the presence of such interfering substances (25).

Batch and Lot Quality Control

Representative plates, panels, or trays from each new reagent batch if prepared in-house or from each new lot if obtained from a commercial source should be subjected to quality control and sterility testing. MICs obtained by testing reference quality control strains should be within acceptable CLSI ranges (27). If such accuracy is not achieved, the batch or lot should be rejected or patient results obtained with the antimicrobial agent(s) in question should not be reported (see below). Similarly, if selected uninoculated plates or trays fail the sterility check after incubation, the batch or lot should be rejected. In addition to these formal quality control procedures that use reference strains, careful review of susceptibility results obtained during daily testing of clinical isolates is important to identify aberrant or unusual susceptibility patterns possibly indicative of reagent or technical problems.

Quality Control Frequency

In addition to batch and lot testing, quality control tests should be performed daily, or at least every day that the plates or trays are being used to test clinical isolates. When quality control is performed on each day of testing, performance is considered satisfactory if no more than 3 out of 30 consecutive results for each drug-reference strain combination are outside the acceptable limits. If this frequency is exceeded, the laboratory must perform corrective action to determine the source of the error and to correct it as described below. However, if daily quality control testing does not reveal an excessive rate of errors, daily testing may be replaced by weekly testing as outlined below (25, 27).

To convert to a weekly quality control testing interval, each drug-reference strain combination is tested for 20 or 30 consecutive testing days to obtain a total of 20 to 30 MICs for each combination. If no more than 1 of 20 or 3 of 30 MICs per combination are outside the accuracy range, weekly testing may replace daily testing. During weekly testing, a single MIC outside the acceptable range requires that daily testing be performed for five consecutive days unless there is an obvious source of error (e.g., contamination, use of an incorrect reference strain or an incorrect inoculum density, testing of an incorrect antimicrobial agent, or use of an incorrect atmosphere for incubation). In such a circumstance, the quality control test need be repeated only once. If no obvious source of error is noted, but all five MICs for a problem drug-organism combination are within the accuracy range, weekly testing may be resumed. If one or more of the five MICs for the problem drug-organism combination are outside the accuracy range, daily testing must be initiated and further means to resolve the problem must be pursued. Returning to weekly testing requires again documenting 20 or 30 consecutive days with no more than one or three MICs outside the accuracy range. If more than the acceptable number of MICs for organism-drug combinations are outside the accuracy range, daily quality control testing must be continued while the problem is being resolved (25, 27).

DISK DIFFUSION TESTING

The disk diffusion method of susceptibility testing allows categorization of most bacterial isolates as susceptible, intermediate, or resistant to a variety of antimicrobial agents. To perform the test, commercially prepared filter paper disks impregnated with a specified single concentration of an antimicrobial agent are applied to the surface of an agar medium that has been inoculated with the test organism. The drug in the disk diffuses through the agar. As the distance from the disk increases, the concentration of the antimicrobial agent decreases logarithmically, creating a gradient of drug concentrations in the agar medium surrounding each disk. Concomitant with the diffusion of the drug, the bacteria that were inoculated onto the surface and are not inhibited by the concentration of the antimicrobial agent continue to multiply until a lawn of growth is visible. In areas where the concentration of the drug is inhibitory, no growth occurs, forming a zone of inhibition around each disk.

The disk diffusion procedure has been standardized primarily for testing common, rapidly growing bacteria (10, 26). This method should not be used to evaluate antimicrobial susceptibilities of bacteria that show marked strain-to-strain variability in growth rates, e.g., some fastidious or anaerobic bacteria. The test, however, has been modified to allow reliable testing of certain fastidious bacteria (discussed in chapter 75 of this Manual).

The diameter of the zone of inhibition is influenced by the rate of diffusion of the antimicrobial agent through the agar, which may vary among different drugs depending upon the size of the drug molecule and its hydrophilicity. The zone size, however, is inversely proportional to the logarithm of the MIC, measured as discussed earlier in this chapter. Criteria currently recommended for interpreting zone diameters and MIC results for commonly used antimicrobial agents are listed in Table 3 and published annually by the CLSI (27).

Establishing Zone-of-Inhibition Diameter Interpretive Criteria

The first step in determining interpretive criteria for the disk diffusion test is selection of MIC breakpoints that define susceptibility and resistance categories for each antimicrobial agent. Zone-of-inhibition diameters that correspond to these breakpoints are initially established by testing 300 or more bacterial isolates by both dilution and disk diffusion methods and correlating zone-of-inhibition diameters and MICs determined for each drug tested (21). Isolates tested should include not only those commonly encountered in clinical laboratories but also those with resistance mechanisms pertinent to the class of antimicrobial agent being tested (21). Organisms evaluated should be those most likely to be tested with the antimicrobial agent in question. The data from these studies are analyzed by preparing a scattergram of values (see the example in chapter 72). By convention, each MIC ($\log_2$ scale) is plotted on the *y* axis, and the corresponding zone-of-inhibition diameter (arithmetic scale) is plotted on the *x* axis. Regression analysis can be performed, and a straight regression line showing the best fit is drawn. From this line, an approximation of the MIC can be inferred from any zone-of-inhibition diameter. For antimicrobial agents to which isolates are either susceptible or resistant and only infrequently intermediate, regression analysis is not valid. In such cases, the data are plotted as a scattergram and the interpretive standards are selected so as to allow optimal separation of resistant and susceptible populations (19, 21, 67). This approach, often called the error-rate-bounded method, may also be employed to minimize interpretive errors that can ensue from strictly applying the linear regression formula to a data set (21).

Antimicrobial Agent Disks

The amounts of antimicrobial agents in the disks used for agar diffusion testing are standardized, and in the United States, only a single disk for each drug is recommended (54). The optimal amount of an antimicrobial agent per disk is determined early in the development of a new drug by testing disks with several different drug contents that can be evaluated by using scattergrams and regression lines (21). The most desirable concentration of a drug per disk is that which produces a zone-of-inhibition diameter of at least 10 mm with resistant isolates and a zone diameter of no larger than 30 mm with susceptible isolates.

Commercially prepared antimicrobial disks usually are supplied in separate containers, each with a desiccant. They must not be used beyond the specified expiration date and should be stored under refrigeration (2 to 8°C) or frozen in a non-frost-free freezer at −20°C or colder until needed. Disks containing a beta-lactam agent should always be stored frozen to ensure that they retain their potency, although a small supply may be stored in the refrigerator for up to 1 week. Unopened disk containers should be removed from the refrigerator or freezer 1 to 2 h before use. This allows the disks to equilibrate to room temperature before the container is opened, thus minimizing the amount of condensation that will occur when warm air contacts the cold disks. A commercially available, mechanical disk-dispensing apparatus can be used and should be fitted with a tight cover, supplied with an adequate desiccant, stored in the refrigerator when not in use, and warmed to room temperature before being opened.

Agar Medium

The recommended medium for disk diffusion testing in the United States is Mueller-Hinton agar (26). This unsupplemented medium has been selected by the CLSI for several reasons: (i) it demonstrates good batch-to-batch reproducibility for susceptibility testing; (ii) it is low in sulfonamide, trimethoprim, and tetracycline inhibitors; (iii) it supports the growth of most nonfastidious bacterial pathogens; and (iv) years of data and clinical experience regarding its performance have been accrued. Fastidious bacteria, such as *Haemophilus* species, *Neisseria gonorrhoeae, Neisseria meningitidis,* and streptococci, do not grow satisfactorily on unsupplemented Mueller-Hinton agar but can be tested by the disk method by using supplemented or modified test media as discussed in chapter 75 of this Manual.

Plates of Mueller-Hinton agar may be purchased, or the agar may be prepared from a commercially available dehydrated base according to the manufacturer's directions. If the agar is prepared, only formulations that have been tested according to and have met acceptance limits recommended by the CLSI should be used (22). The prepared medium is autoclaved and immediately placed in a 45 to 50°C water bath. When cool, it is poured into round plastic flat-bottomed petri dishes on a level surface to give a uniform depth of about 4 mm (60 to 70 ml of medium for 150-mm-diameter plates and 25 to 30 ml for 100-mm-diameter plates) and allowed to cool to room temperature. Agar deeper than 4 mm may cause false resistance results (excessively small zones), whereas agar less than 4 mm deep may be associated with excessively large zones and false susceptibility.

Each batch of Mueller-Hinton agar should be checked when the medium is prepared to ensure that the pH is between 7.2 and 7.4 at room temperature, which means that the pH must be measured after the medium has solidified. This can be done by allowing a small amount of agar to solidify around the tip of a pH electrode in a beaker or a cup, by macerating a sufficient amount of agar in neutral distilled water, or by using a properly calibrated surface electrode. A pH outside the range of 7.2 to 7.4 may adversely affect susceptibility test results. If the pH is too low, drugs such as the aminoglycosides, macrolides, and fluoroquinolones will appear to lose potency whereas others (for example, the penicillins and tetracyclines) may appear to have excessive activity. The opposite effects are possible if the pH is too high.

Freshly prepared plates may be used the same day or stored in a refrigerator (2 to 8°C); if refrigerated, they should be wrapped in plastic to minimize evaporation. Just before use, if excess moisture is visible on the surface, plates should be placed in an incubator (35°C) or, with lids ajar, in a laminar-flow hood at room temperature until the moisture evaporates (usually 10 to 30 min). At the time the medium is to be inoculated, no droplets of moisture should be visible on its surface or on the petri dish cover.

Various components of or supplements to Mueller-Hinton medium may affect susceptibility test results; therefore, appropriate quality control procedures (see "Quality Control" below) must be performed and zone diameters must be within acceptable limits. For example, media containing excessive amounts of thymidine or thymine can reverse the inhibitory effects of sulfonamides and trimethoprim, causing zones of growth inhibition to be smaller or less distinct. Organisms may therefore appear to be resistant to these drugs when in fact they are not. Variation in the concentrations of divalent cations, primarily calcium and magnesium, affects results of aminoglycoside and tetracycline tests with *P. aeruginosa* isolates (26). A cation content that is too high reduces zone sizes, whereas a cation content that is too low has the opposite effect. Sheep blood should not be added to Mueller-Hinton medium for testing of nonfastidious organisms, because the blood can significantly alter the zone diameters with several agents and bacterial species (15).

Inoculation Procedure

To ensure reproducibility of disk diffusion susceptibility test results, the inoculum must be standardized (10, 26). The inoculum may be prepared by the growth method or by direct suspension from colonies on the agar plate, as described above for dilution testing.

When trimethoprim-sulfamethoxazole is tested by the direct inoculum suspension method, colonies from blood agar medium may carry over enough trimethoprim or sulfonamide antagonists to produce a haze of growth inside the zones of inhibition with susceptible isolates.

The Mueller-Hinton agar plate should be inoculated within 15 min after the inoculum suspension has been adjusted. A sterile cotton swab is dipped into the suspension, rotated several times, and gently pressed onto the inside wall of the tube above the fluid level to remove excess inoculum from the swab. The swab is then streaked over the entire surface of the agar plate three times, with the plate rotated approximately 60° each time to ensure even distribution of the inoculum. A final sweep of the swab is made around the agar rim. The lid may be left ajar for 3 to 5 min but no longer than 15 min to allow any excess surface moisture to be absorbed before the drug-impregnated disks are applied.

Antimicrobial Disks

Within 15 min after the plates are inoculated, selected antimicrobial agent disks are distributed evenly onto the surface, with at least 24 mm (center to center) between them. Disks are placed individually with sterile forceps or more commonly with a mechanical dispensing apparatus and then gently pressed down onto the agar surface to provide uniform contact. No more than 12 disks should be placed onto one 150-mm-diameter plate and no more than 5 disks should be placed onto a 100-mm-diameter plate to avoid overlapping zones. Some of the antimicrobial agent in the disk diffuses almost immediately; therefore, once a disk contacts the agar surface, the disk should not be moved.

Incubation

No longer than 15 min after disks are applied, the plates are inverted and incubated at 35°C in ambient air. A delay of more than 15 min before incubation permits excess prediffusion of the antimicrobial agents. The interpretive standards for nonfastidious bacteria are based on results of test samples incubated in ambient air, and the zone-of-inhibition diameters for some drugs, such as the aminoglycosides, macrolides, and tetracyclines, are significantly altered by CO_2; therefore, plates should not be incubated in atmospheres with increased CO_2. Testing isolates of some fastidious bacteria, however, requires incubation in 5% CO_2, and zone diameter criteria for those species have been established on that basis (see chapter 75 of this Manual).

Interpretation and Reporting of Results

Each plate is examined after incubation for 16 to 18 h for all nonfastidious bacterial isolates except staphylococci and enterococci, which must be incubated for a full 24 h to allow detection of resistance to oxacillin and vancomycin (26). If plates are inoculated correctly, the diameters of the zones of inhibition are uniformly circular and the lawns of growth are confluent. Growth that consists of individual isolated colonies indicates that the inoculum was too light, and the test must be repeated. The diameters of the zones of complete inhibition, including the diameter of the disk, are measured to the nearest whole millimeter with calipers or a ruler. With unsupplemented Mueller-Hinton agar, the measuring device is held on the back of the inverted petri dish, which is illuminated with reflected light located a few inches above a black, nonreflecting background.

The zone margin is the area where no obvious growth is visible. When isolates of staphylococci or enterococci are tested, any discernible growth (especially a haze of pinpoint colonies) within the zone of inhibition around the oxacillin disk (for staphylococci) or vancomycin disk (for enterococci) is indicative of resistance. For other bacteria, discrete colonies growing within a clear zone of inhibition may indicate testing of a mixed culture that should be subcultured, reidentified, and retested. However, the presence of colonies within a zone of inhibition may also indicate selection of high-frequency mutants indicative of eventual resistance to that agent, e.g., *Enterobacter* spp. with penicillins and cephalosporins. With *Proteus* species, if a thin film of swarming growth is visible in an otherwise obvious zone of inhibition, the margin of heavy growth is measured and the film is disregarded. With trimethoprim, the sulfonamides, and combinations of the two agents, antagonists in the medium may allow some minimal growth; therefore, the zone diameter is measured at the obvious margin, and slight growth (20% or less of the lawn of growth) is disregarded.

The zone diameters measured around each disk are interpreted on the basis of guidelines published by the CLSI, and the organisms are reported as susceptible, intermediate, or resistant to the antimicrobial agents tested (Table 3) (27). The clinical interpretation of the categories of susceptible, intermediate, and resistant has already been provided above under "Dilution Methods." Computer programs are available that accompany some automated zone size reading devices to allow MICs to be derived from the linear regression equation with selected antimicrobial agents and bacterial isolates (14, 63) (see also chapter 17).

Advantages and Disadvantages

The disk diffusion test has several advantages: (i) it is technically simple to perform and very reproducible, (ii) the reagents are relatively inexpensive, (iii) it does not require any special equipment, (iv) it provides susceptibility category results that are easily understood by clinicians, and (v) it is flexible regarding selection of antimicrobial agents for testing. The primary limitation of the disk diffusion test is the spectrum of organisms for which it has been standardized. There have not been adequate studies to develop reliable interpretive standards for disk testing of bacteria not listed in the CLSI disk diffusion document (26) or the recently developed CLSI guideline for infrequently isolated or fastidious bacteria (24). It is also important to note that only certain drugs have been validated for disk diffusion testing of *Stenotrophomonas maltophilia* and *Burkholderia cepacia* (27). The disk test is inadequate for detection of vancomycin-intermediate *S. aureus* (26, 80, 81), does not detect daptomycin resistance in staphylococci and enterococci (45, 72) or colistin resistance in gram-negative bacilli (44), and in the past was reported to have difficulties in the detection of oxacillin-heteroresistant staphylococci (33, 60) and enterococci with low-level (VanB-type) vancomycin resistance (71, 81). A potential disadvantage of disk diffusion susceptibility testing is that it provides only a qualitative result and a quantitative result indicating the degree of susceptibility (MIC) may be needed in some cases, e.g., those involving penicillin and cephalosporin susceptibilities of *S. pneumoniae* and certain viridans group streptococci (see chapter 75 of this Manual).

Quality Control

The goals of a quality control program for disk diffusion are to monitor the precision and accuracy of the procedure, the performance of the reagents (medium and disks), and the performance of persons who do the testing and read, interpret, and report results. To best achieve these goals, reference strains are selected for their genetic stability and their usefulness in the disk diffusion test.

Reference Strains

Reference strains recommended by the CLSI for quality control of the disk diffusion procedure when nonfastidious bacteria are tested are *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25923 (not the same strain used for quality control of MIC tests), *Enterococcus faecalis* ATCC 29212, and *E. coli* ATCC 35218 (27, 28). *E. coli* ATCC 35218 is recommended as a control only for beta-lactamase inhibitor combinations containing clavulanic acid, sulbactam, or tazobactam. *Enterococcus faecalis* ATCC 29212 can be used to ensure that the levels of inhibitors of trimethoprim or sulfonamides in Mueller-Hinton agar do not exceed acceptable limits and can also be used to control disks containing a high concentration of gentamicin or streptomycin (see chapter 74 of this Manual).

The reference strains listed above should be obtained from a reliable source, and stock cultures should be maintained in such a way that viability is ensured and the opportunity for selection of resistant variants is minimal (28). The procedures for maintaining and storing working stock cultures are described in the CLSI standard (26). If an unexplained result indicates that the inherent susceptibility of the strain has been altered, a fresh subculture of that organism should be obtained.

Zone-of-Inhibition Diameter Ranges

The ranges of zone diameters for reference strains used to monitor performance of the disk diffusion test are updated frequently and published annually; therefore, readers should refer to the most recent CLSI document for this information (27). Generally, results of 1 in every 20 tests in a series of tests might be out of the accepted limits. If a second result falls outside the stated limits, corrective action must be taken. The action taken and the results of that action must be documented.

Frequency of Testing

Each new batch or lot of Mueller-Hinton agar must be tested with the reference strains listed above before the medium is released for use with clinical specimens, and quality control must be done before a new lot of antimicrobial disks is introduced. Appropriate reference strains also should be tested each day that the disk diffusion test is performed. The frequency of testing, however, may be reduced if satisfactory performance is documented for 20 or 30 consecutive days of testing: for each combination of drug and reference strain, no more than 1 of 20 or 3 of 30 zone-of-inhibition diameters may be outside the accepted limits published by the CLSI (27). When this criterion is fulfilled, each reference strain need be tested only once per week and any time a reagent component of the test is changed. However, if a zone-of-inhibition diameter falls outside the acceptable control limits, corrective action must be taken. If the problem appears to be caused by an obvious error such as use of the wrong disk or the wrong reference strain, contamination of the reference strain, or incubation in an incorrect atmosphere, repeating the test with the appropriate parameter is acceptable. However, if a cause of the error is not obvious, quality control must be performed daily for a period that will allow discovery of the source of the aberrant result and documentation of how the problem was resolved. This may be accomplished by the same approach described above under "Quality Control" in "Dilution Methods."

Special Disk Tests

Two specialized applications of the disk test are described in detail in chapter 74. In brief, disk testing with cefoxitin is now the preferred method for detection of *mecA*-mediated oxacillin resistance in both *S. aureus* and coagulase-negative staphylococcal species (26, 27, 78). Cefoxitin serves as a surrogate marker for the principal mechanism of oxacillin resistance in staphylococci and provides more reliable results than oxacillin itself. Secondly, inducible clindamycin resistance is not reliably detected by standard dilution or disk diffusion susceptibility testing without induction of the expression of *erm*-mediated macrolide-lincosamide-streptogramin B resistance in staphylococci and hemolytic streptococci (61). Such strains can be accurately detected only by induction of resistance expression by exposure to a macrolide. A disk approximation test in which erythromycin and clindamycin disks are placed in close proximity allows

recognition of inducible resistance by truncating the clindamycin zone and giving rise to a positive "D-zone test" (27, 42). When recognized through disk approximation testing, such strains should be reported as resistant to clindamycin (27, 61). A similar approach may be taken by incorporating a subinhibitory concentration of erythromycin into broth or agar dilution tests with clindamycin, although this approach is not as convenient as the use of the disk approximation method.

ANTIBACTERIAL SUSCEPTIBILITY TESTING METHODS THAT MAY BE USED OUTSIDE OF THE UNITED STATES

The CLSI is best known for developing laboratory testing standards for use in the United States, including those for antimicrobial susceptibility testing (25, 26, 27). The CLSI standards are recognized as U.S. national standards by the American National Standards Institute and by federal regulations, including the Clinical Laboratory Improvement Amendments (46), and as standard reference procedures by the Food and Drug Administration. However, the CLSI procedures are also used by an increasing number of laboratories outside of the United States, including countries in North and South America and in several areas of Europe, Asia, and Australasia. Some countries have national standards or professional committees comprising their own expert microbiologists that establish methods of susceptibility testing for their countries and interpretive criteria for those tests that may or may not be the same as those of the CLSI (17, 41), as described further in chapter 72.

Several variations on dilution and diffusion methods are used for routine susceptibility testing outside the United States (Table 4). Most non-U.S. methods are specific to individual countries, having developed and evolved locally over many years. Many of the non-U.S. methods differ from the CLSI procedures in the choice of media, inoculum preparation procedures, and, for diffusion methods, disk contents. There are also some variations between these methods in breakpoints and the approaches to establishing the breakpoints (41, 69). Variation in test methods can cause considerable confusion in laboratories, especially if both CLSI and non-CLSI methods are used for different organisms. Thus, it is important that any method should be followed in all its detail for valid application of specific breakpoints.

In the past, there have been limited efforts at harmonizing breakpoints internationally (88, 89). The Europeans through the European Society of Clinical Microbiology and Infectious Diseases have formed the European Union Committee on Antimicrobial Susceptibility Testing (EUCAST). EUCAST has defined reference agar and broth microdilution MIC methods (39, 40) for use in Europe. One encouraging prospect for the future is the establishment for the first time of a global standard reference MIC susceptibility method (broth microdilution) developed through the International Organization for Standards (ISO; Geneva, Switzerland) (50) in concert with the European Committee on Standardization (Brussels, Belgium). The global reference method is in essence the CLSI and EUCAST broth microdilution methods with a few differences harmonized in the ISO standard (50). This will hopefully provide a degree of international standardization for reference MIC determinations and allow a benchmark for assessment of the performance of commercial devices for susceptibility testing. In a second document, the ISO has established criteria for acceptable performance of susceptibility testing devices (51).

International Dilution Methods

Both broth and agar dilution methods have been developed in countries outside the United States. In the past, methods using a limited range of concentrations (often one or two), or so-called breakpoint methods, have been advocated (87). They are the standard form of susceptibility testing advocated by the Japanese Society for Chemotherapy (52, 53) and as an alternative to disk diffusion testing by the British Society for Antimicrobial Chemotherapy (BSAC) (1, 16). Reasonably current published methods and standards include those of the BSAC (1; see also http://www.bsac.org.uk), the Société Française de Microbiologie (CA-SFM; http://www.sfm.asso.fr), the Deutsches Institut für Normung (DIN; documents are available through http://www.beuth.de), and the Swedish Reference Group for Antibiotics (SRGA; http://www.srga.org).

Breakpoint methods may be popular in larger laboratories because large numbers of isolates can be tested cost-effectively using replicators and the methods provide susceptibility category (i.e., qualitative) endpoints. Optical readers are available to facilitate reading of agar dilution plates (e.g., Mastascanelite; Mast Laboratories, Bootle, United Kingdom; see http://www.mastascan.com). However, there are considerable difficulties with quality control, including the lack of

TABLE 4 Non-U.S. disk diffusion methods for susceptibility testing

Method (reference)	Country	Society	Agar medium	Comments
BSAC (2)	United Kingdom	BSAC	Iso-Sensitest	
CA-SFM (65)	France	CA-SFM	Mueller-Hinton	Similar to CLSI
DIN (34, 35)	Germany	DIN	Mueller-Hinton	
SRGA (68, 70)	Sweden	SRGA	Iso-Sensitest or Mueller-Hinton	
Neo-sensitabs	Denmark		Mueller-Hinton	Uses antimicrobials incorporated into compressed tablets rather than paper disks. Size of tablets results in substantially larger zones than conventional 6-mm-diam disks.
Calibrated dichotomous sensitivity (12, 13)	Australia		Sensitest	Dichotomous (no intermediate category). Disk strengths chosen to give annular radius of inhibition of ~6 mm where possible.

appropriate control strains for which the MICs are near the breakpoints and the complexity of quantifying drug concentrations prior to use (64). In addition, problems have been reported in the past with the use of Iso-Sensitest agar (3, 84) and with the incorporation of inhibitors to prevent swarming of *Proteus* spp., such as *p*-nitrophenylglycerol (85), or increased agar content for the same purpose (86). In turn, the choice of Mueller-Hinton by the CLSI has been criticized for not providing luxuriant growth of all organisms (88), suggesting that there is no absolutely ideal medium. As the best-studied, most widely used medium for susceptibility testing, Mueller-Hinton broth was chosen for the ISO reference method (50).

International Diffusion Methods

A wide variety of diffusion methods have been developed in different countries over the years. They are quite diverse in their approaches. Almost all have been maintained primarily because of the widespread popularity of disk testing. None appear to offer any major advantage over the modified Kirby-Bauer method (10) advocated by the CLSI (26). As pointed out above, disk methods are inexpensive and flexible and may become more popular through the use of zone readers and interconnected computers for interpretation of zone diameters (4, 63).

The previously recommended BSAC standard diffusion methods and the comparative and Stokes' methods differed from other diffusion methods in that susceptibility categorization was achieved by comparison with a control strain rather than by reference to a defined set of zone diameters (16). This technique attracted criticism because it was not based upon or derived from correlations with MICs (18) and has now been replaced by a correlated diffusion method (2) which is now well developed and kept up-to-date (http://www.bsac.org.uk/susceptibility_testing.cfm). For the new BSAC method, Iso-Sensitest (Oxoid, Basingstoke, United Kingdom) is the recommended agar, supplemented for fastidious bacteria with whole defibrinated horse blood with or without NAD. Mueller-Hinton supplemented with 5% sodium chloride is recommended for the detection of methicillin and oxacillin resistance in staphylococci. The inoculum is prepared to produce semiconfluent growth only, rather than the confluent growth lawn used in the CLSI method. One important change in the newer British method is the elimination of an intermediate category for most organism-antimicrobial agent combinations (62).

Since 1980, the CA-SFM has put considerable effort into standardization of susceptibility testing, including regular updates with breakpoints for new drugs that are published frequently (65; see also http://www.sfm.asso.fr). Like the CLSI, the CA-SFM has selected Mueller-Hinton as the test medium. For diffusion testing, plates can be inoculated by flooding as well as swabbing. In most other aspects, this method resembles that of the CLSI, including the use of control organisms and the choice of disk strength. The CA-SFM provides zone diameter breakpoints for drugs available in France and elsewhere that are not approved for clinical use in the United States, e.g., fusidic acid and pristinamycin (65).

The German standards organization, DIN, published acceptable methods for diffusion susceptibility testing as early as 1979, with irregular updates since then (34, 35). They too use Mueller Hinton agar but will tolerate the use of other media provided that the MIC-zone diameter relationships have been determined for those media. Like the CA-SFM method, the DIN method has much in common with the CLSI method.

The SRGA (68, 77) method uses Iso-Sensitest, although the SRGA also accepts Mueller-Hinton as an alternative. The SRGA system is based on the methodology developed by the original International Collaborative Study (38) that was the first to provide a sound theoretical basis to diffusion susceptibility testing. The breakpoints for susceptibility were restructured in 1981 into the more conventional susceptible, intermediate, and resistant categories (77) and are updated regularly (70).

A method developed by a commercial firm in Denmark differs technically if not in principle from the other methods. This method employs so-called Neo-sensitabs that are compressed tablets 9 mm in diameter into which the antibiotic has been incorporated (Rosco Diagnostica, Taarstrup, Denmark; http://www.rosco.dk). The method and the interpretive zone diameter criteria are updated and published by the manufacturer periodically. Not only are the tablets larger and thicker than conventional 6-mm-diameter paper disks, they usually contain larger amounts of antibiotic, resulting in significantly larger zones of inhibition with most drugs. This has the disadvantage of reducing the number of tablets that can be put on a single plate and still produce readable zones. However, the system does have the advantage that the tablets can be stored at room temperature for up to 4 years, obviating the need for storage under refrigeration or freezing. This is an obvious benefit for laboratories in developing countries where reliable refrigeration and power can be a problem.

A diffusion method developed in Australia in 1975 (12, 13) and still widely used in that country has a number of unique features. The calibrated dichotomous sensitivity method employs Sensitest agar and an unusual method for inoculum preparation and is unique in defining just two categories of susceptibility: susceptible and resistant. In order to simplify test result reading, each new drug is calibrated against the MIC breakpoint to yield wherever possible a zone diameter of 18 mm. This is achieved by adjusting disk strengths, which in most cases are substantially lower than those used with other methods. The lack of an intermediate category, which increases the risk of serious interpretive errors (e.g., susceptible instead of resistant), the absence of some common drugs from the test range, and some unusual use of surrogate drugs for testing have restricted the adoption of this method outside of Australia. The interpretative criteria are updated regularly and can be found on the Web (http://www.med.unsw.edu.au/pathology-cds/).

Non-CLSI Breakpoints

Each international method has, of necessity, had to develop its own breakpoints because of differences in media, inocula, and, in the case of disk diffusion methods, disk strengths. In addition, some non-U.S. organizations have developed specialized methods for setting breakpoints. The BSAC has developed a formula based on maximum blood concentrations, protein binding, and elimination half-life. This is now integrated with other considerations including pharmacodynamics and clinical outcome data (69). The Japanese Society for Chemotherapy uses both a formula and a comparison with favorable outcome in clinical studies where the MICs for the pathogens have been determined (5, 52, 53, 74, 75). The Spanish Society for Chemotherapy has also published breakpoints which tend to be more conservative as they are based primarily on MIC distributions (66). EUCAST has recently set about the task of establishing breakpoints for Europe (see http://www.eucast.org). A feature of the program has been the collation of large amounts of MIC data to establish wild-type strain distributions and cutoff values as a

prelude to formal breakpoint setting (59). EUCAST integrates wild-type strain distributions and pharmacokinetic-pharmacodynamic information in a manner similar to that used recently by the CLSI. As dosages used in Europe can be different from those used in the United States, some breakpoints will differ from those set by the CLSI. Most other groups have set breakpoints without major reference to pharmacokinetics or pharmacodynamics.

COMMON SOURCES OF ERROR IN ANTIBACTERIAL SUSCEPTIBILITY TESTING

Potential sources of error in antibacterial susceptibility testing may be categorized as those that relate to the test system and its components, those associated with the test procedure, those peculiar to certain organism and drug combinations, and those that relate to reporting (34, 60). The most common sources of error encountered in clinical microbiology laboratories are reviewed in the following paragraphs.

Various components of the susceptibility test system may be a source of error. First, the system itself may have limitations regarding the organisms that should be tested. For example, the disk diffusion method should be used only to test rapidly growing bacterial pathogens that have consistent growth rates (those for which interpretive criteria have been developed and published by the CLSI). Second, the medium used may be a source of error if it fails to conform to recommended composition and performance. Factors common to both agar-based and broth-based systems are the pH of the medium, which for Mueller-Hinton agar or broth should be between 7.2 and 7.4, and its cation content. The concentration of magnesium and calcium in the broth medium should be that recommended by the CLSI to ensure reliable results. For detection of oxacillin resistance in staphylococci by testing of oxacillin, it is essential that the proper amount of sodium chloride be included in the agar or broth used for dilution testing. For agar dilution and disk diffusion, the Mueller-Hinton agar should be 3 to 4 mm deep. Third, the components of the system (antimicrobial disks, agar plates, and trays) must be stored properly, and they should not be used beyond the stated expiration dates.

Steps in the susceptibility test procedure that may be a source of error if they are not performed correctly include inoculum preparation, incubation (conditions and duration), endpoint interpretation, and performance of appropriate quality control. The inoculum must be pure, and it must contain an adequate density of bacteria. With rare exceptions, all systems should be incubated in ambient air at 35°C. The incubation time, however, varies. For conventional dilution and disk diffusion systems, incubation for 16 to 20 h and 16 to 18 h, respectively, is recommended except in tests of staphylococci with oxacillin and vancomycin and enterococci with vancomycin, in which systems must be incubated for a full 24 h (25, 26, 82). The endpoints for all susceptibility tests must be measured accurately, following guidelines published by the CLSI (25, 26). If endpoints are interpreted by an instrument, the reliability of that instrument must be monitored. Moreover, with all susceptibility test systems, appropriate reference strains must be tested at regular intervals, any problems that occur must be thoroughly investigated, and corrective action must be well documented.

Testing of certain antimicrobial agents with some bacteria may yield misleading results, because in vitro results do not necessarily correlate with in vivo activity. Examples include narrow- and expanded-spectrum cephalosporins and aminoglycosides tested with *Salmonella* spp. and *Shigella* spp.; all beta-lactam agents except the penicillinase-resistant penicillins (oxacillin, nafcillin, methicillin, or cefoxitin as a surrogate marker) tested with oxacillin-resistant staphylococci; cephalosporins, aminoglycosides (except concentrations used to detect high-level resistance), clindamycin, and trimethoprim-sulfamethoxazole tested with enterococci; and cephalosporins tested with *Listeria* spp. (25, 26, 27). Therefore, for these combinations of organisms and drugs, results should not be reported. Other potential problems associated with reporting are possible transcriptional errors for laboratories that use a manual recording and reporting system and possible errors in transmission of data for laboratories in which an automated susceptibility test system is interfaced with the laboratory and/or hospital information system.

PROBLEM ORGANISMS AND RESISTANCE MECHANISMS

The dilution and diffusion methods described in this chapter have been developed through careful studies and standardized by national professional organizations and diagnostic device manufacturers. Despite this, there are still some organisms for which methods have not yet been standardized (e.g., corynebacteria and some fastidious bacteria in the case of disk testing) or which fail to provide reliable results with some of the standard tests (e.g., *Stenotrophomonas maltophilia* and *Burkholderia cepacia* in the case of disk diffusion testing of some drugs). Certain other organisms may possess resistance mechanisms that are inducible (VanB-type resistance in some enterococci, ampC beta-lactamase in some gram-negative species) or that result in subtle phenotypic expression under standard inoculum and test conditions (e.g., inducible clindamycin resistance in staphylococci and some streptococci; extended-spectrum beta-lactamases in some gram-negative bacilli). Reliable detection of these subtle resistance traits may require the use of different or modified test methods that are outlined in chapter 74 of this Manual. The CLSI adopted revised MIC and disk diffusion breakpoints for testing coagulase-negative staphylococci with oxacillin in an attempt to avoid errors of false susceptibility (25, 26, 27, 79, 90). However, testing of cefoxitin with both *S. aureus* and coagulase-negative species has more recently been recommended by the CLSI as the simplest and most reliable method for detection of *mecA*-mediated oxacillin resistance (27, 78). The screening tests for detection of extended-spectrum beta-lactamases by using cefpodoxime have been expanded by the CLSI to include testing of *Proteus mirabilis* in addition to *E. coli* and *Klebsiella* spp. (27). There has not previously been uniform agreement regarding what level of accuracy is acceptable when selecting a testing method or system for performing antimicrobial susceptibility testing (55). Recently, general guidelines for acceptable performance (e.g., rates of essential agreement and category agreement) have been developed through an international consensus effort (ISO) (51). However, it is important to keep in mind that new resistance mechanisms or decreases in susceptibility to important therapeutic agents can arise at any time to challenge our methods of susceptibility testing, e.g., in the case of vancomycin-resistant or -intermediate *S. aureus* (20, 47, 80, 81). Thus, susceptibility testing methods must continue to evolve and develop over time as new challenges are presented.

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Special Phenotypic Methods for Detecting Antibacterial Resistance*

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Special phenotypic tests for detecting antibacterial resistance range from the rapid and simple spot β-lactamase test to the more time-consuming and complex minimal bactericidal concentration (MBC) assays. These tests may either supplement or replace traditional testing methods depending on the organism and the assay. This chapter describes tests for detection of high-level aminoglycoside resistance and acquired vancomycin resistance in enterococci; tests for detection of inducible clindamycin resistance in streptococci; tests for detection of oxacillin, vancomycin, and inducible clindamycin resistance in staphylococci; tests for detection of β-lactamases in multiple organisms; and tests for determination of bactericidal activity.

Quality control information is given for all of the tests in each section; however, guidelines for the frequency of quality control testing are not provided, because they have not been determined and may vary depending on laboratory circumstances. A practical approach would be to perform quality control testing each day clinical isolates are tested or less frequently (e.g., weekly) once a laboratory has thoroughly documented that less frequent quality control testing can validate the reliability of the procedures. However, the College of American Pathologists recommends that quality control testing be done daily on β-lactamase tests. Quality control tests should be performed each time new lots of material are put into use.

TESTS TO DETECT RESISTANCE IN ENTEROCOCCI

Systemic enterococcal infections, such as endocarditis, are commonly treated with a cell wall-active agent (either a β-lactam drug or a glycopeptide such as vancomycin) and an aminoglycoside (usually gentamicin or streptomycin). These agents act synergistically to enhance killing (175). However, when an enterococcal strain is resistant to the cell wall-active agent or has high-level resistance (HLR) to the aminoglycoside, there is no synergism and combination therapy does not provide a bactericidal effect (129). Because of this, it is important to determine the susceptibility to both the aminoglycoside and the cell wall-active agent individually in order to predict the likelihood of synergy. Methods for detection of aminoglycoside and vancomycin resistance are discussed here. A discussion of methods for detection of high-level penicillin and ampicillin resistance was included in the last edition of this Manual.

Detection of High-Level Resistance to Aminoglycosides

Because aminoglycosides have poor activity against enterococci (MICs range from 8 to 256 μg/ml), they cannot be used as single agents for therapy (75, 129). This intrinsic, moderate-level resistance is due to poor uptake of the aminoglycoside by the cell (129). Acquired aminoglycoside resistance in enterococci is due either to mutations resulting in decreased binding of the agent to the ribosome, as occurs with streptomycin (called ribosomal resistance), or, more commonly, to the acquisition of new genes that encode enzymes that modify aminoglycosides (called acquired resistance). Acquired aminoglycoside resistance usually corresponds to MICs that are significantly above the concentrations normally tested in routine susceptibility tests, e.g., ≥2,000 μg/ml for streptomycin and ≥500 μg/ml for gentamicin, and is designated HLR (129) (see also chapters 30 and 71).

Synergy between an aminoglycoside and a cell wall-active agent can be determined directly by performing complex time-kill studies (101) or can be predicted by using less cumbersome screening tests. Gentamicin and streptomycin are the only two agents that should be tested on a routine basis. All enterococcal isolates that are resistant to gentamicin are considered resistant to tobramycin and amikacin as well. Resistance to streptomycin is mediated by a different resistance mechanism, and consequently, streptomycin resistance must be determined separately from gentamicin resistance. Isolates of *Enterococcus faecium* are intrinsically resistant to the synergistic actions of amikacin, kanamycin, tobramycin, and netilmicin with cell wall-active agents, irrespective of in vitro testing results for HLR (125). *E. faecalis* strains that are susceptible to gentamicin may be resistant to kanamycin and amikacin. In vitro tests with amikacin cannot reliably predict HLR to amikacin in *E. faecalis,* but kanamycin may be used to predict HLR to amikacin and kanamycin (161), although optimal methods for testing have not been determined. The genes for two additional

* This chapter contains information presented in chapter 74 by Jana M. Swenson, Janet Fick Hindler, and James H. Jorgensen in the eighth edition of this Manual.

enzymes that mediate gentamicin resistance have been described, *aph2″-(Ic)* and *aph2″-(Id),* (28, 199), neither of which is detected using the screening methods described below since their presence seems to lead to intermediate, not high-level, resistance. Only time-kill or molecular studies can detect the presence of these enzymes. Methods for detection of HLR to aminoglycosides are summarized in Table 1 and discussed below.

Agar Dilution Screening Method

Agar plates are prepared with brain heart infusion (BHI) agar supplemented with 500 μg of gentamicin per ml or 2,000 μg of streptomycin per ml. The plates are inoculated by spotting 10 μl of a suspension that is equivalent to a 0.5 McFarland standard prepared from growth on an 18- to 24-h agar plate, giving a final inoculum of 10^6 CFU per spot. The plates are incubated for a full 24 h in ambient air. The presence of more than one colony or a haze of growth should be read as resistant. For streptomycin, the plates should be reincubated for an additional 24 h if there is no growth at 24 h. Mueller-Hinton agar (MHA), MHA plus 5% sheep blood, or dextrose phosphate agar may be substituted for BHI agar, but because growth is better on BHI agar, this is the preferred medium. Commercially prepared agar screen plates are available and have performed well (61, 157, 158). Kanamycin agar screen tests have not been as extensively evaluated and are not standardized, but it has been reported that for determining HLR to both amikacin and kanamycin in *E. faecalis,* kanamycin at 2,000 μg/ml in BHI agar can be used (161).

Broth Microdilution Screening Method

Broth dilution tests are prepared using a single well or a tube containing BHI broth supplemented with 500 μg of gentamicin per ml or 1,000 μg of streptomycin per ml. The final inoculum concentration is that recommended for routine broth microdilution testing, i.e., 5×10^5 CFU/ml. The plates are incubated for 24 h in ambient air. For streptomycin, the plates should be reincubated for an additional 24 h if there is no growth at 24 h. Any growth is interpreted as denoting resistance.

The recommended streptomycin concentration for use in the broth microdilution screen is one-half that used in the agar dilution screen test. Because this test is often included as part of a routine gram-positive MIC panel, the inoculum is that commonly used in broth microdilution testing (5×10^5 CFU/ml). The total number of cells tested in the agar dilution screening procedure (10^6 CFU/spot) is 20-fold larger than that normally used in the broth microdilution test (5×10^4 CFU/0.1-ml well). In order to provide a test that uses a low inoculum and at the same time maximizes the detection of HLR to streptomycin, it was necessary to lower the concentration recommended for testing streptomycin from 2,000 to 1,000 μg/ml in the broth microdilution test. Because of poorer growth and the lower inoculum, Mueller-Hinton broth is inadequate for use in the broth microdilution screen test (183). The performance of other aminoglycosides in this test has not been evaluated.

Disk Diffusion Screening Method

The standard disk diffusion procedure (37) described in chapter 73 (with unsupplemented MHA) is used, except that special high-content disks (gentamicin at 120 μg and streptomycin at 300 μg) are required (162). Zones are measured after 18 to 24 h of incubation in ambient air at 35°C. Isolates for which the zone diameters are ≥10 mm are categorized as susceptible. The absence of a zone of inhibition corresponds to the presence of HLR. Strains for which the zones of inhibition are 7 to 9 mm usually display HLR, but a few are strains for which the MICs are only moderately elevated (183). Therefore, strains for which the zone diameters are 7 to 9 mm should be tested by either the standard agar or the broth microdilution screen method to determine susceptibility or resistance. High-content gentamicin and streptomycin disks are available commercially.

Quality Control

For both gentamicin and streptomycin, *E. faecalis* ATCC 29212 is used as the susceptible control and *E. faecalis* ATCC 51299 is used as the resistant control strain (182).

TABLE 1 Screening methods for detecting vancomycin and high-level aminoglycoside resistance in enterococci

Parameter	Screening procedure			
	Vancomycin agar dilution	Aminoglycoside agar dilution	Aminoglycoside broth microdilution	Aminoglycoside disk diffusion
Medium	BHI agar	BHI agar	BHI broth	MHA
Inoculum	10^5–10^6 CFU/spot	10^6 CFU/spot	5×10^4 CFU/0.1ml	0.5 McFarland[a]
Incubation (h)	24	24[b]	24[b]	18–24
Drug concn				
Gentamicin	NA	500 μg/ml	500 μg/ml	120 μg/disk
Streptomycin	NA	2,000 μg/ml	1,000 μg/ml	300 μg/disk
Vancomycin	6 μg/ml	NA[c]	NA	NA
Endpoint	>1 colony	>1 colony	Any growth	6 mm = resistant, 7–9 mm = inconclusive,[d] ≥10 mm = susceptible

[a] CLSI disk diffusion method (37).
[b] If streptomycin is negative at 24 h, reincubate for an additional 24 h.
[c] NA, not applicable.
[d] If the zone is 7 to 9 mm, the test is inconclusive, and an agar or broth microdilution test should be performed to confirm susceptibility or resistance.

Only *E. faecalis* ATCC 29212 is used for control of disk diffusion tests. The expected quality control limits are 16 to 23 mm for gentamicin (120-μg) disks and 14 to 20 mm for streptomycin (300-μg) disks (38).

Detection of Vancomycin Resistance in Enterococci

As defined by the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS), the MIC interpretive criteria for vancomycin are ≤4 μg/ml for susceptible, 8 to 16 μg/ml for intermediate, and ≥32 μg/ml for resistant. The three most common phenotypes of resistance are (i) high-level vancomycin resistance (MICs, ≥64 μg/ml) with accompanying teicoplanin resistance (MICs, ≥16 μg/ml) (VanA phenotype); (ii) moderate- to high-level vancomycin resistance (MICs, 16 to 512 μg/ml), most commonly without teicoplanin resistance (VanB phenotype); and (iii) intrinsic low-level resistance associated with *Enterococcus gallinarum* and *Enterococcus casseliflavus* (MICs, 2 to 32 μg/ml) (VanC phenotype) (107, 108). Both the VanA and VanB phenotypes are most commonly seen in *E. faecalis* and *E. faecium* but have been found in other species (107). Three additional genotypes have been described recently, *vanD* (137, 144), *vanE* (60), and *vanG* (122). The VanD-type resistance, resulting in HLR to vancomycin (MICs, ≥64 μg/ml) and variable resistance to teicoplanin, has been found only in *E. faecium*. The VanE-type resistance, found in *E. faecalis,* exhibits intermediate vancomycin MICs (16 μg/ml), and the organism remains susceptible to teicoplanin. The VanG phenotype is similar to the VanD phenotype.

Many methods commonly used by clinical laboratories, including disk diffusion and the Vitek Legacy and MicroScan systems, have traditionally had problems detecting low-level vancomycin resistance (both VanB and VanC types) (52, 136, 159, 160, 185, 193, 211). However, systems continue to improve (27, 64, 81, 90, 193, 202) and the newer Vitek 2 and Phoenix systems perform very well for the detection of vancomycin resistance in enterococci (1, 50, 54, 177, 202). Disk diffusion testing requires 24 h of incubation and examination of zones under transmitted light.

The vancomycin agar screening test first described by Willey et al. (211) was adopted by the CLSI in 1993 (29, 181) (Table 1). The sensitivity and specificity levels of 96 to 99% and 100%, respectively, were noted at that time. Commercially prepared plates also perform well (52, 61, 204). However, there may still be some confusion among clinical laboratorians about the characterization of susceptibility or resistance for the *van*C-containing enterococci, *E. gallinarum* and *E. casseliflavus,* because their growth is variable on the agar screen plate. Both of these species intrinsically contain a *van*C gene, but the MICs of vancomycin for them range from 2 to 32 μg/ml (107). Whether the presence of this gene is associated with therapeutic failures is not known. Since the vancomycin MICs for these strains are often >4 μg/ml, the strains are likely to grow on the agar screen plates, where a higher inoculum and a richer medium may promote growth (61, 157, 181). Most strains of *van*C-containing enterococci are motile at 30°C; *E. casseliflavus* is typically yellow pigmented. These characteristics have been used to distinguish *van*C-containing enterococci from other species (14, 61, 62). However, some *E. gallinarum* and *E. casseliflavus* strains may be nonmotile. Because of this, a better test to differentiate them from *E. faecalis* and *E. faecium* is fermentation of 1% methyl-α-D-glucopyranoside (MGP). All *van*C-containing enterococci acidify MGP, whereas *E. faecium* and *E. faecalis* do not (149) (see also chapter 30).

Vancomycin Agar Screen Test

Agar plates are prepared with BHI agar supplemented with 6 μg of vancomycin per ml (BHI-V6). Using growth from an 18- to 24-h agar plate, make a suspension equivalent in turbidity to a 0.5 McFarland standard and inoculate the plates by spotting 1 to 10 μl or swabbing an area 10 to 15 mm in diameter. The final inoculum is 10^5 to 10^6 CFU per spot. After inoculation, incubate the plates for a full 24 h in ambient air at 35°C. The presence of more than one colony or a haze of growth indicates resistance.

Quality Control

For quality control, *E. faecalis* ATCC 29212 (no growth, i.e., susceptible) and *E. faecalis* ATCC 51299 (growth, i.e., resistant) should be tested (182). Plates made with BHI agars from certain manufacturers may allow light growth of *E. faecalis* ATCC 29212, especially if the higher inoculum (10 μl) is used or the plates are held longer than 24 h.

Reporting Resistance in Enterococci

For any serious enterococcal infection, results of the screen for HLR to gentamicin and streptomycin should be reported in concert with the results of the testing of the cell wall-active agent (penicillin, ampicillin, or vancomycin), because synergy would not be expected if any one of the agents reported is resistant. Helpful suggestions on reporting the results of enterococcal tests are given by Hindler and Sahm (75).

TESTS TO DETECT RESISTANCE IN STAPHYLOCOCCI

Detection of Oxacillin Resistance in Staphylococci

Strains of *Staphylococcus aureus* resistant to oxacillin are still referred to as MRSA (for methicillin-resistant *S. aureus*). Even though methicillin is no longer available, the acronym "MRSA" has persisted and therefore will also be used here. MIC interpretive criteria recommended by the CLSI for oxacillin and *S. aureus* are ≤2 μg/ml for susceptible and ≥4 μg/ml for resistant (38). At least three different resistance mechanisms contribute to oxacillin resistance in *S. aureus:* (i) production of a supplemental penicillin-binding protein (PBP), PBP 2a, encoded by a chromosomal *mecA* gene; (ii) inactivation of the drug by increased production of β-lactamase; and (iii) production of modified intrinsic PBPs (MOD-SA) with altered affinity for the drug (23, 25, 42, 69, 197). Studies to determine the prevalence of the different resistance mechanisms have not been done; however, it is assumed that the last two occur only rarely. Strains that possess *mecA* (the classic resistance) are either heterogeneous or homogeneous in their expression of resistance. With homogeneous expression, virtually all cells express resistance when tested by standard in vitro test methods. However, testing of a heteroresistant isolate results in some cells that appear to be susceptible and others that appear to be resistant. Often only 1 in 10^4 to 1 in 10^8 *mecA*-positive cells in the test population expresses resistance (71, 156, 198). Heterogeneous expression occasionally

results in MICs that appear to be borderline, i.e., oxacillin MICs of 2 to 8 μg/ml, and consequently, the isolates may be misinterpreted as susceptible (MICs ≤2 μg/ml) (164). Isolates that have classic resistance are usually resistant to other agents such as erythromycin, clindamycin, chloramphenicol, tetracycline, trimethoprim-sulfamethoxazole, older fluoroquinolones, or aminoglycosides. However, some MRSA isolates, such as those described in community-associated infections (referred to as CA-MRSA), are not multiply resistant (18, 138, 216). Resistance mediated by β-lactamase or the presence of modified PBPs (MOD-SA) also results in borderline resistance. β-Lactamase-mediated resistance can usually be distinguished from the classic type (*mecA* positive) of resistance or MOD-SA resistance by the addition of a β-lactamase inhibitor (e.g., clavulanic acid) to the oxacillin MIC test, which lowers the MIC by 2 dilutions or more. Isolates that are resistant by either the β-lactamase or the MOD-SA mechanism usually do not have multiple drug resistance, similar to CA-MRSA.

Reference dilution methods recommended by the CLSI are generally reliable for detection of oxacillin resistance in *S. aureus,* and since lowering of the MIC breakpoints for coagulase-negative staphylococci in 1999 (32), the sensitivity of detection in that group has improved. However, recent studies have found that correlation of these interpretive criteria with the presence or absence of *mecA* is optimal only for *S. epidermidis, S. haemolyticus,* and, possibly, *S. hominis* (66, 80, 115). For other species of coagulase-negative staphylococci (e.g., *S. saprophyticus* and *S. lugdunensis*) the new breakpoints tended to be less specific, i.e., many *mecA*-negative strains were categorized as resistant by disk and MIC methods (59, 80, 115, 121, 150). In 2005, the CLSI revised the staphylococcus tables to group *S. lugdunensis* strains with *S. aureus,* which has helped to resolve some of these problems (35). More recently, it was shown (J. Swenson, unpublished data) that there are still *mecA*-negative *S. lugdunensis* strains categorized as oxacillin resistant by disk diffusion using the 1-μg oxacillin disk (a major interpretive error). Because of this, the CLSI now recommends that testing of *S. lugdunensis* by disk diffusion be done using cefoxitin as a surrogate for oxacillin (see below); oxacillin interpretive criteria for *S. lugdunensis* no longer appear in CLSI M100 tables for staphylococci (38).

Despite continued improvements in reference testing methods with oxacillin, other phenotypic tests have been studied. The use of cefoxitin has recently been highlighted by many investigators as an excellent surrogate for use in detecting *mecA*-mediated resistance in staphylococci. Special phenotypic methods using cefoxitin as well as other methods are discussed below.

Detection Using Cefoxitin as a Surrogate for Oxacillin

Within the past several years, there have been multiple reports of the use of cefoxitin as a surrogate marker for detection of *mecA*-mediated oxacillin resistance. Cefoxitin is a more potent inducer of the *mecA* regulatory system than are other penicillins (123). Following recent studies including a multilaboratory study (187), the CLSI has adopted a cefoxitin disk diffusion method that is equal in sensitivity and specificity to oxacillin disk diffusion for detecting *mecA*-mediated resistance in *S. aureus.* For coagulase-negative staphylococci, the cefoxitin disk diffusion test is equal in sensitivity but greater in specificity when compared to disk diffusion with oxacillin (37). The major drawback of the test is the failure to detect *mecA*-positive strains of *S. simulans* (187).

The cefoxitin disk test is performed using the routine disk diffusion procedure, except that modified interpretive criteria are used: for *S. aureus,* results of ≤19 mm are reported as oxacillin resistant and results of ≥20 mm are considered oxacillin susceptible; for coagulase-negative staphylococci, ≤24 mm is resistant and ≥25 mm is susceptible. The test is easy to read using reflected light and does not require careful examination of the disk diffusion zones for light growth or small colonies. The use of cefoxitin in an agar dilution screen similar to the oxacillin salt-agar screen has also been investigated (58, 187) and warrants further study.

Oxacillin-Salt Agar Screening Test

Although the oxacillin-salt agar screen test has been widely used, recent studies have questioned its sensitivity, especially for heterogeneously resistant strains (17). It is less sensitive than the cefoxitin disk diffusion test (16, 57, 188). The oxacillin-salt agar screen test cannot be used for detecting oxacillin resistance in coagulase-negative staphylococci because it uses 6 μg of oxacillin per ml (190). Consequently, the CLSI eliminated recommendations for using the agar screen to test coagulase-negative staphylococci.

Test Method

MHA supplemented with 4% sodium chloride and 6 μg of oxacillin per ml is used for the agar screen method recommended by the CLSI (36, 37). Plates containing methicillin are not recommended. For the test, inoculum suspensions are prepared by selecting colonies from overnight growth on a nonselective agar plate. The colonies are transferred to broth (e.g., tryptic soy broth) or saline to produce a suspension that matches the turbidity of a 0.5 McFarland standard. This suspension is used to inoculate the oxacillin agar screen plate by either (i) dipping a cotton swab into the test suspension, expressing the excess liquid from the swab, and inoculating an area 10 to 15 mm in diameter (or streaking the swab onto a quadrant of the agar surface) or (ii) spotting an area 10 to 15 mm in diameter with a 1-μl loop that has been dipped in the suspension (186). Test plates are incubated for a full 24 h at 35°C (no higher) in ambient air and examined for growth of more than one colony, which indicates resistance. Once again, the test is currently not recommended for coagulase-negative staphylococci (36, 37).

Quality Control

S. aureus ATCC 29213 (oxacillin susceptible) and *S. aureus* ATCC 43300 (oxacillin resistant) are the recommended quality control strains.

Other Tests for Detection of Oxacillin Resistance in Staphylococci

Several commercial rapid methods that detect oxacillin resistance in staphylococci are available. They include latex agglutination tests that detect the presence of PBP 2a, the MRSA-Screen test (Denka-Seikin Co., Ltd., Tokyo, Japan), the PBP 2′ latex agglutination test (Oxoid Limited, Basingstoke, United Kingdom), the Mastalex test (Mast Diagnostics, Bootle, United Kingdom) (11), and the Slidex MRSA Detection test (bioMérieux, Marcy l'Etoile, France). Only the first two tests have been cleared by the U.S. Food and Drug Administration (FDA). The MRSA-Screen test, cleared for use only with *S. aureus,* has been widely

evaluated and has high sensitivity and specificity for that species (17, 116, 164, 188, 203, 205, 214). Detection of resistance in coagulase-negative staphylococci by the MRSA-Screen test has been less successful, requiring either induction, an increased inoculum, or an increased agglutination time for adequate sensitivity (77, 79, 115, 213). The Oxoid PBP 2′ latex agglutination test was approved by the FDA for testing both *S. aureus* and coagulase-negative staphylococci, the latter requiring induction with oxacillin or cefoxitin. Although not extensively evaluated, its use for same-day reporting of MRSA from blood cultures has been described (119). When confirmation of the presence of the *mecA* gene is required, besides these commercial rapid methods, molecular analysis may also be performed by standard PCR methods (see chapter 16).

Limitations of Methods for Detection of Oxacillin Resistance

Growth of an *S. aureus* isolate on an oxacillin agar screen plate generally means that the isolate is *mecA* positive. When performed properly, the oxacillin agar screen method detects most *mecA*-positive *S. aureus* strains. Occasionally a heteroresistant *mecA*-positive strain is not detected; this may be due in part to a low frequency of resistance expression (17, 153) or to lot-to-lot or manufacturer-to-manufacturer variation in the test medium (74, 76). When the oxacillin agar screen test was compared directly to the use of cefoxitin by disk diffusion, investigators found increased sensitivity in detecting *mecA*-mediated resistance by using cefoxitin disk diffusion, although the statistical significance of this finding was not determined (16, 207).

The oxacillin agar screen test generally does not detect borderline-resistant strains, whether caused by *mecA*-mediated heteroresistance or by the other mechanisms responsible for borderline resistance. Although MOD-SA isolates, particularly those associated with MICs of ≥8 μg/ml, may grow on agar screen plates (186), isolates with borderline resistance due to β-lactamase are usually associated with oxacillin MICs of ≤4 μg/ml and do not usually grow on the screen plates. Cefoxitin disk diffusion testing performs well for detection of borderline resistance caused by *mecA* (57, 187) but does not detect other types of borderline resistance. MOD-SA strains which do not contain *mecA* are categorized as susceptible by cefoxitin disk diffusion despite the oxacillin MIC that they express (187). Since borderline resistance caused by β-lactamase or MOD-SA is infrequently encountered in clinical specimens of *S. aureus,* the possibility of their presence minimally affects the utility of these tests. Testing by a dilution method may also fail to detect extremely heteroresistant, *mecA*-positive *S. aureus* strains (164), which often have borderline resistance. However, if dilution testing is performed so that oxacillin MICs below 2 μg/ml can be detected, MIC testing can be used to discern isolates with borderline resistance, i.e., those with oxacillin MICs of 2 to 8 μg/ml.

Reporting Results

The CLSI recommends that oxacillin-resistant staphylococci be reported as resistant to all β-lactam agents, including penicillins, cephems (i.e., all cephalosporins, including cephamycins), β-lactam/β-lactamase inhibitor combination agents, and carbapenems. These agents are clinically ineffective against oxacillin-resistant staphylococcal infections, even though they may demonstrate in vitro activity (36, 37). Consequently, an isolate that grows on the oxacillin agar screen plate or demonstrates a cefoxitin zone diameter in the resistant range should be considered resistant to these agents as well as to all penicillinase-stable penicillins. Isolates of *S. aureus* that appear oxacillin resistant by an alternative test method but fail to grow on the agar screen plate are probably borderline resistant and lack *mecA* (102, 127, 153). Much less is known about borderline resistance than about *mecA*-positive resistance because there have been few clinical studies (120); however, in animal model studies, isolates with β-lactamase-mediated resistance appear to be effectively treated with β-lactam agents (24, 25, 143, 194). If a phenotypically oxacillin-resistant *S. aureus* strain is isolated from a seriously ill patient and it does not contain *mecA,* this information should be conveyed to the patient's physician. The incidence of phenotypically susceptible *mecA*-positive strains of *S. aureus* is not known. However, should phenotypically susceptible strains be isolated from serious infections in patients with a history of MRSA infection, confirmation by one of the rapid molecular methods such as detection of PBP 2a by latex agglutination should be considered (154, 163, 164).

Detection of Vancomycin Resistance in Staphylococci

Vancomycin resistance in staphylococci is a rapidly evolving subject. Previously, vancomycin-intermediate (MIC of 8 to 16 μg/ml) *S. aureus* (VISA) and *Staphylococcus* species other than *S. aureus* (VISS) were identified and characterized (174). In addition, *S. aureus* strains with reduced susceptibility to vancomycin (MIC, 4 μg/ml) were described and were associated with the same risk factors as VISA (63). More recently, vancomycin-resistant *S. aureus* (VRSA) strains with *vanA*-mediated resistance (MICs, 32 to 1,024 μg/ml) were isolated from four patients in the United States (20–22; T. Madhavan, D. Sievery, J. Rudrik, J. Torresan, and D. Schulman, *Abstr. 43rd IDSA Ann. Meet.,* abstr. 1073, 2005). Until January 2006, the CLSI interpretive criteria for vancomycin and *S. aureus* were as follows: ≤4 μg/ml, susceptible; 8 to 16 μg/ml, intermediate; and ≥32 μg/ml, resistant. As of January 2006, the susceptible category is ≤2 μg/ml, 4 to 8 μg/ml is intermediate, and ≥16 μg/ml is resistant (38). It is important to note that the CLSI interpretive criteria for vancomycin susceptibility in coagulase negative staphylococci (CoNS) were not changed and thus are different from the criteria for *S. aureus.* No *van* gene-mediated vancomycin resistance has been reported for CoNS, but there are reports of isolates with elevated vancomycin MICs (3, 47, 103, 165, 169, 206).

Some routine susceptibility testing methods fail to detect vancomycin-intermediate or vancomycin-resistant staphylococci (191; R. B. Carey, J. B. Patel, S. McAllister, A. Thompson, P. Raney, F. C. Tenover, C. Ginocchio, D. Bopp, N. Dumas, and D. Kohlerschmidt, *Abstr. 44th Intersci. Conf. Antimicrob. Agents Chemother.,* abstr. D-66, 2004; Madhavan et al., Abstr. 43rd IDSA Ann. Meet.). The CLSI reference broth microdilution method and E test are reliable for detection of VISA and VRSA. However, the disk diffusion test cannot detect VISA or VISS because these isolates produce zone sizes within the susceptible range (≥15 mm). In contrast, the disk diffusion test does detect VRSA. An evaluation of automated susceptibility testing systems revealed that not all systems were able to detect one or more VRSA (Carey et al., 44th ICAAC). Some manufacturers of automated susceptibility testing have made adjustments in their testing systems to address this problem.

The BHI-V6 screen agar, used to detect vancomycin-resistant enterococci, was evaluated for detection of

vancomycin-intermediate or -resistant staphylococci in two studies (191; R. B. Carey, D. Lonsway, S. McAllister, J. Patel, P. Raney, and A. Thompson, unpublished data). In both studies, all isolates for which the vancomycin MICs were ≥8 μg/ml grew on the screen agar. Isolates for which the MICs were 4 μg/ml demonstrated variable growth. In both studies, the agar was inoculated with a suspension of organism equal to a 0.5 McFarland standard. In the study by Tenover et al., the plates were inoculated using a 10-μl drop from a calibrated pipette. Carey et al. evaluated the amount and method of inoculum delivery. Delivery of a 10-μl drop from a calibrated pipette was the most sensitive method, demonstrating confluent growth at 24 h for all isolates with MICs ≥8 μg/ml. Inoculation of the agar using a swab immersed in the inoculum and then expressed also resulted in growth of isolates for which the MICs were 8 μg/ml, although for some isolates the growth was a haze or isolated colonies. Other inoculation methods, 10 μl delivered by calibrated loop, 1 μl delivered by calibrated pipette, and 10 μl of a suspension prepared using the Prompt Inoculation System-D (3M Company, St. Paul, Minn.), all demonstrated significantly less sensitivity. In both studies, isolates with a vancomycin MIC ≤2 μg/ml did not grow on commercially prepared media. These studies demonstrate that the BHI-V6 agar is a sensitive method for detection of staphylococci for which the vancomycin MICs are ≥8 μg/ml, using either the 10-μl drop or swab inoculation methods. Additional studies with more isolates are needed to evaluate this agar for detection of *S. aureus* isolates for which the MICs are 4 μg/ml.

Although reduced susceptibility to vancomycin can occur in both *S. aureus* and CoNS, the occurrence in *S. aureus* is more concerning because this species is more frequently a cause of serious infection. It is for this reason that the Centers for Disease Control and Prevention recommended use of the screen agar for detection of *S. aureus* with reduced susceptibility to vancomycin (22). This recommendation is for laboratories that may miss these isolates when using their primary susceptibility testing method. However, some laboratories may consider limiting this testing to methicillin-resistant *S. aureus* strains since these are the isolates most likely to develop reduced susceptibility to vancomycin. Use of the BHI-V6 agar for *S. aureus* is described in the CLSI documents (36, 37).

Test Method

The recommended method for inoculating the agar is a 10-μl drop of a 0.5 McFarland suspension delivered with a micropipette. Alternatively, a swab can be used to spot an area 10 to 15 mm in diameter. The agar plate should be read after 24 h of incubation and carefully examined with transmitted light. Greater than one colony of growth or a light film of growth is considered positive (36, 37).

Quality Control

As mentioned above, the BHI-V6 plate is the same screening agar used to detect vancomycin-resistant enterococci (VRE) and the same quality control strains recommended for the VRE test can also be used for the *S. aureus* test (38). These strains are vancomycin-susceptible *E. faecalis* ATCC 29212 and *vanB*-mediated, vancomycin-resistant *E. faecalis* ATCC 51299. Since the amount of inoculum applied for the VRE test (1 to 10 μl of a 0.5 McFarland suspension) spans the recommended inoculum for the *S. aureus* test, a laboratory that uses this medium for both purposes need perform quality control only once, either daily or weekly.

Reporting Results

Growth of *S. aureus* on the BHI-V6 agar is presumptive for either VISA or VRSA (36, 37) (http://www.cdc.gov/ncidod/dhqp/ar_visavrsa_algo.html). Additional testing is needed to confirm vancomycin resistance. First, the identity and purity of the isolate should be confirmed. Then, the MIC of the isolate should be determined using a validated method. For many laboratories the most available method is the E test. It is recommended that any *S. aureus* isolate for which the vancomycin MIC is ≥4 μg/ml be sent to a reference laboratory for confirmation. In the United States, several states request that VISA and/or VRSA be reported to the public health authority.

Detection of Inducible Clindamycin Resistance by Use of the D-Zone Test

Although erythromycin and clindamycin are in separate antimicrobial agent classes, macrolides and lincosamides, respectively, their mechanisms of action (inhibition of protein synthesis) and mechanisms of resistance are similar. The two main mechanisms of resistance are (i) an efflux pump and (ii) a methylase enzyme that alters the ribosomal binding site of the antimicrobial agents. The first type, which confers resistance to macrolides only (designated M-type, for macrolide), is mediated in staphylococci by *msrA*. The second type, which confers resistance to macrolides, lincosamides, and streptogramin B agents (designated MLS_B type, for macrolide-lincosamide-streptogramin B), is mediated by an *erm* gene. In staphylococci, the MLS_B-type resistance can be either constitutive or inducible; if inducible, the isolate appears to be susceptible to the lincosamide (i.e., clindamycin) when using routine testing methods, unless induced by a macrolide (i.e., erythromycin). It is important to determine if resistance (whether inducible or constitutive) to clindamycin exists when it is being considered for therapy. Detailed explanation of these resistance mechanisms can be found in chapter 71.

Phenotypically, if an isolate has M-type resistance it is resistant to erythromycin but susceptible to clindamycin. If an isolate has MLS_B-type resistance, then it is erythromycin resistant and may be susceptible or resistant to clindamycin because it is either constitutive or inducible to that drug. In strains that are erythromycin resistant but clindamycin susceptible, it is important to determine if inducible clindamycin resistance exists (and an *erm* gene is present) or if the strain remains clindamycin susceptible (and an efflux gene is present).

Test Method

Detection of inducible clindamycin resistance can be easily accomplished using a disk diffusion procedure by placing a 15-μg erythromycin disk adjacent to a 2-μg clindamycin disk and looking for a flattening of the clindamycin zone, which looks like the letter D and is therefore referred to as a D zone. For laboratories that are already performing disk diffusion, this may be done by placing the disks from 15 to 26 mm apart (55) with the other disks tested on an MHA plate. Some disk dispensers may position disks more than 26 mm apart even if the disks are placed in adjacent positions. Therefore, because dispensers may vary and the distance is critical, the distance should be verified before being adopted as a standard procedure. Placing the disks 15 to 20 mm, rather than 26 mm, apart may make the test easier to interpret (179, 215), although to accomplish this the disks must be placed on the plates by hand instead of with a

disk dispenser. For laboratories that routinely perform antimicrobial susceptibility methods other than disk diffusion, Jorgensen et al. have shown that the test can be performed on a standard blood agar plate used for purity checks (94) by streaking one-third of the plate for confluent growth and then streaking for isolation on the rest of the plate. In that study, the investigators showed that dilutions of the 0.5 McFarland inoculum of up to 1:250 can be used. The disks are placed 15 mm apart on the portion of the plate where confluent growth would occur. In a later study, it was determined that the BBL Prompt system (BD, Sparks, Md.) should not be used to inoculate the purity plate for D-zone determination (215). In that study, the investigators suggested that no more than a 1:20 dilution of a 0.5 McFarland suspension should be used to inoculate the purity plate. However, if the Prompt inoculum wand was used directly to inoculate the plate, it may have delivered inadequate inoculum. More recently the Prompt System was successfully used for this purpose (J. F. Hindler and D. A. Bruckner, *Abstr. 105th Gen. Meet. Am. Soc. Microbiol.,* abstr. C-325, 2005) by transferring inoculum from the Prompt reservoir tray, using a 10-μl loop, and streaking one-third of the plate to obtain confluent growth. For either method, organisms that show a blunting or flattening of the clindamycin zone are considered D-zone test positive; those that show no flattening are D-zone test negative.

Quality Control and Quality Assessment

Two strains have been designated for quality assessment purposes (i.e., for training, competency assessment, or test evaluation): *S. aureus* BAA-977, which contains inducible *ermA*-mediated resistance; and *S. aureus* BAA-976, which contains *msrA*-mediated resistance to erythromycin only. *S. aureus* ATCC 25923 should be used as the routine quality control strain for daily or weekly quality control testing of clindamycin and erythromycin disks using MHA. If the test is performed as part of the purity check procedure, the CLSI recommends that the disk content should be verified using *S. aureus* ATCC 25923 on MHA (38). However, this would require that laboratories that do not do disk diffusion routinely would have to have a supply of MHA plates on hand for quality control purposes only. An alternative to this would be to use the two quality assessment strains, BAA-977 and BAA-976, as quality control organisms at daily or weekly intervals based on CLSI recommendations when using the purity plate method.

Reporting Results

The incidence of inducible clindamycin resistance in staphylococci can be highly variable, both by geographic area and by organism group (i.e., hospital-associated MRSA, community-associated MRSA, and coagulase-negative staphylococci). The CLSI now recommends that isolates that are D-zone test positive should be reported as clindamycin resistant (36, 37). However, there is some controversy about this, given that clindamycin has been effective in some situations where inducible resistance was demonstrated (112). As a conservative approach, the CLSI has suggested that inducibly clindamycin-resistant strains could be reported as resistant with a comment stating, "This isolate is presumed to be resistant based on detection of inducible clindamycin resistance. Clindamycin may still be effective in some patients." If the test is not offered routinely, it should be available by request for cases where clindamycin is being considered for therapy (36, 37).

TESTS TO DETECT RESISTANCE IN STREPTOCOCCI

Oxacillin Disk Screen Test for Detection of Penicillin Resistance in Pneumococci

A screening test in which a 1-μg oxacillin disk is used to detect penicillin resistance in pneumococci was first described following an outbreak caused by *Streptococcus pneumoniae* resistant to multiple antimicrobial agents in South Africa in the 1970s (45, 82, 184). Since then, this test has been used extensively and shown to be highly sensitive but not specific for detection of penicillin-nonsusceptible pneumococci (46). Strains identified as nonsusceptible by this method may be penicillin susceptible, intermediate, or resistant. Penicillin MIC tests must be performed with any strain that produces a zone diameter of $\leq$19 mm to determine if it is resistant (46). MIC tests rather than the oxacillin disk screen should be used routinely on strains isolated from cerebrospinal fluid and blood.

Detection of Inducible Clindamycin Resistance by Use of the D-Zone Test

The importance of determining erythromycin and clindamycin resistance phenotypes and the detection of inducible clindamycin resistance follows the same logic as that described for staphylococci (see "Detection of Inducible Clindamycin Resistance by Use of the D-Zone Test" above); however, the gene responsible for the M phenotype (i.e., erythromycin resistance and clindamycin susceptibility) in streptococci is *mef*(A). The MLS_B phenotype exists in isolates of beta-hemolytic streptococci, *S. pneumoniae,* and viridans group streptococci; however, in pneumococci and viridans streptococci it is usually the constitutive type and only rarely inducible (128, 201). Therefore, inducible clindamycin resistance needs to be determined only in isolates of beta-hemolytic streptococci.

Test Method

Detection of inducible clindamycin resistance in beta-hemolytic streptococci can be accomplished using a disk diffusion procedure similar to that for staphylococci by placing a 15-μg erythromycin disk adjacent to a 2-μg clindamycin disk and looking for a flattening of the clindamycin zone, which looks like the letter D and is therefore referred to as a D zone. For laboratories that are already performing disk diffusion, the disks must be placed by hand at a distance of 12 mm from each other (151) on an MHA plate with 5% sheep blood along with other disks being tested. A disk dispenser cannot be used to place the two disks since they must not be farther away than 12 mm (151). For laboratories that routinely perform other antimicrobial susceptibility methods, the purity plate method suggested by Jorgensen et al. can also be performed on a standard blood agar plate used for purity checks (94, 151) by streaking one-third of the plate for confluent growth and then streaking for isolation on the rest of the plate. The disks are then placed 12 mm apart on the portion of the plate where confluent growth would occur. Only the use of a 0.5 McFarland suspension has been evaluated for inoculation of the purity plate (151). For either method, organisms that show a blunting or flattening of the clindamycin zone are considered D-zone test positive; those that show no flattening are D-zone test negative.

Quality Control and Quality Assessment

Two strains have been designated for quality assessment (i.e., for training, competency assessment, or test evaluation)

of the D-zone test: *S. aureus* BAA-977, which contains inducible *ermA*-mediated resistance to clindamycin, and *S. aureus* BAA-976, which contains *msrA*-mediated resistance to erythromycin only. *S. pneumoniae* ATCC 49619 should be used as the routine quality control strain for daily or weekly quality control testing of clindamycin and erythromycin disks, using MHA with 5% sheep blood. If the test is performed as part of the purity check procedure, ideally the disk content should be verified using *S. pneumoniae* ATCC 49619 on MHA with 5% sheep blood. However, this would require that laboratories that do not do disk diffusion routinely would have to have a supply of MHA with 5% sheep blood on hand for quality control purposes only. An alternative to this would be to use the two quality assessment strains, BAA-977 and BAA-976, as quality control organisms at daily or weekly intervals based on CLSI recommendations when using the purity plate method.

Reporting Results

The incidence of inducible clindamycin resistance in beta-hemolytic streptococci can be highly variable, both by geographic area and organism group (44, 106). However, when inducible clindamycin resistance is detected, the CLSI recommends that, as for staphylococci, isolates shown to be D-zone test positive should be reported as clindamycin resistant. As a conservative approach, the CLSI has suggested that inducibly clindamycin-resistant strains could be reported as resistant with a comment stating, "This isolate is presumed to be resistant based on detection of inducible clindamycin resistance. Clindamycin may still be effective in some patients." If the test is not offered routinely, it should be available by request for cases where clindamycin is being considered for therapy.

DETECTION OF ENZYMES MEDIATING RESISTANCE

β-Lactamase Tests

In the clinical laboratory, β-lactamase tests must be used only when they can provide clinically useful information, and the definitions of positive or negative reactions must not be extended beyond their intended meanings. For example, a β-lactamase-positive result for a *Neisseria gonorrhoeae* isolate means that the isolate is resistant to penicillin but does not imply that the isolate is resistant to the extended-spectrum cephalosporin group of β-lactam agents. Similarly, direct β-lactamase tests for members of the family *Enterobacteriaceae* or for *Pseudomonas* spp. (all of which produce a variety of β-lactamases that result in various susceptibilities to β-lactam agents) have little clinical value and should not be used for these species. A list of the organisms for which β-lactamase tests are useful is given in Table 2.

Direct Tests for β-Lactamase Activity

In the direct β-lactamase test, a positive reaction indicates that the isolate is resistant to the β-lactam agents noted in Table 2, but a negative reaction is inconclusive. For example, most ampicillin-resistant *Haemophilus influenzae* isolates produce β-lactamase, which can be detected by direct β-lactamase tests; however, rare strains are ampicillin resistant but β-lactamase negative (8, 46, 56). For the latter, conventional disk diffusion or dilution tests are needed to detect the resistance (see chapter 73). Three direct β-lactamase assays, the acidimetric, iodometric, and chromogenic methods, have been widely used (73, 110). Each method involves testing bacteria grown on nonselective media, and the results are available within 1 to 60 min. The acidimetric and iodometric methods use a colorimetric indicator to detect the presence of penicilloic acid in the reaction vessel following β-lactamase hydrolysis of penicillin. In the acidimetric method, the substrates are citrate-buffered penicillin and a phenol red indicator. A decreasing pH associated with the presence of penicilloic acid results in a color change from red (negative result) to yellow (positive result) (53). The substrates in the iodometric test are phosphate-buffered penicillin plus a starch-iodine complex. Penicilloic acid, if present, reduces the iodine and prevents it from combining with starch, resulting in a colorless reaction (positive); a bluish purple color corresponds to a negative result (15).

The chromogenic cephalosporin nitrocefin can be used in a test tube assay (135) but has been incorporated into

TABLE 2 Bacteria for which β-lactamase tests have been used in the clinical laboratory

Species	Method(s) commonly used	Predicted resistance[a]
Bacteroides spp. and other gram-negative anaerobes, except *B. fragilis* group	Direct β-lactamase tests[b]	Penicillins[c]
Enterococcus spp.	Direct β-lactamase tests	Penicillins[c]
Haemophilus influenzae	Direct β-lactamase tests	Penicillins[c]
Moraxella catarrhalis	Direct β-lactamase tests (nitrocefin only)	Penicillins[c]
Neisseria gonorrhoeae	Direct β-lactamase tests	Penicillins[c]
Staphylococcus spp.	Direct β-lactamase tests with prior induction	Penicillins[c]
Escherichia coli	NCCLS ESBL screening and confirmation tests	Penicillins, cephems, and aztreonam
Klebsiella pneumoniae and *K. oxytoca*	NCCLS ESBL screening and confirmation tests	Penicillins, cephems, and aztreonam

[a] A positive result indicates resistance; however, a negative result is inconclusive, since other resistance mechanisms may occur.

[b] Includes chromogenic cephalosporin, acidimetric, and iodometric tests.

[c] A positive result indicates resistance to all penicillinase-labile penicillins, including amoxicillin, ampicillin, azlocillin, carbenicillin, mezlocillin, piperacillin, and ticarcillin.

several filter paper-type disk or strip products that are commercially available and widely used in clinical laboratories. β-Lactamase hydrolysis of the chromogenic cephalosporin molecule causes an electron shift that results in a colored product (135). Although the acidimetric and iodometric methods have varied in performance, perhaps due in part to lack of experience with these methods, the chromogenic method has been reliable in detecting β-lactamases produced by all of the organisms indicated in Table 2 (92, 134).

The colorimetric β-lactamase tests rely on visualization of a colored product that presumably results from β-lactamase destruction of the substrate β-lactam molecule. However, these tests are not 100% specific, and other substances may yield colored end points. Serum may cause a colored reaction with the nitrocefin test (135), and, if reagents are not stored properly, spontaneous degradation of penicillin may produce false-positive acidimetric or iodometric β-lactamase reactions.

While some bacteria (e.g., *H. influenzae, N. gonorrhoeae,* and enterococci) constitutively produce β-lactamase, others (e.g., staphylococci) may produce detectable amounts of enzyme only after exposure to an inducing agent, which is generally a β-lactam (48). If staphylococci produce a positive β-lactamase result without induction, the results can be reported. However, if no β-lactamase is detected, then the test must be performed with cells that have been exposed to an inducing agent before a negative result is reported. This can be done by testing organisms that have been grown in the presence of subinhibitory concentrations of a β-lactam agent (e.g., 0.25 μg of cefoxitin per ml) in a broth or agar system. Alternatively, growth from around the periphery of the zone surrounding a β-lactam disk (e.g., a 1-μg oxacillin or 30-μg cefoxitin disk) can be tested. A positive result may take longer to develop for staphylococci than for other organisms, and the test should not be considered negative until it has been allowed to react for at least 60 min.

β-Lactamase testing by the chromogenic nitrocefin method with anaerobic gram-negative bacilli other than those from the *Bacteroides fragilis* group may be performed prior to susceptibility testing (33). Members of the *B. fragilis* group characteristically produce β-lactamase, and they should be considered penicillin resistant. As with aerobes, resistance to β-lactam drugs is not always mediated by β-lactamase production (e.g., in some strains of *Bacteroides distasonis* and *B. fragilis*) (2, 33, 83).

The *S. aureus* strains recommended by the CLSI for quality control of routine disk diffusion and dilution tests (36–38) can be used for quality control of β-lactamase tests. *S. aureus* ATCC 25923 is β-lactamase negative, whereas *S. aureus* ATCC 29213 is β-lactamase positive.

Tests for Extended-Spectrum β-Lactamases

The genes generally responsible for β-lactamase-mediated ampicillin resistance in *Escherichia coli, Klebsiella* spp., *Proteus mirabilis,* and some other genera of the family *Enterobacteriaceae* can undergo simple point mutations that result in the production of novel β-lactamases that are capable of hydrolyzing extended-spectrum cephalosporins (e.g., cefotaxime, ceftriaxone, ceftizoxime, and ceftazidime) and aztreonam, as well as older β-lactam drugs. These enzymes are referred to as extended-spectrum β-lactamases (ESBLs) (9, 12, 85, 113, 147, 196) and are discussed in chapter 71. More than 200 different types of ESBLs have been noted to occur in several gram-negative species and are associated with a variety of in vitro antimicrobial susceptibility profiles (12, 196; http://www.lahey.org/Studies/).

The in vitro susceptibility results obtained with an isolate that produces ESBLs often defy typical "hierarchy" rules of β-lactam (particularly cephem) activity. Sometimes the more narrow spectrum cephems (particularly the cephamycins, such as cefoxitin or cefotetan) are more active than broad-spectrum agents (26, 126). Several reports suggest that ESBL-producing isolates should be considered resistant to all extended-spectrum penicillins, cephalosporins, and monobactams even if they appear to be susceptible to these agents in vitro (139, 140, 173). The β-lactamase-stable carbapenems (ertapenem, imipenem, meropenem) are active in vitro (4, 84, 91, 148, 171, 172, 212) and appear to be clinically effective against ESBL producers (88, 124, 139, 142). Some ESBL-producing isolates are susceptible to β-lactam/β-lactamase inhibitor combination agents in vitro, but the effectiveness of these agents in vivo is uncertain, particularly if there is a high concentration of organisms at the infection site (86). The genes that code for production of ESBLs are often linked to other resistance genes, so that ESBL-producing isolates are often multiply resistant (e.g., resistant to aminoglycosides and trimethoprim-sulfamethoxazole) (96).

Routine disk diffusion and MIC tests using traditional breakpoints may not always identify isolates that produce ESBLs (87, 89, 96, 126). Thus, the CLSI has developed MIC and disk diffusion screening breakpoints for aztreonam, cefotaxime, cefpodoxime, ceftazidime, and ceftriaxone for *E. coli, Klebsiella oxytoca,* and *Klebsiella pneumoniae* and, more recently, for cefpodoxime, ceftazidime, and cefotaxime for *Proteus mirabilis* that aid in detecting ESBL-producing isolates (38) of those species. ESBL-producing clinical isolates may demonstrate high-level resistance to one or more of the screening drugs (88, 96, 114, 124, 171, 210). Thus, the sensitivity of the screen test increases when more than one screening drug is used (51, 126, 212). Cefpodoxime is most likely to detect isolates producing ESBLs, but this agent lacks specificity, particularly for *E. coli* (65, 126, 195). However, the CLSI has modified the screening breakpoints for cefpodoxime for *E. coli* and *Klebsiella* spp. in order to increase the specificity with a minimal effect on the sensitivity (34). Only cefpodoxime, cefotaxime, and ceftazidime have been validated for use with *P. mirabilis* (38).

Unlike inducible AmpC β-lactamases, ESBLs are inhibited by clavulanic acid, and this property is used in laboratory tests to identify ESBLs in the clinical laboratory. These tests are based on enhanced activity when a β-lactam (usually ceftazidime or cefotaxime) is tested with clavulanic acid compared to the activity when the β-lactam is tested alone. The CLSI describes standard disk diffusion and broth microdilution MIC tests to be used as phenotypic confirmatory tests for the presence of ESBLs in *E. coli, K. oxytoca, K. pneumoniae,* and *P. mirabilis*. For broth microdilution, cefotaxime and ceftazidime are tested with and without 4 μg of clavulanic acid per ml. A decrease in the MIC of ≥3 dilutions for the agents tested in combination with clavulanic acid compared to the values obtained for the agents tested alone indicates the presence of an ESBL. For disk diffusion, the same agents incorporated into disks with and without 10 μg of clavulanic acid are tested. An increase in the zone diameter of ≥5 mm for either of the disks with clavulanic acid indicates the presence of an ESBL. *K. pneumoniae* ATCC 700603 should be included for quality control purposes; accepted ranges are given in the current CLSI M100 tables (38). Isolates of *E. coli, Klebsiella* spp., and *P. mirabilis* confirmed to be ESBL producers should be reported as resistant to penicillins (not including β-lactam/β-lactamase inhibitor combinations), cephalosporins (which excludes the

cephamycins, cefoxitin and cefotetan), and aztreonam (36–38). For screen-positive strains that do not show a clavulanic acid effect, there are insufficient data to justify modifying reports at present; these isolates should be reported as they test. One of the limitations of the phenotypic confirmatory test is that some ESBL-producing strains may demonstrate a negative confirmatory test result, which may occur as a result of decreased porin production, hyperproduction of a TEM-1 or SHV-1 β-lactamase, production of additional β-lactamases (e.g., AmpC) that are not inhibited by clavulanic acid, or a combination of these (12, 196). Steward et al. (180) showed in a recent study that cefepime with and without clavulanic acid is superior to the current confirmatory test agents (both cefotaxime and ceftazidime with and without clavulanic acid) in detection of ESBLs in some strains that have multiple β-lactam resistance mechanisms. The CLSI has not addressed the utility of the screening or phenotypic confirmatory tests for detecting ESBLs in members of the family *Enterobacteriaceae* other than *E. coli, Klebsiella* spp., and *P. mirabilis.* In theory, however, they should detect ESBLs in other genera that are normally susceptible to extended-spectrum cephalosporins.

Typically, screening for and confirmation of ESBL production is a two-step process; however, there are several FDA-cleared commercial products that incorporate a confirmatory test on a primary susceptibility test panel. The laboratory's ability to detect and report the presence of ESBLs in a timely manner will likely affect the selection of appropriate therapy for patients with infections caused by ESBL-producing strains. Consequently every laboratory should have a procedure to address the detection of ESBLs (105, 140, 148, 173, 192). There is some debate as to the significance of ESBLs in isolates from noninvasive infections, such as uncomplicated urinary tract infections (51, 141). However, regardless of the source, ESBL-producing isolates are important from an infection control perspective.

Tests for Detection of Plasmid-Mediated AmpC-Type β-Lactamases

Inducible AmpC-type β-lactamases occur on the chromosome of several gram-negative bacilli such as *Enterobacter cloacae, Citrobacter freundii, Serratia marcescens,* and *Pseudomonas aeruginosa* (113). These enzymes confer resistance to a wide spectrum of β-lactams, and they are resistant to the commonly used β-lactamase inhibitors (see details in chapter 71). AmpC-type β-lactamases may be located on transmissible plasmids in several bacterial species that lack an inducible chromosomal enzyme, including *E. coli, Klebsiella* spp., *Salmonella* spp., and *P. mirabilis* (146). These plasmids often carry resistant determinants for multiple classes of antimicrobial agents. AmpC-type enzymes in porin-deficient strains can result in carbapenem resistance (10, 176). Therefore, detection of plasmid-mediated AmpC-type enzymes may be important for treatment decisions, infection control, and epidemiological investigations (19, 131, 132).

Isolates with a plasmid-mediated AmpC-type enzyme are usually resistant to cephamycins (e.g., cefoxitin and cefotetan), which is a useful phenotypic marker for the β-lactamase. However, loss of a porin can also result in cephamycin resistance (72), so a more specific test is necessary. Several tests have been proposed for detecting this type of resistance. Two assays, the modified three-dimensional extract test and the cefoxitin-agar medium-based assay, are too labor-intensive for routine use in the clinical laboratory (39, 133). The more promising assay is a disk diffusion assay similar to the CLSI disk diffusion confirmatory test for ESBL detection. This test incorporates 20 μg of β-lactamase inhibitor, 48-1220 or LN-2-128, into a 30-μg cefotetan disk. In one study (7), a 4-mm reduction in zone size around the disk with cefotetan plus 48-1220 when compared to the zone size around the disk with cefotetan only correlated well with the presence of a plasmid-mediated AmpC-type enzyme in isolates of *Klebsiella* spp. and *E. coli*. Although this test holds promise for phenotypic detection of AmpC β-lactamases, these β-lactamase inhibitors are not commercially available so the test cannot yet be used by clinical laboratories.

DETERMINATION OF BACTERICIDAL ACTIVITIES OF ANTIMICROBIAL AGENTS

Most in vitro susceptibility test methods measure the abilities of antimicrobial agents to inhibit the growth of bacteria. It is also possible to assess the ability of a drug to provide a bactericidal effect on an isolate or group of isolates. This may be useful in managing serious infections that require bactericidal action for optimal efficacy such as endocarditis and osteomyelitis (152), for immunosuppressed patients (170), and to study the pharmacodynamic properties of new or established antimicrobial agents or drug combinations (6, 111, 178). The tests most often performed include determination of the MBC, serum bactericidal titers (SBT), and kinetic time-kill assays. Even in the most sophisticated clinical laboratories serving large tertiary-care medical centers, determinations of bactericidal activity probably account for fewer than 1% of all susceptibility tests performed. Thus, these are highly specialized tests performed for very limited indications.

Tests of bactericidal activity are primarily used for selection and monitoring of therapy for patients with infective endocarditis (41, 93, 145, 152, 155, 167, 168, 208), osteomyelitis (93, 130, 145, 152, 209), meningitis (145, 155, 166), or for neutropenic cancer patients (97, 100, 170). These tests may be favored by certain physicians or specialty groups (e.g., infectious disease specialists) for the management of their patients' very complicated infections but do not represent standard care in most medical centers. Data in support of the use of bactericidal activity determinations for patient management are limited, and early studies were often performed using nonstandardized methods (41, 93, 117, 167, 189). Prior reviews have highlighted the technical problems and lack of expert consensus for interpreting the results of bactericidal determinations (117, 118, 152). The perception that the tests are not reproducible may have stifled the development of consensus criteria for when and how these tests can best be applied. The lack of standardization of the specific assays has been addressed by the CLSI through the publication of standard methods for determining bactericidal activity (30, 31). This section discusses bactericidal testing in a clinical laboratory setting and reflects the consensus standards developed by the CLSI.

Clinical Use of Bactericidal Testing

For those who favor the use of bactericidal tests for management of serious infections, a typical sequence is to first determine the MICs and MBCs of a limited number of agents that could be used to treat the patient in order to select the agent with the most favorable (i.e., lowest) MBC relative to the safely achievable levels of the drug. Determination of the MBC provides an opportunity to detect possible tolerance of the patient's isolate to drugs that would normally be considered bactericidal against the species being tested and might

result in clinical failure (43, 70, 78, 130, 200). Once one or more drugs have been selected on the basis of the MIC and MBC results and the patient has been started on standard doses of the agent(s), the second step is to assess the bactericidal activity achieved in the patient by performance of the serum bactericidal test(s). The goal is to ensure that the dosage regimen provides sufficient bactericidal activity in that patient's blood or, possibly, cerebrospinal fluid. The body fluid (usually serum) is serially diluted and inoculated with the patient's infecting organism. The SBT test result is expressed as a titer indicating the dilution of serum or body fluid that is bactericidal; a higher titer indicates better activity. If the SBT is deemed to be too low, it may be possible to alter the dosage regimen or to select an alternative regimen in an effort to avoid a poor therapeutic outcome (152, 208, 209). Performance of the SBT test can also provide an assessment of the effectiveness of drug combinations intended to provide a synergistic effect in order to treat a highly resistant bacterial isolate or an organism not killed by single-agent therapy (e.g., *Enterococcus* spp.) (30).

Performance of bactericidal tests requires considerable technical proficiency on the part of the laboratory and a highly informed and experienced clinician to make use of the data. The interpretation of the SBT must take into account knowledge of the key pharmacodynamic property of the antimicrobial agent (i.e., concentration-dependent versus time-dependent killing activity), the location and severity of the infection, the pathogenic potential of the infecting organism, and the potential toxicity and cost of the treatment regimen. Bactericidal testing should not be performed unless there is close communication between the clinical microbiologist and the requesting clinician. Potential pitfalls include selecting potentially more toxic or expensive drugs on the basis of a low MIC or MBC (unless the physician is very knowledgeable about various classes of antimicrobial agents) and raising antibiotic levels to potentially toxic levels in order to achieve a higher or more favorable SBT. Therefore, laboratories should undertake bactericidal testing only after thorough discussions with their clinician customers.

Determination of the MBC

There are several basic requirements for standardized determination of the MBC. The test may be performed by either the tube broth macrodilution method or the broth microdilution procedure. A detailed description of the methods can be found in the CLSI document that deals specifically with bactericidal tests (31). In brief, the test usually uses standard cation-adjusted Mueller-Hinton broth (with lysed horse blood supplementation if needed for fastidious organisms) dispensed in 1-ml aliquots in glass tubes (13 by 100 mm) or in 0.1-ml volumes in plastic microdilution trays. A significant component in performance of the MBC test is the inoculum preparation and inoculation procedure. An actively growing (log-phase) inoculum is used (31, 189) rather than an inoculum that is allowed to grow to the stationary phase or fresh colonies suspended in broth or saline, as for a standard MIC test (36). Use of a log-phase inoculum is necessary to accurately assess the bactericidal properties of drugs that require actively growing cells to exert a bactericidal effect. In addition, it is important to dispense the standardized inoculum suspension below the meniscus of the broth carefully in each tube or well to prevent splashing that can lead to organisms sticking to the wall of the tube or well above the meniscus rather than being in contact with the drug in the test medium (31). If splashing or aerosolization of the inoculum occurs, viable organisms recovered at the time of the final subculture may represent bacteria that were not sufficiently exposed to the drug during the course of the test and thus would give the erroneous perception that the drug was not bactericidal (31, 98, 189).

After determination of the MIC following incubation at 35°C, quantitative subcultures (usually 0.01 ml) are performed at 24 h (following vortexing or shaking at 20 h) with samples from each tube or well with concentrations at and above the MIC of the drug being tested. The aliquots are streaked across the entire surface of a plate containing a standard growth medium (e.g., sheep blood agar). By transferring a small sample volume and spreading it across the entire plate, possible carryover effects of the antibiotic are minimized. In order to calculate the degree of killing at each antibiotic concentration, it is necessary that colony counts be performed on the positive control tube or well at the time of initial inoculation of the MIC test. As in the standard MIC procedure, the target inoculum density is 5×10^5 CFU/ml (31, 36). After the subculture plates are incubated for 24 to 48 h, any visible colonies are counted on each subculture plate. The standard definition of the MBC is the reduction of the initial inoculum by $\geq$99.9% (3 logs) (31). In practice, it is advisable to correct for pipetting error and to account for the Poisson distribution of bacterial cells in a liquid by using statistically derived rejection value tables (31). The tables define the number of colonies that can be tolerated based on the initial inoculum density of each test. If the number of colonies on a particular plate exceeds the rejection value for that test, the antibiotic did not achieve the strict definition of a bactericidal effect at that given concentration of the drug. It is then hoped that the next higher concentration of the drug will have fewer surviving colonies to fulfill the definition of a bactericidal effect. The MBC of an antibiotic should be reported in micrograms per milliliter in the same manner as the MIC is reported. A given MBC test should be considered invalid if there is evidence of contamination of the inoculum or the subcultured tubes or wells, if there is no growth in the positive control well or tube or there is growth in the sterility well or tube, or if there are several skipped dilutions in the MIC portion of the test (31, 67).

Assessment of Bactericidal Activity by the Time-Kill Method

Measuring the rate of bactericidal activity by time-kill analysis provides the opportunity to assess the speed with which killing may occur at a given antibiotic concentration. It is a more laborious approach than determination of the MBC and thus may be best reserved for research purposes. It is also possible to assess the activities of drugs in combination in order to recognize synergistic, indifferent, or antagonistic effects of two or more drugs tested together (31, 99, 104). This approach appears to correlate better with in vivo studies of combined drug effects than the alternative checkerboard titration method (5, 13, 40). The details of performance of the time-kill test have been defined in the CLSI guideline (31). Many of the same critical factors that affect the outcome of the MBC test apply to the performance of time-kill assays, e.g., preparation of an actively growing inoculum and use of quantitative subcultures from antibiotic-containing tubes. The time-kill method requires that multiple samples be removed at various times (e.g., 0, 4, 8, 12, and 24 h) for colony counts, and thus, the volume of

medium for each antibiotic concentration tested is usually ≥10 ml contained in a glass test tube or flask (31). A given antibiotic is often tested at more than one concentration that relates to the previously determined MIC of that agent (e.g., the MIC, two times the MIC, or four times the MIC). When high concentrations (four or more times the MIC) of a drug are tested by the time-kill method, it is necessary to demonstrate that antibiotic carryover has not falsely diminished the number of colonies on subculture plates (31). The results of a time-kill assay are frequently depicted graphically by plotting the colony counts of each antibiotic and concentration tested over time (Fig. 1). A bactericidal effect is again defined as ≥99.9% killing at a specified time (31). When drugs are tested in combination by the time-kill method, synergy is often defined as a ≥2 log decrease in the number of CFU achieved with a drug combination compared to that achieved with the most active drug tested alone (31).

Performance of the Serum Bactericidal Test

The determinations of the serum inhibitory and bactericidal titers are performed in a manner analogous to the MIC and MBC procedures, except that the patient's serum, rather than preweighed and prediluted concentrations of the drug, is used as the source of antibiotic. A guideline that describes the details of the serum bactericidal test has been published by the CLSI (30). The principal steps in performing the test include (i) obtaining one or more blood samples from the patient at specified intervals; (ii) preparing twofold dilutions of the patient's serum in pooled and pretested human serum in glass tubes or plastic microdilution trays; (iii) inoculating each tube or well with a standardized, actively growing inoculum (as described above for the MBC test) of the patient's own infecting organism in an appropriate growth medium (usually cation-adjusted Mueller-Hinton broth); and (iv) incubating for 20 to 24 h (30). Pooled human serum is recommended as the diluent of the patient's serum in each tube or well in order to make protein binding consistent throughout the range of serum dilutions, especially for drugs that are highly protein bound. The pretesting of the pooled human serum includes screening for human immunodeficiency virus type 1 and hepatitis B and C viruses, for antibiotic activity, and for optical clarity (30). In addition, the pooled serum should be heat inactivated immediately prior to use, and the pH should be adjusted to the correct range of 7.2 to 7.4 (30). An alternative to the laborious serum preparation step just described is the creation of a serum ultrafiltrate that precludes the need for a serum diluent (30, 109).

The blood samples for testing are usually obtained from the patient shortly after the antibiotic is administered, at the time of the presumed peak level in serum (usually 30 to

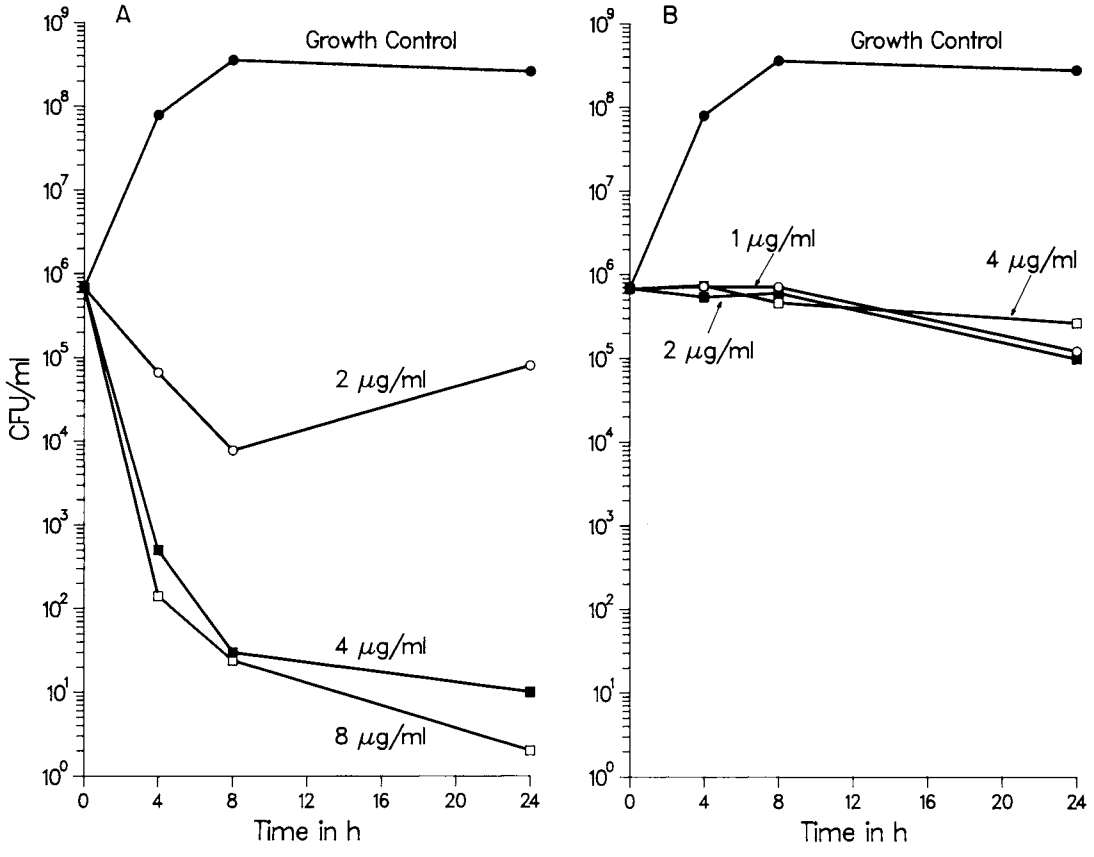


FIGURE 1 Results of time-kill assay of an *Enterococcus faecium* isolate. (A) Daptomycin tested at the MIC (2 μg/ml) and at two and four times the MIC, demonstrating a bactericidal effect at two and four times the MIC. (B) Vancomycin tested at the MIC (1 μg/ml) and at two and four times the MIC, without evidence of a bactericidal effect. Reproduced from reference 95 with permission.

60 min after administration), and often immediately prior to administration of the next drug dose (trough level) (30). The end points of the test are indicated by the highest dilution (titer) of the patient's serum sample that prevents visible growth following the incubation period (serum inhibitory titer). Similar to the MBC determination, quantitative subcultures are performed from each dilution of the patient's serum that has prevented visible growth. The SBT is defined as that dilution (titer) that provides a ≥99.9% reduction of the original inoculum of the test based upon the standard rejection value tables (30). Thus, the results of the test are reported as dilutions (the titer) of the patient's serum rather than as a drug concentration (in micrograms per milliliter) as in the MIC and MBC tests.

Interpreting the Results of Bactericidal Determinations

The interpretation of the results of the bactericidal determinations described above has not received the same degree of critical scrutiny or level of consensus standards that have been achieved for interpretation of MICs. However, a few general statements can be made. A favorable MBC would typically be the same as or perhaps 1 or 2 dilutions greater than the MIC of agents that are normally considered bactericidal (e.g., β-lactams, glycopeptides-lipopeptides, aminoglycosides, and fluoroquinolones). A principal rationale for performing MBC or kinetic time-kill studies would be to detect an isolate that is not effectively killed by a typically bactericidal drug. This failure to exert a bactericidal effect is often described as tolerance and is defined as an MBC ≥32-fold the MIC of a particular agent (31, 70). Failure to achieve at least a 3-$\log_{10}$ reduction of CFU in the kinetic time-kill assay could likewise signify a tolerant strain (31).

Results of the serum bactericidal test that reflect a high titer of antibacterial activity in the serum suggest that the patient has been dosed adequately, has not experienced abnormal elimination of the antibiotic, and does not have a tolerant bacterial isolate. This perception can be quantified by comparing the peak and trough titers obtained with those that have been shown to correlate with rapid organism eradication in collaborative studies (152, 208, 209). For rapid clearance of bacteremia and optimal time to sterilization of cardiac vegetations in endocarditis, a peak SBT of ≥1:64 and a trough SBT of ≥1:32 should be achieved whenever possible (152, 208). However, lower bactericidal titers do not necessarily signify a poor clinical outcome. In patients with chronic osteomyelitis, limited data have suggested that peak SBTs of ≥1:16 and trough SBTs of ≥1:4 predict clinical cure, while titers as low as 1:2 at the trough have been shown to be satisfactory in acute osteomyelitis (152, 209). Titers of ≥1:8 have generally (80% of cases) been associated with satisfactory outcomes for cancer chemotherapy patients (100).

Limitations of Bactericidal Determinations

As stated earlier, clinical microbiologists and infectious disease specialists are not uniformly convinced of the importance of bactericidal determinations as a routine part of even highly complicated bacterial infections. Part of the controversy over the use of these tests may stem from the fact that the procedures themselves have only rather recently been standardized to a significant degree. Indeed, there are still several procedural steps in these tests that can be difficult to control (Table 3), not the least of which are the critical steps of inoculum preparation and dispensing and the final step of accurately counting the surviving bacteria. Despite full attention to the critical details of these test procedures, confusing results may still occur in the form of the skip tube phenomenon (67) or trailing bactericidal end points due to persisters (31). Persisters are thought to represent a fraction of the bacterial population and occasionally exceed 0.1% of the population due to metabolically inactive cells not effectively eradicated during the test (68). A point of occasional concern is the paradoxical or Eagle effect, in which a bactericidal effect can be demonstrated at a relatively low drug concentration, but cells are found not to be killed at very high drug concentrations relative to the MIC (31). This phenomenon has been attributed to secondary effects of some cell wall-active agents on intracellular processes such as protein synthesis, thereby slowing the growth rate and preventing the drug's principal effect on cell wall synthesis of rapidly growing cells (49). A major contribution of the CLSI guidelines is the provision of suggested quality control measures, including established MBC ranges for commonly used control organisms (31, 36). Despite these vigorous efforts to standardize these determinations, it is likely that bactericidal tests will continue to be a subject of discussion and be performed at relatively few institutions for purposes of patient management.

TABLE 3 Technical factors that are critical for reliable determination of MBCs and SBTs and potential pitfalls in those procedure steps

Procedure step	Potential pitfall
Preparation of an actively growing, log-phase inoculum	Use of stationary-phase inoculum will increase the percentage of persisters and potentially result in false tolerance with antibiotics that require rapidly growing cells to exert a bactericidal effect
Delivery of the inoculum aliquot below the meniscus of the broth	Splashing of the inoculum suspension onto the wall of the tube or microdilution well will prevent some cells from contact with the antibiotic and lead to an inaccurate number of colonies upon final subculture
Quantitative subculture of tubes or wells containing drug concentrations at the MIC or higher following incubation	Subculture of too large a volume may cause the problem of antibiotic carryover and persistent inhibition; subculture of an insufficient volume precludes accurate counting of surviving organisms
Determination of ≥99.9% reduction in CFU/ml	Inaccurate pipetting or failure to compensate for pipetting error or Poisson distribution of bacteria will compromise calculation of the bactericidal end point
Failure to account for protein binding in the SBT test	SBT may be falsely elevated with drugs that are highly protein bound (e.g, >90%)

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Susceptibility Test Methods: Fastidious Bacteria*

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Most fastidious bacteria do not grow satisfactorily in standard in vitro susceptibility test systems that use unsupplemented media. For certain fastidious species that are more frequently encountered, such as *Haemophilus influenzae, Neisseria gonorrhoeae, Streptococcus pneumoniae, Neisseria meningitidis,* and other *Streptococcus* spp., slight modifications have been made to standard Clinical and Laboratory Standards Institute (CLSI), formerly NCCLS, disk diffusion and MIC methods to allow reliable testing of these bacteria. The modifications generally involve the use of a test medium with added nutrients and sometimes extended incubation times and/or incubation in an atmosphere with increased levels of CO_2 (Table 1). Specific zone diameter and MIC interpretive criteria have been developed by CLSI for these bacteria, as have acceptable ranges for recommended quality control (QC) strains. CLSI also describes a standard MIC method for testing *Helicobacter pylori* by using an agar dilution procedure. Standard MIC methods for testing potential agents of bioterrorism to include *Bacillus anthracis, Yersinia pestis, Burkholderia mallei, Burkholderia pseudomallei, Francisella tularensis,* and *Brucella* spp. have been developed, and conditions for testing these are listed in Table 2 (41). Most recently, CLSI has published a proposed guideline for testing infrequently isolated or fastidious bacteria including *Abiotrophia* spp., *Granulicatella* spp., *Aeromonas* spp., *Plesiomonas* spp., *Bacillus* spp. (not *B. anthracis*), *Campylobacter jejuni/coli, Corynebacterium* spp., *Erysipelothrix rhusiopathiae,* the *HACEK* group, *Lactobacillus* spp., *Leuconostoc* spp., *Listeria monocytogenes, Moraxella catarrhalis, Pasteurella* spp., *Pediococcus* spp., *Vibrio* spp., (not *V. cholerae*) (38). Interpretive criteria for these organisms were primarily adapted from those for organisms included in CLSI standards (41), taking into consideration information in the literature and the experiences of authors of the guideline. This is in contrast to the extensive microbiological, clinical, and pharmacodynamic databases normally used for development of interpretive criteria that appear in CLSI standards. The classification of M45 as a guideline rather than a standard was based on this fact. A summary of the testing conditions for these organisms appears in Table 3.

There are no specific recommendations for other fastidious bacteria such as *Bordetella* spp. and *Legionella* spp. This is because in part (i) infections caused by these bacteria usually respond to drugs of choice, (ii) isolates are infrequently encountered, and (iii) isolates are often difficult to grow and special media are required.

In addition to conventional MIC test methods (e.g., agar dilution or broth dilution methods), the E-test MIC determination method (AB Biodisk, Solna, Sweden) has been used to test many types of fastidious bacteria. The E-test approach allows placement of strips on special media and the use of various incubation conditions. The limitations of this method include its cost and lack of clearance by the U.S. Food and Drug Administration (FDA) for testing many less commonly encountered fastidious bacteria. Prior to use of the E test for clinical testing in the United States, the FDA clearance status for the particular organism-antimicrobial agent combination should be known. If FDA clearance has not been granted, the results should be interpreted with caution and should be qualified on the patient report.

This chapter summarizes the standard methods recommended by CLSI for antimicrobial susceptibility testing of *Streptococcus* spp. (including *S. pneumoniae*), *H. influenzae, N. gonorrhoeae, N. meningitidis,* and *H. pylori.* The standards for testing potential agents of bioterrorism are also discussed. Methods for testing the infrequently isolated or fastidious bacteria included in the new CLSI M45 guideline are summarized. The incidence of resistance, test methods, and indications for testing and the reporting of results are provided.

S. PNEUMONIAE

Incidence of Resistance

Since it was first reported in 1967, penicillin resistance in pneumococci has been steadily increasing worldwide (15, 62, 63, 114, 216). Interpretive criteria for penicillin and *S. pneumoniae* were originally developed for isolates associated with meningitis and are defined by the CLSI as follows: susceptible, ≤0.06 μg/ml; intermediate, 0.12 to 1 μg/ml; and resistant, ≥2 μg/ml (41). Infections outside the central nervous system, such as pulmonary infections, due to strains for which penicillin MICs are in the intermediate range may be treatable with penicillin (62, 146). It has been suggested

*This chapter contains information presented in chapter 71 by Janet Fick Hindler and Jana M. Swenson in the eighth edition of this Manual.

TABLE 1 Disk diffusion and MIC testing conditions and recommended QC strains for select fastidious bacteria

Organism(s)	Method	Medium[a]	Inoculum source[b]	Incubation atmosphere[c]	Incubation length (h)	Recommended QC strain(s)
S. pneumoniae and *Streptococcus* spp.	Disk diffusion	MHA + 5% sheep blood	18–20-h growth (from SBA)	5–7% CO_2	20–24	*S. pneumoniae* ATCC 49619
	Broth microdilution	CAMHB-LHB	18–20-h growth (from SBA)	Ambient air	20–24	*S. pneumoniae* ATCC 49619
H. influenzae and *Haemophilus parainfluenzae*	Disk diffusion	HTM agar	20–24-h growth (from CHOC)	5–7% CO_2	16–18	*H. influenzae* ATCC 49247, *H. influenzae* ATCC 49766[d]
	Broth microdilution	HTM broth	20–24-h growth (from CHOC)	Ambient air	20–24	*H. influenzae* ATCC 49247, *H. influenzae* ATCC 49766[d]
N. gonorrhoeae	Disk diffusion	GC agar base + supplement	20–24-h growth (from CHOC)	5–7% CO_2	20–24	*N. gonorrhoeae* ATCC 49226
	Agar dilution	GC agar base + supplement	20–24-h growth (from CHOC)	5–7% CO_2	20–24	*N. gonorrhoeae* ATCC 49226
N. meningitidis	Disk diffusion	MHA + 5% sheep blood	20–24-h growth (from CHOC)	5–7% CO_2	20–24	*S. pneumoniae* ATCC 49619
	Broth microdilution	CAMHB-LHB	20–24-h growth (from CHOC)	5–7% CO_2	20–24	*S. pneumoniae* ATCC 49619
	Agar dilution	MHA + 5% sheep blood	20–24-h growth (from CHOC)	5–7% CO_2	20–24	*S. pneumoniae* ATCC 49619
H. pylori	Agar dilution	MHA + 5% aged (≥2-wk-old) sheep blood	72-h growth (from SBA)[e]	Microaerobic; produced by gas-generating system for campylobacters	~72	*H. pylori* ATCC 43504

[a] HTM, GC agar base, and CAMHB-LHB are defined in the text.
[b] Inoculum suspension is in Mueller-Hinton broth or 0.9% NaCl standardized to a 0.5 McFarland standard for disk diffusion. For broth dilution, final organism concentration is 5×10^5 CFU/ml; for agar dilution, final organism concentration is 10^4 CFU/spot; CHOC, chocolate agar; SBA, sheep blood agar.
[c] Incubation temperature, 35°C.
[d] In addition, *H. influenzae* ATCC 10211 can be used to assess growth-supporting capabilities of HTM. *H. influenzae* ATCC 49766 is used for QC of select cephalosporins (e.g., cefaclor, cefamandole, and cefuroxime).
[e] Suspension is in 0.9% NaCl standardized to a 2.0 McFarland standard.

that penicillin MIC interpretive criteria be revised to reflect this (68, 89, 146). The rates for strains not susceptible to penicillin exceed 50% in some parts of the world (15, 99, 104), and in a recent U.S. survey (25), most of the strains not susceptible to penicillin demonstrated high-level resistance (MICs ≥2 μg/ml) rather than intermediate resistance (21.2% versus 14.2%). Some of the highest penicillin resistance rates occur in Asia, where 16.2% of the isolates were reported to be intermediately resistant and 44.1% were highly resistant in 2001–2002 (104). Strains of pneumococci that are susceptible to penicillin generally are susceptible to other β-lactam agents; however, as the penicillin MIC increases, the MICs of other β-lactam agents increase also (25, 99, 114, 160). Pneumococcal strains resistant to cefotaxime or ceftriaxone were not reported until the early 1990s (23, 179), and current rates of resistance to the extended-spectrum cephalosporins vary by location (15, 99, 104, 160). In a recent international survey of over 21,000 isolates, 96.5% of all isolates were susceptible to ceftriaxone (MIC, ≤1.0 μg/ml) whereas among isolates not susceptible to penicillin (MIC, ≥0.12 μg/ml), 89.1% were susceptible to ceftriaxone (160).

Resistance has been described for all classes of antimicrobial agents that are usually considered for treating pneumococcal infections, except for the glycopeptides and linezolid (15, 25, 63, 99, 104, 120, 160). In a recent U.S. survey (24), 31% of isolates were macrolide resistant. Resistance to macrolides in Asian-Pacific countries is much higher, exceeding 75% in some areas (104). Erythromycin MICs for pneumococci with macrolide-lincosamide-streptogramin B (MLS)-type resistance (encoded by the *ermB* gene) are usually ≥64 μg/ml, and clindamycin MICs are ≥8 μg/ml, whereas for isolates of the M phenotype (encoded by the *mef*A gene), erythromycin MICs are in the range of 1 to 32 μg/ml and clindamycin MICs are ≤0.25 μg/ml (54, 99). Susceptibility and resistance to azithromycin, clarithromycin, and dirithromycin among *S. pneumoniae* isolates can be predicted by testing erythromycin (41). Telithromycin, a ketolide, is considerably more active than macrolides against *S. pneumoniae,* with fewer than 1% of isolates showing resistance in recent North American studies (25, 52, 114).

Resistance rates for trimethoprim-sulfamethoxazole (TMP-SMX) range from 35 to 47% in the United States and are similar to those outside the United States (15, 25, 104). Resistance rates for fluoroquinolones remain low (24, 25, 104, 114); however, a higher prevalence of resistance to fluoroquinolones has been shown in other countries (92, 212). Resistance has only been rarely reported for linezolid (6, 25), and resistance rates for quinupristin-dalfopristin are <1% (6, 54) among *S. pneumoniae* strains. Resistance to tigecycline has not been described (69).

Multidrug resistance in *S. pneumoniae* is defined as resistance to three or more antimicrobial classes, commonly penicillins, TMP-SMX, and macrolides (114, 120). As many

TABLE 2 Broth microdilution MIC testing conditions, recommended QC strains, and drugs recommended for testing potential agents of bioterrorism

Organism(s)	Medium[a]	Inoculum source[b]	Incubation atmosphere[c]	Incubation length (h)	Recommended QC strain(s)	Drugs recommended for testing
B. anthracis	CAMHB	16–18-h growth (from SBA)	Ambient air	16–20	*Escherichia coli* ATCC 25922, *S. aureus* ATCC 29213	Ciprofloxacin, doxycycline, penicillin, tetracycline
Brucella spp.	Brucella broth, pH adjusted to 7.1 ± 0.1	48-h growth (from SBA)	Ambient air	48	*E. coli* ATCC 25922, *S. pneumoniae* ATCC 49619	Doxycycline, gentamicin, tetracycline, TMP-SMX, streptomycin
B. mallei, *B. pseudomallei*	CAMHB	16–18-h growth (from SBA)	Ambient air	16–20	*E. coli* ATCC 25922, *E. coli* ATCC 35218, *P. aeruginosa* ATCC 27853	Amoxicillin-clavulanic acid, ceftazidime, doxycycline, imipenem, tetracycline, TMP-SMX (*B. pseudomallei* only)
F. tularensis	CAMHB with 2% defined growth supplement, pH adjusted to 7.3 ± 0.1	24-h growth (from CHOC)	Ambient air	48	*E. coli* ATCC 25922, *S. aureus* ATCC 29213, *P. aeruginosa* ATCC 27853	Chloramphenicol, ciprofloxacin, doxycycline, gentamicin, levofloxacin, streptomycin, tetracycline
Y. pestis	CAMHB	24-h growth (from SBA)	Ambient air	24[d]	*E. coli* ATCC 25922	Chloramphenicol, ciprofloxacin, doxycycline, gentamicin, streptomycin, tetracycline, TMP-SMX

[a] CAMHB, cation-adjusted Mueller-Hinton broth.
[b] Inoculum suspension is in CAMHB standardized to a 0.5 McFarland standard. Final organism concentration is 5×10^5 CFU/ml. CHOC, chocolate agar; SBA, sheep blood agar.
[c] Incubation temperature, 35°C.
[d] If unacceptable growth in the control well, reincubate for an additional 24 h.

as 25% of *S. pneumoniae* isolates in the United States have been reported to be multidrug resistant, and rates are higher in other countries (15, 104, 120).

Reference Test Methods

CLSI describes both a broth microdilution method and a disk diffusion method for the testing of pneumococci (39-41). Details of these procedures are listed in Table 1. The broth microdilution method may be used to test all of the antimicrobial agents recommended by CLSI for pneumococci. With the exception of the oxacillin disk screening test for penicillin susceptibility, the disk diffusion method does not work for testing the β-lactam agents, including the cephalosporins (40, 41, 188). When used to predict penicillin susceptibility, oxacillin disk diffusion zone diameters of ≥20 mm indicate that the isolate is susceptible to penicillin (41). However, strains with zone diameters of ≤19 mm cannot be readily categorized as resistant, since a strain with an oxacillin zone diameter of ≤19 mm may be penicillin susceptible, intermediate, or resistant (53) when the actual penicillin MIC for the strain is determined. Therefore, if the oxacillin screen test is used without follow-up MIC testing for strains with zones of ≤19 mm, the potential for overstating penicillin resistance is high.

Commercial Methods for Testing

Several options are available for determining MICs of various antimicrobial agents for pneumococci. The E-test method has been extensively evaluated, and a recent evaluation was performed by Tenover et al. (191). All drugs recommended by CLSI for testing against pneumococci except clindamycin and telithromycin have been cleared by the FDA for testing by the E test. The accuracy of the E test has been reported to be >90% for most relevant drugs;

TABLE 3 Disk diffusion and MIC testing conditions, recommended QC strains, and agents to consider for primary testing for infrequently isolated or less common fastidious bacteria

Organism(s)	Method	Medium[a]	Inoculum source[b]	Incubation atmosphere[c]	Incubation length (h)	Recommended QC strain(s)	Agents to consider for primary testing
Abiotrophia spp., *Granulicatella* spp.	Broth microdilution	CAMHB-LHB[c] +0.001% pyridoxal HCl	20–24-h growth, from CHOC containing cysteine	Ambient air	20–24	*S. pneumoniae* ATCC 49619	Cefotaxime or ceftriaxone, penicillin, vancomycin
Aeromonas hydrophila complex, *Plesiomonas shigelloides*	Broth microdilution	CAMHB	16–18-h growth (from SBA)	Ambient air	16–20	*E. coli* ATCC 25922, *E. coli* ATCC 35218[d]	Amoxicillin-clavulanic acid, broad-spectrum or extended-spectrum cephalosporins, fluoroquinolones, TMP-SMX
	Disk diffusion	MHA	16–18-h growth (from SBA)	Ambient air	16–18	*E. coli* ATCC 25922, *E. coli* ATCC 35218	
Bacillus spp. (not *B. anthracis*)	Broth microdilution	CAMHB	16–18-h growth (from SBA)	Ambient air	16–20	*S. aureus* ATCC 29213	Clindamycin, gentamicin (for combined therapy, vancomycin)
C. jejuni, *C. coli*	Broth microdilution	CAMHB-LHB	24–48-h growth (from SBA)	10% CO_2, 5% O_2, 85% N_2 (microaerobic)	48 at 36°C or 24 at 42°C	*C. jejuni* ATCC 33560	Ciprofloxacin, erythromycin
Corynebacterium spp.	Broth microdilution	CAMHB-LHB	24–48 h growth, (from SBA or CHOC)	Ambient air	48	*S. pneumoniae* ATCC 49619	Erythromycin, gentamicin, penicillin, vancomycin
E. rhusiopathiae	Broth microdilution	CAMHB-LHB	18–24-h growth (from SBA)	Ambient air	20–24	*S. pneumoniae* ATCC 49619	Penicillin or ampicillin
HACEK group	Broth microdilution	CAMHB-LHB	24–48-h growth, (from CHOC)	Ambient air	24–48	*S. pneumoniae* ATCC 49619, *E. coli* ATCC 35218	Ampicillin, amoxicillin-clavulanic acid, ceftriaxone or cefotaxime, ciprofloxacin or levofloxacin, imipenem, TMP-SMX

Lactobacillus spp.	Broth microdilution	CAMHB-LHB	18–24-h growth (from SBA)	Ambient air	20–24	*S. pneumoniae* ATCC 49619	Penicillin or ampicillin, gentamicin (for combined therapy)
Leuconostoc spp.	Broth microdilution	CAMHB-LHB	18–24-h growth (from SBA)	Ambient air	20–24	*S. pneumoniae* ATCC 49619	Penicillin or ampicillin
L. monocytogenes	Broth microdilution	CAMHB-LHB	18–24-h growth (from SBA)	Ambient air	20–24	*S. pneumoniae* ATCC 49619	Penicillin or ampicillin, TMP-SMX
M. catarrhalis	Broth microdilution	CAMHB	18–24-h growth (from SBA)	Ambient air	20–24	*S. aureus* ATCC 29213, *E. coli* ATCC 35218	Amoxicillin-clavulanic acid, cefaclor or cefuroxime, TMP-SMX
Pasteurella spp.	Broth microdilution	CAMHB-LHB	18–24-h growth (from SBA)	Ambient air	18–24	*S. pneumoniae* ATCC 49619, *E. coli* ATCC 35218	β-Lactam/β-lactamase inhibitor combinations, cephalosporins, fluoroquinolones, macrolides, penicillins, tetracyclines, TMP-SMX
	Disk diffusion	MHA with 5% sheep blood	18–24-h growth (from SBA)	Ambient air	18–24	*S. pneumoniae* ATCC 49619, *E. coli* ATCC 35218, *S. aureus* ATCC 25923 (disk diffusion)	
Pediococcus spp.	Broth microdilution	CAMHB-LHB	18–24-h growth (from SBA)	Ambient air	20–24	*S. pneumoniae* ATCC 49619	Penicillin, gentamicin (for combined therapy)
Vibrio spp. (not *V. cholerae*)[e]	Broth microdilution	CAMHB	16–18-h growth (from SBA)	Ambient air	16–20	*E. coli* ATCC 25922, *E. coli* ATCC 35218	Cefotaxime, ceftazidime
	Disk diffusion	MHA	16–18-h growth (from SBA)	Ambient air	16–18	*E. coli* ATCC 25922, *E. coli* ATCC 35218	Tetracycline, fluoroquinolones

[a] CAMHB-LHB and MHA are defined in the text.
[b] Suspension is in Mueller-Hinton broth or 0.9% NaCl standardized to a 0.5 McFarland standard for disk diffusion. For broth dilution, final organism concentration is 5×10^5 CFU/ml. CHOC, chocolate agar; SBA, sheep blood agar.
[c] Incubation temperature, 35°C (except for *C. jejuni* and *C. coli*).
[d] *E. coli* ATCC 35218 is used for quality control when testing β-lactam/β-lactamase inhibitor combination drugs.
[e] For halophilic species, prepare inoculum in 0.85% NaCl (normal saline).

however, the number of minor errors with penicillin is relatively high. E-test penicillin MICs are slightly lower than those determined by reference broth microdilution (110). Since the E test uses incubation in CO_2, the MICs of the macrolides and ketolides tend to be 1 to 2 dilutions higher than those by the broth tests, in which incubation is done in ambient air (21). This is because these agents are less active at lower pH, which occurs with CO_2 incubation.

Other commercially available FDA-cleared panels or systems specifically designed for testing pneumococci include MicroScan (Dade Behring MicroScan Inc., West Sacramento, Calif.), Phoenix (BD Diagnostic Systems, Sparks, Md.), Sensititre (Trek Diagnostic Systems, Inc, Westlake, Ohio), and Vitek 2 (bioMerieux, Inc., Hazelwood, Mo.). The Pasco, MicroScan MICroSTREP, Sensititre, and Vitek 2 systems have been evaluated and found to produce MICs that are comparable to those obtained with the CLSI broth microdilution reference method (83, 107, 108, 142).

Strategies for Testing and Reporting of Results

CLSI stipulates that *S. pneumoniae* isolates from cerebrospinal fluid (CSF) be routinely tested by a reliable MIC method to determine susceptibilities to penicillin and cefotaxime or ceftriaxone, and meropenem should be added if it is on the institution's formulary. In addition, vancomycin should be tested by either an MIC or a disk diffusion method (41). Along with penicillin, CLSI recommends primary testing and reporting of erythromycin and TMP-SMX for isolates from non-CSF sources. Both drugs can be tested by either an MIC or a disk diffusion method. However, in communities where penicillin resistance is high and resistance to other agents is likely, MIC testing of penicillin, cefotaxime or ceftriaxone, meropenem, vancomycin, and one representative each of the macrolide, fluoroquinolone, and tetracycline classes would be appropriate for testing of strains isolated from non-CSF sources. Other drugs that might warrant testing if being considered for treatment of infections other than meningitis include cefepime, clindamycin, and telithromycin. Infections, such as pneumonia, caused by strains for which the MICs of penicillin are in the intermediate range may be treatable with penicillin or an extended-spectrum cephalosporin if maximal doses are used (68, 89, 146), and it may be appropriate to include a comment to this effect on the patient report. CLSI standards include a second set of nonmeningitis MIC breakpoints for cefotaxime and ceftriaxone that apply to infections other than meningitis (41). For CSF isolates, only interpretations using the original meningitis breakpoints should be reported; for isolates from other sites, both meningitis and nonmeningitis interpretations should be reported (41).

The oxacillin screening procedure should be used only for isolates from patients with non-life-threatening infections. If the zone diameter is ≤19 mm, at a minimum the MICs of penicillin and an extended-spectrum cephalosporin should then be determined (41).

STREPTOCOCCI OTHER THAN PNEUMOCOCCI

Incidence of Resistance

Because there are significant differences in susceptibility of β-lactam agents in viridans group versus beta-hemolytic streptococci, there are separate ampicillin, penicillin, cefotaxime, ceftriaxone, and cefepime interpretive criteria for the two organism groups. As defined by CLSI (41), the members of the viridans group include strains identified as *Streptococcus mitis, S. oralis, S. sanguinis, S. salivarius, S. intermedius, S. constellatus, S. mutans,* and *S. bovis,* as well as the small-colony-forming beta-hemolytic strains with group A, C, F, or G antigens (the *S. anginosus* group, which was previously called *S. milleri*). The beta-hemolytic group includes the large-colony-forming pyogenic strains with group A *(S. pyogenes)*, C, or G antigens and strains with group B *(S. agalactiae)* antigen.

Although beta-hemolytic streptococci have been uniformly susceptible to penicillin, two reports have described penicillin MICs of 0.25 to 0.5 μg/ml for group B streptococci (17) and group C streptococci (194). The clinical significance of these elevated MICs is not known. High-level aminoglycoside resistance has been described for group B streptococci (26). The MLS-type macrolide resistance due to *erm* genes in beta-hemolytic streptococci may be either inducible or constitutive (45). If the resistance is inducible, the strains will be resistant to erythromycin (and the other 14- and 15-membered macrolides) but appear to be susceptible to clindamycin unless resistance is induced. Resistance rates for erythromycin vary for group A streptococci and were recently reported to be as high as 32% in parts of California (213). In a large Canadian study conducted in 2002–2003, the predominant mechanism for erythromycin resistance in group A streptococcus was *mef*A whereas that in group B streptococcus was *erm* (47). Resistance rates among streptococci vary for tetracyclines and are low for fluoroquinolones (6, 25, 69). In several recent large surveillance studies, resistance was not observed for daptomycin (101), linezolid, or quinupristin-dalfopristin (6, 69).

Penicillin resistance in viridans group streptococci has been described, and resistance rates can be >50% (2, 69), particularly for *S. mitis* and *S. sanguinis*. Ahmed et al. noted 67.5% penicillin resistance among 40 blood culture isolates from bacteremic children with cancer (2). Resistance has been described for some viridans group isolates for macrolides, lincosamides, tetracyclines, quinupristin-dalfopristin, and fluoroquinolones (2, 3, 69, 195). Single reports of resistance to vancomycin have been noted for *S. bovis* (161) and for *S. mitis* (124), and resistance to linezolid has been reported on at least one occasion (172). In a recent study, all 92 viridans group streptococci selected to represent more resistant strains were susceptible to daptomycin (101). High-level aminoglycoside resistance has been described for viridans group streptococci (95).

Reference Test Methods

Studies by CLSI to determine interpretive criteria for both MIC and disk diffusion testing have resulted in breakpoints for streptococci that were initially published in 1995. Penicillin and select cephalosporin breakpoints were subsequently modified as mentioned above. Details for MIC and disk diffusion tests are described in Table 1. The disk diffusion test may be used to determine the penicillin susceptibility of beta-hemolytic streptococci; however, it is unreliable and should not be used for viridans group streptococci. Other agents may be tested by either the MIC or the disk method. Aminoglycoside MICs of >1,000 μg/ml for streptococci (207) have been described; however, there are no published methods for screening for high-level aminoglycoside resistance in streptococci.

Susceptibility testing of members of the genera *Abiotrophia* and *Granulicatella* (formerly known as "nutritionally deficient

streptococci") are included in the new CLSI M45 document as described below. Recent CLSI standards describe a disk approximation test for inducible resistance to clindamycin in beta-hemolytic streptococci whereby erythromycin is used to induce resistance to clindamycin. Mueller-Hinton agar (MHA) supplemented with 5% sheep blood is inoculated as for a disk diffusion test, and a 15-μg erythromycin disk and a 2-μg clindamycin disk are placed approximately 12 mm apart on the agar surface. The plate is incubated at 35°C overnight, and flattening of the clindamycin zone indicates inducible clindamycin resistance (41).

Commercial Test Methods

There have been few reports of evaluations of commercial susceptibility test systems for streptococci other than pneumococci, although it might be expected that systems capable of testing pneumococci would also perform adequately for other streptococci. The MicroScan and Phoenix systems are cleared by the FDA for the testing of *Streptococcus* spp. The Vitek 2 system is FDA cleared for testing of group B streptococci. The E test has not been extensively evaluated but does appear to be a possible alternative to the reference methods for testing *Streptococcus* spp. (106, 173).

Strategies for Testing and Reporting of Results

Routine testing of β-hemolytic streptococci is unnecessary since there have been only sporadic reports of decreased susceptibility to penicillin, primarily in group B streptococci (17, 194). However, if erythromycin is being used to treat infections caused by group A streptococci and treatment failure is suspected, testing might be considered. Similarly, if erythromycin is being considered for prophylaxis of pregnant women who are highly allergic to penicillin, in an effort to prevent perinatal group B streptococcal disease, testing should be done. If clindamycin is being considered for β-hemolytic streptococci that test clindamycin susceptible and erythromycin resistant, the determination of inducible clindamycin resistance should be considered. For viridans group streptococci, penicillin MICs should be determined for strains isolated from blood, especially for patients with infective endocarditis. Disk diffusion is not reliable for testing penicillin in viridans group streptococci (10).

H. INFLUENZAE

Incidence of Resistance

Ampicillin resistance in *H. influenzae* is most commonly mediated by a plasmid-borne β-lactamase, TEM-1 being the most common enzyme. The incidence of β-lactamase-producing *H. influenzae* organisms ranges from 5% to nearly 40% in various geographic areas (15, 25, 63, 100, 105, 183, 217). Recently, a decrease in β-lactamase-producing isolates has been documented in North America (90). Ampicillin resistance can result from altered penicillin-binding proteins in β-lactamase-negative, ampicillin-resistant (BLNAR) strains, but this mechanism of resistance appears to be uncommon. In a recent survey, Brown and Rybak noted that 0.4% of 3,296 isolates from throughout the United States were BLNAR (25). Among 395 isolates collected from patients with meningitis during 1999 through 2002 in Japan, 13.9% were shown to be BLNAR (86). However, in a respiratory surveillance study conducted during a similar time in Japan, only one BLNAR strain among 281 isolates was found (98). Compared with β-lactamase-producing or ampicillin-susceptible *H. influenzae,* BLNAR isolates are less susceptible to amoxicillin-clavulanic acid and to various cephalosporins such as cefaclor, cefuroxime, cefixime, and cefotaxime (13). Use of a 2-μg rather than a 10-μg ampicillin disk and a 1/2 μg rather than a 10/30 μg amoxicillin-clavulanic acid disk with the standard CLSI disk diffusion method has been suggested as a method to better identify BLNAR strains (115).

Resistance among *H. influenzae* isolates to broad-spectrum oral cephalosporins (e.g., cefixime and cefpodoxime) and to extended-spectrum cephalosporins (e.g., cefotaxime) is rare (217). Resistance to expanded-spectrum cephalosporins (e.g., cefuroxime) is uncommon, <3% in a recent survey (102). The narrower-spectrum, β-lactamase-labile cephalosporins (e.g., loracarbef and cefprozil) are less active, with resistance rates ranging from 3 to nearly 30% (25, 102, 105, 183, 217).

Resistance to TMP-SMX among *H. influenzae* organisms occurs in approximately 20% of isolates from North America (93, 100, 102, 217), but resistance rates as high as 60% have been noted in other parts of the world including Southeast Asia (64). Resistance to tetracyclines is <3% (25, 102, 217), and resistance to fluoroquinolones is rare (25, 60, 93). Although erythromycin has not been considered a drug of choice for the treatment of infections caused by *H. influenzae,* some of the newer macrolides are more active than erythromycin and may be effective as treatment. Less than 2% of isolates examined in various studies were resistant to azithromycin; however, clarithromycin resistance rates approached 20% (25, 98, 100, 102, 105, 183, 217). Telithromycin is highly active against *H. influenzae* with resistance rates of approximately 1% (25, 98, 217).

Reference Test Methods

β-Lactamase production in *H. influenzae* can easily be detected by the chromogenic cephalosporin, acidometric, or iodometric β-lactamase test methods (see chapter 74).

CLSI has developed broth microdilution MIC and disk diffusion methods for testing *H. influenzae,* and these methods can also be used to test *H. parainfluenzae*. Testing of the more unusual *Haemophilus* spp. including *H. aphrophilus, H. paraphrophilus,* and *H. segnis* are now addressed in the new CLSI M45 document. Specific variables related to each of the methods for testing *H. influenzae* and *H. parainfluenzae* are listed in Table 1. *Haemophilus* test medium (HTM) is recommended and consists of a Mueller-Hinton base, 15 μg of hematin per ml, 15 μg of NAD per ml, and 5 mg of yeast extract per ml. Cation-adjusted Mueller-Hinton broth (CAMHB) is used with the components listed above for the preparation of HTM broth, which also contains 0.2 IU of thymidine phosphorylase per ml. Although HTM agar is transparent, some investigators have reported difficulties in measuring zones and poor growth of some strains (88, 141). Broth microdilution tests with HTM generally give clearer endpoints. The problems most often noted with both the disk diffusion and the broth microdilution methods are equivocal endpoints with BLNAR strains with several β-lactams. Because of this, CLSI recommends that BLNAR strains (which are best detected by tests with ampicillin) be considered resistant to amoxicillin-clavulanic acid, ampicillin-sulbactam, cefaclor, cefamandole, cefetamet, cefonicid, cefprozil, cefuroxime, loracarbef, and piperacillin-tazobactam and that the activities of these agents against BLNAR strains not be tested (41).

Commercial Test Methods

Currently, the only FDA-cleared broth microdilution system for testing *Haemophilus* spp. is Sensititre. The E test has been

cleared by the FDA for testing *H. influenzae* with most drugs that would be used for treating *Haemophilus* infections, and results with E test have been shown to be comparable to those obtained by CLSI reference methods (111).

Strategies for Testing and Reporting of Results

β-Lactamase testing will detect the most common type of clinically significant resistance in *H. influenzae*. β-Lactamase-positive isolates are ampicillin and amoxicillin resistant. To detect BLNAR strains, an ampicillin disk diffusion or MIC test is required. However, since the incidence of BLNAR is low in many geographic areas, such tests may not be routinely needed, and for practical purposes, a negative β-lactamase test result translates into ampicillin susceptibility. Frequently, the β-lactamase test is the only test routinely performed with clinical isolates. Because of increasing resistance to TMP-SMX, testing should be considered and CLSI recommends that this agent be tested and reported routinely (41). Other oral agents that might be considered, such as amoxicillin-clavulanic acid, the oral cephalosporins, newer macrolides, and quinolones, are predictably active against *H. influenzae* and are often prescribed empirically. Consequently, routine testing of these drugs is generally not useful. However, these and other agents may be tested for surveillance or epidemiological purposes (41).

N. GONORRHOEAE

Incidence of Resistance

During the past 2 decades, increasing rates of penicillin and tetracycline resistance among *N. gonorrhoeae* isolates have led to recommending ceftriaxone, cefixime, ciprofloxacin, ofloxacin, or levofloxacin as the primary agents for uncomplicated gonorrhea instead of penicillin or tetracycline (30). Penicillin resistance is due to the production of a plasmid-associated TEM-1 type β-lactamase (penicillinase-producing *N. gonorrhoeae* [PPNG]) or to mutations in chromosomal genes that result in altered penicillin-binding proteins or diminished outer membrane permeability (chromosomally mediated resistant *N. gonorrhoeae* [CMRNG]). The activities of other β-lactams against PPNG are generally unaltered; however, CMRNG may show decreased susceptibility to other β-lactams (49). Tetracycline resistance in *N. gonorrhoeae* can be plasmid- or chromosomally mediated, with plasmid-mediated resistance resulting in a higher level of resistance.

The Gonococcal Isolate Surveillance Program, which tests urethral isolates from male clients visiting sexually transmitted disease clinics throughout the United States, reported that 16.4% of 6,552 isolates were resistant to penicillin or tetracycline or both in 2003. The incidence of PPNG peaked at 11.0% in 1991 and was only 1% in 2003. The incidence of tetracycline resistance has remained fairly stable and was 3.7% in 2003. All 6,552 isolates tested in 2003 were susceptible to ceftriaxone and cefixime; however, there appears to be a subtle shift towards higher MICs for both these agents. Nevertheless, only 4 isolates had elevated ceftriaxone MICs of 0.5 μg/ml and 45 isolates had elevated cefixime MICs of 0.5 to 2.0 μg/ml (29).

Fluoroquinolone resistance has become widespread throughout Asia and is increasing in the United States. In a study of 115 isolates *of N. gonorrhoeae* from female sex workers in the Philippines, 49% of isolates had ciprofloxacin MICs of ⩾4 μg/ml and 63% of isolates had MICs ⩾1 μg/ml (8). In Japan in 2002, 70% of 87 *N. gonorrhoeae* isolates from males with urethritis were ciprofloxacin resistant and only 1 isolate produced β-lactamase (177). The Gonococcal Antimicrobial Susceptibility Program conducted by the World Health Organization noted that ciprofloxacin resistance rates for gonococci from patients in India in 2001 varied from 10.6 to 100% among 10 regions studied (165). In the 2003 Gonococcal Isolate Surveillance Program study, 0.9% of the isolates exhibited intermediate susceptibility and 4.1% were resistant to ciprofloxacin (29). Newman et al. noted that 17% of isolates from 117 patients seen at a public sexually transmitted disease clinic in Hawaii in 2001 were ciprofloxacin resistant (150).

Reference Test Methods

Routine β-lactamase tests readily detect PPNG and can reliably be performed by either the chromogenic cephalosporin, acidimetric, or iodometric method.

CLSI recommends the use of GC agar base for disk diffusion and agar dilution MIC testing. For both tests, a 1% defined growth supplement must be added; however, in agar dilution tests with imipenem and with clavulanate, the growth supplement must be free of cysteine to avoid inhibition of the activities of these two agents (39-41). The agar dilution method is preferred to the broth dilution method for MIC testing because *N. gonorrhoeae* has a tendency to autolyse in liquid media. For other details of testing, see Table 1.

Commercial Test Methods

The only commercial method currently available for susceptibility testing of *N. gonorrhoeae* is E test, which has been shown to produce results comparable to those of conventional reference agar dilution methods (18).

Strategies for Testing and Reporting Results

Generally, there is no need for routine clinical laboratories to perform antimicrobial susceptibility tests with *N. gonorrhoeae* unless there are unusual circumstances. These might include the patient's intolerance to the drugs of choice, treatment failure (assuming that compliance was not an issue), or a disseminated gonococcal infection. Additionally, if fluoroquinolone resistance is noted in a particular geographic area, testing may be warranted if fluoroquinolones are being prescribed. Some laboratories may perform β-lactamase tests for all isolates if β-lactamase results are requested by the local public health departments for epidemiological purposes. However, many public health departments have eliminated this requirement. Surveillance for established and emerging resistance for *N. gonorrhoeae* is generally performed by designated state and local public health agencies.

N. MENINGITIDIS

Incidence of Resistance

Increasing numbers of β-lactamase-negative *N. meningitidis* isolates from the United States and elsewhere have reduced susceptibility to penicillin (MICs, 0.12 to 1.0 μg/ml), although the frequency of these isolates in different regions varies widely (85, 167, 200).

A study of 400 *N. meningitidis* isolates obtained in 1998 and 1999 in Spain showed that 37% of them had penicillin MICs of 0.12 to 1.0 μg/ml (200). In a study of 53 invasive isolates collected in 1998–99 in the United States, 30.2% had penicillin MICs of 0.12 to 0.25 μg/ml (167). There have been two recent reports from Europe of *N. meningitidis* isolates with penicillin MICs of 1.5 μg/ml and 2.0 μg/ml; E test was used for testing the first isolate, and agar dilution was used for the

second (59, 80). The clinical significance of isolates for which penicillin MICs are elevated is uncertain, and penicillin remains the drug of choice for the treatment of meningococcal disease (74, 138). Many infections caused by isolates with reduced susceptibility to penicillin have successfully been treated with high doses of penicillin (186, 208); however, rare reports cited clinical failure (meningitis) when a lower than recommended dose of penicillin was used (198). The broad-spectrum cephalosporins (e.g., ceftriaxone) remain highly active against isolates with elevated penicillin MICs (85, 164, 167, 200). In the 1980s, four isolates of β-lactamase-producing *N. meningitidis* were reported (20, 50, 67), and one isolate was reported in 1996 (201); additional isolates have not been noted.

Regarding agents used for prophylaxis, resistance to sulfonamides occurs frequently, and resistance to rifampin has been documented on several occasions (109, 164, 171, 200). There are two reports of *N. meningitidis* isolated from CSF with decreased susceptibility to ciprofloxacin (MIC, 0.12 and 0.25 μg/ml), one from Australia and another from Spain; however, *N. meningitidis* is typically very susceptible to this agent (MIC, ≤0.03 μg/ml) (4, 178).

Strategies for Testing and Reporting of Results

CLSI recently published standards for susceptibility testing of *N. meningitidis* that utilizes either the broth microdilution method and CAMHB with 2 to 5% lysed horse blood (CAMHB-LHB) or the agar dilution method and MHA with 5% sheep blood, both with incubation in 5% CO_2 (41, 109). Interpretive criteria are listed for agents such as penicillin, cefotaxime, and ceftriaxone, which might be prescribed for treating a meningococcal infection. In addition, interpretive criteria are listed for agents such as ciprofloxacin and rifampin, which might be used for prophylaxis of meningococcal case contacts. CLSI also approved a disk diffusion method for testing *N. meningitidis* which utilizes MHA with 5% sheep blood and CO_2 incubation. As with MIC tests, interpretive criteria are listed for several therapeutic and prophylactic agents. Unfortunately, these do not include breakpoints for ampicillin and penicillin, as results obtained with these agents were unreliable (41). Several investigators have examined modified penicillin disk diffusion methods by using 2- and 10-IU penicillin disks and 1-μg oxacillin disks and also determined that these cannot reliably distinguish *N. meningitidis* isolates that are susceptible from those that have decreased ampicillin and penicillin susceptibility (19, 27, 157). Therefore, disk diffusion testing should not be used for these drugs.

Because of the lack of clinical failures with the drugs of choice for the treatment of meningococcal infections, susceptibility testing is not warranted in most situations (170, 171). Although the E test has not yet been cleared by the FDA for testing *N. meningitidis,* several studies have shown it to be reliable for testing meningococci and to perform best by using MHA with 5% sheep blood incubated in CO_2 (1, 136, 155, 157, 199). However, in a 14-lab study, Vazquez et al. noted some difficulties in obtaining reliable results for rifampin with E test (199).

H. PYLORI

Incidence of Resistance

The rates of resistance for *H. pylori* vary considerably among the agents recommended for therapy (e.g., amoxicillin, clarithromycin, metronidazole, and tetracycline), with resistance to metronidazole being highest. Using the CLSI reference method, Osato et al. reported approximately 35% resistance to metronidazole among 3,193 isolates in the United States (152). In a Korean study that used similar methodologies, 40.6% of 652 isolates were metronidazole resistant (118); however, a study in Japan reported only 12.4% metronidazole resistance among 388 isolates but noted significant differences from various geographic areas (116). Clarithromycin resistance rates were lower, with approximately 11% resistance in the U.S. study and 5.9 and 12.9% resistance observed in the Korean and Japanese studies, respectively. The incidence of resistance to tetracycline and amoxicillin is currently very low (118, 126, 152). Kim et al. recently identified discordant susceptibility results for clarithromycin, metronidazole, and tetracycline when testing multiple isolates from different locations in the stomach of individual patients (117). A similar finding was reported by Osato et al. (151) for metronidazole susceptibility. These results suggest that resistant isolates are not evenly distributed throughout the stomach and susceptibility results for isolates from a single biopsy may not be representative of the entire population.

Reference Test Method

CLSI describes an agar dilution MIC method for testing *H. pylori*. The test medium is MHA supplemented with aged (≥2-week-old) sheep blood. The inoculum is prepared from 72-h-old growth on a blood agar plate to obtain a final concentration of bacteria approximating 10^5 CFU/spot. Incubation is for 3 days at 35°C in a microaerobic atmosphere produced by a gas-generating system typically used for campylobacters. *H. pylori* ATCC 43504 has been designated as a QC strain, and currently there are only interpretive criteria for clarithromycin. Other antimicrobial agents that have been studied and for which there are CLSI QC ranges include amoxicillin, metronidazole, telithromycin, and tetracycline (41).

Commercial Test Methods

Although no commercial methods are FDA cleared for antimicrobial susceptibility testing of *H. pylori,* several investigators have examined the E test. In one study the percent agreement (i.e., results within ±1 doubling dilution) between E test and agar dilution was 84.6% for amoxicillin, 94.1% for clarithromycin, 89.9% for metronidazole, and 89.1% for tetracycline (159). In a multilaboratory study, laboratories correctly identified clarithromycin- and metronidazole-susceptible and -resistant strains 93% of the time using E test (16). However, two studies reported significant discrepancies for metronidazole susceptibility by agar dilution and E test (139, 151). All of these E-test evaluations report differences in methods for agar dilution and E test, such as media, inoculum, and incubation conditions, which may account for the different results.

Strategies for Testing and Reporting of Results

Because of growth requirements and complex antimicrobial susceptibility testing recommendations, testing of *H. pylori* is not practical for the routine clinical laboratory. However, because of the significant resistance noted for metronidazole and clarithromycin, testing may be required in select situations, in which case a reliable reference laboratory should be used. Although there are currently no CLSI interpretive criteria for metronidazole, investigators have used an MIC of >8 μg/ml for resistance (118, 126, 151, 152).

POTENTIAL BACTERIAL AGENTS OF BIOTERRORISM

Several bacterial agents are identified as potential agents of bioterrorism by the Centers for Disease Control and Prevention (http://www.bt.cdc.gov/agent/index.asp). These include *Bacillus anthracis, Yersinia pestis, Francisella tularensis, Burkholderia mallei, Burkholderia pseudomallei,* and *Brucella* spp. Until recently, standardized antimicrobial susceptibility testing methods and interpretive criteria did not exist for any of these bacteria. The antimicrobial susceptibility patterns for most of them are predictable, so susceptibility testing of naturally occurring isolates is often not necessary. However, it is possible for antimicrobial resistance to spontaneously occur among these organisms and, in the case of a bioterrorism event, there is the possibility of engineered resistance. To detect any possible resistance, standardized broth microdilution susceptibility testing methods and interpretive criteria were established and published by CLSI (41).

Acquired resistance to antimicrobial agents commonly used for treatment of infections caused by *B. anthracis, Y. pestis, F. tularensis, B. mallei, B. pseudomallei,* and *Brucella* spp. is rare. Isolates of *B. anthracis* may contain inducible β-lactamases, which can confer resistance to penicillins and cephalosporins (32, 56, 130). Because of this potential resistance, penicillin is recommended for anthrax prophylaxis only when the organism burden would be low and this recommendation is limited to young children and pregnant women who should not receive other prophylaxis therapies such as fluoroquinolones and tetracyclines (28). Laboratory-generated fluoroquinolone resistance in *B. anthracis* was reported, but fluoroquinolone resistance in clinical isolates has not been reported (34, 163). There are reports of two spontaneously occurring drug-resistant *Y. pestis* isolates recovered in Madagascar (71, 81). Both isolates acquired conjugative plasmids. In one isolate, the plasmid carried resistant determinants for ampicillin, chloramphenicol, tetracycline, kanamycin, streptomycin, and sulfonamide. In the other isolate, the plasmid conferred resistance to streptomycin. Susceptibility studies of *B. pseudomallei* and *B. mallei* indicate that most isolates are susceptible to amoxicillin-clavulanic acid, ceftazidime, imipenem, tetracycline, doxycycline, and TMP-SMX, using CLSI interpretive criteria (192). However, some isolates of *B. pseudomallei* have elevated ceftazidime MICs (i.e., MIC ≥8 μg/ml) (97, 192). Clinical isolates of *Brucella* spp. with reduced susceptibility to rifampin and TMP-SMX have been reported (14, 119, 140). In these studies, the susceptibility testing methods used were either not clearly defined or different from the methods currently recommended by CLSI. Also, resistant isolates were not characterized for resistance determinants or mutations. Therefore, the definition of resistance to rifampin or TMP-SMX among *Brucella* spp. is unclear. Isolates of *F. tularensis* demonstrate β-lactamase activity, and they are resistant to β-lactams including cephalosporins and carbapenems (12, 96). Resistance to aminoglycosides, tetracyclines, fluoroquinolones, and chloramphenicol has not been reported.

B. anthracis, B. mallei, B. pseudomallei, and Y. pestis demonstrate sufficient growth with commonly used broth microdilution MIC testing medium and incubation conditions (Table 2). However, the recommended testing medium for *F. tularensis* is CAMHB with 2% of a defined growth supplement. This is the same cysteine-containing defined growth supplement that is added to GC agar base for susceptibility testing of *Neisseria gonorrhoeae*. The supplement is commercially available from two manufacturers: IsoVitalex Enrichment (BD Diagnostics, Franklin Lakes, N.J.) and XV Factor Enrichment (PML Microbiologicals, Wilsonville, Oreg.). Some isolates of *Brucella* spp., particularly isolates of *B. abortus,* require CO_2 incubation. Studies are under way to determine if incubation in CO_2 significantly changes MIC values for antimicrobial agents that should be tested such as aminoglycosides, tetracyclines, or TMP-SMX. For the slower-growing organisms, *F. tularensis, Y. pestis,* and *Brucella* spp., 48 h of incubation may be needed to achieve sufficient growth.

There are few studies comparing alternative susceptibility testing methods to the standard CLSI reference broth microdilution methods. One study compared E test to broth microdilution for testing *B. anthracis* (143). In this study, E-test MICs were within a single doubling dilution of the broth microdilution MICs for ceftriaxone, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, rifampin, tetracycline, and vancomycin, but not for penicillin. Penicillin MIC values for E test tended to be lower than those obtained by broth microdilution. A similar trend for penicillin MICs was noted for *B. anthracis* in a study comparing E test to agar dilution (197). These results suggest that E test is acceptable for susceptibility testing of *B. anthracis* with antimicrobial agents other than penicillin. Similar studies are needed to establish alternative methods for the other potential agents of bioterrorism so that testing is available in more laboratories.

Susceptibility testing of potential agents of bioterrorism raises safety concerns for clinical microbiology laboratories. All of the above-mentioned organisms require at least biosafety level (BSL) 2 practices, containment, and facilities. BSL 3 conditions are recommended for activities with a high potential for aerosol production (166). Since the inoculum preparation for susceptibility testing has the potential for aerosol production, all susceptibility testing procedures must be performed in a BSL 3 facility. Laboratories without BSL 3 facilities should send isolates to an appropriately equipped reference laboratory that has select agent clearance status, such as that at reference laboratories within the Laboratory Response Network and at the Centers for Disease Control and Prevention (http://www.cdc.gov/od/sap/ and http://www.bt.cdc.gov/lrn/biological.asp).

ABIOTROPHIA SPECIES AND *GRANULICATELLA* SPECIES

For *Abiotrophia* spp. and *Granulicatella* spp., resistance has been reported to agents from several different antimicrobial classes including penicillins, cephalosporins, carbapenems, macrolides, lincosamides, fluoroquinolones, and tetracyclines. In a study of 20 blood isolates by Murray et al., resistant rates for specific agents were as follows: penicillin, 5%; cefotaxime, 35%; cefepime, 47%; meropenem, 10%; erythromycin, 30%; clindamycin, 5%; and tetracycline, 10% (145). These results are consistent with those previously reported (73, 196). Murray et al. also noted a levofloxacin-resistant *Abiotrophia elegans* (currently known as *Granulicatella elegans*) which was isolated from a patient who had received 10 days of levofloxacin prophylaxis (145). Little is known about mechanisms of resistance in these bacteria. There is one report of an *Abiotrophia defectiva* isolate with a conjugative, Tn*916*-like, transposon, Tn*3872* (162). The transposon carried *ermB* and *tetM* resistance determinants, which conferred resistance to erythromycin and tetracycline, respectively. This finding demonstrates, that these bacteria are able to

acquire and transfer mobile genetic elements which confer antimicrobial resistance.

Because *Abiotrophia* spp. and *Granulicatella* spp. are fastidious, it is recommended that these be tested in CAMHB-LHB supplemented with 0.001% pyridoxal hydrochloride and incubated in CO_2 (Table 3) (38, 193). In a report by Namdari et al., a procedure for adding pyridoxal to an inoculum prepared with the Prompt system and a MicroScan dried gram-positive panel is described (147). It should be noted that susceptibility testing of aminoglycosides against *Abiotrophia* spp. and *Granulicatella* spp. is not recommended even though aminoglycosides are commonly used in combination therapy with an agent active against the cell wall, such as penicillin or vancomycin, for treating serious infections caused by these organisms. Since the aminoglycoside acts synergistically with the agent, in vitro susceptibility to aminoglycosides does not predict in vivo susceptibility (22).

AEROMONAS HYDROPHILA COMPLEX (*A. CAVIAE, A. HYDROPHILA, A. JANDAEI, A. SCHUBERTII,* AND *A. VERONII*) AND *PLESIOMONAS SHIGELLOIDES*

Aeromonas spp. are typically resistant to ampicillin, show variable results with β-lactam/β-lactamase inhibitor combinations, and are resistant to narrow-spectrum cephalosporins (122, 154, 203, 204). Inducible β-lactamases in *Aeromonas* spp. have been noted, and resistance may emerge during therapy with a β-lactam agent (123). A recent study showed that all 18 isolates from patients with traveler's diarrhea were susceptible to cefotaxime and ciprofloxacin and were variably resistant to chloramphenicol, tetracycline, and TMP-SMX (204). In Taiwan, where *Aeromonas* spp. are frequently encountered, results from 234 isolates in 1996 demonstrated that most isolates were susceptible to expanded-spectrum cephalosporins, fluoroquinolones, and aminoglycosides and approximately one-half of the isolates were susceptible to tetracycline and TMP-SMX. These authors remarked that there appeared to be species differences and *A. sobria* was more susceptible than either *A. hydrophila* or *A. caviae* (122). Similar results were noted by Vila et al. from Spain in 2002 (203).

Plesiomonas spp. are typically resistant to ampicillin and other penicillins including piperacillin and susceptible to β-lactamase inhibitor combinations and several other classes of antimicrobial agents (37, 78, 113, 185). However, Stock and Wiedemann noted ceftazidime resistance for 20 of 52 human isolates when an inoculum of 10^6 CFU/ml was used with a standard CLSI broth microdilution method. In another study, these same authors saw considerable variations in MICs when different media and inoculum concentrations from 10^4 to 10^7 were used to test 30 different β-lactams (184).

Although *Aeromonas* spp. and *Plesiomonas shigelloides* cause diarrheal disease, otherwise healthy individuals with diarrhea due to these bacteria usually recover spontaneously without treatment (78, 203) and routine antimicrobial susceptibility testing of isolates from fecal specimens is not recommended (38). However, both genera can cause a variety of other infections where treatment may be necessary (9, 78, 122, 123, 185, 204). Consequently, for isolates from extraintestinal sources, antimicrobial susceptibility testing may be warranted. These organisms share growth characteristics similar to those of *Enterobacteriaceae,* which led CLSI to recommend broth microdilution MIC testing using a method similar to that used for *Enterobacteriaceae*.

BACILLUS SPECIES

Bacillus spp. other than *B. anthracis* are common contaminants in the clinical laboratory, but these bacteria can cause serious disease. Since the susceptibility profiles of *Bacillus* spp. are quite variable, susceptibility testing should be considered when *Bacillus* spp. are isolated from normally sterile body sites. Isolates of *Bacillus* spp. can have β-lactamases that confer resistance to penicillins and possibly cephalosporins (174). *B. cereus* is the most common species found in clinical specimens, and most of these isolates are resistant to penicillin and extended-spectrum cephalosporins, although penicillin resistance does not always predict cephalosporin resistance (197, 205). Penicillin and cephalosporin resistance also occurs in other species of *Bacillus,* but it is less common. Resistance to several other agents has been reported. These agents include aminoglycosides, erythromycin, clindamycin, chloramphenicol, tetracycline, fluoroquinolones, TMP-SMX, and vancomycin. Resistance to carbapenems has not been reported for *Bacillus* spp. (197, 205; F. Cockerill, unpublished data).

Standard susceptibility testing methods for nonfastidious bacteria can be used for testing *Bacillus* spp. CLSI provides guidelines for broth microdilution testing (Table 3) (38). Agar dilution and E-test methods using MHA were compared in a study by Turnbull et al. (197). In both studies E-test MICs were similar to agar dilution MICs for several agents including cefotaxime, ciprofloxacin, erythromycin, gentamicin, penicillin, tetracycline, and vancomycin. Two commercial β-lactamase tests, Nitrocefin (Oxoid, Basingstoke, England) and Intralactam strips (Mast Diagnostics, Merseyside, United Kingdom) were evaluated in a study by Andrews and Wise (7); both tests failed to detect β-lactamase activity in four of five penicillin-resistant isolates of *B. cereus*. Therefore, β-lactamase testing is not recommended for predicting penicillin susceptibility in *Bacillus* spp.

C. JEJUNI

Resistance to macrolides and fluoroquinolones, the drugs of choice for treating gastrointestinal infections caused by *C. jejuni* and *C. coli,* is being reported with increasing frequency for human and animal isolates. The National Antimicrobial Resistance Monitoring System noted no ciprofloxacin resistance among *C. jejuni* human isolates in 1990, but a resistance rate of 12% occurred in 1997 and this increased to 18% in 2001. Erythromycin resistance was 1% in 1997 and 2% in 2001 (82). Smith et al. examined nearly 5,000 *C. jejuni* isolates from feces of Minnesota residents and noted an increase in the incidence of ciprofloxacin resistance from 1.3% in 1992 to 10.2% in 1998 (180). Of 537 *C. jejuni* isolates from human fecal specimens obtained during 1997 and 1998 in Spain, 75% were resistant to ciprofloxacin and 3.2% were resistant to erythromycin (175). A recent study noted that patients with invasive illness due to resistant isolates were more likely to have adverse effects than those infected with susceptible isolates (91). Resistance among animal isolates, which may subsequently be transmitted to humans, has been associated with the addition of macrolides and fluoroquinolones to animal food as growth-promoting agents (57).

Because of the increasing incidence of resistance, testing of isolates from individual patients with severe illness or

prolonged symptoms may be warranted or isolates might be tested for epidemiologic purposes. Susceptibility testing by broth microdilution in CAMHB-LHB and incubation in a campylobacter environment (Table 3) is suggested. Agar dilution using MHA supplemented with 5% sheep blood has been shown to be satisfactory and is listed as an alternative method by the CLSI (41). Disk diffusion methods have been used successfully by some investigators (72, 175), although CLSI has not yet identified a reliable disk diffusion method. The E test correlated well with agar dilution in a study by Baker when ciprofloxacin, erythromycin, and tetracycline were tested (11), and Hayward et al. had similar success when testing gatifloxacin (87).

CORYNEBACTERIUM SPECIES

The many *Corynebacterium* spp. may exhibit a variety of susceptibility profiles. Most isolates of *C. amycolatum, C. jeikeium,* and *C. urealyticum* are multiresistant to antimicrobial agents that are often considered for therapy of gram-positive infections, including penicillins, cephalosporins, macrolides, aminoglycosides, quinolones, tetracyclines, and clindamycin. These species remain susceptible to teicoplanin and vancomycin. Considerable variation in activities of antimicrobial agents occurs with the various other species of *Corynebacterium,* and many strains may be highly susceptible (70, 127, 158, 169, 182, 206). Virtually all isolates of *C. urealyticum* are resistant to quinolones, which has important implications for empiric therapy for urinary tract infections due to this species (65). Significant antimicrobial resistance remains rare for *C. diphtheriae,* and penicillin resistance has not been reported to date (58). However, among 15 isolates from Vietnamese children with diphtheria, 4 were resistant to erythromycin (121), and 5 of 410 isolates from around the world tested by Engler et al. showed reduced susceptibility to macrolides and ketolides (58).

Growth characteristics among the *Corynebacterium* spp. may vary, and previous studies have used a variety of methods, media, and incubation conditions for antimicrobial susceptibility testing. Prior to the publication of CLSI M45, the absence of interpretive criteria specifically for *Corynebacterium* spp. led to inconsistencies in interpreting results when CLSI interpretive criteria for other gram-positive organisms (e.g., streptococci and staphylococci) were applied (58, 70, 127, 206). CLSI recommends broth microdilution with CAMHB-LHB and incubation in ambient air (Table 3). Some strains grow satisfactorily after 24 h of incubation, but others need 48 h. The 48 h is particularly important for detecting resistance to β-lactams, and it is recommended, if growth is not satisfactory or if an isolate appears susceptible to β-lactams at 24 h, that the test be reincubated and final results be read after 48 h of incubation (38). Currently, disk diffusion testing of *Corynebacterium* spp. is not recommended by CLSI. Several investigators have used E test satisfactorily for testing *Corynebacterium* spp. (215), and Engler et al. used E test to confirm the reduced susceptibility to macrolides and ketolides that was initially identified by agar dilution testing of *C. diphtheriae* (58). Susceptibility testing of *Corynebacterium* spp. is warranted when the organisms are isolated from normally sterile sites.

ERYSIPELOTHRIX RHUSIOPATHIAE

Erysipelothrix rhusiopathiae is an intrinsically vancomycin-resistant gram-positive bacillus. Other agents that have little to no activity against isolates of this species are aminoglycosides and TMP-SMX. Antimicrobial susceptibility studies of isolates from a number of sources have shown all isolates of *E. rhusiopathiae* to be susceptible to penicillins, cephalosporins, carbapenems, and fluoroquinolones (66, 79, 189, 202, 209). Resistance to clindamycin, erythromycin, and tetracycline does occur. In one study of 214 isolates from pigs and swine in Japan the occurrence of resistance was as follows: erythromycin, 2%; clindamycin, 1%; and tetracycline, 53% (209). The tetracycline-resistant isolates were positive for *tet*M (210).

Isolates of *E. rhusiopathiae* are slightly fastidious, so CAMHB-LHB is recommended for broth microdilution MIC testing (38). There are also reports of agar dilution using either MHA supplemented with 5% horse blood or unsupplemented MHA (66, 189, 209).

HACEK GROUP

There are limited antimicrobial susceptibility test data on the HACEK organisms (i.e., *Haemophilus, Actinobacillus, Cardiobacterium, Eikenella,* and *Kingella* spp.) in part because these organisms are infrequently encountered and often difficult to grow. Members of HACEK are susceptible to extended-spectrum cephalosporins and fluoroquinolones and are often susceptible in vitro to ampicillin and penicillin (10, 43, 61, 125). Occasional isolates of *Cardiobacterium hominis, Eikenella corrodens,* and *Kingella* spp. produce β-lactamase (75, 128, 132, 181); however, β-lactamase production in *Actinobacillus actinomycetemcomitans* has not been documented (133, 156). Recent American Heart Association recommendations for treatment of patients with endocarditis caused by HACEK organisms suggest that because of difficulties in performing antimicrobial susceptibility testing and potential failure to identify ampicillin-resistant strains, HACEK organisms should be considered ampicillin resistant and ampicillin should not be used for patients with endocarditis due to HACEK organisms (10).

Broth microdilution with CAMHB-LHB can be used for testing HACEK organisms. Some isolates may require 48 h of incubation to obtain adequate growth as evidenced by substantial turbidity in the positive growth control well. Some isolates may not grow satisfactorily in CAMHB-LHB. Susceptibility testing may be warranted for isolates from normally sterile sites, and β-lactamase testing should be performed on HACEK isolates (38).

LACTOBACILLUS, PEDIOCOCCUS, AND *LEUCONOSTOC* SPECIES

Lactobacillus spp., *Pediococcus* spp., and *Leuconostoc* spp. are intrinsically vancomycin-resistant gram-positive bacteria. It is important to note that some species of *Lactobacillus* are vancomycin susceptible; they include *L. gasseri, L. delbrueckii, L. casei, L. paracasei, and L. acidophilus* (46). Isolates of all three genera are susceptible to penicillin, ampicillin, and amoxicillin (42, 44, 187, 211), but their susceptibility to cephalosporins is variable. For example, in one study the ceftriaxone MIC ranges were as follows: *Leuconostoc,* ≤0.25 to 128 μg/ml ($n = 47$); *Pediococcus,* ≤0.25 to 16 μg/ml ($n = 24$); and *Lactobacillus,* 1 to >128 μg/ml ($n = 13$) (187). Most isolates are susceptible to carbapenems, but isolates with elevated imipenem MICs (>0.5 μg/ml) have been reported for all three genera (42, 76, 187, 211). There is one report of an imipenem

therapeutic failure for a central nervous system infection due to *Leuconostoc* with an imipenem MIC of 4 μg/ml (48). Ciprofloxacin has little activity for all three genera. In a report by Swenson et al. (187) the ciprofloxacin MIC_{90} of *Leuconostoc* and *Lactobacillus* isolates was 4 μg/ml, and for *Pediococcus* the MIC_{90} was 16 μg/ml. Isolates of *Lactobacillus* and *Leuconostoc* are susceptible to clindamycin and erythromycin, but isolates of *Pediococcus* can be resistant to both agents (187, 211). Gentamicin, which may be used in combination therapy, has good activity against isolates of all three genera. In one study, the MIC_{90} was 0.5 μg/ml for *Lactobacillus* and *Leuconostoc* and 2 μg/ml for *Pediococcus* (187).

The medium recommended by CLSI for broth microdilution testing of *Lactobacillus* spp., *Pediococcus* spp., and *Leuconostoc* spp. is CAMHB-LHB (Table 3). There are several reports of susceptibility testing by agar dilution using either unsupplemented MHA or MHA with 5% sheep blood (42, 44, 76). If testing by agar dilution, incubation in air with 5% CO_2 may be necessary to achieve sufficient growth.

L. MONOCYTOGENES

Clinical isolates of *L. monocytogenes* remain susceptible to the drugs of choice including ampicillin (or penicillin) and TMP-SMX. With the exception of occasional resistance to tetracyclines (31), *L. monocytogenes* is typically susceptible in vitro to other agents active against gram-positive bacteria, including chloramphenicol, vancomycin, and macrolides (84, 135, 137, 176); however, these have not proved to be as useful in vivo as the aforementioned agents (94, 103). Although *L. monocytogenes* may appear susceptible to cephalosporins in vitro, these agents are not effective clinically.

Listeria infections are generally treated empirically, and antimicrobial susceptibility testing is usually not necessary. Only criteria for susceptibility (MIC, ≤2.0 μg/ml) are listed for ampicillin and penicillin in CLSI guidelines because clinical isolates of *L. monocytogenes* have not been noted to have results other than those indicating susceptibility (38). *L. monocytogenes* is not truly fastidious, and testing in Mueller-Hinton broth without the blood supplement has been done satisfactorily (137). However, CLSI recommends using CAMHB-LHB. It is important to remember that cephalosporins should not be tested or reported for *L. monocytogenes* as these results might indicate false susceptibility (5). This cautionary note is emphasized in CLSI documents (38) and illustrates why it is inappropriate to indiscriminately report susceptibility results for any agent without knowing if it would be a reasonable therapeutic option. Because cephalosporins are frequently used empirically for the treatment of meningitis, the laboratory should quickly communicate smear or culture findings suspicious for *Listeria* whenever they occur.

M. CATARRHALIS

More than 90% of *M. catarrhalis* isolates produce β-lactamase (15, 63, 93, 100, 102, 183, 217) and are resistant to amoxicillin, ampicillin, and penicillin. These isolates remain susceptible to amoxicillin-clavulanic acid, which is often prescribed for *M. catarrhalis* infections. Most clinical isolates produce one of two types of chromosomally mediated β-lactamases: BRO-1 or BRO-2 (112, 148, 168). BRO-1-producing strains are 10-fold or more prevalent than BRO-2-producing strains, and ampicillin and penicillin MICs for BRO-1 strains appear to be higher (e.g., ≥4.0 μg/ml) than those for BRO-2 strains (e.g., ≤0.5 μg/ml) (51, 112, 168). Because of the low MICs for the latter strains, their clinical significance in response to β-lactamase-labile penicillins is questionable. Resistance rates for TMP-SMX are low in most studies (69, 93, 217); however, Johnson et al. reported over 10% resistance in isolates from Latin America (102). Resistance to the other recommended drugs for treating *M. catarrhalis* infections remains less than 1% (63, 69, 93, 102, 183, 217).

Only the chromogenic cephalosporin method has reliably detected the β-lactamases produced by *M. catarrhalis* (55). Routine β-lactamase testing may not be necessary because of the high incidence of β-lactamase-positive strains. Nevertheless, some advocate reporting of β-lactamase results to highlight the fact that this pathogen is generally unresponsive to some agents (e.g., amoxicillin) commonly prescribed for the treatment of respiratory tract infections. MIC testing without β-lactamase testing can be problematic as there is some overlap in ampicillin and amoxicillin MICs in β-lactamase-positive and -negative strains (168). Since *M. catarrhalis* typically responds to the drugs of choice, testing beyond the β-lactamase test is rarely indicated (38). CLSI document M45-P addresses MIC testing of *M. catarrhalis,* and the recommended test medium is CAMHB (Table 3). Despite the lack of disk diffusion testing recommendations, Doern and Tubert (55) demonstrated that disk diffusion interpretive criteria for nonfastidious bacteria published by NCCLS in 1984 appear to be satisfactory, at least as they pertain to the susceptible category for amoxicillin-clavulanic acid, cephalothin, chloramphenicol, erythromycin, tetracycline, and TMP-SMX (149). However, because of the absence of frank resistance to these agents among the 74 clinical isolates examined, the investigators were unable to make definitive recommendations pertaining to disk diffusion testing.

PASTEURELLA SPECIES

Human isolates of *Pasteurella* spp. are generally susceptible to penicillin with MICs ≤0.5 μg/ml (36, 77, 144); however, β-lactamase production in an isolate from a respiratory tract infection has been documented. β-Lactamase was detected with the nitrocefin-impregnated disk method, and the isolate had an amoxicillin MIC of 8 μg/ml and an amoxicillin-clavulanic acid MIC of 0.25 μg/ml (131). *Pasteurella* spp. are generally very susceptible to many other antimicrobial agents, and no resistance has been documented for parenteral cephalosporins, quinolones, tetracyclines, chloramphenicol, or TMP-SMX (36, 77, 144). Resistance to erythromycin can occur, and there is at least one report of failure of erythromycin to cure a cat bite victim who went on to develop meningitis (129).

Broth microdilution and disk diffusion methods are described by CLSI for testing *Pasteurella* spp. (Table 3). Due to the absence of nonsusceptible strains, there are only susceptible interpretive criteria for agents that might be tested, with the exception of erythromycin. Susceptibility testing may be warranted for isolates from normally sterile sites, and β-lactamase testing should be done on these isolates as well as those from respiratory sources (38). Testing of isolates from bite wounds is not necessary since bite wound infections are generally treated empirically with agents that would cover a variety of organisms likely to be implicated in the infection.

VIBRIO SPECIES (NOT *V. CHOLERAE*)

The non-cholera *Vibrio* spp. are generally susceptible to most antimicrobial agents, including newer cephalosporins,

aminoglycosides, fluoroquinolones, and tetracyclines; however, as with other organisms, resistance to one or more of these can occur (33, 35, 134, 153, 190). One study noted ampicillin resistance for 5 of 6 environmental isolates of *Vibrio vulnificus* (214), but Chuang et al. recently reported that all 42 clinical isolates of *V. vulnificus* were ampicillin susceptible (35). Chuang et al. also demonstrated synergistic killing of *V. vulnificus* with a combination of minocycline and cefotaxime (35), and this finding has contributed to current recommendations for treating *V. vulnificus* infections with doxycycline and ceftazidime (74).

Although the non-cholera *Vibrio* spp. cause diarrheal disease, otherwise healthy individuals with diarrhea due to these bacteria usually recover spontaneously without treatment. When *Vibrio* spp. are isolated from sources associated with serious infections, antimicrobial susceptibility testing of them is warranted (33, 134). CLSI suggests use of broth microdilution or disk diffusion. For testing of these halophilic organisms, some have suggested use of MHA containing 1% NaCl. However, CLSI recommends preparation of the inoculum suspension in 0.85% NaCl solution for both disk diffusion and broth microdilution MIC testing with unsupplemented MHA or CAMHB, respectively (38).

CONCLUSION

Infections caused by many of the fastidious bacteria discussed in this chapter are treated empirically as these bacteria are often susceptible to the drugs of choice. Consequently, susceptibility testing is infrequently needed. In certain circumstances testing may be warranted, such as when there appears to be clinical failure, patient intolerance to the drug(s) of choice, or serious infections for which there are several appropriate drugs that might be prescribed. Additionally, susceptibility testing may aid in species identification (e.g., differentiating *C. jeikeium* from other *Corynebacterium* species). If a physician seems unsure about requesting antimicrobial susceptibility testing on a fastidious organism, he/she should be encouraged to seek assistance from an infectious diseases clinician or pharmacist.

The E test has been examined for testing a variety of fastidious bacteria. For the less common species, many of the data generated have been obtained from comparisons with nonstandardized in vitro test methods with limited examination of the clinical correlation of the results. Consequently, the results for E test should be interpreted with caution in the clinical setting. Requests for susceptibility testing of fastidious bacteria are often for isolates associated with serious infections, and MIC results are likely to be more useful than qualitative results. The disk diffusion test should be used only for bacteria for which there are CLSI interpretive criteria. It is important for laboratory workers to maintain an awareness of the methods available for testing fastidious bacteria and their strengths and limitations. If testing must be performed, it should be done by a laboratory familiar with these limitations.

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Susceptibility Test Methods: Anaerobic Bacteria

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The importance of anaerobes as the cause of significant infections, as well as the benefits of specific antimicrobial treatment and prophylaxis against anaerobic bacteria, is well recognized (24, 26). In general, performance of antimicrobial susceptibility testing is viewed as a necessity for effective guidance of antimicrobial therapy. However, when and how susceptibility testing of anaerobes should be performed has been the subject of debate, due in part to several confounding factors and misconceptions (6, 22, 25, 83). For example, specimens obtained from most infections involving anaerobes are polymicrobic, making recovery and identification of individual isolates slow and causing antimicrobial susceptibility results to be unacceptably delayed so that a consistent impact on individual clinical outcomes is difficult to achieve. For the clinician, the combination of surgical management and use of empiric broad-spectrum antimicrobial therapy has limited the correlation of potential antimicrobial resistance with outcome. Such observations have led many laboratories away from the performance of susceptibility testing. However, there is substantial evidence that antimicrobial resistance is significant among many anaerobes worldwide and that inappropriate therapy can result in poor clinical responses and increased mortality (59, 67, 69). Antimicrobial susceptibility data have also revealed significant differences among individual hospitals on a regional and local basis, suggesting that one medical center's patterns are not applicable to organisms from other institutions (45, 72, 73). Thus, the need for susceptibility testing of anaerobes is considerably more important now than in the past.

If possible, individual hospitals should establish patterns of resistance for some anaerobes on a periodic basis, with individual patient isolates being tested as needed to assist in patient care. For surveillance purposes, the testing of 75 to 100 isolates representing anaerobes with known resistance, such as members of the *Bacteroides fragilis* group, *Prevotella* spp., *Fusobacterium* spp., and *Clostridium* spp., should be considered. Preferably, 30 isolates should be from the *B. fragilis* group, and at least 10 of each of the other genera should be tested. Alternatively, cumulative susceptibility results from individual patient isolates should be included in the hospital antibiogram. Antimicrobial agents to be tested should generally be based upon the hospital's formulary, although one agent from each antimicrobial class should be included, even if it is not on the hospital formulary, to detect resistance. These data would be important as part of the cumulative susceptibility report if the formulary were changed and could aid in the choice of empiric therapy. For individual patient management, susceptibility should be performed when (i) selecting an active agent is critical for disease management, (ii) there is consideration for long-term therapy, (iii) anaerobes are isolated from specific body sites (e.g., blood, brain, bone, or joint), or (iv) there is the failure of a usual regimen (Table 1).

This chapter describes currently available methodologies and their interpretation for susceptibility testing of anaerobes. The Clinical Laboratory Standards Institute (CLSI, formerly NCCLS) anaerobe working group has established an agar dilution reference method using brucella blood agar as the testing medium (15–17). This method is not considered simple or economical to perform but will serve as the method to which other more practical methods can be compared (Table 2). At present, alternative testing methods include the limited agar dilution, broth microdilution (for the *B. fragilis* group), and the E test. β-Lactamase testing provides very limited data but can be useful if penicillin therapy is being considered. Broth disk elution and disk diffusion tests are not considered appropriate for anaerobic susceptibility testing because their results do not correlate with those of the agar dilution reference method (14).

CURRENT PATTERNS OF ANTIBIOTIC RESISTANCE

Susceptibility testing of anaerobes has not been routinely performed at most hospitals (39). As a result, most of the published literature reporting susceptibility of anaerobes is generated by reference laboratories testing a limited number of isolates from one or more medical centers (1–3, 31, 37, 44, 45, 72, 73). Over the last decade, significant variations in susceptibility results for anaerobes have been reported from different countries, geographic locations within countries, and even hospitals within the same cities (45, 47, 53, 54, 56, 72, 79). Of particular note, the incidence of clindamycin resistance has increased from <10 to >40% for the *B. fragilis* group at some hospitals (45, 73), while resistance to cephalosporins and cephamycins is also rising (45, 72–74, 79). Some differences in susceptibility results among various reports may be accounted for by the use of different testing methods, lack of uniformity in interpretive breakpoints among countries, and clustering of MICs at the breakpoint for some species with

TABLE 1 Indications for susceptibility testing of anaerobic bacteria

Indication	Examples[a]
Surveillance	
Annual monitoring of isolates at individual medical centers	*B. fragilis* group, *Prevotella* spp., *Fusobacterium* spp., *Clostridium* spp., *B. wadsworthia*
Clinical	
Known resistance of a particular species	*B. fragilis* (clindamycin, cephamycins, piperacillin, fluoroquinolones); *Prevotella* spp. and *Fusobacterium* spp. (penicillin, clindamycin)
Failure of a usual therapeutic regimen	Any anaerobe
Pivotal role of antimicrobial agent in clinical outcome	*B. fragilis* group (osteomyelitis, joint infection)
Need for long-term therapy	*B. fragilis* group, *Prevotella* spp. (osteomyelitis, endocarditis, brain abscess, liver abscess, lung abscess)
Infections of specific body sites	Any anaerobe (brain abscess, endocarditis, prosthetic devices or graft, bacteremia)

[a] Examples only, and not intended as all-inclusive. See the text for specific recommendations.

some antimicrobial agent combinations (4, 42). Regardless, it is clear from recent publications that resistance to many classes of antimicrobial agents among anaerobes is increasing, and clinicians and laboratories can no longer assume susceptibility of anaerobes to these agents without testing. Further, neither national nor even local data from other institutions are sufficient to predict susceptibility of anaerobes to antimicrobial agents at one's own hospital (45, 72, 73). A general outline of current resistance patterns for anaerobic bacteria is provided below (40).

Gram-Negative Bacilli and Cocci

B. fragilis Group

Among the 13 members of the *B. fragilis* group, *B. fragilis* is generally the most susceptible, although greater than 95% of all species are resistant to penicillin and ampicillin. The carboxy- and ureidopenicillins, ticarcillin, and mezlocillin are somewhat more active than penicillin, but <50% of isolates are susceptible (2, 9, 79). Piperacillin is the most active of the ureidopenicillins against the *B. fragilis* group, although susceptibility has fallen from approximately 90 to 70% over the last 8 to 10 years (2, 41, 72, 73). The isoxazolyl penicillins, such as oxacillin and nafcillin, are not active against these organisms. The principal mechanism of resistance to penicillins is β-lactamase production. Thus, β-lactam–β-lactamase inhibitor combinations, such as ampicillin-sulbactam, amoxicillin-clavulanic acid, ticarcillin-clavulanic acid, and piperacillin-tazobactam, are active against nearly all strains of *B. fragilis* group, with <2% resistance reported in most studies (3, 9, 41, 44, 47, 53, 72, 73, 79).

Among the cephalosporins and cephamycins, cefoxitin remains very active against members of the *B. fragilis* group, with 80 to 90% of isolates susceptible. Cefotetan demonstrates activity against *B. fragilis* similar to that of cefoxitin, but it is much less active against the other members of the *B. fragilis* group (2). With the exception of ceftizoxime, broad-spectrum cephalosporins generally have poor activity against most members of the *B. fragilis* group, inhibiting <50% of isolates (73). Susceptibility to ceftizoxime varies widely among published studies (60 to 90%), probably due to differences in testing methods (4). Narrow-spectrum cephalosporins, such as cefazolin, are not active against members of the *B. fragilis* group.

A marked decrease in susceptibility to clindamycin among *Bacteroides* spp. has become widely recognized worldwide, as noted above (1, 3, 9, 44, 45, 47, 53, 72, 73, 79). The clindamycin resistance determinants include several *erm* genes that are frequently located on transferable plasmids and are often linked to transferable tetracycline resistance (65, 82). Among other agents, chloramphenicol, metronidazole, and carbapenems (imipenem, ertapenem, and meropenem) are nearly uniformly active against all members of the *B. fragilis* group in the United States (2, 36, 72), although imipenem-resistant strains have been reported from Japan, the United States, Hungary, Kuwait, and other countries (5, 63, 75). Of note, imipenem resistance is mediated by a zinc metalloenzyme that confers resistance to all current β-lactam and β-lactam–β-lactamase inhibitor combination agents and has

TABLE 2 Methods for susceptibility testing of anaerobic bacteria

Method	Medium	Inoculum	Incubation time (h)	Advantages	Disadvantages
Agar dilution[a]	Brucella blood agar	10^5 cells/spot	48	Reference method, multiple isolates tested/antibiotic	Labor intensive, expensive
Broth microdilution[b]	Supplemented brucella broth	10^6 cells/ml (10^5/well)	48	Economical, commercial panels available, multiple antibiotics/isolate	Limited shelf life of frozen panels, poor growth by some strains
E test	Brucella blood agar	0.5 McFarland, swab plate	24–48	Precise MIC value, convenient for individual patient isolates	Expensive for surveillance use

[a] Media are commercially available.
[b] Frozen panels available from PML Microbiologicals, Inc. (Wilsonville, Oreg.); lyophilized panels are available from Trek Diagnostic Systems.

been reported to be transferable (64, 75). Of more concern, however, strains resistant to metronidazole in France have been reported, associated with a transferable plasmid (64), and in recent years, additional resistant strains carrying *nim* genes have been reported in Washington state, the United Kingdom, and other countries in Europe (27, 55, 70). *nim* genes encode a nitroimidazole reductase which converts nitroimidazoles to aminoimidazole, preventing the formation of the active toxic nitroso residue (11). Among fluoroquinolone agents, trovafloxacin (a trifluoronaphthyridone) had excellent activity against most members of the *B. fragilis* group; however, it is no longer marketed in most countries, including the United States (15, 43, 44, 46, 71, 72). Other fluoroquinolones such as moxifloxacin, recently approved by the FDA for skin structure infections and intra-abdominal infections, and gatifloxacin also exhibit good activity against a broad range of anaerobes (9, 23, 48, 71). Resistance to fluoroquinolones in *Bacteroides* and other anaerobes is increasing with the widespread use of this class of antimicrobial agents (29, 61, 72; D. M. Citron, *Abstr. 45th Intersci. Conf. Antimicrob. Agents Chemother.*, abstr. E-1440, 2005). Tigecycline, a glycylcycline derivative of minocycline, was recently approved by the FDA for intra-abdominal and skin and soft tissue infections. Although breakpoints for anaerobes have not yet been defined by CLSI, several studies describe its activity against anaerobic organisms (10, 49).

Prevotella and *Porphyromonas*

Recent taxonomic changes for this group of anaerobes are provided in reference 51. In general, data on the susceptibility of these organisms (mostly former *Bacteroides* species) are more limited than those of the *B. fragilis* group. Overall, both genera are more susceptible than the *B. fragilis* group. Currently, about 50% of *Prevotella* spp. are resistant to penicillin and ampicillin due to β-lactamase production, with susceptibility to piperacillin, cefoxitin, and cefotetan ranging from 70 to 90% in most published studies (30, 35, 44, 53, 57). Eight percent of *Porphyromonas* strains were reported to produce β-lactamase in a survey from Japan (78), as were 17% of strains recovered from serious pelvic infections (62). Susceptibility of *Porphyromonas* isolates is rarely reported separately in most published literature from the United States, but β-lactamase production is considered rare at present (36). As in the case of the *B. fragilis* group, both genera are nearly uniformly susceptible to carbapenems, metronidazole, and chloramphenicol, although clindamycin resistance has been observed in a small percentage of strains (36).

Other Gram-Negative Bacilli

Penicillin resistance among isolates of the genus *Fusobacterium* has been observed; *Fusobacterium nucleatum* strains from saliva in infants show increasing rates of β-lactamase production related to age, day care attendance, and exposure to antimicrobial agents (60), and a β-lactamase-producing strain was responsible in a case of fatal sepsis in a compromised patient (38). In general, >90% of *Fusobacterium* spp. are susceptible to cephalosporins and cephamycins (36, 50, 53, 81). *Campylobacter rectus* and *Campylobacter curvus* (formerly *Wolinella*) vary in their susceptibility to β-lactams but remain very susceptible to chloramphenicol, metronidazole, and clindamycin (81). *Bilophila wadsworthia* is a gram-negative anaerobe from the gastrointestinal tract that frequently produces β-lactamase and therefore is resistant to penicillin and ampicillin. High MIC_{90} values are also seen when testing piperacillin, with values clustering near the breakpoints. *B. wadsworthia* is susceptible to clindamycin, cefoxitin, β-lactam–β-lactamase inhibitor combinations, carbapenems, and metronidazole (7). *Campylobacter gracilis* (formerly *Bacteroides gracilis*) was previously considered to be resistant to many β-lactam agents. However, data suggest that when properly identified and tested, this organism is susceptible to most agents tested, including β-lactam–β-lactamase inhibitor combinations, cefoxitin, and clindamycin (58). In fact, a newly described but more resistant organism, *Sutterella wadsworthensis,* was often isolated from the same samples and identified as *C. gracilis*. *S. wadsworthensis* may demonstrate resistance to clindamycin, piperacillin, and/or metronidazole (58).

Gram-Positive Bacilli and Cocci

Non-Spore-Forming Gram-Positive Bacilli

The *Eubacterium* group, *Actinomyces, Propionibacterium,* and *Bifidobacterium* are usually susceptible to β-lactam agents, including the penicillins, cephalosporins and cephamycins, carbapenems, and β-lactam–β-lactamase inhibitor combinations. *Lactobacillus* spp. are variably susceptible to cephalosporins and may be inhibited effectively only by penicillin (36). Vancomycin is active against all *Propionibacterium* spp., *Actinomyces* spp., *Eubacterium* spp., peptostreptococci, and some *Lactobacillus* spp., but *Lactobacillus casei* and several other species are usually resistant (36). The newer antimicrobial agents such as linezolid, daptomycin, dalbavancin, telavancin, and ramoplanin with activity against gram-positive aerobic organisms also exhibit excellent in vitro activity against most anaerobic gram-positive species (8, 12, 32–34). Most non-spore-forming gram-positive anaerobes are resistant to metronidazole.

Spore-Forming Gram-Positive Bacilli

Clostridium perfringens is generally very susceptible to most antianaerobic agents and to fluoroquinolones (9, 44, 46). However, non-perfringens *Clostridium* spp. and *Clostridium difficile* have variable susceptibility (19, 35–37, 44, 66, 79). Drugs to which resistance among non-perfringens species is observed include clindamycin, fluoroquinolones, and β-lactams; however, chloramphenicol and metronidazole remain active. *C. difficile* may be resistant to many β-lactams, including cephalosporins, fluoroquinolones, and clindamycin, but retains susceptibility to metronidazole and vancomycin, while *Clostridium innocuum* has vancomycin MICs of 8 to 32 μg/ml (35–37, 66).

Gram-Positive Cocci

At present, only *Peptococcus niger* remains in this genus, and several of the species in the genus *Peptostreptococcus* have been reclassified into other genera (76). In general, these organisms are highly susceptible to all β-lactams, β-lactam–β-lactamase inhibitors, cephalosporins, carbapenems, chloramphenicol, and metronidazole (36, 46, 53, 66, 79, 81). Fluoroquinolone resistance is increasing among the species isolated from skin and soft tissue infections (Citron, 45th ICAAC), and some strains may be resistant to clindamycin. Occasionally, microaerophilic streptococci including *Abiotrophia* and *Granulicatella* species are initially identified as *Peptostreptococcus* spp. and reported to be resistant to metronidazole. The presumptive identification of a metronidazole-resistant *Peptostreptococcus* sp. should prompt further identification of the organism because such isolates are rare. The *nimB* gene coding for metronidazole resistance has been demonstrated in two highly resistant strains of *Finegoldia magna,* although it appears to be prevalent in susceptible strains of other gram-positive cocci as well (80).

DESCRIPTION OF TEST METHODS

Agar Dilution

Media

The recommended medium is supplemented brucella blood agar, which supports the growth of essentially all anaerobes (17). Brucella base agar is supplemented with hemin (5 μg/ml) and vitamin K_1 (1 μg/ml) prior to autoclaving and 5% defibrinated or laked sheep blood after cooling to 48 to 50°C. To prepare hemin stock solution (5 mg/ml), dissolve 0.5 g of hemin in 10 ml of 1 N NaOH and bring to a 100-ml volume with distilled water. Sterilize by autoclaving at 121°C for 15 min. Add 1 ml of agar per liter. The stock solution may be stored at 4 to 8°C for 1 month. Vitamin K_1 stock solution (10 mg/ml) is prepared by mixing 0.2 ml of Vitamin K_1 (3-phytyl menadione) with 20 ml of 95% ethanol, and it is added to the agar base to achieve a final concentration of 1 μg/ml prior to autoclaving. The stock solution can be stored for up to 6 months at 4°C in a dark bottle. Lysed (laked) sheep blood is prepared by a single cycle of alternate freezing and thawing and does not require clarification by centrifugation. Laked blood may be stored at –20°C for up to 6 months.

The agar is dispensed in 17-ml volumes in test tubes prior to autoclaving. After autoclaving, these tubes may be stored at 4 to 8°C for up to 1 month. On the day of the test, the agar is melted by heating and then cooled in a water bath to 48 to 50°C. Laked blood (1 ml) and the antimicrobial dilutions (2 ml) are added, and after mixing by gently inverting the tubes twice, the plates are poured. After they have solidified, the plates are dried in the incubator by inverting them with the lids ajar for 45 min. The CLSI recommends that plates not be stored any longer than 7 days in closed containers at 4 to 8°C. However, for research and precise evaluation purposes, storage for no longer than 72 h is recommended. Due to instability, plates containing imipenem (but not meropenem and ertapenem) or clavulanic acid must be used on the day of preparation.

Inoculum Preparation

The inoculum may be prepared by suspending colonies taken from a 24- to 72-h brucella blood agar plate into brucella broth or other clear broth medium to a density equal to the 0.5 McFarland standard. Alternatively, the initial suspension may be prepared by inoculating five or more colonies into enriched thioglycolate or other broth medium that supports good growth and by incubating for 4 to 24 h to obtain adequate turbidity (dilution may be required) (17). Equivalence to a 0.5 McFarland standard can be measured visually or by using a colorimeter or a simple photometer device (e.g., Vitek, Hazelwood, Mo.; Microscan, West Sacramento, Calif.; Trek Diagnostic Systems, Inc., Cleveland, Ohio). Although it is more accurate than visual inspection, the use of different broth media can affect photometer readings, requiring the user to verify the size of the inoculum by performing colony counts. Species with large cells, such as *C. perfringens,* require fewer cells to achieve this turbidity and will show correspondingly lower colony counts, while the converse is true for organisms with smaller cells, such as *Veillonella* or *Micromonas micros.*

The organism suspensions are dispensed by pipette into the wells of the replicator head (32 to 36 wells) and applied to the plates with a multipronged replicator device that delivers approximately 0.001 ml per spot (10^5 CFU). The drug-free control plates are inoculated first, followed by the antimicrobial plates, starting with the lowest drug concentration. The plates should be marked to ensure proper orientation.

Contamination by aerobic bacteria during the inoculation procedure can be detected by inoculating drug-free plates and incubating them in an aerobic environment. If using thioglycolate or other agar-containing broth media for inoculum preparation, an additional control plate may be inoculated and refrigerated to distinguish the inoculum residue that occurs at the time of setting up the MIC testing plates. Agar dilution plates can be inoculated in an aerobic environment, although the exposure time prior to incubation should be minimized. After stamping the plates, the inoculation spots should absorb for 10 to 15 min into the medium, at which time the plates are stacked upside down (to prevent condensation from falling on spots) and immediately placed into an anaerobic environment for incubation.

Incubation Conditions

An anaerobic chamber or anaerobic jars equipped with disposable hydrogen/carbon dioxide generators and palladium-coated catalyst pellets or ascorbic acid envelopes are recommended for incubating agar dilution plates. The incubation atmosphere should contain approximately 5% CO_2, and an indicator of anaerobiasis should be included. Incubation is at 35 to 37°C for 44 to 48 h (17).

Interpretation of Results

Since 1993, the CLSI has defined the endpoint for agar dilution testing as the concentration at which the change from the growth control is the most marked (14). This change is defined as no growth or lighter growth, a haze, multiple tiny colonies, or one to several normal-sized colonies. The technologist should take care to discern a few colonies that may be present as the result of a "splash" from a resistant neighboring isolate from the few colonies that precede full growth of the strain. Endpoints can be difficult to interpret when testing some gram-negative organisms with β-lactams, particularly ceftizoxime and piperacillin. This is especially problematic with many strains of fusobacteria that produce L forms that appear as transparent hazes in the presence of even very high concentrations of β-lactam agents. The current CLSI document for susceptibility testing of anaerobes now includes a color figure illustrating the endpoints described above and should be utilized as an additional guide when using this test method (17). It is important to compare the drug-containing plates to the drug-free control plate when reading the tests, because spots of different species of anaerobic bacteria can have very different appearances, ranging from mucoid-opaque, as with the *B. fragilis* group, to gray-transparent, as with *Bacteroides ureolyticus*.

The interpretation of MIC results should be done according to criteria recommended by the CLSI (Table 3) (17). In 1993, an intermediate category was established for anaerobic bacteria (14). For many antimicrobial agents used against anaerobes, a significant percentage of strains have susceptibility test endpoints that cluster at or near the suggested breakpoints. In the twofold-dilution method, the degree of acceptable variation of endpoints (usually plus or minus 1 twofold dilution) does not permit adequate distinction of the qualitative categories. If an intermediate value is determined for any anaerobe, the CLSI recommends maximum dosages of the antimicrobial agent for therapy. With such dosages, it is believed that organisms with susceptible or intermediate endpoints are amenable to therapy. This recommendation is predicated upon the presumed surgical intervention that frequently accompanies infections involving these organisms.

TABLE 3 Interpretive categories for MICs for anaerobic bacteria[a]

Antimicrobial agent	MIC (μg/ml)		
	Susceptible	Intermediate	Resistant
Amoxicillin-clavulanic acid	≤4/2	8/4	≥16/8
Ampicillin[b]	≤0.5	1	≥2
Ampicillin-sulbactam	≤8/4	16/8	≥32/16
Cefotetan	≤16	32	≥64
Cefoxitin	≤16	32	≥64
Chloramphenicol	≤8	16	≥32
Clindamycin	≤2	4	≥8
Ertapenem	≤4	8	≥16
Imipenem	≤4	8	≥16
Metronidazole	≤8	16	≥32
Meropenem	≤4	8	≥16
Penicillin[b]	≤0.5	1	≥2
Piperacillin	≤32	64	≥128
Piperacillin-tazobactam	≤32/4	64/4	≥128/4
Tetracycline	≤4	8	≥16
Ticarcillin-clavulanic acid	≤32/2	64/2	≥128/2
Trovafloxacin	≤2	4	≥8

[a] Adapted from reference 17 with permission of the publisher.

[b] Members of the *B. fragilis* group are presumed to be resistant. Other gram-negative anaerobes may be screened for β-lactamase activity by using a chromogenic cephalosporin test if penicillin therapy is contemplated. Higher levels in blood are achievable; infection with non-β-lactamase-producing organisms with higher MICs might be treatable.

Broth Microdilution Test

The broth microdilution procedure has been validated by CLSI for testing only members of the *B. fragilis* group. Other, more-oxygen-sensitive anaerobes did not grow consistently when inoculated on the bench. Testing in an anaerobic chamber using reduced MIC trays may improve growth and make this method suitable for other species of anaerobes. The anaerobe working group of CLSI is evaluating additional antimicrobial agents and other species for correlation of the broth microdilution method to the reference standard.

Media

Brucella broth supplemented with hemin (5 μg/ml), vitamin K_1 (1 μg/ml), and lysed horse blood (5%) is the recommended medium. Microdilution trays may be prepared fresh, frozen after preparation, or purchased commercially as lyophilized or frozen panels. Following the manufacturer's recommendations for storage of commercially prepared panels is recommended, whereas in-house-prepared trays may be kept at −70°C for up to 6 months if stored in sealed plastic bags. Antimicrobial agents should be diluted according to the scheme described by the CLSI (17) and prepared in large volumes, 15 to 100 ml, depending on the device used to simultaneously dispense aliquots of 0.1 ml per well into the standard 96-well panels. If the inoculum is to be added by pipette, the antimicrobial solutions are prepared at twice the desired concentration and the wells are filled with 0.05 ml, after which 0.05 ml of inoculum is added to each well for a final volume of 0.1 ml. Final volumes of less than 0.1 ml are not recommended for testing anaerobes due to loss of liquid by evaporation. Inoculum effects may be exaggerated when smaller volumes are used.

Inoculum Preparation

Inoculum preparation is similar to that of the agar dilution procedure. Organisms may be suspended into a clear broth to equal the turbidity of the 0.5 McFarland standard, or the isolate can be grown in a supplemented thioglycolate or other broth that supports growth of the organism for 4 to 24 h and then diluted to the turbidity of the 0.5 McFarland standard (approximately 1.5×10^8 CFU/ml). The final concentration of the organism is 1×10^6 to 2×10^6 CFU/ml (10^5 CFU/well). Depending on the method of tray inoculation, the dilution technique will differ. If 10 μl is added to each well, then the 0.5 McFarland suspension is diluted 1:10. If 50 μl is added, then the 0.5 McFarland suspension is diluted 1:50. If a lyophilized tray is used, the 0.5 McFarland suspension is diluted 1:100.

Inoculation Procedure

Frozen trays should be brought to room temperature prior to inoculation. The inoculation can be accomplished by using a disposable, hand-held, 96-prong inoculator, a mechanized dispenser, or a multichannel pipettor, depending on the preparation method of the panel, within 15 min after inocula are prepared. While the members of the *B. fragilis* group are relatively oxygen tolerant, reducing the trays in an oxygen-free environment prior to inoculation (2 to 4 h) may enhance the growth of certain fastidious anaerobes and reduce the "edge" effect of outer rows being reduced more rapidly than inner wells (52). Trays should be reduced prior to inoculation if metronidazole is to be tested, since the antimicrobial activity of metronidazole is dependent on the formation of an active intermediate that requires a reduced atmosphere (52). False resistance can occur with nonfastidious, rapidly growing strains that produce significant growth before metronidazole is reduced to its active form. Control wells should include a well with broth but no drug (growth control) and an uninoculated well as a sterility check. This well may also be used as a negative control well for visual comparison with growth in inoculated wells. Alternatively, an uninoculated tray may be incubated as a sterility check, especially if the trays are prepared in-house.

It is advisable to perform a colony count and a purity check of the inoculum. This is accomplished by removing 10 μl from the growth control well, diluting it into 10 ml of saline (1:100), and spreading 0.1 ml onto the surface of a nonselective blood agar plate for anaerobic incubation. The presence of 100 to 200 colonies indicates an inoculum of 1×10^6 to 2×10^6 CFU/ml. A small amount of the growth control well can be inoculated onto a quadrant of a blood agar plate for aerobic incubation to detect aerobic contamination.

Incubation Conditions

Trays are most conveniently incubated in an anaerobic chamber for 40 to 48 h. Alternatively, they can be placed into large anaerobic jars, regular anaerobic jars laid on their side, or anaerobic pouches with the appropriate anaerobic gas generator. No more than four trays should be stacked on top of each other to ensure uniformity of heating and gas exchange. Trays should not be sealed with sealing tape (unless perforated) if set up on the bench, as this will decrease the rate of diffusion of anaerobic gases to the inoculum and may result in poor growth or false resistance to metronidazole.

Interpretation of Results

The plates may be examined with reflective light by using a viewing device, such as a stand with a magnifying mirror. Broth microdilution MIC determinations require criteria similar to those for the agar dilution procedure for reading endpoints: the concentration at which the most significant

reduction in growth is observed is interpreted as the MIC. Similar to that of agar dilution, this decrease in growth may include a tiny, gradually diminishing button of growth, with trailing endpoints also being observed (17). If the growth in the drug-free growth control well is poor, the test should not be read. At this time, breakpoints for broth microdilution are similar to those for agar dilution (17).

E Test

The E test (AB BIODISK, Solna, Sweden) has been used more frequently for testing anaerobic organisms in recent years, primarily because of its convenience. Several studies have demonstrated its utility and indicate that results correlate well with the CLSI reference approved agar dilution method (13, 68). Rosenblatt has noted that some strains of *Prevotella* and *Bacteroides* spp. show very major errors (false susceptibility) with penicillin and ceftriaxone; however, a β-lactamase test would provide the correct result in these instances. In addition, false resistance to metronidazole among anaerobes has been reported when using the E test. This phenomenon can be the result of test conditions and medium quality and is generally eliminated if test plates are reduced in an anaerobic atmosphere overnight prior to their use (18).

Procedure

The E test consists of plastic strips coated with a gradient of antimicrobial on one side and an MIC interpretive scale on the other side. The method consists of streaking to confluence (three directions) a 0.5 to 1 McFarland standard of the test organism on a 150-mm-diameter supplemented brucella blood agar plate. Up to six E-test strips can be applied to the surface of the plate in a radial fashion with the lowest concentration toward the center. If large plates are not available, two strips, one on each half, may be placed on a standard-size plate with the high concentrations of the strips opposite each other. Following 24 to 48 h of anaerobic incubation, an elliptical zone of inhibition is formed. MICs are read at the point of intersection of the ellipse with the interpretive scale of MICs.

Validation for most antimicrobial agents recommended for testing of anaerobes has been confirmed for the E test (68). The complete list of FDA-approved antimicrobial agents for anaerobe testing is found in the E-test package insert. The E test provides a flexible and simple procedure that is well suited for individual isolate testing in smaller laboratories or for those labs that do not perform batch testing of anaerobe susceptibility. Its main drawback is the relatively high cost for each strip. This can be alleviated somewhat by limiting the number of antimicrobial agents being tested to a few relevant drugs from the hospital's formulary.

β-Lactamase Testing

β-Lactamase testing of anaerobes can be performed as described by the CLSI (17). Two easily performed methods include a nitrocefin disk assay (Cefinase; BD Diagnostics Systems, Sparks, Md.) and the S1 chromogenic cephalosporin disk (International BioClinical, Inc., Portland, Oreg.). Both tests should be performed according to the manufacturers' directions. Hydrolysis of the β-lactam ring by β-lactamases causes a color change on the disks from yellow to red. Most reactions occur within 5 to 10 min, but some β-lactamase-positive strains of *Bacteroides* spp. may react more slowly (up to 30 min) (21). When testing *Bilophila wadsworthia,* 1% pyruvate should be added to the testing growth medium for consistent results (77).

β-Lactamase testing has limited utility in detecting resistance to certain β-lactam agents among anaerobes. While a chromogenic cephalosporin test is simple and quick and generally detects β-lactamases produced by species of *Prevotella, Porphyromonas, Bacteroides,* and other anaerobes, resistance to β-lactam drugs is not always mediated by β-lactamase production (e.g., some strains of *Bacteroides distasonis* and *B. fragilis* are resistant because of alterations of penicillin-binding proteins) (20, 28). Therefore, β-lactamase test results are limited in their clinical application. A positive test does, however, provide clinically relevant information quickly in some situations and can predict resistance to penicillin G and ampicillin.

QUALITY CONTROL

A quality control program is designed to monitor the accuracy and precision of a susceptibility test procedure, the performance of reagents and equipment, and the performance of persons who conduct the tests. Quality control must be performed to demonstrate that any new medium used adequately supports the growth of the test organisms and that antimicrobial agents have not deteriorated during shipping or storage. These tests must be a part of any testing program using any of the methods described above. Ideally, the quality control strain(s) that most closely resembles the tested organism(s) should be included. The recommended quality control strains are *B. fragilis* ATCC 25285, *Bacteroides thetaiotaomicron* ATCC 29741, and *Eubacterium lentum* ATCC 43055. The CLSI is currently evaluating a nontoxigenic strain of *C. difficile* (ATCC 700057) for replacement of the *E. lentum* strain. Two quality control strains should be used for each assessment when using the agar dilution procedure. When testing an individual strain using broth microdilution or E test, one quality control strain should be included. Expected values for quality control strains are published by the CLSI (17). For some antimicrobial agent-quality control organism combinations, no quality control ranges are recommended because of the difficulty in reading of endpoints.

CONCLUSIONS

Increasing antimicrobial resistance among anaerobes has become a significant problem in recent years, underscoring the need for more antimicrobial susceptibility testing. Current methodologies allow for accurate surveillance or individual isolate testing by most laboratories. Future studies comparing broth microdilution to the reference agar method and the anticipated development of an improved microdilution system will result in better standardization of the more user-friendly method and possibly more widespread commercial availability.

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Susceptibility Test Methods: Mycobacteria, *Nocardia*, and Other Actinomycetes*

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In his review article entitled "The White Plague," M. F. Perutz recounted that Cardinal Richelieu (1585 to 1642), Heinrich Heine (1797 to 1856), Frédéric Chopin (1810 to 1849), Anton Chekhov (1860 to 1904), Franz Kafka (1883 to 1924), George Orwell (1903 to 1950), and Eleanor Roosevelt (1884 to 1962) all had a common fate (112). Each of them died of tuberculosis. For many of these famous cases, it is unknown if the disease was only poorly understood or if management of the disease was simply inadequate or even inappropriate. For some, effective antimicrobial agents for the treatment of tuberculosis had not been discovered or were discovered too late. Streptomycin and *p*-aminosalicylic acid were not introduced until the late 1940s, and isoniazid, ethambutol, and rifampin were not used to treat tuberculosis until 1952, 1961, and 1968, respectively. Therefore, it is also possible that the more contemporary of these patients were early victims of antimicrobial resistance. In the same article, Professor Perutz related the response of the Nobel laureate Gerhard Domagk (1895 to 1964) to Otto Warburg's (1883 to 1970) comment that Domagk "deserved monuments in each valley and every mountain." Domagk, who in 1935 discovered Prontosil (a precursor of sulfanilamide), Conteben (thiosemicarbzone), and Neoteben (isonicotinic acid hydrazide), replied to Warburg that "no one is interested any longer in diseases that can be cured." Domagk could not have anticipated the extent to which antibiotic resistance would come to complicate the chemotherapy of all infectious diseases, especially tuberculosis.

ANTIMICROBIAL AGENTS

Although a variety of antimicrobial agents are available for the treatment of mycobacterial diseases, not all agents are suitable for treating all types of infections. Furthermore, in the face of antimicrobial resistance, the choice of alternative therapies can be problematic and clinical experience becomes a prevailing factor. For other uncommon mycobacterial infections, the physician is not infrequently faced with a dilemma in choosing a treatment regimen because of a lack of clinical precedence or unclear efficacy. The situation is confounded further by the need to treat mycobacterial infections with a combination of agents to improve efficacy, to prevent resistance, or to overcome intrinsic resistance (34).

The antimicrobial agents that are used in the treatment of mycobacterial infections are discussed below and, for the most commonly encountered species, the primary agents are summarized in Table 1 (75, 160). For tuberculosis, first-line drugs are those usually used to treat uncomplicated cases. The Clinical and Laboratory Standards Institute (CLSI, formerly National Committee for Clinical Laboratory Standards [NCCLS]) document on drug susceptibility testing of mycobacteria (28) currently recommends that first-line testing include ethambutol (EMB), rifampin (RMP), two levels of isoniazid (INH), and pyrazinamide (PZA). In the United States, streptomycin (SM) has been moved from first-line to second-line testing (28). Second-line drugs are those used when first-line therapy fails or is inappropriate. These agents are often accompanied by more severe side effects.

Drug resistance in *Mycobacterium tuberculosis* occurs randomly and at a low frequency and is usually a result of single-step mutations. There are two types of drug resistance seen in *M. tuberculosis:* primary and acquired. Primary drug resistance occurs when drug-resistant organisms are found in a previously untreated person. Acquired resistance, the most common form of resistance, can emerge against any of the antituberculosis agents during chemotherapy (111).

Antibiotic resistance in bacteria can occur by a number of mechanisms (summarized for *M. tuberculosis* in Table 2). Five of these categories are found in mycobacteria: decreased uptake of drug such as seen in dormant acid-fast bacilli; drug inactivation by production of β-lactamases; increased efflux as seen in fluoroquinolone resistance; alteration of the target as described below with RMP and INH; and reduced prodrug-activating enzymes as exhibited in PZA resistance (157).

Isoniazid

Isoniazid (INH) (isonicotinic acid hydrazide), a synthetic antimicrobial agent introduced in 1952 for the treatment of tuberculosis, is remarkably specific and potently bactericidal for tubercle bacilli. INH has comparatively low toxicity and is active against virtually all wild-type strains of *M. tuberculosis.* The mechanism of action of INH occurs in three steps: (i) activation of the drug, (ii) binding of the "active" form to the target(s) of inhibition, and (iii) exertion of an inhibitory effect. In turn, the mechanism of INH resistance can involve alterations in each of these steps, alone or in combination. While the mechanism of INH activation is not completely resolved, it is likely that the process involves an oxidation

*This chapter contains information presented in chapter 73 by Clark B. Inderlied and Gaby E. Pfyffer in the eighth edition of this Manual.

TABLE 1 Antimicrobial agents recommended for primary treatment of common mycobacterial infections

Mycobacterium species	Site of infection: antimicrobial agents
M. tuberculosis complex[a]	Any site: isoniazid + rifampin + ethambutol + pyrazinamide
M. avium complex	Pulmonary: clarithromycin (or azithromycin) + rifampin + ethambutol; disseminated: clarithromycin (or azithromycin) + ethambutol ± rifabutin
M. kansasii	Pulmonary: isoniazid + rifampin + ethambutol
M. abscessus, M. chelonae, M. fortuitum[b]	Nonpulmonary: clarithromycin (if susceptible) + ≥1 other drug, based on susceptibility test results
M. abscessus[c]	Pulmonary: multidrug regimen, based on susceptibility test results, that includes clarithromycin
M. marinum	Clarithromycin, doxycycline or minocycline, trimethoprim-sulfamethoxazole, or rifampin + ethambutol

[a] Almost all (>95%) *M. bovis* isolates are resistant to pyrazinamide.
[b] Surgical debridement often is important for successful therapy.
[c] Currently there are no drug regimens of proven efficacy. Antimicrobial therapy may provide symptomatic improvement and disease regression. Surgical resection of limited disease (if possible) and multidrug therapy are optimal.

reaction catalyzed by a catalase-peroxidase encoded by the *kat*G gene (182). The oxidized form of INH can then covalently bind to the nicotinamide moiety of NAD(H) to form INH-NAD(H) adducts, which in turn compete with NAD(H) for binding to an enoyl-acyl carrier protein reductase encoded by the *inh*A gene (76).

The primary effect of INH is on mycolic acid synthesis, as evidenced by the increased fragility of the mycobacterial cell, increased intracellular viscosity, decreased cellular hydrophobicity, and loss of acid fastness of INH-resistant isolates (169). The mycolic acids are produced by the fatty acid synthesis (FAS) I and II enzyme systems. It is the FAS II system that synthesizes the long-chain mycolic acids that are species specific. Activated INH targets one of the FASII enzymes, the enoyl-acyl carrier protein (ACP) reductase of InhA. However, InhA is not the only target, because activated INH also targets the fatty acid elongation enzyme ketoacyl-ACP synthase A or KasA. In addition, INH appears to interfere with NAD metabolism, energy metabolism, and macromolecular synthesis (36, 170, 171).

The mechanism of action of INH correlates with the known mechanisms of INH resistance. Banerjee et al. (6) reported that INH and ethionamide resistance in *M. tuberculosis* correlated with a missense mutation in the *inh*A gene. Other studies showed that *kat*G mutations account for 30 to 60% of INH resistance (65, 101) and that *inh*A mutations confer a low level of INH resistance that may not always be clinically significant (89). Mutations in two other genes may confer some INH resistance: the *ahp*C gene, which encodes an alkyl hydroperoxide reductase subunit (38, 80, 142, 168, 181), and the *kas*A gene, which encodes the previously mentioned KasA enzyme (90). The degree to which mutations in *ahp*C and *kas*A contribute to INH resistance is not clear, although the role of *ahp*C appears to be minor. In aggregate, however, mutations in these four genes (*kat*G, *inh*A, *ahp*C, and *kas*A) may account for ~90% of INH resistance (Table 2) (39, 149).

INH is active only against replicating tubercle bacilli in the presence of oxygen; slowly replicating bacilli in the caseous lesions are not readily killed by INH, and dormant bacilli under anaerobic conditions are unlikely to be affected. INH resistance develops rapidly when patients are given monotherapy, and the frequency of INH resistance within a population of tubercle bacilli ranges from 10^{-5} to 10^{-6} (33). Wild-type isolates of *M. tuberculosis* are inhibited by INH at concentrations of <0.2 μg/ml (93). Other slowly growing mycobacteria, such as *M. kansasii* and *M. xenopi,* are inhibited by 1 to 5 μg/ml. Most other nontuberculous mycobacteria, including the *M. avium* complex (MAC), *M. marinum,* and all rapidly growing mycobacteria, are resistant to INH.

TABLE 2 Mechanisms of drug resistance in *Mycobacterium tuberculosis*[a]

Antimicrobial agent	Gene involved	Target	Mechanism of action
RMP	*rpoB*	β Subunit of RNA polymerase	Inhibits transcription
INH	*kat*G	Catalase-peroxidase	Inhibits mycolic acid biosynthesis; other multiple effects on DNA, lipids, carbohydrates, and NAD metabolism
	inhA[b]	EnvM analog 3-ketoacyl-acyl carrier protein reductase analog	
	kasA[b]	B-Keroacyl ACP synthase	
	ahpC[b]	Subunit of alkyl hydroperoxide reductase	
EMB	*embB*	Arabinosyltransferase	Inhibits arbinogalactan synthesis
SM	*rpsL*	Ribosomal protein S12	Inhibits protein synthesis
		16S rRNA	Inhibits protein synthesis
PZA	*pncA*	Pyrazinamidase	Acidifies cytoplasm and de-energizes membrane
Ethionamide	*inhA*		Inhibits mycolic acid biosynthesis
Fluoroquinolones	*gyrA*	DNA gyrase A subunit	Inhibits DNA gyrase
Amikacin, kanamycin	*rrs*	16S rRNA	Inhibits protein synthesis

[a] Adapted from reference 157.
[b] Mechanism of action statement similar to that of *kat*G.

INH is well absorbed when administered orally or intramuscularly; it is distributed throughout the body, and the levels in cerebrospinal fluid (CSF) may equal the levels in plasma in patients with meningeal inflammation or 20% of the levels in plasma in patients without inflammation (160). INH is metabolized in the liver and intestines, primarily by acetylation by an *N*-acetyltransferase, which can vary significantly from person to person. However, the acetylator phenotype of an individual does not appear to influence either the efficacy of INH or the risk of hepatotoxicity. Adverse drug reactions include infrequent, age-related hepatitis and, less frequently, peripheral neuropathy, hypersensitivity reactions such as fever and rash, and arthralgias.

Rifampin

Rifampin (RMP) (rifampicin) was introduced in 1968 as a potent antituberculosis agent. It affects intracellular, slowly replicating bacilli in caseous lesions as well as the actively replicating bacilli in open cavities. RMP is active against a wide variety of non-acid-fast bacteria and several other slowly growing mycobacteria, notably *M. leprae, M. kansasii, M. xenopi,* and *M. marinum,* but has variable activity in vitro against MAC and is inactive against the rapidly growing mycobacteria. It easily diffuses through the mycobacterial cell membrane due to its lipophilic nature and binds to the bacterial RNA polymerase, thus inhibiting RNA synthesis (156). RMP inhibits the prokaryotic DNA-dependent RNA polymerase by binding to the β subunit at the presumed catalytic center of the enzyme. The mammalian RNA polymerase is inhibited by RMP only at significantly higher concentrations than is the prokaryotic enzyme. The RNA polymerase of MAC isolates appears to be susceptible to RMP; therefore, the primary mechanism of intrinsic resistance is most probably impermeability.

Telenti et al. (150) first showed that RMP resistance correlated with changes, primarily amino acid substitutions, within a conserved region of the *rpoB* gene that encodes the β subunit of the *M. tuberculosis* RNA polymerase. Subsequent studies showed that >96% of RMP resistance could be attributed to mutations within an 81-bp region of the *rpoB* gene (103). Rifamycin resistance that is due to mutations outside this "hot spot" are rare, but such mutations appear to cluster at codon 176 at the beginning of the *rpoB* gene (62). In two strains of *M. avium* with high MIC values, missense mutations were detected, but in two other strains, no mutations were detected. This agrees with the observations of Guerrero et al. (54), who showed that mutations in the *rpoB* gene in *M. avium* and *M. intracellulare* are rare and that high RMP MIC values most probably are a reflection of impermeability to the drug.

RMP is well absorbed from the gastrointestinal tract, and peak concentrations of 5 to 10 μg/ml are reached 1 to 2 h after an oral dose of 600 mg; concentrations in CSF reach 50% of the levels in plasma in patients with meningeal inflammation. RMP is available in combination with INH as a single capsule (Rifamate) containing 300 mg of RMP and 150 mg of INH and in combination with INH and pyrazinamide as a single capsule (Rifater). An RMP concentration of 0.5 μg/ml is bactericidal for wild-type isolates of *M. tuberculosis.* Adverse drug reactions include hepatotoxicity, gastrointestinal and hypersensitivity reactions, and a red-orange discoloration of urine, tears, other body fluids, and soft contact lenses. RMP also induces increased hepatic metabolism of several other drugs, including methadone and birth control pills. Of particular concern is the interaction of RMP and, to a somewhat lesser degree, rifabutin with protease inhibitors (saquinavir, ritonavir, and indinavir), which leads to enhanced hepatic metabolism and may result in subtherapeutic levels of the antiviral agents (9).

Rifabutin and Rifapentine

Rifabutin (ansamycin), a spiropiperidyl rifamycin, and rifapentine, a cyclopentyl rifamycin, have potent in vitro activity against *M. tuberculosis*. Rifabutin also is very active against MAC (64, 129). The mode of action and mechanism of resistance of both drugs appear to be identical to those of RMP; however, approximately 30% of RMP-resistant *M. tuberculosis* isolates are susceptible to rifabutin and rifapentine. The latter observation correlates with certain specific mutations in the *rpoB* gene (14). Yang et al. (178) analyzed clinical strains of *M. tuberculosis* for cross-resistance to rifamycins. Alterations at codons 513 and 531 correlate with resistance to RMP, rifabutin, and rifalazil (KRM-1648). Point mutations at codons 516 and 529, deletion at codon 518, and insertion at codon 514 influence susceptibility to RMP but not to rifabutin or KM-1648, while alteration at codons 515, 521, and 533 did not influence susceptibility to RMP, rifabutin, and KR-1648 (178). These findings were confirmed by analyzing recombinant *M. tuberculosis* clones containing plasmids with specific mutations (166). As with RMP, both rifabutin and rifapentine are metabolized to the corresponding biologically active 25-desacetyl metabolite. Rifabutin decreases the incidence of disseminated MAC disease in human immunodeficiency virus (HIV)-infected patients when used as a prophylactic agent, and it is approved for that indication (87, 107). The role of rifabutin as a therapeutic agent for MAC disease is unclear, but there may be a significant dose effect (73). In addition to being more active than RMP on a weight basis, rifabutin has a long elimination half-life in humans and concentrates in tissues, notably lung tissues, where the levels are 10-fold higher than in serum. This may account for the reported effectiveness of rifabutin in the therapy of MAC pulmonary infections.

Rifabutin is absorbed from the gastrointestinal tract and reaches peak levels of 0.5 μg/ml in serum about 4 h after a 300-mg dose. Adverse drug reactions with rifabutin are similar to those observed with RMP, including the above-mentioned important adverse interactions with antiretroviral agents (9). Some unique rifabutin toxicities include leukopenia, thrombocytopenia, arthralgias, and uveitis when coadministered with clarithromycin.

Rifapentine was approved by the U.S. Food and Drug Administration (FDA) for the treatment of tuberculosis in 1998 (5). In a study of 722 patients, 361 received rifapentine plus INH, PZA, and EMB while the remaining patients received RMP in place of rifapentine along with the other drugs (5). In the intensive phase of the study, rifapentine was administered twice a week while RMP was administered daily. In the continuation phase, rifapentine and INH were administered once a week and RMP and INH were administered twice a week. Sputum conversion was somewhat higher in the rifapentine group than in the RMP group: 87 and 81%, respectively. However, relapse was somewhat higher in the rifapentine group than in the RMP group: 10 and 5%, respectively. Rifapentine reaches a peak concentration of 15 μg/ml in serum 5 to 6 h after a 600-mg dose, with a half-life of about 13 h.

Pyrazinamide

Pyrazinamide (PZA) is a synthetic derivative (pyrazine analog) of nicotinamide and, in combination with INH, is rapidly bactericidal for replicating and nonreplicating forms of *M. tuberculosis,* with an average MIC of 20 μg/ml (157). PZA is totally inactive against other *Mycobacterium* species,

including *M. bovis,* MAC, and the rapidly growing mycobacteria. PZA is active only at an acidic pH; therefore, the pH of the growth medium must be adjusted for accurate measurements of the in vitro activity of the drug. It is most likely that PZA is active only in the acidic milieu of the phagolysosome and, depending on the concentration achieved at the site of the infection, may be bacteriostatic or bactericidal. PZA is hydrolyzed in the liver to the active metabolite pyrazinoic acid, and although the mechanism of action of PZA is unknown, its activity depends on this conversion. *M. tuberculosis* produces a pyrazinamidase, and most strains of PZA-resistant *M. tuberculosis* lack this enzyme; however, some PZA-resistant isolates retain enzyme activity, suggesting that there are other mechanisms of resistance such as drug efflux (183, 184).

The lack of pyrazinamidase activity and its correlation with PZA resistance have been associated with mutations in the *pncA* gene that encodes the enzyme (135, 136, 143). Indeed, it now appears that 72 to 97% of PZA resistance can be attributed to mutations in the *pncA* gene (Table 2). Data from one study suggest that this correlation can be used to distinguish between *M. tuberculosis* and *M. bovis* (134); however, PZA-susceptible *M. bovis* isolates, although uncommon, have been described.

PZA is well absorbed from the gastrointestinal tract and widely distributed throughout the body, with maximum levels in serum of approximately 45 μg/ml 1 to 4 h following an oral dose of 1 g (20 to 25 mg/kg of body weight). Hepatotoxicity occurs in a small number of patients; photosensitivity and rash are rare. Gout is an important contraindication because of the hyperuricemia associated with PZA therapy. PZA therapy is usually discontinued after the first 2 months of short-course treatment for tuberculosis, whereas INH and RMP treatment is continued for an additional 4 months.

Ethambutol

Ethambutol (EMB) [dextro-2,2-(ethylenediimino)-di-1-butanol-dihydrochloride] is a synthetic antituberculosis compound that was introduced in 1961. The MIC values of EMB tested against wild-type isolates of *M. tuberculosis* range from 1 to 5 μg/ml, but the activity of the drug against other slowly growing nontuberculous mycobacteria is more variable. The drug is active only against growing bacilli and has no activity against nongrowing mycobacteria. The primary mechanism of action of EMB is bacteriostatic inhibition of cell wall synthesis, while evidence points to a specific effect on arabinogalactan synthesis (147). The frequency of mutation to EMB resistance in *M. tuberculosis* is on the order of 10^{-5}, and there is evidence that EMB resistance in *M. tuberculosis* correlates with a specific mutation (at codon 306) in the *embB* gene, which encodes an arabinosyltransferase (144). Mutations in this codon have been associated with MIC values of 20 to 40 μg/ml for several EMB-resistant isolates of *M. tuberculosis* (1). While mutations in the *embB* gene result in high-level resistance in *M. tuberculosis,* this mutation accounts for only 70% of resistant strains; thus, other gene mutations are likely to play an additional role (157). Although many MAC isolates have high EMB MIC values, combinations of EMB and other agents, notably quinolones and macrolides, are synergistic (66, 78), and it has been shown that the MIC value does not correlate with clinical response (141). It appears that EMB affects the permeability of the MAC cell wall and perhaps increases the intracellular concentration of the other potentially more active drugs (67).

Peak concentrations of EMB in serum are 5 μg/ml 2 to 4 h after a 25-mg/kg dose. The most important adverse effect associated with EMB is decreased visual acuity due to optic neuritis that is related to both the dose and duration of treatment. EMB is not recommended for the treatment of children too young to be monitored for changes in vision unless no other drug is appropriate because of resistance. The effects on vision are generally reversible when the drug is discontinued.

Aminoglycosides

The aminoglycosides that are used for the treatment of tuberculosis and other mycobacterial infections include streptomycin (SM), kanamycin (a glycoside of 2-deoxystreptamine), and amikacin, which is a derivative of kanamycin. In addition, tobramycin is active against *M. chelonae,* and capreomycin and viomycin, basic peptide antibiotics with a mechanism of action similar to that of the aminoglycosides, are active against *M. tuberculosis* and certain other species of mycobacteria. Gentamicin is inactive against mycobacteria at the usual concentrations attained in serum.

The primary mechanism of action of the aminoglycosides is inhibition of the post- to pretranslocation step of protein synthesis by blocking the binding of the aminoacyl-tRNA (e-type binding). Viomycin also blocks aminoacyl-tRNA translocation, and viomycin resistance crosses to capreomycin, suggesting that the mechanism of action is the same. SM MIC values for wild-type isolates of *M. tuberculosis* are usually well below the peak concentration in serum of 25 to 50 μg/ml 1 to 2 h after a 1-g intramuscular dose. Amikacin is the most potent of the aminoglycosides. There is comparatively little clinical experience with amikacin in the treatment of tuberculosis because the drug is expensive and inconvenient to administer, but amikacin often is used in combination with one or more other agents for treatment of serious infections caused by rapidly growing mycobacteria. Amikacin also is active against MAC; the majority of isolates have an MIC of <32 μg/ml, which approaches the maximum concentration in serum. In an early uncontrolled trial of disseminated MAC in HIV-infected patients, amikacin was shown to be the active component of a multiple-drug regimen (26). In that trial, treatment was associated with a positive microbiological and clinical response, but the clinical utility of amikacin for disseminated MAC is uncertain (4). The drug may be useful in an "induction" regimen to clear a bacteremia.

The molecular basis of SM resistance (Table 2) in *M. tuberculosis* results from mutations in the gene that encodes ribosomal protein S12 or from mutations in the 16S rRNA region, which is structurally linked to the S12 protein in the assembled ribosome (47, 81, 104). Finken et al. (47) showed that mutations in the *rpsL* gene coding for the S12 protein were present in 20 of 38 SM-resistant strains and that there was a mutation in the *ssr* gene, encoding 16S rRNA, in 9 strains. Nair et al. (104) determined the nucleotide sequence of the *rpsL* gene and showed that SM resistance, in a small number of isolates, appeared to be a result of point mutations at codon 43 of this gene, a site of SM resistance in *Escherichia coli.* Adverse drug reactions associated with aminoglycosides and peptide antibiotics include hearing loss, tinnitis, loss of balance, and renal failure.

Cycloserine

D-Cycloserine (4-amino-3-isooxazolidinone) is an analog of D-alanine that inhibits the synthesis of D-alanyl-D-alanine, an essential component of the mycobacterial cell wall. Cycloserine is active against all mycobacteria as well as several other types of bacteria. Although it is one of the secondary

drugs for treatment of tuberculosis, in vitro susceptibility testing is not recommended due to technical problems with the test (28, 113). Peak levels in serum of 20 to 40 μg/ml are achieved 4 h following an oral dose of 250 mg. The drug is widely distributed through the body, including the CSF. There are significant adverse drug reactions associated with cycloserine treatment, notably peripheral neuropathy and central nervous system dysfunction including seizures and psychotic disturbances.

Ethionamide

Ethionamide (2-ethyl-pyridine-4-carbonic acid thioamide) is a derivative of isonicotinic acid and, like INH, blocks mycolic acid synthesis. However, isolates of *M. tuberculosis* that are resistant to high concentrations of INH are susceptible to ethionamide, suggesting that the site of action may be different from that of INH. However, mutations in the *inhA* gene have been associated with ethionamide resistance (6). The average MIC for *M. tuberculosis* is 0.6 to 2.5 μg/ml, and levels of 2 to 20 μg/ml in serum are achieved 3 to 4 h following an oral dose of 0.5 to 1 g. Side effects associated with ethionamide include gastrointestinal irritation with nausea, vomiting, and cramps and neurologic symptoms.

Dapsone

Dapsone (diaminodiphenyl sulfone) is a synthetic compound that was shown to be active against *M. leprae* in the early 1940s. Dapsone is an antifolate that, like other inhibitors of folic acid synthesis, exerts primarily a bacteriostatic effect and is only weakly bactericidal. Dapsone is administered orally and is well absorbed and distributed throughout the body. Levels in tissue are approximately 2 μg/ml following a 200-mg dose. The drug has a long half-life in serum, of 10 to 50 h depending on the individual patient. Common adverse drug reactions include nausea, vomiting, anorexia, and methemaglobinemia; hematuria, rash, pruritus, and fever also can occur. Traditionally, dapsone is used in combination with RMP and clofazimine for the treatment of leprosy. Acedapsone is a diacetylated form of dapsone with an extraordinarily long half-life of 46 days; as a result, this drug is administered infrequently (e.g., five injections per year), with peak concentrations in tissue occurring 20 to 35 days after administration. Acedapsone is relatively inactive against *M. leprae,* but in vivo it is deacetylated to the parent compound.

Macrolides and Ketolides

Azithromycin and clarithromycin are the most important agents in the treatment of all forms of MAC disease and are effective and approved prophylactic agents for preventing disseminated MAC disease in HIV-infected persons (58, 117, 180). These drugs also are useful in the treatment of disease caused by *M. marinum, M. haemophilum, M. kansasii, M. simiae,* and rapidly growing mycobacteria other than *M. fortuitum.* Indeed, they are viewed as potential cornerstones in the treatment of nontuberculous mycobacterial infections (4). Azithromycin, an azalide (a subclass of macrolides), and clarithromycin are structurally similar to erythromycin and have modifications that improve their acid stability and increase their potency, half-life, achievable concentrations in tissue, and bioavailability without causing toxicity. These macrolides are bacteriostatic agents and inhibit the growth of microorganisms by binding to the 50S subunit of the prokaryotic ribosome, blocking protein synthesis at the peptidyltransferase step. Meier et al. (92) showed that both clarithromycin- and azithromycin-resistant mutants of *M. intracellulare* have a single-base mutation at adenine-2058 in the 23S rRNA gene, a site of mutation or methylation that has been associated with macrolide resistance in other bacteria (106). The same genetic basis for macrolide resistance was found in *M. chelonae* and *M. abscessus* (158).

The in vitro activity of azithromycin against MAC appears to be quite modest, with MIC values 32- to 64-fold above the maximum concentration in serum (74). The remarkable ability of azithromycin to concentrate in tissues most likely accounts for its therapeutic activity in animal studies and humans (76, 83). Azithromycin is rapidly absorbed from the gastrointestinal tract and widely distributed throughout the body. It has a terminal half-life of 68 h. The peak concentration in serum following a 500-mg dose is 0.4 to 0.6 μg/ml; however, the drug concentrates in tissues to high levels. In a small study with humans, the levels of azithromycin in polymorphonuclear neutrophils were nearly 1,000-fold higher than the levels in serum (3, 133).

Clarithromycin inhibits 90% of MAC isolates with MIC values of 0.25 to 0.5 μg/ml when measured by a radiometric broth macrodilution method at a neutral to slightly alkaline pH (the activities of all macrolides are strongly influenced by pH). Peak levels in serum of 2 to 3 μg/ml are achieved 5 to 6 h following a 500-mg dose; the concentrations in tissue are 4 to 5 times greater than the concentrations in serum, and the concentration in macrophages is 20 to 30 times greater. The elimination half-life is 5 to 7 h following a 500-mg dose twice a day.

The ketolides are semisynthetic derivatives of erythromycin with structural changes that improved antimicrobial activity and pharmacokinetics. Telithromycin, a ketolide licensed in the United States and Europe, is active against mycobacteria in vitro but has not been evaluated in a clinical trial in humans (46, 121).

Quinolones

Ciprofloxacin, ofloxacin, levofloxacin, gatifloxacin, and moxifloxacin are fluorinated carboxyquinolones with good in vitro activity against multidrug-resistant and pansusceptible *M. tuberculosis* (15, 70, 75). MIC values of ciprofloxacin and ofloxacin against susceptible isolates of *M. tuberculosis* range from 0.25 to 3 μg/ml and from 0.5 to 2.5 μg/ml, respectively. Measurements of the early bactericidal activity of ciprofloxacin in patients with pulmonary tuberculosis suggested that the drug is effective at a high dosage (1,000 mg/day) (140). Fluoroquinolones have variable activity against MAC (179). Most *M. fortuitum* group isolates are susceptible to ciprofloxacin (currently the class representative for fluoroquinolones), whereas activity against *M. abscessus* and *M. chelonae* is more variable.

The CLSI recommends that ofloxacin be included as the class representative for fluoroquinolones in the panel of secondary antituberculosis drugs (28). The suggested susceptibility breakpoint is 2 μg/ml for the agar proportion method (28). However, levofloxacin (the L-isomer of ofloxacin) is nearly twice as active against *M. tuberculosis* as ofloxacin in vitro. For this reason, protocols for susceptibility testing of levofloxacin are desirable. Data from a recent study support susceptibility breakpoints of 2 μg/ml for BACTEC TB460 and BACTEC MGIT 960 and 1 μg/ml for agar proportion (132).

The mechanism of action of all fluorinated quinolones is inhibition of DNA synthesis as a result of binding to the DNA gyrase (bacterial topoisomerase II) (123). Although this is the presumed mechanism of action in mycobacteria, fewer studies have been performed with mycobacteria than with other microorganisms. Takiff et al. (148) showed that quinolone resistance in *M. tuberculosis* can be ascribed to mutations in

the *gyrA* and *gyrB* genes, which encode the DNA gyrase subunits (Table 2). In *M. tuberculosis,* fluoroquinolones function by binding to the bacterial enzyme-DNA complex, with suggested mechanisms being strand-breakage, SOS-mediated autolysis, and replication blockage. When combined with various first-line antimycobacterial drugs, fluoroquinolones have shown greater reductions in CFUs within infected macrophages than the individual drugs alone. While there are no reports of cross-resistance between quinolones and other classes of antimycobacterial agents, there is cross-resistance within the quinolone class, such that reduced susceptibility to one quinolone may likely confer reduced efficacy to all quinolones (51).

Recently, moxifloxacin has received attention as being useful in the treatment of tuberculosis, especially multidrug cases. Although slightly delayed in the first few days of response, the antimycobacterial effect of moxifloxacin appears to be similar to that of INH after 5 days of treatment (119). The newer quinolones (sparfloxacin, gatifloxacin, and moxifloxacin) have lower MICs than levofloxicin, ciprofloxacin, and ofloxacin. Treatment studies with mice have shown moxifloxacin has the most bactericidal efficacy against *M. tuberculosis,* followed by sparfloxacin, levofloxicin, and ofloxacin (in that order). Against *M. tuberculosis,* moxifloxacin has a reported MIC of 0.12 to 0.5 μg/ml (51).

Ciprofloxacin is well absorbed from the gastrointestinal tract and is rapidly distributed throughout the body. Maximum concentrations in serum of 2.4 or 4.3 μg/ml are achieved 1 to 2 h after an oral dose of 500 or 750 mg, respectively. The elimination half-life of ciprofloxacin is 4 h in subjects with normal renal function. Maximum ofloxacin concentrations in serum are achieved 1 to 2 h after an oral dose. Following a single 400-mg dose, the concentration of ofloxacin in serum is 2.9 μg/ml; after a steady-state dose, it is 4.6 μg/ml. The maximum concentration of levofloxacin in serum is approximately twice that of ofloxacin at the same dosage. The efficacy of fluoroquinolones in the treatment of pulmonary tuberculosis may relate, in part, to the observation that these quinolones concentrate in lung tissue to levels at least four times greater than the concentration in serum. Adverse effects with ciprofloxacin and other fluoroquinolones may be less severe than with the other secondary agents (12).

p-Aminosalicylic Acid

p-Aminosalicylic acid (PAS) is an antifolate that is active against *M. tuberculosis* but inactive against most other mycobacteria. There is some evidence that PAS also may affect iron transport in *M. tuberculosis* and salicylic acid metabolism. The average MIC for susceptible isolates of *M. tuberculosis* is 1 μg/ml, and peak levels of 7 to 8 μg/ml are achieved in serum 1 to 2 h after a 4-g dose. PAS is incompletely absorbed in the gastrointestinal tract and is associated with significant gastrointestinal side effects; in combination with the need for large dosages (10 to 12 g/day), this leads to frequent problems with treatment adherence.

Clofazimine

Clofazimine [3-(*p*-chloroanilino)-10-(*p*-chlorophenyl)-2, 10-dihydro-2-isopropyliminophenazine] is a substituted iminophenazine, bright red dye. It has weak bactericidal activity against *M. leprae* but is used in combination with RMP and dapsone as a conventional treatment regimen for leprosy. However, it may take up to 50 days of treatment before there is evidence of tissue antimicrobial activity, which may influence the length of time before there is a clinical response in the treatment of leprosy. Clofazimine has good in vitro activity against MAC, but despite this, it appears to offer little in the treatment of disseminated MAC (137). Indeed, treatment regimens that contained clofazimine have been associated with higher mortality than those that did not (24). The drug also has good in vitro activity against *M. tuberculosis,* but there is little or no information on the in vivo activity.

The mechanism of action of clofazimine is unknown; however, it is highly lipophilic and binds preferentially to mycobacterial DNA. The absorption of clofazimine following an oral dose is variable and ranges from 45 to 60%. The average concentrations in serum are 0.7 to 1.0 μg/ml following a dose of 100 to 300 mg. The half-life is extraordinarily long (estimated to be 70 days), and the drug tends to be deposited in fatty tissues and cells of the reticuloendothelial system. Adverse drug reactions are limited primarily to a pink or red discoloration of the skin, conjunctiva, cornea, and body fluids and gastrointestinal intolerance including pain, diarrhea, nausea, and vomiting.

Amithiozone

Amithiozone (Thiacetazone, Tibione, or Panthrone) is a thiosemicarbazole that is active against *M. tuberculosis,* with an average MIC for wild-type strains of 1 μg/ml. Resistance develops quickly when monotherapy is given. Peak levels in serum of 1 to 4 μg/ml are achieved 1 to 2 h following an oral dose of 150 mg. Adverse drug reactions are gastrointestinal irritation and bone marrow suppression, and hepatotoxicity can occur in patients receiving concomitant INH. Additionally, there appears to be an association of Stevens-Johnson syndrome and severe epidermal necrolysis in HIV-infected patients with tuberculosis treated by regimens containing amithiozone (42, 53). Consequently, the World Health Organization recommends that amithiozone not be used to treat patients known or suspected to be infected with HIV (109). Amithiozone is not available in the United States and is not used in Europe because of the adverse effects, but it has been used successfully in combination with INH for the treatment of tuberculosis in some African countries, where adverse effects are believed to be less severe.

M. TUBERCULOSIS COMPLEX

Drug Resistance

In the early 1960s, the World Health Organization organized two meetings that led to the description of reliable criteria and techniques for testing mycobacteria for resistance to antituberculosis drugs (20, 21). The critical proportion for resistance on Löwenstein-Jensen slants varied according to the drug, e.g., 1% for INH and RMP and 10% for SM, EMB, PZA, ethionamide, kanamycin, and cycloserine. However, based on the experience of Russel and Middlebrook with 7H10 agar (128), the Centers for Disease Control and Prevention (CDC) recommended Middlebrook 7H10 agar and 1% as the critical proportion for all drugs (85).

Resistance is fundamentally a phenomenon linked to large initial bacterial populations. In pulmonary tuberculosis, the greatest populations are those prevailing in cavities, which can contain 10^7 to 10^9 organisms, whereas the populations found in hard caseous foci, the most common type of lesion, generally do not exceed 10^2 to 10^4 organisms (19). The greater frequency of resistance during treatment of cavitary tuberculosis was shown as early as 1949 (68, 69). David at CDC (33) demonstrated the probability distribution of drug-resistant mutants and in a fluctuation test showed that *M. tuberculosis* spontaneously mutated to resistance to INH, SM,

EMB, and RMP. The average mutation rates for INH, SM, EMB, and RMP were calculated to be 3×10^{-8}, 3×10^{-8}, 1×10^{-7}, and 2×10^{-10} mutation per bacterium per generation, respectively. Thus, the mutation rate for resistance to two drugs is theoretically less than 10^{-15}. Implicit in all the studies of the genetic basis of antimicrobial resistance in *M. tuberculosis* is that the multiple-drug-resistance (MDR) phenotype (minimally defined as simultaneous resistance to INH and RMP) is the result of accumulative mutations rather than the acquisition of an MDR transfer factor (103).

Special-Population Hypothesis

According to the generally accepted theory, resistance appearing during drug treatment is due to selection and multiplication of the resistant mutants preexisting in the tubercle bacillus population of the host. Inasmuch as the susceptible bacilli are the predominant part of the population, initial killing involves a greater number of microorganisms. The consequence is a sharp fall in the population of bacilli during the initial period of treatment. The rise due to multiplication of the resistant mutants occurs later. This "fall-and-rise" phenomenon, as demonstrated in the patient's sputum, was described in the late 1940s (32, 120). In 1979, Mitchison (97) suggested the "special-populations" hypothesis to explain the action of the major antituberculous drugs against the various subpopulations of tubercle bacilli (Fig. 1). The subpopulations include (i) rapidly growing bacilli in the pulmonary lesions; (ii) bacilli that grow in short metabolic spurts and that might be susceptible to RMP but not INH; (iii) bacilli that reside in the acidic environment of the caseous lesions; and (iv) dormant, nonreplicating bacilli. The hypothesis was developed to explain in part the basis of the early bactericidal activity and the later sterilizing activity of antituberculosis agents. Each drug in the conventional multiple-drug treatment regimen for tuberculosis is more or less effective in eradicating tubercle bacilli within each of these special populations. Thus, the use of multiple drugs in the treatment of tuberculosis is aimed at both preventing drug resistance and achieving a maximum therapeutic effect. Figure 1 depicts the special-populations hypothesis, as presented by Mitchison in 1992 (98) in his Garrod lecture, altered to include a reference to the observation by Wayne and Sramek (165) that dormant *M. tuberculosis* is susceptible to nitroimidazoles. This study was based on the assumption that dormant tubercle bacilli are in an anaerobic metabolic state and therefore would be susceptible to agents such as metronidazole.

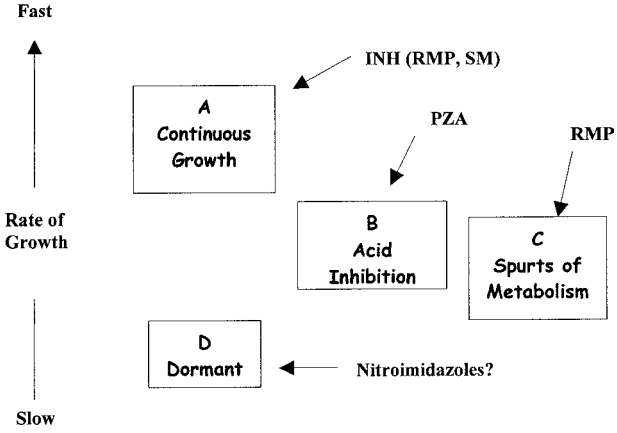


FIGURE 1 Schematic representation of the special-populations hypothesis of Mitchison (98). The model accounts for the action of antimycobacterial agents on different populations of *M. tuberculosis* bacilli in various sites in the infected host. (A) INH, RMP, and SM are active primarily against bacilli in a continuous state of growth, which are the majority population at the start of treatment. (B) PZA is active only against bacilli within the acidic early caseous lesions that develop within the first 2 months of chemotherapy (98). (C) The comparatively rapid bactericidal activity of RMP is important for the eradication of bacilli that grow for short periods (not long enough to be affected by INH). (D) Some tubercle bacilli may be sequestered in lesions in a metabolically inactive and dormant state; however, there is evidence that these dormant bacilli may actually be in an anaerobic state and susceptible to nitroimidazoles such as metronidazole (165). (Reprinted from reference 98 with permission of Oxford University Press.)

Critical Concentrations

The criteria for defining drug-resistant *M. tuberculosis* were established on an empirical basis, i.e., that there is a certain proportion of drug-resistant mutants above which therapeutic success is less likely to be realized. The procedures used to perform drug susceptibility tests and the criteria for interpreting the results take into account two factors: (i) the critical proportion of drug-resistant mutants and (ii) the critical concentration of the drug in the test medium. On the basis of clinical and bacteriologic studies, the significant proportion of bacilli resistant to an antituberculosis drug, above which a clinical response is unlikely, was generally set at 1%. The critical concentration of a drug is the concentration that inhibits the growth of most cells within the population of a wild-type strain of tubercle bacilli without appreciably affecting the growth of the resistant mutant cells that might be present. In other words, if the proportion of tubercle bacilli that are resistant to the critical concentration of a drug exceeds 1%, it is unlikely that the use of that drug will lead to a therapeutic success. It should be noted that this concentration may not have a direct relationship to the peak level of the drug in serum. The critical concentrations of antituberculosis drugs, in different media, are given in Table 3 (28, 82).

Low versus High Critical Concentrations

On occasion, both the agar proportion and BACTEC 460TB methods may indicate that an *M. tuberculosis* isolate is resistant to INH, EMB, or SM at the low critical concentration of the drug (Table 3) but susceptible to the same drug at the high concentration. Indeed, it is unclear if testing the low concentration of EMB has any merit, especially because recent evidence indicates that mutations in the *embB* gene are associated only with resistance at the high concentration (1, 144). Similar studies have not been performed with SM; however, the merit of testing SM at the low concentration must be questioned. If the low concentration of the drug only were tested, the report of "resistant" could potentially mislead a clinician to believe that the drug has no value in the treatment of tuberculosis. On the other hand, low-level resistance to INH may be suggestive of resistance to ethionamide (157). Therefore, it is important to test both concentrations of INH or, if only the low concentration is tested, to reflex test the higher concentration when an isolate is resistant to the lower concentration, recognizing that there is not uniform consensus regarding the clinical relevance of the results of testing at a higher concentration when two concentrations are used (28). The clinician should be alerted that when an isolate is resistant to the low concentration but susceptible to the high concentration, therapeutic effect may be achieved with an

TABLE 3 Critical concentrations of antimycobacterial agents to test against *M. tuberculosis* by radiometric (BACTEC 460TB) or agar proportion methods

Antimicrobial agent	Typical MIC (μg/ml) for susceptible strains	Concn in serum (μg/ml)[e]	Medium and concn (μg/ml)[f]					
			BACTEC 460TB 12B low	BACTEC 460TB 12B high	7H10 low	7H10 high	7H11 low	7H11 high
Primary agents								
INH	0.05–0.2	7	0.1	0.4	0.2	1	0.2	1
RMP	0.5	10	2		1		1	
PZA[a]	20	45	100		NR[i]	NR	NR	
EMB	1–5	2–5	2.5[g]	7.5	5	10	7.5	
Secondary agents								
SM	8	25–50	2[g]	6	2	10	2	10
Capreomycin	1–50	30	1.25[h]		10		10	
Kanamycin[b]	5	14–29	5[h]		5		6	
Ethionamide	0.6–2.5	2–20	1.25[h]		5		10	
PAS	1	7.5	4[h]		2		8	
Rifabutin[c]	0.06–8	0.2–0.5	0.5[h]		0.5/1/2		0.5	
Ofloxacin[d]	0.5–2.5	3–11	2[h]		2		2	

[a] PZA tested at pH 6 in BACTEC 12B medium.
[b] Kanamycin is the class representative for amikacin.
[c] RMP-susceptible versus RMP-resistant isolates; about 30% of RMP-resistant isolates are rifabutin susceptible.
[d] Ofloxacin is the class representative for fluoroquinolones.
[e] Concentrations in serum 1 to 4 h after administration of the usual dosage.
[f] Unless otherwise stated, the concentrations shown are from references 28, 63, and 106.
[g] Woodley (172) showed that 2.5 μg of EMB per ml and 2.0 μg of SM per ml tested against susceptible and resistant isolates agreed with the 7H10 proportion method 97 to 99 and 100%, respectively.
[h] According to reference 113.
[i] NR, no recommendation.

adjustment in dosage (e.g., INH). The CLSI recommends that the following comment be appended to the results:

> These results indicate low-level resistance to INH. Experts believe that patients infected with strains exhibiting this level of INH resistance may benefit from continuing therapy with INH. A specialist in the treatment of tuberculosis should be consulted concerning the appropriate therapeutic regimen and dosages (28).

Extent of Service

In the United States, mycobacteriology laboratories can be classified by the levels or extents of services provided based on criteria proposed by CDC and the American Thoracic Society (ATS). In general, the criteria emphasize the importance of the number of specimens processed, the expertise of the laboratory staff, and the cost-effectiveness of the procedures and protocols (61, 85). The ATS recognizes three levels of service (levels I, II, and III), ranging from specimen referral, to identification and drug susceptibility testing of *M. tuberculosis,* to identification of other mycobacteria. It is recommended that a laboratory should perform susceptibility testing only if the laboratory is capable of identifying the isolate to species and if it regularly performs susceptibility tests on that type of isolate (e.g., at least 10 tests per week). Because certain drugs are tested infrequently (e.g., cycloserine or amikacin), must be prepared from reference powders (e.g., rifabutin or ciprofloxacin), or are problematic (e.g., cycloserine [113]), second-line drug testing should be referred to a laboratory with specific expertise in testing these agents.

When To Perform Susceptibility Tests

The approved CLSI standard on susceptibility testing of mycobacteria (28) states that the first isolate of *M. tuberculosis* obtained from each patient should be tested. This ensures the most effective treatment for a patient and contributes to the surveillance database for tuberculosis control. Susceptibility testing should be repeated if cultures fail to convert to negative after 3 months of therapy or if there is clinical evidence of failure to respond to therapy. If a laboratory is unable to provide this service, then isolates should be referred to another laboratory for timely testing.

Methods To Test the Susceptibility of *M. tuberculosis*

Methods that are commonly used to test rapidly growing aerobic and facultative anaerobic bacteria are, for a variety of reasons, unsuitable for testing most mycobacterial species. The methods generally accepted for determining the antimicrobial susceptibility of mycobacteria are based on the growth of the microorganisms on solid or in liquid medium containing a specified concentration of a single drug. In the past, four growth-based methods have been described: the proportion method, the radiometric or BACTEC 460TB (Becton Dickinson Microbiology Systems, Sparks, Md.) method (a modified proportion method), the absolute-concentration method, and the resistance ratio method (56, 63, 75, 138). Only the agar proportion, BACTEC 460TB, and the commercial nonradiometric broth systems are discussed further in this chapter; the last two are described elsewhere (63). These nonradiometric methods, either manual or fully automated, have been created mainly to overcome the safety and regulatory principles associated with radiolabeled substrates. They include VersaTREK (formerly called the ESP Culture System II; Trek Diagnostic Systems, Inc., Cleveland, Ohio) (176), the Mycobacteria Growth Indicator Tube (MGIT; Becton Dickinson) (57, 115), automated MGIT 960 (Becton Dickinson), and the MB/BacT ALERT 3D (BioMérieux, Durham, N.C.) (118). Descriptions of luciferase

reporter mycobacteriophages (125) and molecular beacons (86) await further review of broader testing at this time.

Based on the current CLSI guidelines (28), a rapid susceptibility testing method should be used in conjunction with rapid methods of primary culture and identification to allow the earliest possible detection of resistance. The recommended rapid broth methods for susceptibility testing of *M. tuberculosis* are commercial systems that have been cleared by the FDA. At present, three such FDA-approved systems are available, i.e., the BACTEC 460TB, the ESP Culture System II, and, more recently, BACTEC MGIT 960. Most importantly, any such rapid method utilized should have been previously demonstrated to generate results that correlate with those obtained with the standardized agar proportion method. Optimally, susceptibility results for *M. tuberculosis* should be reported within 28 days of receipt of the specimen in the laboratory (13, 152). There are no commercially available broth systems cleared by the FDA for testing nontuberculous mycobacteria.

Source of Inoculum for Susceptibility Testing

The source of the inoculum for a susceptibility test may be either a smear-positive specimen (direct method) or growth from a primary culture or subculture (indirect method). The direct method is used when antimicrobial resistance is known or suspected. However, the indirect method is considered the standard method for inoculum preparation, and results of the direct method are usually confirmed by subsequent testing by the indirect method. With both the direct and the indirect methods, the inoculum must be a pure culture and careful attention must be given to avoid over- or underinoculation.

For the direct method, the inoculum is either a digested, decontaminated clinical specimen or an untreated, normally sterile body fluid, in which acid-fast bacilli are seen in stained smears. To ensure adequate but not excessive growth in the direct susceptibility test on solid medium, specimens are diluted according to the number of organisms observed in the stained smear of the clinical specimen. A typical dilution scheme is shown in Table 4. Theoretically, this type of inoculum is more representative of the population of the tubercle bacilli in a particular lesion in the host. It is prudent to include an undiluted inoculum if the smear-positive specimen is from a patient who is receiving antimicrobial therapy, because a significant proportion of the bacilli seen on the smear may be nonviable. The direct susceptibility test has two major advantages. First, results can be reported within 3 weeks from the time of specimen receipt in the laboratory for a majority of smear-positive specimens. Second, the proportion of resistant bacteria better represents the patient's bacterial population. Use of the direct method may be warranted in situations where there is a high prevalence of drug resistance (49). However, cost can become a critical factor when there is a high incidence of smear-positive specimens containing nontuberculous mycobacteria. Also, it is not possible to accurately calibrate the inoculum, which may result in insufficient or excessive growth on drug-free quadrants. In addition, if contaminants grow, results are not interpretable. The rate of failure for direct susceptibility testing can reach 15% or more, necessitating retesting by the indirect method. The direct method is not recommended for routine use with the BACTEC 460TB or any of the newer, nonradiometric, liquid-medium-based procedures at this time.

TABLE 4 Guidelines for selection of the dilution of a specimen concentrate prior to inoculation of 7H10 medium for susceptibility testing using the direct method

Dilutions to test[a]	No. of acid-fast bacilli observed with:	
	Carbol fuchsin stain	Fluorochrome stain
Undiluted, 10^{-2}	0	<25
10^{-1}, 10^{-2}	1–10	25–250
10^{-2}, 10^{-3}	>10	>250

[a] Dilutions of concentrated specimen are prepared based on the number of bacilli observed in the initial acid-fast smear. Sterile distilled water is used to prepare the dilutions; the carbol fuchsin stain is examined with the oil immersion objective (1,000×), and the fluorochrome stain is examined with the high-dry objective (450×). If the patient is receiving therapy, not all bacilli observed in the smear may be viable; therefore, the undiluted specimen should be tested as well as the appropriate dilution based on the microscopic criteria given in this table.

For the indirect method, the source of the inoculum is a pure subculture, usually from the primary isolation media. Careful attention should be paid to the selection of colony types so that the final inoculum is representative of all types present to ensure that there is a balance of potentially resistant and susceptible bacilli. The source of inoculum for the liquid medium-based susceptibility testing can be growth on a solid medium or in broth. When using turbid growth in a liquid medium (such as Middlebrook 7H9 broth, BACTEC 12B, MGIT, MB/BacT, or VersaTREK), it is important to do a purity check because a mixed culture (tubercle bacilli and nontuberculous organisms) can give rise to apparent resistance.

Media Used for Susceptibility Testing

Although a variety of media have been used for drug susceptibility testing of slowly growing mycobacteria, including Middlebrook 7H10 or 7H11 agar and Löwenstein-Jensen media, there is considerable variability in the results. Egg-based media are unsuitable for susceptibility testing, not only because of uncertainty about the potency of the drugs after inspissation but also because phospholipids, proteins, and certain amino acids present in the medium affect some drugs. In an effort to provide uniformity in the testing of mycobacteria, CDC (85) and CLSI (28) recommend that Middlebrook 7H10 agar supplemented with oleic acid-albumin-dextrose-catalase (OADC) be used as the standard medium for susceptibility testing of slowly growing mycobacteria by the agar proportion method. A majority of *M. tuberculosis* clinical isolates grow on this medium, and under a dissecting microscope, the transparency of 7H10 agar facilitates the recognition of mixed mycobacterial species or the presence of contaminants. Occasionally, there is insufficient growth of drug-resistant strains of *M. tuberculosis* on 7H10 medium for the test to be valid. With these isolates, 7H11 medium may be substituted for 7H10 agar, but it is necessary to use higher concentrations of some drugs, as shown in Table 3 (59, 75). At present there is no standard formulation for Middlebrook 7H10 agar or OADC (such as there is for Mueller-Hinton medium), and it has been demonstrated that quality control of the medium, especially the OADC supplement, is critical (55).

The medium used for studies with mycobacteria in the BACTEC 460TB instrument is an enriched Middlebrook 7H9 broth containing 4 μCi of [^{14}C] palmitic acid per vial, which is referred to as 7H12 or BACTEC 12B medium.

Drugs Used for Susceptibility Testing

Antimicrobial agents for susceptibility testing (reference powders) can be obtained directly from the manufacturer or from commercial sources. In the United States, most antimicrobial agents are also available from U.S. Pharmacopoeial

Convention, Inc., Reference Standards Order Department, 12601 Twinbrook Parkway, Rockville, MD 20852. The reference powder should be accompanied by information about its assay potency (in micrograms per milligram), expiration date, and lot number, as well as the stability and solubility of the agent. Preparations formulated for therapeutic use in humans or animals should not be used. Unopened vials of powders should be stored as specified by the manufacturer, and opened containers should be stored in a desiccator at the recommended temperature. Stock solutions of most agents at 1,000 μg/ml or greater remain stable for at least 6 months at –20°C and for 1 year at –70 to –80°C. Directions provided by the drug manufacturer should be followed in addition to these general recommendations. Paper disks impregnated with standardized amounts of the primary and secondary drugs are available from commercial sources for use in the disk elution modification of the proportion method. Use of these disks obviates errors in weighing and dilution, as well as errors in labeling, because the disks are coded with the drug name and concentration. This technique provides results equivalent to those obtained with solutions prepared from reference powders. Quality control (QC) testing should be performed with each new batch of antimicrobial agent.

Agar Proportion Method

The agar proportion method for susceptibility testing of slowly growing mycobacteria was developed in the early 1960s by G. Canetti (19, 21). The method was modified and standardized in the United States and has been considered the standard method of drug testing for *M. tuberculosis* complex to all drugs except PZA in the United States and many European countries for many years. The preferred medium for the proportion test is Middlebrook 7H10 agar. Drugs can either be prepared from reference powders (agar diffusion) or be added as drug-impregnated disks (disk elution) (56). With regard to testing PZA, the BACTEC 460TB is considered the reference method (28).

Inoculum and Incubation

The inoculum can be prepared by the direct or indirect method. When the direct method is used, cultures should be examined weekly for 3 weeks, and even though mature colonies may appear on control media in less than 3 weeks, a report of "susceptible" should not be submitted until week 3. Resistant strains of *M. tuberculosis* complex may be slow to produce visible growth because of metabolic differences compared with susceptible wild-type isolates. If cultures are incubated beyond 3 weeks, degradation of the antimicrobial compound may permit the appearance of colonies of organisms that were susceptible to the initial concentration of the drug. Because the controls are inoculated with a processed specimen, isolated colonies should also be examined for colony morphology and pigmentation differences, as well as for the presence of mixed species of mycobacteria or contaminants. Small colonies of rapid growers, as well as the rough, dry colonies of some MAC strains, are similar in appearance to *M. tuberculosis* colonies on 7H10 agar. Also, rapidly growing mycobacteria may be slow to develop on primary isolation media and rapidly growing mycobacteria will appear as MDR when tested against primary antituberculosis agents. For a test to be valid, the control must show good growth (at least 50 to 150 colonies). Susceptibility testing results must never be reported without a preliminary identification.

When using the indirect method, a sufficient number of colonies must be selected to make a suspension that is equivalent to a 1.0 McFarland standard or, if there is insufficient growth, a sufficient number should be subcultured into Dubos Tween-albumin broth and incubated at 35 to 37°C until the turbidity matches a 1.0 McFarland standard. The suspension should not contain clumps of organisms. The actual number of CFU per milliliter is likely to vary from suspension to suspension, so that two dilutions (usually 10^{-2} and 10^{-4}) of the suspension must be used to inoculate two sets of media. If the source of the inoculum is old or if there is scant growth, 10^{-1} and 10^{-3} dilutions should be used or the isolate should be subcultured in broth. QC strains should be tested at dilutions of 10^{-2} and 10^{-4}. Quadrant plates are commonly used for the agar proportion method, and 0.1 ml (about 3 drops from a Pasteur pipette) of each dilution of the inoculum is placed into each quadrant, using 1 dilution per set of plates. The plates are allowed to dry thoroughly after inoculation, placed in individual CO_2-permeable polyethylene bags (clear sandwich bags), and incubated at 35 to 37°C in 5 to 10% CO_2 in air. The plates must be protected from light during storage and incubation to prevent the formation of formaldehyde from the medium ingredients. To check strains for purity, blood agar and 7H10 agar plates are inoculated with 1 or 2 drops of the suspension and streaked for isolation.

Reading, Interpreting, and Reporting Results

Purity control plates are examined for contamination after 1 to 2 days; the 7H10 plate should be examined throughout the incubation period, and the blood agar plate should be examined for 1 week. Once the "no-drug" control quadrant of the test isolate or control strain shows at least 50 to 150 colonies, the plates can be read and interpreted. If there are fewer than 50 colonies in the no-drug quadrant by 3 weeks, the test is repeated. Use of the designations 1+ to 4+ to report the amount of growth is discouraged. This grading scheme may be helpful, however, for in-house evaluation of appropriate inoculum size. The reference agar proportion method employs a percentage calculation to detect resistance or susceptibility. The number of colonies observed in each quadrant is reported as a percentage of the number of colonies in the no-drug control quadrant. If the percentage of colonies exceeds 1%, the isolate should be reported as resistant to that drug at the concentration tested. The percentage of resistant colonies can be used as an indicator of therapeutic efficacy and should immediately be reported to the physician, along with the drug concentration and test method.

Microcolonies may be observed when using the agar proportion method, especially when testing against EMB. Several possibilities may account for the presence of microcolonies: growth of susceptible organisms in the presence of degraded drug, true resistance, or partial resistance. One study reported that most strains that had microcolonies in the EMB quadrant had EMB-susceptible results when tested with the BACTEC 460 method (J. Ridderhof, I. George, and W. Gross, *Abstr. Intersci. Conf. Antimicrob. Agents Chemother., 1999*, abstr. 865, 1999). The significance of microcolonies is unknown, and each laboratory should determine how to best report these findings, based on its own in-house experience.

Quality Control

M. tuberculosis H37Rv (ATCC 27294), which is susceptible to all primary and secondary antituberculosis drugs, is recommended for QC. Strains of *M. tuberculosis* that are resistant to INH, RMP, and/or other drugs are available from the American Type Culture Collection; however, these strains are resistant to high concentrations of the respective drugs and are not ideal for QC testing. Ideally, a strain of *M. tuberculosis* that shows resistance at or near the cutoff values of a particular drug

should be used for QC. A number of strains used in the Centers for Disease Control and Prevention's Model Performance Evaluation Program offer these characteristics. Also, for safety considerations, it is not advisable to use a single *M. tuberculosis* strain that is resistant to more than two drugs (28). Aliquots of suspensions of QC strains of *M. tuberculosis,* adjusted to match a 1.0 McFarland standard in 7H9 broth, can be stored at −70°C for up to 6 months. QC testing should be performed with each new batch of medium or antimicrobial agent, and media should be checked for sterility and shown to support adequate growth. Guthertz et al. (55) demonstrated the importance of controlling for all components of 7H10 agar including lots of Middlebrook 7H10 agar, glycerol, and especially OADC. The issue of which QC results should be available before releasing patient test results is discussed below.

BACTEC 460TB Method

Although nonradiometric systems are cleared by the FDA for susceptibility testing of *M. tuberculosis* to primary drugs, the BACTEC 460TB method continues to be important in drug testing. Mycobacteria easily grow in the BACTEC 12B medium containing [^{14}C]palmitic acid. This compound is metabolized, resulting in the production of $^{14}CO_2$; therefore, growth or inhibition of growth in BACTEC 12B medium is measured as changes in the growth-dependent production of CO_2. The instrument quantitatively detects the amount of $^{14}CO_2$ released, expressed in terms of a growth index (GI), and then automatically replaces the headspace with 5 to 10% unlabeled CO_2 in air, thereby maintaining the recommended CO_2 atmosphere. The rate and amount of $^{14}CO_2$ produced are directly proportional to the rate and amount of growth.

The BACTEC 460TB system can be used to test all primary drugs (RMP, INH, PZA, and EMB), as well as the secondary drugs including the newer quinolones and rifamycins (113). The rapid availability of results with the BACTEC 460TB procedure may take precedence over cost considerations. Irrespective of its rapidity, it is prudent to consider the limitations of the BACTEC 460TB method. In some clinical situations, the method might be best considered a screening test, because it does not allow an estimate of the percentage of resistant bacilli and is vulnerable to major errors (false susceptibility or resistance) due to the use of mixed populations of mycobacterial species. Indeed, when an MDR isolate of *M. tuberculosis* is detected for the first time by the BACTEC 460TB method, the identity of the isolate should be confirmed and the presence of a contaminant or mixed culture should be ruled out before secondary agents are tested. While the importance of promptly reporting an MDR isolate of *M. tuberculosis* cannot be overstated, the consequences of a false report of multiple resistance must be recognized as well (108). Clinician concerns about discrepancies between susceptibility test results and clinical response or status must be communicated back to the laboratory as part of an effective quality assurance program.

Three of the primary drugs (INH, RMP, and EMB) and SM are available as lyophilized powders in the BACTEC SIRE kit, designed specifically for use with 12B medium and the BACTEC 460TB instrument. Alternatively, stock solutions of these agents can be prepared from reference powders. Lyophilized vials of PZA are also available for the pH 6.0 modified BACTEC PZA test medium. The secondary drugs must be prepared from reference powders. Critical concentrations for most of these drugs have been established, and the results of the radiometric and proportion methods correlate well (113).

Inoculum and Incubation

The source of inoculum for susceptibility testing should be fresh growth of actively growing bacilli. If growth from a primary BACTEC 12B vial is used for the inoculum, the daily GI reading should be at least 500 or, even better, between 800 and 900. The higher the GI, the shorter the period until the control vial reaches >30 and the earlier the test results can be interpreted. If the primary vial is past the GI peak or if the vial was kept at room temperature for more than 2 days, a fresh 12B vial should be inoculated. Other types of broth culture (Dubos, Middlebrook 7H9, Septichek AFB, VersaTREK Myco, MGIT, or MB/BacT medium) can be used as a source of inoculum; however, it is necessary to ensure that the broth culture is not contaminated or not mixed with other mycobacterial species. A variety of procedures may be used for mycobacterial purity checks (28). After the inoculum is thoroughly mixed to break up clumps, the turbidity should be adjusted to match a 0.5 McFarland standard rather than a 1.0 McFarland standard. This inoculum is then used to inoculate the drug-containing vials. A control vial (without drugs) is inoculated with a 1:100 dilution of the inoculum. All dilutions should be prepared with diluting fluid or distilled water, but not a broth or other substrate-containing medium. Additionally an undiluted control may be used to monitor the growth kinetics of the inoculum and also can serve as a source of inoculum if a test must be repeated.

Also, fresh growth on any solid media may serve as an inoculum source. It is important to harvest several (5 to 10) colonies in order to ensure a representative sample of growth (harvesting from only 2 or 3 single colonies is inadequate), and it is very important to thoroughly homogenize the inoculum in a tube containing 3 to 5 ml of diluting fluid and glass beads. After being vortexed for 1 to 2 min, the tube is left to stand undisturbed for 30 min to allow larger particles to settle, and then the top half of the supernatant is transferred into a new tube. The turbidity is adjusted to match a 0.5 McFarland standard, and this suspension is used to inoculate the drug-containing vials. Blood agar and 7H10 plates are inoculated with a few drops of the inoculum suspension to check for purity.

As safety precautions, disposable tuberculin syringes with a permanently attached needle should be used; rubber gloves, N95 mask, and a gown must be worn; and all steps involving the preparation of inocula and inoculation of vials should be performed in a class II or III biological safety cabinet.

Prior to inoculation, BACTEC 12B vials must be prerun in the BACTEC 460TB instrument to establish a gas phase of 5% CO_2 in air in the headspace of the vial; any vial with an initial GI of 20 must be rejected. Each prerun vial is inoculated with 0.1 ml of the adjusted suspension of the test or QC isolate, and the vials are incubated at 37 ± 1°C (the temperature of incubation is critical) in the dark.

Reading, Interpreting, and Reporting Results

Vials are read on the BACTEC 460TB instrument at intervals of 24 ± 1 h for a minimum of 4 days and until the control vial reaches a GI of 30. For laboratories with limited weekend coverage, an alternative schedule is to inoculate the vials on Friday and first read them on Monday (60). The Monday reading is disregarded, because it reflects 2 days of CO_2 production, and the vials are read daily for at least 3 days (5 days total). Batch testing is discouraged unless the susceptibility test can be started during the same week that the isolate is identified to species, i.e., identified as *M. tuberculosis* complex. In those situations, to avoid any delay, the isolate should be sent to a reference laboratory that works 7 days a week.

When the GI of the control is 30 after a minimum of 4 days, the difference in GI from one day to the next, designated the ΔGI, should be interpreted as follows:

$$\Delta GI \text{ of control} > \Delta GI \text{ of drug} = \text{susceptible}$$

$$\Delta GI \text{ of control} < \Delta GI \text{ of drug} = \text{resistant}$$

If the GI is 500 and >500 on the next reading, the isolate should be considered resistant to that drug regardless of the ΔGI (it is necessary to check that the GI of the control was not >30 in less than 3 days to exclude overinoculation). Continued incubation for a few days may allow one to resolve borderline results, but the incubation should not be extended beyond 3 days after the control vial is positive. Susceptibility test results should never be reported without identification of the isolate as *M. tuberculosis* complex or *M. tuberculosis,* especially when the isolate is resistant to a drug(s). Usually, BACTEC 460TB results are straightforward; however, if the ΔGIs are close (within 10%), a preliminary report should be issued and the test should be repeated. Secondary drugs should be tested if the isolate is resistant to RMP or any two primary drugs (28). Use of the term "borderline," as recommended by the BACTEC manufacturer, is not encouraged, because the definition of this term is unclear and may mislead the clinician into believing that a drug has no use in the treatment of tuberculosis. Susceptibility test results should be reported without delay. The report should include the test method, the name of the drug, the concentration tested, and the result (susceptible or resistant). It is not possible to report the percent resistance by using the BACTEC 460TB method. Drug resistance should be reported by telephone and/or fax to the requesting physician, the infection control program, and the local tuberculosis control program, and a follow-up hard-copy report should be submitted. It is prudent to confirm the receipt of a fax report in lieu of direct communication with the physician or public health official.

Quality Control

Reference strains with known susceptibility patterns should be tested with each new batch of drug and BACTEC 12B medium. In addition, QC tests should be performed at least once a week in laboratories that perform tests daily or weekly or when a patient isolate is tested if tests are performed less frequently. The H37Rv strain of *M. tuberculosis* (ATCC 27294), which is susceptible to all standard antituberculosis agents, is commonly used for QC. Mutants of H37Rv selected for in vitro resistance to single or multiple drugs are also available; however, these strains are resistant to very high concentrations and are not particularly suitable for QC purposes. In addition, reporting the test results for a patient isolate should not be delayed because of the pending test results for a resistant QC strain. Suspensions of QC strains may be prepared in BACTEC diluting solution and frozen at −70°C in 1-ml test samples for up to 6 months (138). A single tube is thawed for QC testing, and the same procedure as that for clinical isolates is used. At present, there is no published consensus on which QC results should be available before the test results on a patient isolate may be released. However, it seems reasonable that the QC test results for medium components be available and acceptable, as well as the results for the "susceptible" QC strain (e.g., ATCC 27294).

Testing PZA

Reliable testing of PZA by the radiometric method is different from testing by the BACTEC 460TB method for the other primary drugs (96), because PZA activity is best measured at pH 5.5 rather than pH 6.8, the usual pH of the growth medium. However, most strains of *M. tuberculosis* grow poorly at pH 5.5 and some fail to grow altogether. As a compromise between testing at the pH for optimum PZA activity and testing at the pH for optimum growth, Salfinger et al. (131) recommended that PZA be tested at pH 5.9 ± 0.1. In addition, to accurately and reliably test PZA, several other adjustments to the standard radiometric method are made. (i) To prepare the inoculum, each isolate must be subcultured in a fresh BACTEC 12B vial supplemented with BACTEC reconstituting fluid, which is an aqueous solution of polyoxyethylene stearate (POES) (139). The isolate is tested daily, and once the GI is 300 to 500, this culture is used for susceptibility testing. Alternatively, a 1:10 dilution of the inoculum (GI = 999 or 0.5 McFarland standard) used for testing the other primary drugs is used and the suspension is vigorously homogenized before use. (ii) Either the lyophilized PZA is reconstituted with POES or PZA reference powder is dissolved in POES to achieve a final concentration of 100 μg/ml, the critical concentration for the BACTEC method, which is equivalent to 25 to 50 μg/ml (used in the proportion method) (138). (iii) A special BACTEC 12B medium at pH 6.0 supplemented with POES is used to test PZA. (iv) The PZA-containing vial and the control vial are inoculated with the same suspension; i.e., the inoculum for the control vial should not be diluted. (v) The vials are tested daily until the control vial reaches a GI of 200; if the GI fails to reach 200 within 14 days, the test is uninterpretable. (vi) The isolate is considered susceptible to PZA if the GI of the PZA vial is <10% of the GI of the control vial. If the GIs of the test and control vials are very close, the result is considered borderline and the test must be repeated. (vii) In addition to the use of H37Rv (PZA susceptible) for QC testing, *M. bovis* BCG (PZA resistant) can be used as a control for PZA resistance. If an isolate tests resistant to PZA, especially if it is resistant to PZA alone, it should be identified to the species level, because *M. bovis* and *M. bovis* BCG are PZA resistant whereas almost all *M. tuberculosis* isolates are PZA susceptible. This is especially important if the laboratory identifies isolates only to the level of the *M. tuberculosis* complex. Some *M. tuberculosis* isolates grow poorly at pH 6.0 (130), and other isolates may be inhibited by the POES supplement (95).

VersaTREK

VersaTREK (fomerly called ESP Culture System II; Trek Diagnostic Systems, Inc.) is a fully automated, continuously monitoring system for growth and detection of mycobacteria (176), which allows testing of the susceptibility of *M. tuberculosis* to major primary drugs (i.e., INH, RMP, EMB, and PZA) as well as the secondary agent SM. The VersaTREK technology is based on detection of pressure changes (gas production or gas consumption due to microbial growth) within the headspace above the broth culture medium in a sealed bottle. The culture medium consists of a Middlebrook 7H9 broth, which has been enriched with glycerol and Casitone, and contains a cellulose sponge. Before the medium is inoculated with a test strain, growth and antibiotic supplements must be added.

Inoculum and Incubation

VersaTREK Myco antibiotics are supplied as lyophilate by the manufacturer. Preparation of the bottles requires the addition of 1.0 ml of antibiotic solution. The final concentrations in the Myco bottles are as follows: INH, 0.1 μg/ml; RMP, 1.0 μg/ml; EMB, 5.0 μg/ml; and SM, 8.0 μg/ml. INH

and EMB can be tested at higher concentrations (0.4 and 8.0 μg/ml, respectively). For the inoculation of organisms into the VersaTREK Myco bottles, 0.5 ml of a 1:10 dilution from a McFarland standard of 1 must be added to the drug-containing bottles. The inoculum can be prepared from either solid (e.g., Löwenstein-Jensen) or liquid (e.g., VersaTREK Myco) medium. Cultures are kept in the instrument at 35°C, and growth is automatically monitored.

Reading, Interpreting, and Reporting Results

At the end of the specified incubation period, as determined by the drug-free control bottle for each isolate that is tested, the isolate is manually determined to be susceptible or resistant to a drug. If the control bottle (without drugs) signals positive, the time to detection is calculated. Drug-containing bottles are monitored for another 3 days after the control bottle has turned positive. Results are interpreted according to the following rules: a strain is considered resistant if the time of detection is the same time as or up to 3 days after that for the control bottle; a strain is considered susceptible if no growth occurs in the drug-containing bottle or if the time of detection is more than 3 days after that for the control bottle. The few studies demonstrate good agreement of the VersaTREK (formerly called ESP Culture System II) with the BACTEC 460TB and the agar proportion method and detection times, which are very comparable to those obtained by the radiometric method (10, 126, 176).

Quality Control

Each new VersaTREK Myco Susceptibility Kit and/or lots of medium and growth supplement should be tested with strains of *M. tuberculosis* appropriate for QC. A pansusceptible strain of *M. tuberculosis* should be included with each testing run (28).

MGIT (Manual and Automated MGIT 960) Systems

In 1995, the MGIT was introduced for the growth and detection of mycobacteria from clinical specimens (57, 115). Each MGIT tube contains modified Middlebrook 7H9 broth, and in the bottom is a fluorescence-quenching-based oxygen sensor (silicon rubber impregnated with a ruthenium pentahydrate). The fluorescent compound is sensitive to the presence of oxygen dissolved in the medium. The fluorescence of the indicator is quenched in the presence of oxygen, but fluorescence increases as soon as actively respiring microorganisms utilize the dissolved oxygen. Fluorescence is detected using a 365-nm UV transilluminator. The MGIT system is available as a manual system or a fully automated, continuously monitoring system called BACTEC MGIT 960. Apart from using nonradiometric medium, it frees the technician from using syringes. In addition to detecting and monitoring growth, the system can be used for testing of susceptibility to INH, RMP, EMB, PZA, and SM. Fluorescence of the drug-containing tubes is compared with that of the drug-free growth control tube. When using the instrument, the ratio of relative growth between the drug-containing tube and the growth control tube is determined by software algorithms. If the relative growth in the drug tube equals or exceeds that in the growth control tube, the organism is considered resistant to the drug; if the relative growth is less than that in the growth control tube, the organism is considered susceptible.

Five drugs (INH, RMP, EMB, PZA, and SM) are designed specifically for use with the BACTEC MGIT 960 instrument. Stock solutions of these drugs also can be prepared from reference powders. Lyophilized vials of PZA are available for testing susceptibility based on an adapted protocol utilizing BACTEC MGIT 960 PZA medium at pH 5.9. Similar to the BACTEC 460TB procedure, the BACTEC MGIT 960 employs a two-tier test, with the first tier comprising the four SIRE drugs being tested at drug concentrations equivalent to the critical concentrations of the agar proportion method (i.e., SM, 1.0 μg/ml; INH, 0.1 μg/ml; RMP, 1.0 μg/ml; and EMB, 5.0 μg/ml). If there is resistance to INH, EMB, and/or SM, a second test that involves a higher concentration of the drug(s) (SM, 4.0 μg/ml; INH, 0.4 μg/ml; and EMB, 7.5 μg/ml) may be used. There appears to be good correlation between the MGIT and the BACTEC 460TB system or the agar proportion method (8, 114, 127). The time to results is comparable to that for the radiometric technique. A multicenter report suggests that an overall correlation between the BACTEC 460 and the agar proportion method could be achieved with amikacin, kanamycin, and ofloxacin; however, inconsistent results in both methods were obtained with cycloserine (113). A recent report validated the use of BACTEC 460 and MGIT 960 for testing the susceptibility of *M. tuberculosis,* including multidrug-resistant strains, to levofloxacin (132).

Inoculum and Incubation

Inoculum (0.5 ml) for drug susceptibility testing may be prepared from either liquid or solid media. To ensure viability of the colonies, accuracy of results, and reproducibility of dilutions, strict adherence to the guidelines of the manufacturer is imperative. Prior to inoculating the AST tubes, 0.8 ml of the BACTEC MGIT 960 drug susceptibility testing supplement (OADC) and 100 μl of the drug solution (INH, RMP, or EMB) are added to the MGIT. With a liquid source of inoculum, the MGIT must nominally be used no sooner than the day following the instrument's positivity (day 1) and no later than 5 days following the day of the instrument's positivity. If it is older, the isolate must first be subcultured again. With solid media, the isolate can be used no longer than 14 days after the first appearance of colonies on the plate or slant.

If growth from a primary MGIT is taken for susceptibility testing, the suspension can immediately be used for testing. Appropriate dilutions (1:5) have been made for day 3 through 5 positive MGIT 960 tubes prior to inoculation of the drug set to avoid overinoculation. When working with a solid medium source, inoculum preparation includes vortexing with glass beads and two specified settling times to ensure homogeneity of the final cell suspension. The suspension has to be adjusted to a McFarland standard of 0.5 equivalent and then diluted 1:5 before inoculation of the MGIT 960 AST set. For the growth control tube, 100 μl of a positive MGIT 960 broth is pipetted into 10 ml of sterile saline to prepare a 1:100 dilution of the growth suspension. From this suspension, 0.5 ml is inoculated into a fresh MGIT without drug. Cultures are kept at 35°C, and growth is automatically monitored.

Reading, Interpreting, and Reporting Results

Drug results are listed automatically on the data report form (S, susceptible; R, resistant; X, invalid result), together with growth units and time to result. As with all drug susceptibility testing, the report should indicate the method used and the concentration of drug tested. Interpretive comments, such as those suggested by the CLSI (28), should be considered.

Quality Control

Reference strains with known susceptibility patterns should be tested with each new batch of drug and MGIT medium and included with each test run (28).

Testing PZA

PZA can reliably be tested by using the novel BACTEC MGIT PZA tube, which contains 7 ml of MGIT broth, modified with a reduced pH (114). Inocula can be prepared either from MGIT medium or from solid medium, such as Löwenstein-Jensen medium. Prior to inoculating the PZA set tubes, 0.8 ml of MGIT PZA supplement and 100 μl of PZA solution (100 μg/ml) are added. MGIT cultures are used no sooner than the day following the instrument's positivity (day 1) and no later than 5 days following the day of positivity. On days 1 and 2 following positivity, the inoculum is used undiluted, while on days 3 to 5, suspensions are diluted 1:5 with sterile saline. Then 0.5 ml is inoculated into the MGIT PZA tubes. The growth control tube is inoculated with 0.5 ml of a 1:10 dilution of the respective suspension. Cultures grown on Löwenstein-Jensen medium are used no later than 14 days after the first colonies appear on the slant. To ensure homogeneity, a suspension adjusted to a McFarland standard of 0.5 equivalent is prepared by using glass beads and then diluted 1:5 prior to inoculation of the MGIT PZA set. All inoculated PZA sets must be loaded into the instrument within 8 h of inoculation. Again, using predefined algorithms, readings are automatically interpreted by the instrument. In contrast to BACTEC 460TB, the PZA "borderline" category no longer exists with this method.

MB/BacT ALERT 3D

The MB/BacT ALERT 3D System (bioMerieux), a fully automated, nonradiometric system, can also be used for mycobacterial culture susceptibility testing of *M. tuberculosis* outside the United States (118). With this technology, carbon dioxide is released into the medium by actively metabolizing microorganisms and is detected by a gas-permeable sensor containing an indicator embedded at the bottom of the culture bottles. Color changes are monitored by a reflectometric detection unit, and data are compiled by an internal database management system.

Inoculum and Incubation

Following the instructions of the manufacturer, the MB/BacT process bottles containing a modified Middlebrook 7H9 medium (with casein, bovine serum albumin, and catalase) are supplemented with the following final drug concentrations: INH, 1 μg/ml; RMP, 1 μg/ml; SM, 1 μg/ml; and EMB, 2 μg/ml. Subsequently, bottles are inoculated with 0.5 ml of a suspension of *M. tuberculosis* adjusted to a McFarland standard of 2. Two bottles without drugs serve as growth controls, one inoculated with 0.5 ml of the cell suspension adjusted to a McFarland standard of 2 (control 1) and one with 0.5 ml of the cell suspension (McFarland standard of 2) diluted 1:100 (control 2).

Reading, Interpreting, and Reporting Results

At the time that the MB/BacT System recognizes growth in the control 2 bottle, testing is terminated and the growth status of the bottles containing the drugs is determined. An isolate is considered resistant if the drug-containing bottle is positive before or on the same day as the corresponding diluted control bottle (control 2). Susceptibility is defined as the drug-containing bottle remaining negative or becoming positive later than the control 2 bottle. On the basis of preliminary studies, there is excellent agreement of the MB/BacT results with susceptibility data generated by the agar proportion method and BACTEC 460TB (18, 40, 118), in particular for INH and RMP. The manufacturer increased the EMB concentration to 2.5 μg/ml in order to improve the correlation with the results of the agar proportion method (138). The time necessary to determine the susceptibility patterns of *M. tuberculosis* is within what has been observed for the BACTEC 460TB System.

Quality Control

Reference strains with known susceptibility patterns should be tested with each new batch of drug and MB/BacT medium, and with each test run (28).

M. AVIUM COMPLEX

Clinical Significance

MAC is commonly isolated from a variety of clinical specimens and in many clinical laboratories is the most frequently encountered *Mycobacterium* species. Isolation of MAC from blood or other sterile sites is almost always clinically significant and is especially important in HIV-infected patients with profound CD4 T-cell lymphocytopenia, i.e., ≤75 CD4 T lymphocytes per μl. Although disseminated MAC infection remains a complication in HIV-infected patients, the incidence has dramatically decreased due to the introduction of effective prophylaxis and immune system restoration associated with the use of highly active antiretroviral therapy. Also, disseminated MAC disease still occurs in patients who fail to respond to antiretroviral treatment because of drug resistance, drug intolerance, or lack of compliance. In many of these patients, MAC infection represents a relapse of MAC disease rather than a new infection. MAC is also a cause of chronic pulmonary lung disease and is the leading cause of lung disease due to nontuberculous mycobacteria in the United States. The incidence of MAC lung disease has been steadily increasing in patients with underlying bronchiectasis, chronic obstructive pulmonary disease, or cystic fibrosis. Nevertheless, the detection of MAC in respiratory or gastrointestinal tract specimens can be difficult to interpret, especially in patients who are not clearly at risk for MAC disease. Clinical, histopathologic, and/or radiologic evidence of MAC disease, repeated isolation of MAC, increasing numbers of bacilli in sequential specimens, or the absence of other identifiable causes of signs and symptoms are important factors to consider in assessing the clinical significance of MAC isolates.

Drug Resistance

Most MAC isolates are intrinsically resistant to INH and PZA, and these antimicrobial agents play no role in the treatment of MAC infection (4). The intrinsic resistance to INH most likely is due to a lack of a complete antimicrobial effect (7, 91). Cell-free in vitro studies have shown that certain drug targets (e.g., ribosomes, ribosomal subunits, and RNA polymerase) bind the corresponding drugs (e.g., macrolides, aminoglycosides, and rifamycins) and that the target functions are inhibited, yet many of these compounds are ineffective against whole cells. MAC isolates have variable MIC values to aminoglycosides (amikacin, kanamycin, and SM) and rifamycins (RMP and rifabutin), and one or both of these agents are often used in combination with other drugs for treatment of MAC disease.

Correlation between in vitro MAC susceptibility test results and clinical response has been demonstrated in a controlled clinical trial only with azithromycin and clarithromycin (23). Although wild-type MAC isolates are uniformly susceptible to macrolides, resistance develops quickly with macrolide monotherapy and may eventually develop with combination therapy. An analysis of macrolide-resistant MAC isolates

showed that over 95% of clinically significant macrolide resistance in MAC is a consequence of mutations in the V-domain of the 23S rRNA gene (92, 106). Therefore, clinically significant macrolide resistance can be defined as a clarithromycin MIC of ≥32 μg/ml or an azithromycin MIC of ≥256 μg/ml (75, 106). Predicting in vivo susceptibility is always less reliable; however, a positive clinical and microbiological response is expected if the MIC values of clarithromycin and azithromycin are ≤4 and ≤32 μg/ml, respectively. Clarithromycin and azithromycin are approved by the FDA for the treatment of MAC disease, but either agent must be combined with at least one and often two additional agents, commonly EMB and RMP (24). Clarithromycin, azithromycin, and rifabutin are approved as prophylactic agents for disseminated MAC infection in HIV-infected patients; however, their mechanism of action as prophylactic agents is unclear.

When To Perform Susceptibility Tests

Universally accepted guidelines regarding indications for susceptibility testing of MAC do not exist. However, testing should be considered for clinically significant isolates from patients on prior macrolide therapy, from patients who become bacteremic while on macrolide preventive therapy, and from patients who relapse while on macrolide therapy. Testing initial isolates from blood or tissue of patients with disseminated disease or respiratory samples from patients with pulmonary disease to establish baseline values also may be valuable. Susceptibility testing should be repeated after 3 months for patients with disseminated disease and after 6 months for patients with chronic pulmonary disease if the patient does not improve clinically and remains culture positive.

Test Methods

Broth microdilution and macrodilution and agar dilution methods that measure, in a quantitative manner, the in vitro activity of antimycobacterial agents against MAC have been described. In Europe, an agar dilution method with Mueller-Hinton agar supplemented with OADC is used; however, in the United States, there is consensus that both radiometric broth macrodilution and broth microdilution methods are accurate and reliable and that agar-based methods should not be used. The current recommendations of the CLSI Subcommittee on Antimycobacterial Susceptibility Testing indicate that susceptibility testing for MAC should be restricted to clarithromycin or azithromycin (28). Clarithromycin results predict those of azithromycin; therefore, testing both agents is not necessary. BACTEC 12B medium can be used for macrodilution testing, and either Middlebrook 7H9 broth with casein or Mueller-Hinton broth supplemented with OADC or OAD is acceptable for microdilution tests. The concentration ranges of clarithromycin and azithromycin to test are shown in Table 5.

The issue of pH remains somewhat controversial. One view is that testing at pH 7.2 to 7.4 is potentially misleading because MAC isolates tend to grow more slowly, and some fail to grow, at this pH. Thus, MIC values measured in this pH range may reflect synergy between a suboptimal pH for growth and inhibitory drug activity (72; S. Beaty, S. Siddiqi, and M. Gnacek, *Abstr. 92nd Gen. Meet. Am. Soc. Microbiol. 1992,* abstr. U-102, 1992). Also, BACTEC 12B medium is unstable at pH 7.2 to 7.4 because of the poor buffering capacity of the medium at pH values above 7.2. In addition, the intracellular environment of MAC-infected macrophages is pH 6.0 to 6.5, which suggests that macrolides should be tested under the mildly acidic, more clinically relevant conditions. Although MAC can block phagolysosome fusion, the intracellular pH remains in the range of pH 6.0 to 6.5 (110, 122, 146). Blocking phagolysosome fusion prevents a drop to pH 5.0 or lower but does not result in an increase in the intracellular pH to 7.2 to 7.4. The CLSI does not make a specific recommendation but states that pH 6.8 and 7.4 are both acceptable, providing that the recommended interpretive criteria are followed (Table 5). Data from a multicenter study (175) showed that testing in broth at either pH is satisfactory, supporting the CLSI view.

Inoculum and Incubation

Currently there are no specific recommendations regarding inoculum preparation. The options include the use of "seed" BACTEC vials (subcultures of fresh growth) for the radiometric method or the preparation of a suspension of mycobacteria taken directly from an agar plate or a broth culture for microdilution. However, if possible, only transparent colony types should be tested because this variant generally is more virulent and more resistant to antimicrobial agents than the opaque variant. The inoculum should be between 10^4 and 10^5 CFU/ml for the radiometric broth macrodilution test and approximately 5×10^5 CFU/ml for the broth microdilution test. Tween 80 or other surfactants should not be used to disperse clumps of bacilli because of the potential synergistic effect of the surfactant activity of Tween 80 and antimicrobial agents.

Incubation is at 37°C. For the radiometric test, incubation should not extend beyond 10 days, and the "no-drug" control should not exceed a GI of 999 in less than 4 days. For broth microdilution, trays are incubated for 7 to14 days. The endpoint for the radiometric test is defined by the GI value for the inoculum diluted 1:100, whereas the endpoint for the broth microdilution assay is visible turbidity.

TABLE 5 Susceptibility testing of clarithromycin and azithromycin against *M. avium* complex[a]

Antimicrobial agent	Concn range (μg/ml) for:		pH	Interpretive criteria			QC (*M. avium* ATCC 700898)	
	BACTEC	Microtiter		Susceptible (μg/ml)	Intermediate (μg/ml)	Resistant (μg/ml)	Concn range (μg/ml)	Expected endpoints (μg/ml)
Clarithromycin	2–64 or 4, 16, and 64	1–64	6.8	≤16	32	≥64	1, 2, and 4	1–4
Clarithromycin	2–64 or 4, 8, and 32	2–64	7.3–7.4	≤4	8–16	≥32	0.5, 1, and 2	0.5–2
Azithromycin	16–512 or 32, 128, and 512	NR[b]	6.8	≤128	256	≥512	8, 16, and 32	8–32

[a] Data from reference 28.
[b] NR, no recommendation.

Reporting Results

When testing clarithromycin or azithromycin, both the MIC value and an interpretation, based on the pH of the medium and the breakpoints listed in Table 5, should be reported (28). Because untreated wild strains of MAC rarely, if ever, are intermediate or resistant to macrolides, the CLSI subcommittee recommends that laboratories confirm such results by repeat testing (28). A confirmed intermediate result may suggest that the patient has a mixed population of MAC organisms and should be monitored for emerging resistance. Testing agents other than the macrolides (e.g., amikacin, ciprofloxacin, or moxifloxacin) should be restricted to situations in which the clinician has extensive experience in the use of these agents in the treatment of MAC disease or for research purposes. Testing EMB or RMP should be discouraged because MIC values do not necessarily correlate with clinical response and, therefore, could be misleading. If drugs other than macrolides are tested, only the MIC values should be reported without an interpretation. However, it is important to recognize that disseminated MAC disease is principally an infection of macrophages in the blood, bone marrow, spleen, and other tissues. Thus, clinical effectiveness is likely to relate to both potent activity and the ability of drugs to accumulate in cells and tissues to levels above the MIC for the infecting microorganism.

OTHER SLOWLY GROWING MYCOBACTERIA

In the mid-1980s, Woods and Washington (177) reviewed the nontuberculous mycobacterioses and concluded that no clear recommendations could be made for treatment of these infections and that the need for susceptibility testing was difficult or impossible to assess. Now, many years later, the situation has not appreciably changed. Nevertheless, for certain species of nontuberculous mycobacteria, there has been an accumulation of information and experience that can be the basis for some recommendations (28). Susceptibility testing is often helpful with these less frequently encountered species because of the paucity of information about treatment options, and in vitro results provide some rational basis for guiding treatment (52). Furthermore, baseline information may be useful if there is a microbiologic relapse. However, the vagaries of testing and interpretation of results mandate that the testing be performed in an experienced laboratory and, to better ensure consistency of results, that follow-up testing be performed by the same laboratory.

M. kansasii

Isolates of *M. kansasii* from patients not previously treated with RMP are predictably susceptible to RMP, and patients have been successfully treated with RMP, INH, and EMB, with or without SM (4). However, resistance to RMP can develop during therapy, and a patient's history of RMP therapy may be unknown or unclear. Therefore, CLSI recommends that all initial isolates of *M. kansasii* be tested for susceptibility to rifampin only, by agar proportion, as described for *M. tuberculosis*, or a broth-based method that yields comparable results (28). Susceptibility testing should be repeated if cultures remain positive after 3 months of appropriate therapy.

Untreated wild strains of *M. kansasii* are susceptible to the critical concentrations of RMP and EMB used to test *M. tuberculosis* (discussed earlier in the chapter) but are resistant to the critical concentration of INH (0.2 μg/ml by agar proportion) and show variable susceptibility to the higher concentration (1.0 μg/ml). Despite these in vitro results, INH appears clinically active. Therefore, because in vitro results do not correlate with clinical outcome, testing susceptibility of *M. kansasii* to INH should not be done. Isolates of *M. kansasii* that are resistant to RMP (MIC ≥1 μg/ml) should be tested against the following secondary drugs: rifabutin, EMB, SM, clarithromycin, amikacin, ciprofloxacin (as the class representative for the older fluoroquinolones), trimethoprim-sulfamethoxazole or sulfamethoxazole, and moxifloxacin. Unfortunately, however, for most of these agents data regarding both concentrations to be tested and optimal testing conditions are limited. Tentative testing and interpretive guidelines for these agents are shown in Table 6 (28).

M. marinum

Routine susceptibility testing of *M. marinum* is not recommended and should be discouraged. Isolates are consistently susceptible to several clinically useful antimicrobial agents, including RMP, EMB, doxycycline (or minocycline), trimethoprim-sulfamethoxazole, and clarithromycin (28). Additionally, the risk of acquired mutational resistance to one or more of these agents is extremely low. Successful treatment may require surgical excision or debridement as well as antimicrobial therapy. However, if a patient fails to respond clinically after several months of appropriate therapy and remains culture positive, susceptibility testing of *M. marinum* should be considered. Agar proportion, agar disk elution, and broth microdilution have been used, but there are insufficient data to recommend any one method. Drugs suggested by CLSI to test are RMP, EMB, clarithromycin (the class agent for newer macrolides), amikacin, and sulfamethoxazole or trimethoprim-sulfamethoxazole (28).

M. haemophilum

M. haemophilum is an opportunistic pathogen in immunocompromised patients, and there is some correlation between susceptibility test results and clinical efficacy, although virtually all treatment regimens examined included combinations of agents (145). Wild-type isolates of *M. haemophilum* appear to be susceptible to quinolones, rifamycins, clarithromycin, and azithromycin and resistant to PZA and EMB; they are likely to be resistant to INH and SM (11, 145).

M. xenopi, M. szulgai, and *M. malmoense*

In vitro susceptibility test results for *M. xenopi, M. szulgai,* and *M. malmoense* may be helpful because of the paucity of

TABLE 6 Tentative breakpoint concentrations for antimicrobial agents that might be tested against *M. kansasii* and selected slowly growing mycobacteria other than *M. tuberculosis*[a]

Antimicrobial agent	Resistance breakpoint (μg/ml)
RMP	1–2
Rifabutin	0.5–2
EMB	5
INH[b]	5
SM	10
Clarithromycin	32
Amikacin	10
Ciprofloxacin	2
Trimethoprim-sulfamethoxazole	2/38

[a] Data from reference 28.
[b] Not recommended for testing *M. kansasii*.

information about the susceptibility patterns of these species. For example, susceptibility testing has been reported to be important to the management of infections caused by *M. xenopi* (153), but an earlier report indicated that the correlation between susceptibility test results and therapeutic response was inconsistent (177). Pulmonary infections by these three species have been successfully treated with INH, EMB, and RMP, and data from at least one report indicated that clarithromycin was effective in the treatment of *M. xenopi* infections (4). Candidate drugs for testing with these species are the same as for *M. kansasii* (Table 6). The test method should be either the BACTEC radiometric, broth microdilution, or agar proportion; however, there may be advantages to defining the in vitro susceptibility in terms of an MIC. Extrapolation of the *M. tuberculosis* "critical concentrations" to these species of mycobacteria may be misleading, because there is some evidence that infections caused by these species can be successfully treated with higher concentrations of INH, SM, and EMB than might be used for *M. tuberculosis* (4).

M. gordonae

Testing *M. gordonae* is inappropriate because isolates almost always represent contaminants; *M. gordonae* rarely causes actual disease (164). Before testing *M. gordonae* isolates, one should ask whether there is convincing evidence that the isolate is playing a role in the disease process. Until these questions are answered in the affirmative, susceptibility testing is inappropriate because the results could be misleading and the patient may be misdiagnosed and inappropriately treated.

The most prudent approach to the testing of slowly growing mycobacteria other than *M. tuberculosis* complex is to restrict it to reference laboratories with extensive experience working with these species. Interpretation of the susceptibility test results requires good communication between the laboratory and the clinician.

RAPIDLY GROWING MYCOBACTERIA

Clinical Significance

Rapidly growing mycobacteria are defined as acid-fast bacilli that form visible colonies from a dilute inoculum on a solid medium within 5 to 7 days. It is important to note that this definition does not necessarily apply to the original culture of a patient's specimen. Although there are over 30 species of rapidly growing mycobacteria, most human disease is caused by *M. abscessus, M. chelonae,* or *M. fortuitum* group (17). These three species/groups are important causes of skin and soft tissue infections, especially following penetrating trauma with possible soil or water contamination. They also cause nosocomial infections (e.g., catheter-related bacteremia and wound infections following augmentation mammaplasty or cardiac bypass surgery), and *M. abscessus* can cause pulmonary disease, especially in patients with cystic fibrosis. Disseminated disease is uncommon and is usually associated with cellular immune deficiency, particularly that due to corticosteroid therapy, although it is rare in HIV-infected patients. Several other rapidly growing mycobacteria are infrequent to rare causes of disease in humans, including *M. mucogenicum, M. immunogenum, M. neoaurum, M. mageritense, M. smegmatis, M. goodii,* and *M. wolinskyi* (17, 161, 162), and at least two species are animal pathogens (51). Clinical isolates of rapidly growing mycobacteria should be identified to species, because the antimicrobial susceptibility patterns differ for the different species. For example, *M. abscessus* and *M. chelonae* are always resistant to trimethoprim-sulfamethoxazole, whereas *M. fortuitum* group virtually always is susceptible.

Susceptibility testing should be performed for any clinically significant, rapidly growing mycobacteria. This includes isolates from blood, sterile body fluids, tissue, and material collected from skin and soft tissue lesions, and multiple isolates from sputum (or other respiratory specimens). The recovery of rapidly growing mycobacteria from sputum or other respiratory specimens is more difficult to interpret, because frequently these isolates are not clinically significant. However, the repeated isolation of a rapidly growing mycobacterium from a respiratory specimen, especially one that is smear positive for acid-fast bacilli, is highly suggestive of true pulmonary disease. In patients with wound infections, debridement and excision of the infected tissue are frequently necessary adjuncts to antimicrobial therapy, although cutaneous infections occasionally resolve spontaneously with neither surgical intervention nor antimicrobial therapy (159).

Methods

Broth microdilution is recommended for susceptibility testing of the *Mycobacterium fortuitum* group, *Mycobacterium chelonae*, and *Mycobacterium abscessus* and, theoretically, also should be appropriate for testing other clinically significant, rapidly growing mycobacteria, although data to support this are not currently available (28). The E test has been evaluated for testing rapidly growing mycobacteria, but the method did not yield results comparable to those of broth microdilution (173) and, therefore, cannot be recommended at this time. As mentioned earlier, for optimal interpretation of MIC results, isolates should be identified to the species level. If cultures (from any site except respiratory) are positive after 6 months of appropriate antimicrobial therapy, susceptibility testing should be repeated, and the species identity should be confirmed.

Broth Microdilution Method

The broth microdilution method for testing rapidly growing mycobacteria is essentially a modification of the CLSI standard method for non-acid-fast organisms that grow aerobically (27); it has been described in detail by Brown et al. (16). Antimicrobial agents (and concentrations in serial twofold dilutions) that should be tested are amikacin (1 to 128 μg/ml), cefoxitin (2 to 256 μg/ml), ciprofloxacin (0.125 to 16 μg/ml), clarithromycin (0.06 to 64 μg/ml), doxycycline (or minocycline) (0.25 to 32 μg/ml), imipenem (1 to 64 μg/ml), and sulfamethoxazole (or trimethoprim-sulfamethoxazole) (1 to 64 μg/ml). Additionally, for isolates of *M. chelonae* only, tobramycin (1 to 32 μg/ml) should be tested. Testing linezolid and one of the newer fluoroquinolones (e.g., moxifloxacin) also should be considered. Commercially manufactured broth microdilution panels are available (Trek Diagnostic Systems). Alternatively, broth microdilution plates can be prepared by the method described by Brown et al. (16); once prepared, the plates can be sealed in plastic bags and stored at −70°C for up to 6 months.

Inoculum

Fresh growth is prepared by inoculating a blood agar plate with the test organism and incubating the plate at 30°C for 72 h. The current recommendation is to prepare the inoculum by collecting growth from the blood agar plate with a sterile cotton swab and making a suspension in sterile distilled water that is equivalent to the density of 0.5 McFarland turbidity standard. The final inoculum should be approximately 5×10^5 CFU/ml or 5×10^4 CFU/100 μl/well of a microtiter plate. The purity of the inoculum should be

checked for each isolate, and periodically the size of the inoculum should be verified by quantitative plate culture.

Incubation and Reading

The microtiter plates are sealed and incubated for 3 to 5 days at 30°C in air. Plates must not be incubated beyond 5 days because of drug instability. If there is not sufficient growth after 5 days of incubation to interpret the results, the test should be repeated. Three days typically is sufficient for *M. fortuitum* complex; *M. chelonae* and *M. abscessus* usually require 4 days (occasionally 5 days). The MIC is defined as the lowest concentration of antimicrobial agent that completely inhibits visible growth. As with other types of bacteria, "trailing" is common when testing sulfonamides, and the MICs of these agents should be read at approximately 80% inhibition of growth. Trailing also is often seen with isolates of *M. fortuitum* group and clarithromycin; when this occurs, the endpoint is read at the end of the trailing.

Reporting Results

MIC values and an interpretation, based on the breakpoints for the rapidly growing mycobacteria listed in Table 7 (28), are reported for each drug, with the following exceptions. Imipenem results should not be reported for *M. chelonae* or *M. abscessus.* This recommendation is based on data from a multicenter evaluation (174), which showed that imipenem MIC values for these two species (which usually are at the resistance breakpoint of 8 to16 μg/ml) were not reproducible, resulting in major category changes of susceptibility. As previously indicated, tobramycin results should be reported only for *M. chelonae*. Because tobramycin is the aminoglycoside of choice for *M. chelonae* infections, amikacin results need be reported only if the isolate of *M. chelonae* is tobramycin resistant.

Quality Control

The QC strain recommended for monitoring test performance and for verifying the concentration of antimicrobial agents is *M. peregrinum* ATCC 700686. *Staphylococcus aureus* ATCC 29213 is an acceptable alternative. QC tests should be performed on each new batch of test plates and once each week or each time testing is performed. Currently, interlaboratory proficiency testing available for testing rapidly growing mycobacteria is limited. Therefore, laboratories are encouraged to submit isolates to reference laboratories with extensive experience in testing these mycobacteria for confirmatory tests in lieu of a formal proficiency test program.

Alternative Susceptibility Testing Methods

In the last decade, drug susceptibility testing of mycobacteria has become a very dynamic field, spawning many new technologies that may prove successful in a clinical laboratory. They all comply with the CDC goal that susceptibility test results be available within a mean of 28 days from the time the specimen arrives in the laboratory (13). Some of these techniques are based on improved methods for measuring inhibition of growth, while others are based on molecular assays for both analytes that correlate with growth or direct detection of mutations associated with resistance (2). Methods that distinguish viable from nonviable tubercle bacilli include tetrazolium dye reduction (29, 48, 102), flow cytometry (99), and a particle-counting immunoassay to quantify mycobacterial antigen (41). Jacobs et al. (77) described a method based on the use of a luciferase reporter mycobacteriophage. The premise was that viable mycobacteria support the infection and multiplication of phages, whereas inhibited or killed mycobacteria do not. Therefore, mycobacteria susceptible to INH or RMP supported the phage infection and expressed luciferase. When exposed to INH or RMP, the cells were killed and no light was produced. This assay was modified to allow easy detection of the emitted light with a Polaroid film box (also known as the "Bronx box") (125). Cooksey et al. (30) introduced the luciferase gene into *M. tuberculosis* H37Ra by electroporation of a plasmid containing the luciferase gene linked to the *hsp60* promoter. The results of a broth microtiter assay with the luciferase-H37Ra strain were comparable to the results of a conventional broth microdilution assay. Cangelosi et al. (22) described an alternative approach based on the measurement of the precursor rRNA. In this assay, *M. tuberculosis* nucleic acid is probed with specific pre-16S rRNA stem sequences in the presence or absence of drugs that have a direct or indirect effect on rRNA synthesis. Responses to RMP and ciprofloxacin were

TABLE 7 Antimicrobial agents to test against rapidly growing mycobacteria by broth microdilution method[a]

Antimicrobial agent	Concn range (μg/ml)	MIC (μg/ml) for:			QC concn range (μg/ml) for:	
		Susceptible strains	Intermediate strains	Resistant strains	*M. peregrinum* ATCC 700686[g]	*S. aureus* ATCC 29213[g]
Amikacin[b]	1–128	≤16	32	≥64	≤1–4	1–4
Cefoxitin	2–256	≤16	32–64	≥128	16–32	1–4
Ciprofloxacin	0.125–16	≤1	2	≥4	≤0.125–0.5	0.125–0.5
Clarithromycin[c]	0.06–64	≤2	4	≥8	≤0.06–0.5	0.125–0.5
Doxycycline[d]	0.25–32	≤1	2–8	≥16	≤0.125–0.5	0.125–0.5
Imipenem[e]	1–64	≤4	8	≥16	2–16	
Sulfamethoxazole	1–64	≤32		≥64	≤1–4	32–128
Tobramycin[f]	1–32	≤4	8	≥16	4–8	0.125–1

[a] Table and footnotes are adapted from reference 28.

[b] An amikacin MIC of ≥64 μg/ml for *M. abscessus* is unusual and must be confirmed.

[c] Class agent for all newer macrolides. *M. chelonae* and *M. abscessus* should be read at 3 days but not more than 4 days. Trailing across a breakpoint should be interpreted as resistance with *M. fortuitum*.

[d] Doxycycline and minocycline are interchangeable.

[e] Do not report for *M. chelonae* or *M. abscessus*. An imipenem MIC of ≥8 μg/ml for *M. fortuitum* is unusual and should be confirmed.

[f] Report results only for *M. chelonae*.

[g] Expected range for QC purposes. Potencies of drug preparations also can be tested with *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 and compared with expected ranges as described in CLSI document M7 (27).

detected within 24 and 48 h, respectively. Detectable pre-rRNA was depleted in susceptible cells but remained abundant in resistant cells. The ability of RMP to block the lytic cell cycle of bacteriophage D29 within susceptible *M. tuberculosis* and hence the production of plaques on a lawn has led to another bacteriophage-based assay for susceptibility testing of *M. tuberculosis* to a variety of drugs (43, 44).

Mutations in genes that encode targets of antimycobacterial agents (Table 2) can be detected by a variety of methods. A particular focus of such studies has been RMP resistance because of the pivotal role of RMP in the treatment of tuberculosis and other mycobacterial infections and the conserved nature of the genetic basis for resistance (>96% of RMP resistance correlates with mutations in an 81-bp segment of the *rpoB* gene). The methods used to detect *rpoB* mutations include PCR amplification of the target sequence and detection by DNA sequencing (71, 79, 150; V. Sintchenko, P. J. Jelfs, W. K. Chew, and G. L. Gilbert, Letter, *J. Antimicrob. Chemother.* **44:**294–295, 1999), the line probe assay (31, 37, 88), single-strand conformation polymorphism (45, 151), dideoxy fingerprinting (45), the mismatch RNA-RNA protection assay (105), the heteroduplex generator assay (167), the use of molecular beacons synthesized from modified oligonucleotides (86, 116), and PCR–enzyme-linked immunosorbent assay (50). Similar approaches have been developed for detecting mutations involved in INH (124), EMB and RMP (163), and PZA (35, 100) resistance. Torres et al. (154) have proposed real-time PCR coupled to fluorescence detection for a rapid detection of RMP and INH resistance associated with mutations in *M. tuberculosis*. DNA microarray technology described for mycobacterial identification has also been applied to the efficient detection of mutations associated with resistance to TB drugs (94, 155). Undoubtedly, the application of these assays for routine use in the clinical mycobacteriology laboratory is likely to require technical simplification or automation as well as outcome analysis to justify the anticipated increased costs compared with conventional approaches. Indeed, even if these goals are achieved, it is likely that there are many more mutations to be discovered before antimicrobial resistance in *M. tuberculosis* and other mycobacteria can reliably be detected by molecular assays.

NOCARDIA AND OTHER AEROBIC ACTINOMYCETES

Clinical Significance

Nocardia spp. and other aerobic actinomycetes (*Actinomadura, Rhodococcus, Gordonia, Tsukamurella,* and *Streptomyces* spp.) can cause serious disease in healthy hosts, and infections by these microorganisms in immunocompromised hosts can be systemic and life threatening (84). Combination antimicrobial therapy for several months is often necessary to achieve a positive outcome. In vitro susceptibility testing may be of great value in guiding treatment and monitoring for resistance.

Testing Method

The antimicrobial agents that are recommended for primary and secondary in vitro susceptibility testing are listed in Table 8. The broth microdilution method described for rapidly growing mycobacteria is recommended for testing *Nocardia* and other aerobic actinomycetes (28). The inoculum should

TABLE 8 Antimicrobial agents to test against *Nocardia* and other aerobic actinomycetes[a]

Antimicrobial agent	Concn range (μg/ml)	MIC (μg/ml) for:			QC concn (μg/ml) for[e]:	
		Susceptible strains	Intermediate strains	Resistant strains	*S. aureus* ATCC 29213	*E. coli* ATCC 35218
Primary						
Amikacin[b]	1–16	≤8		≥16	1–4	
Amoxicillin-clavulanic acid	2/1–32/16	≤8/4	16/8	≥32/16	0.12/0.06–0.5/0.25	4/2–16/8
Ceftriaxone	4–64	≤8	16–32	≥64	1–8	
Ciprofloxacin	0.25–4	≤1	2	≥4	0.12–0.5	
Clarithromycin[c]	0.5–8	≤2	4	≥8	0.12–0.5	
Imipenem	1–16	≤4	8	≥16	0.016–0.06	
Linezolid[d]	0.5–8	≤8			1–4	
Minocycline[b]	0.5–8	≤1	2–4	≥8	0.06–0.5	
Sulfamethoxazole[b] or Trimethoprim-sulfamethoxazole	4–64 / 0.25/4.75–4/76	≤32 / ≤2/38		≥64 / ≥4/76	32–128 / ≤0.5/9.5	
Tobramycin	1–16	≤4	8	≥16	0.12–1	
Secondary						
Cefepime	2–32	≤8	16	≥32	1–4	
Cefotaxime	4–64	≤8	16–32	≥64	1–4	
Doxycycline	0.5–8	≤1	2–4	≥8	0.12–1	
Gentamicin	1–16	≤4	8	≥16	0.12–1	

[a] Table and footnotes are adapted from reference 28.
[b] The breakpoints for these antimicrobial agents are different from the current CLSI recommendations for organisms that grow aerobically.
[c] Used as a class agent for newer macrolides.
[d] Proposed breakpoint. No *Nocardia* isolates with linezolid MICs of >8 μg/ml have been reported.
[e] At present there are no standard quality control strains of *Nocardia* or other aerobic actinomycetes. These standard *S. aureus* and *E. coli* strains should be used for QC of the concentration of antimicrobial agents.

be prepared as a suspension of microorganisms harvested from a blood agar plate that was incubated for 1 to 3 days at 37°C in air. Some species may require incubation for up to 5 days in order to have sufficient growth. Presently there are no standard strains available for QC, but standard strains of *S. aureus* and *E. coli* can be used for QC of the drug preparations.

Reporting of Results

MIC values and an interpretation, based on the breakpoints for *Nocardia* spp. and other aerobic actinomycetes listed in Table 8 (28), are reported for each drug. The interpretive criteria recommended for the aerobic actinomycetes by the CLSI in document M24-A for amikacin, minocycline, and sulfamethoxazole are different from the criteria for rapidly growing aerobic bacteria recommended by the CLSI in document M7-A5 (27).

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Detection and Characterization of Antimicrobial Resistance Genes in Pathogenic Bacteria

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For the last several years, innovative new technologies for clinical microbiology laboratories have focused on decreasing the time required to detect pathogenic microorganisms directly in clinical samples. However, the dramatic increase in multidrug resistance among a plethora of bacterial genera has necessitated the development of tests specifically designed to detect resistant strains, particularly to guide infection control efforts in health care facilities. Rapid tests that identify patients colonized or infected with organisms, such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci, can save infection control programs thousands of dollars each year by permitting isolation rooms to be utilized more effectively than would otherwise be the case (208, 247). Yet, multidrug resistance is not an issue just among health care-associated pathogens. Multidrug-resistant *Salmonella* species (258) and pneumococci (123) are among the community-associated pathogens that pose therapeutic challenges.

A wide variety of DNA probes and PCR assays focused on the detection of acquired resistance genes have been described over the last two decades (42, 229). Within the last few years, reports of several novel methods of detecting resistance genotypes have appeared in the literature. These include real-time PCR assays with molecular beacons, peptide-nucleic acid fluorescent in situ hybridization probes, microarrays, and pyrosequencing methods. In addition, sets of PCR primers designated analyte-specific reagents (ASRs) are available commercially. This chapter will focus specifically on genotypic methods for detecting and characterizing antimicrobial-resistant bacterial pathogens. Molecular methods for characterizing viruses and fungi are covered elsewhere in this Manual.

RATIONALE FOR USING GENETIC TESTS TO DETECT RESISTANCE GENES

There are four major reasons to use genetic tests to identify antimicrobial resistance genes or mutations associated with resistance. First, genetic methods can be used to detect resistance genes, or mutations that are associated with resistance, in organisms directly in clinical specimens. These results may be used to guide therapy or to decide whether to place a patient in a hospital isolation room.

Second, genetic methods can arbitrate MIC results that are at or near the breakpoint for resistance for bacterial species. For example, isolates of *S. aureus* for which the oxacillin MICs are between 2 and 8 μg/ml (borderline resistant) may contain the *mecA* (methicillin) resistance gene determinant or may represent a falsely resistant result from an automated susceptibility testing method (76, 222). A test indicating the absence of *mecA* in an isolate of *S. aureus* suggests that a physician could use an antimicrobial agent other than vancomycin to treat the infection.

Third, genetic tests are more accurate than analysis of resistance phenotypes for monitoring the epidemiologic spread of a particular resistance gene in a hospital or community setting. For example, tracking the spread of the *vanA* vancomycin resistance gene in enterococci or *S. aureus* with PCR assays has been helpful in documenting the spread of multiresistant enterococci in Europe (1, 83) and vancomycin-resistant *S. aureus* (VRSA) in the United States (33).

Fourth, genetic tests can be used as the "gold standard" for resistance tests in evaluating the accuracy of new susceptibility testing methods (54, 221).

However, there are several potential pitfalls associated with using genetic tests to detect resistant organisms. These include the lack of expression of resistance genes that are detected (28), the problem of mixed or normal flora that may contain resistance genes (such as *vanB* in *Clostridium* species in fecal samples) (14, 15), mutations in target organisms that alter sequences used for PCR primers and lead to false-negative results, and the emergence of novel resistance genes that are not detected by existing genetic assays. Each of these factors can affect the sensitivity or specificity of molecular assays (42).

GENETIC TESTS FOR RESISTANCE GENES AND DNA SEQUENCING STRATEGIES TO DETECT MUTATIONS ASSOCIATED WITH RESISTANCE

General Guidelines

The ideal genetic test targets nucleic acid sequences within the open reading frame (or coding region) of the resistance gene and avoids sequences outside of the gene that may contain insertion elements or promoter sequences that may be present in susceptible strains or strains with other types of resistance genes. Among the primers that have been described for studying antibacterial resistance are those directed to β-lactamase genes and the genes that encode resistance to aminocyclitols,

aminoglycosides, chloramphenicol, glycopeptides, isoniazid, macrolides, mupirocin, oxazolidinones, quinolones, rifampin, sulfonamides, tetracyclines, and trimethoprim. Examples of PCR primers that target resistance genes or mutations associated with resistance are shown in Table 1. In addition, multiplex PCR assays for the detection of aminoglycoside resistance genes in enterococci, plasmid-carried *amp*C genes in *Enterobacteriaceae,* and glycopeptide resistance genes in enterococci and staphylococci are shown in Table 2. The tables are not meant to be exhaustive but rather give an indication of the types of assays that have been described previously. Appropriate specificity controls (organisms that have a similar resistance pattern but contain resistance genes other than the target gene) should always be included in all reactions using these primers. Examples of DNA probes that can be used to detect resistance genes have been described in previous editions of this Manual (229).

DNA Sequencing

DNA sequence analysis has been particularly helpful for identifying point mutations associated with resistance to fluoroquinolones (54, 86, 104), extended-spectrum β-lactamases (19, 183, 184), oxazolidinones (257), and antimycobacterial drugs (139, 224). There are a number of mutations associated with resistance to isoniazid, rifampin, and streptomycin (10, 69, 266). Unfortunately, additional genetic loci associated with resistance must also be involved since only 60 to 90% of resistance can be explained by these loci, although pyrosequencing assays have improved detection (10, 266). Other novel pyrosequencing assays that target mutations associated with resistance, such as changes in rRNA sequences associated with linezolid resistance, can provide high-throughput results in a few hours (205).

In-House Assays and ASRs

Utilization of home-brew PCR assays for detecting resistance genes or implementing assays reported in the literature in a clinical microbiology laboratory has always been a problem. In-house validation of research protocols can be time-consuming. Yet, many of these assays provide significant advances in turnaround time or cost-effectiveness over those of traditional test methods. A recent advancement is the availability of ASRs. In the United States, reagents for nucleic acid amplification-based technologies may be available from manufacturers in the form of ASRs. ASRs are produced under "good manufacturing practices"; however, unlike Food and Drug Administration (FDA)-cleared diagnostic kits, the clinical utility of the ASRs does not have to be validated in prospective multisite trials before marketing. The process of making materials available through ASRs has its advantages and drawbacks. Because time-consuming clinical trials are obviated, the ASRs can be available to clinical laboratories much sooner than diagnostic kits. From the manufacturers' perspective, the process allows a product to be evaluated by a number of users to ensure optimal performance. Therefore, if optimization of an ASR is indicated, this can be accomplished before time-consuming and costly clinical trials are undertaken for an FDA-approved kit version. A major drawback of ASRs, in contrast to FDA-approved kits, is that FDA guidelines prohibit the provision of testing protocols for the ASRs by the manufacturer. Manufacturers can only refer customers to protocols published in the medical literature or to scientists or practitioners who have established working protocols in their laboratories. Another drawback of ASRs relates to the extent of verification required by certifying agencies before tests can be implemented in clinical practice. For example, the Clinical Laboratory Improvement Amendments of 1988 require a more extensive verification process for ASRs than for FDA-approved kits (44). The FDA requires laboratories to add a disclaimer on reports for tests that use ASRs. The mandatory language for this disclaimer is, "This test was developed and its performance characteristics determined by the (laboratory name). It has not been cleared or approved by the U. S. FDA" (45).

SPECIFIC APPLICATIONS

The following sections review applications of molecular diagnostic methods for specific classes of resistance determinants.

AMINOGLYCOSIDE RESISTANCE GENES

The diversity of known aminoglycoside resistance genes, which are common in both gram-positive and gram-negative organisms (173, 201, 260), continues to expand with the recognition of additional acetyltransferases, adenylyltransferases, and phosphotransferases. Unfortunately, the lack of consensus sequences among the acetyltransferase and adenylyltransferase genes prohibits detection of multiple determinants with a single PCR primer set (201), making it difficult to use amplification-based tests to predict aminoglycoside resistance, especially in gram-negative organisms. However, multiplex assays for high-level gentamicin resistance in enterococci (Table 2) are available to predict the effectiveness of combination therapy (synergy) with aminoglycosides and cell wall-active agents (116, 239). Among the newer aminoglycoside resistance genes reported to be found in gram-negative organisms is the *arm*A resistance determinant (75, 82, 261).

DETECTING GENES ASSOCIATED WITH RESISTANCE TO β-LACTAM DRUGS

Oxacillin Resistance in Staphylococci

Detection of oxacillin resistance in staphylococci, which is mediated primarily by the *mec*A determinant (32), by phenotypic methods continues to be a problem (222), particularly with coagulase-negative staphylococci (228). A *mec*A PCR assay can differentiate those isolates that are borderline resistant to oxacillin due to the modification of penicillin binding proteins (PBPs; the so-called MOD strains) or the production of large quantities of β-lactamase from isolates that are resistant due to the presence of the *mec*A determinant (132). The rare strains of *S. aureus* that are resistant to oxacillin by virtue of containing modified PBPs with reduced affinity for oxacillin may be misclassified as oxacillin susceptible by the *mec*A gene test since these strains are truly oxacillin resistant but do not contain the *mec*A gene (231, 247). A latex agglutination assay to identify the *mec*A gene product, i.e., PBP 2a, can be used for isolated colonies of *S. aureus* (28, 99, 241). These tests provide an alternative to phenotypic tests for the clinical microbiology laboratory, presuming that the caveats of their use are understood.

An increasing body of evidence demonstrates that identification and isolation of hospitalized patients who are nasal carriers of MRSA can reduce the incidence of health care-associated infections caused by these organisms and can reduce costs (161). Therefore, emphasis has been placed on the importance of active microbiology surveillance programs for the detection of nasal carriers of MRSA in the health care

TABLE 1 PCR assays for antimicrobial resistance genes

Antimicrobial agent and gene	Primers (5′ → 3′)	Product size	Use	Reference(s)
Aminoglycosides				
Aminoglycoside-modifying enzymes				
aac(6′)-Ia	ATG AAT TAT CAA ATT GTG TTA CTC TTT GAT TAA ACT	558 bp	Detection, probe	168[a]
aac(6′)-Ic	CTA CGA TTA CGT CAA CGG CTG C TTG CTT CGC CCA CTC CTG CAC C	130 bp	Detection	91[b]
aac(3)-Ia	ACC TAC TCC CAA CAT CAG CC ATA TAG ATC TCA CTA CGC GC	169 bp	Detection	240[c]
aac(3)-Ic	GAT GAT CTC TAC TCA AAC C TTA GGC AGC AGG TTG AGG	472 bp	Cloning, sequencing	188
aac(3)-IV	GTT ACA CCG GAC CTT GGA AAC GGC ATT GAG CGT CAG	675 bp	Detection	88
aphA-3	GGG ACC ACC TAT GAT GTG GAA CG CAG GCT TGA TCC CCA GTA AGT C	595 bp	Detection	80
aph(3′)-VIa	ATA CAG AGA CCA CAT ACA GT GGA CAA TCA ATA ATA GCA AT	235 bp	Detection	245
aad(2″)-Ia	ATG TTA CGC AGC AGG GCA GTC G CGT CAG ATC AAT ATC ATC GTG C	188 bp	Detection	242
aac(6′)-Ie-aph(2″)-Ia	GAG CAA TAA GGG CAT ACC AAA AAT C CCG TGC ATT TGT CTT AAA AAA CTG G	485 bp	Detection	108
aac(6′)-Iih	GGA TAG CGG ATG ATT ATC A TAA GAG TTT AAT GAA TAA TTA	856 bp	Sequencing	55
aph(2″)-Ib	TAT GGA TCC ATG GTT AAC TTG GAC GCT GAG ATT AAG CTT CCT GCT AAA ATA TAA ACA TCT CTG CT	920 bp	Detection	108
aph(3′)-IIIa	GGC TAA AAT GAG AAT ATC ACC GG CTT TAA AAA ATC ATA CAG CTC GCG	523 bp	Detection	217, 239
ant(4′)-Ia	CAA ACT GCT AAA TCG GTA GAA GCC GGA AAG TTG ACC AGA CAT TAC GAA CT	294 bp	Detection	217, 239
aadA	TGA TTT GCT GGT TAC GGT GAC CGC TAT GTT CTC TTG CTT TTG	284 bp	Detection, probe	38
aadE	ACT GGC TTA ATC AAT TTG GG GCC TTT CCG CCA CCT CAC CG	597 bp	Detection, probe	38
aad-6	AGA AGA TGT AAT AAT ATA G CTG TAA TCA CTG TTC CCG CCT	978 bp	Detection	118
strA-strB	TAT CTG CGA TTG GAC CCT CTG CAT TGC TCA TCA TTT GAT CGG CT	519 bp	Detection	216
16S rRNA methylases				
armA	AGG TTG TTT CCA TTT CTG AG TCT CTT CCA TTC CCT TCT CC	590 bp	Detection, sequencing	260
rmtA	CTA GCG TCC ATC CTT TCC TC TTT GCT TCC ATG CCC TTG CC	635 bp	Detection	264
rmtB	ATG AAC ATC AAC GAT GCC CT CCT TCT GAT TGG CTT ATC CA	769 bp	Detection, sequencing, probe	261
Spectinomycin				
rrs (*Neisseria meningitidis, N. gonorrhoeae*)	CTT ACC TGG TCT TGA CA CGA TTA CTA GCG ATT CC	373 bp	Sequencing	74
β-lactams				
mecA	TGG CTA TCG TGT CAC AAT CG CTG GAA CTT GTT GAG CAG AG	310 bp	Detection	243
bla_{SHV}	GCC GGG TTA TTC TTA TTT GTC GC TCT TTC CGA TGC CGC CGC CAG TCA	1,017 bp	Sequencing	148

(Continued on next page)

TABLE 1 *(Continued)*

Antimicrobial agent and gene	Primers (5′ → 3′)	Product size	Use	Reference(s)
bla_{SHV}	GGT TAT GCG TTA TAT TCG CC ATC TTT CGC TCC AGC TGT TC	275 bp	Probe[d]	184[e]
bla_{SHV}	GGT TAT GCG TTA TAT TCG CC TTA GCG TTG CCA GTG CTC	867 bp	Detection	184
bla_{TEM}	ATG AGT ATT CAA CAT TTC CG TTA CTG TCA TGC CAT CC	351 bp	Detection, probe	183
bla_{TEM}	ATG AGT ATT CAA CAT TTC CG CTG ACA GTT ACC AAT GCT TA	867 bp	Detection	184
bla_{CTX-M}	CGC TTT GCG ATG TGC AG ACC GCG ATA TCG TTG GT	550 bp	Detection, probe	19
$bla_{CTX-M-2}$	ATG ATG ACT CAG AGC ATT CG TTA TTG CAT CAG AAA CCG TG	884 bp	Detection	167
$bla_{CTX-M-9}$	GTG ACA AAG AGA GTG CAA CGG ATG ATT CTC GCC GCT GAA GCC	857 bp	Detection	194
$bla_{CTX-M-10}$	GCT GAT GAG CGC TTT GCG TTA CAA ACC GTT GGT GAC G	684 bp	Detection	150
bla_{GES-1}	ATG CGC TTC ATT CAC GCA C CTA TTT GTC CGT GCT CAG G	864 bp	Probe[d]	170
$bla_{GES/IBC}$	GTT TTG CAA TGT GCT CAA CG TGC CAT AGC AAT AGG CGT AG	371 bp	Detection, sequencing	249
bla_{PER-1}	ATG AAT GTC ATT ATA AAA GC AAT TTG GGC TTA GGG CAA GAA A	926 bp	Detection, probe	238
bla_{PER-2}	CGC TTC TGC TCT GCT GAT GGC AGC TTC TTT AAC GCC	469 bp	Detection	16
bla_{PSE}	ACC GTA TTG AGC CTG ATT TA ATT GAA GCC TGT GTT TGA GC	321 bp	Detection	17[f]
bla_{ROB-1}	TGT TTG CAA TCG CTG CC TTA TCG TAC ACT TTC CA	400 bp	Detection	107
bla_{TLA-1}	TCT CAG CGC AAA TCC GCG CTA TTT CCC ATC CTT AAC TAG	974 bp	Detection	4
bla_{VEB-1}	CGA CTT CCA TTT CCC GAT GC GGA CTC TGC AAC AAA TAC GC	643 bp	Detection	141
bla_{KPC}	TGT CAC TGT ATC GCC GTC TAT TTT TCC GAG ATG GGT GAC	331 bp	Detection	262
bla_{KPC-2}	GCT ACA CCT AGC TCC ACC TTC ACA GTG GTT GGT AAT CCA TGC	989 bp	Sequencing	137
bla_{IMI-1}	ATA GCC ATC TTG TTT AGC TC TCT GCG ATT ACT TTA TCC TC	818 bp	Probe[d]	12
bla_{NmcA}	TGC AGC TTA ATT ATT TTC AGA TTA G ATT TTT TTC ATG ATG AAG TTA AGC C	2,122 bp	Sequencing	175[g]
bla_{SME-1}	AAC GGC TTC ATT TTT GTT TAG GCT TCC GCA ATA GTT TTA TCA	830 bp	Detection	180
bla_{IMP}	CTA CCG CAG CAG AGT CTT TG AAC CAG TTT TGC CTT ACC AT	587 bp	Detection	200
bla_{VIM}	TCT ACA TGA CCG CGT CTG TC TGT GCT TTG ACA ACG TTC GC	748 bp	Detection	171
bla_{VIM}	AGT GGT GAG TAT CCG ACA G ATG AAA GTG CGT GGA GAC	261 bp	Detection, probe	77
bla_{SPM-1}	CCT ACA ATC TAA CGG CGA CC TCG CCG TGT CCA GGT ATA AC	649 bp	Detection	195
ampC (promoter, *Escherichia coli*)	GAT CGT TCT GCC GCT GTG GGG CAG CAA ATG TGG AGC AA	271 bp	Sequencing	30
bla_{ACC}	AAC AGC CTC AGC AGC CGG TTA TTC GCC GCA ATC ATC CCT AGC	346 bp	Detection	163[h]
bla_{ACT-1}	ATT CGT ATG CTG GAT CTC GCC ACC CAT GAC CCA GTT CGC CAT ATC CTG	396 bp	Detection	48
bla_{DHA}	CCG TCA CTC ACA CAC GGA AGG CGT ATC CGC AGG GGC CTG TTC	1,199 bp	Detection, sequencing	136[i]
bla_{FOX}	TGT GGA CGG CAT TAT CCA G AAA GCG CGT AAC CGG ATT G	868 bp	Detection, sequencing	138

(Continued on next page)

TABLE 1 PCR assays for antimicrobial resistance genes *(Continued)*

Antimicrobial agent and gene	Primers (5′ → 3′)	Product size	Use	Reference(s)
bla$_{OXA-1}$	CCA AAG ACG TGG ATG GTT AAA TTC GAC CCC AAG TT	540 bp	Detection	207
bla$_{OXA-2}$	TTC AAG CCA AAG GCA CGA TAG TCC GAG TTG ACT GCC GGG TTG	703 bp	Detection	214
bla$_{OXA-10}$	CGT GCT TTG TAA AAG TAG CAG CAT GAT TTT GGT GGG AAT GG	652 bp	Detection	214
bla$_{OXA-1}$ group	TTT TCT GTT GTT TGG GTT TC TTT CTT GGC TTT TAT GCT TG	447 bp	Detection	17
bla$_{OXA-2}$ group	AAG AAA CGC TAC TCG CCT GC CCA CTC AAC CCA TCC TAC CC	486 bp	Detection	17
bla$_{OXA-10}$ group	TCA ACA AAT CGC CAG AGA AG TCC CAC ACC AGA AAA ACC AG	276 bp	Detection	17
bla$_{OXA-23}$	CCT CAG GTG TGC TGG TTA TTC CCC AAC CAG TCT TTC CAA AA	513 bp	Detection	29
bla$_{OXA-24}$	TTC CCC TAA CAT GAA TTT GT GTA CTA ATC AAA GTT GTG AA	1,020 bp	Detection, sequencing	20
Chloramphenicol/ florfenicol				
catA1	CCA CCG TTG ATA TAT CCC CCT GCC ACT CAT CGC AGT	623 bp	Detection	87
cmlA	TGT CAT TTA CGG CAT ACT CG ATC AGG CAT CCC ATT CCC AT	456 bp	Detection	88
flo	CAC GTT GAG CCT CTA TAT GG ATG CAG AAG TAG AAC GCG AC	869 bp	Detection	144
Glycopeptides				
vanA	GCT ATT CAG CTG TAC TC CAG CGG CCA TCA TAC GG	783 bp	Detection	196[j]
vanA	GGG AAA ACG ACA ATT GC GTA CAA TGC GGC CGT TA	732 bp	Detection	65
vanB	CGC CAT ATT CTC CCC GGA TAG AAG CCC TCT GCA TCC AAG CAC	667 bp	Detection	120
vanC1	GAA AGA CAA CAG GAA GAC CGC ATC GCA TCA CAA GCA CCA ATC	796 bp	Detection	37
vanC2/3	CGG GGA AGA TGG CAG TAT CGC AGG GAC GGT GAT TTT	484 bp	Detection	110
vanC3	GCC TTT ACT TAT TGT TCC GCT TGT TCT TTG ACC TTA	224 bp	Detection	39
vanD	TAA GGC GCT TGC ATA TAC CG TGC AGC CAA GTA TCC GGT AA	461 bp	Detection	165
vanE	TGT GGT ATC GGA GCT GCA G GTC GAT TCT CGC TAA TCC	513 bp	Detection, probe	68
vanG	CGG TTG TGC CGT ACT TGG C GGG TAA AGC CAT AGT CTG GGG C	811 bp	Detection	134
Macrolides, lincosamides, and streptogramins				
ermA	CTT CGA TAG TTT ATT AAT ATT AGT TCT AAA AAG CAT GTA AAA GAA	645 bp	Detection	219
ermA (ermTR)	AGA AGG TTA TAA TGA AAC AGA A GGC ATG ACA TAA ACC TTC AT	212 bp	Detection	186
ermB	GAA AAG GTA CTC AAC CAA ATA AGT AAC GGT ACT TAA ATT GTT TAC	639 bp	Detection	219
ermC	TCA AAA CAT AAT ATA GAT AAA GCT AAT ATT GTT TAA ATC GTC AAT	642 bp	Detection	219
ermF	GCA GAC AGG CGC AAG CAG CAA ACC ACG TTC CCA TGA GTG GTA TGG	606 bp	Detection	191

(Continued on next page)

TABLE 1 *(Continued)*

Antimicrobial agent and gene	Primers (5′ → 3′)	Product size	Use	Reference(s)
ermG	AGG GAA AGG TCA TTT TAC TGC CCC TAC CTA TAA CTA AAC ATT	664 bp	Detection	186
mefA/mefE	AGT ATC ATT AAT CAC TAG TGC TTC TTC TGG TAC TAA AAG TGG	348 bp	Detection	219
mefA	GAC CAA AAG CCA CAT TGT GGA CCT CCT GTC TAT AAT CGC ATG	1,431 bp	Restriction enzyme analysis	154
mefE	CTA TGC GAT TTT GGG ACC TG GAA AGC CCC ATT ATT GCA CA	801 bp	Detection	128
ereA	AGT CGG CGG TTA TTT CAT TGC TCC CTC ATT TTC ATT TA	746 bp	Detection	204
ereB	CGG ATA AAG AAG CAC TAC AC AAC GAC CTC AGA TAC AGA TG	788 bp	Detection	204
mphA	AAC TGT ACG CAC TTG C GGT ACT CTT CGT TAC C	837 bp	Detection	219
msrA/msrB	GTC AAA AAC TGC TAA CAC AAG AAT AAT ACT GCT AAC GAT AAT	343 bp	Detection	204
smp	AAA TTG TTT AAA AAG AAA TC TTT GAA CCA TAA TAT TCA TC	616 bp	Detection, probe	220
vat	CAA TGA CCA TGG ACC TGA TC AGC ATT TCG ATA TCT CC	615 bp	Detection	6
vatB	CCT GAT CCA AAT AGC ATA TAT CC CTA AAT CAG AGC TAC AAA GTG	601 bp	Detection	5
satG (vatE)	CTA TAC CTG ACG CAA ATG C GGT TCA AAT CTT GGT CCG	511 bp	Detection	250
vga	TCT AAT GGT ACA GGA AAG ACA ACG ATC GTG AGA TAC AAA GAT TAT	399 bp	Detection	219
linA/linA′	GTA GAT GTA TTA ACT GGA A GAA AAA GAA GTT GAG CTT C	325 bp	Detection, probe	147
linB	CCT ACC TAT TGT TTG TGG AA ATA ACG TTA CTC TCC TAT TC	944 bp	Detection	22
Mupirocin				
Isoleucyl-tRNA synthetase (IRS) gene	CCA TGC CTT ACC AGT TGA ATT GGA TCC CCG AGC ACT ATC CGA	1.65 kb	Probe[d]	81
mupA	CCC ATG GCT TAC CAG TTG A CCA TGG AGC ACT ATC CGA A	1.65 kb	Detection, probe	182
mupA	TGA CAA TAG AAA AGG ACA GG CTC TAA TTC AAC TGG TAA GCC	190 bp	Detection	157
ileS-2	GTT TAT CTT CTG ATG CTG AG CCC CAG TTA CAC CGA TAT AA	237 bp	Detection	149
Quinolones				
gyrA (Mycobacterium tuberculosis)	CAG CTA CAT CGA CTA TGC GA GGG CTT CGG TGT ACC TCA T	320 bp	Sequencing	109
gyrA (Acinetobacter baumannii)	AAA TCT GCC CGT GTC GTT GGT GCC ATA CCT ACG GCG ATA CC	343 bp	Sequencing	244
gyrA (E. coli)	ACG TAC TAG GCA ATG ACT GG AGA AGT CGC CGT CGA TAG AAC	190 bp	Sequencing	66[k]
gyrA (S. pneumoniae)	TTC TCT ACG GAA TGA ATG GAT ATC ACG AAG CAT TTC CAG	272 bp	Sequencing	104
gyrB (S. pneumoniae)	TTC TCC GAT TTC CTC ATG AGA AGG GTA CGA ATG TGG	458 bp	Sequencing	158
parC (S. pneumoniae)	TGG GTT GAA GCC GGT TCA CAA GAC CGT TGG TTC TTT C	361 bp	Sequencing	104
parE (S. pneumoniae)	CCA ATC TAA GAA TCC TG GCA ATA TAG ACA TGA CC	357 bp	Sequencing	166
qnrA	TCA GCA AGA GGA TTT CTC A GGC AGC ACT ATT ACT CCC A	627 bp	Detection	193

(Continued on next page)

TABLE 1 PCR assays for antimicrobial resistance genes *(Continued)*

Antimicrobial agent and gene	Primers (5′ → 3′)	Product size	Use	Reference(s)
qnr	CCG TAT GGA TAT TAT TGA TAA AG CTA ATC CGG CAG CAC TAT TA	661 bp	Detection	126
Sulfonamides				
*sul*A	AGC CAA TCA TGC AAA GAC AG ATT TTC CGC TTC ATC AGC CAG	916 bp	Sequencing	131
*sul*I	CTT CGA TGA GAG CCG GCG GC GCA AGG CGG AAA CCC GCG CC	437 bp	Detection	88
Dihyropteroate synthase (DHPS) gene *(Pneumocystis carinii)*	TTA CTC CTG ATT CTT TTT TCG ATG GG GCC TTA ATT GCT TGT TCT GCA ACC	259 bp	SSCP[l] assay	125
Tetracycline				
tet(A)	GTA ATT CTG AGC ACT GT CCT GGA CAA CAT TGC TT	954 bp	Probe[d]	92[m]
tet(B)	CAG TGC TGT TGT TGT CAT TAA GCT TGG AAT ACT GAG TGT AA	528 bp	Detection, sequencing	191
tet(E)	GTG ATG ATG GCA CTG GT TGC TGT ACA TCG CTC TT	1,196 bp	Probe[d]	73[n]
tet(G)	GCT CGG TGG TAT CTC TGC TC AGC AAC AGA ATC GGG AAC AC	468 bp	Detection	144
tet(K)	GTA GGA TCT GCT GCA TTC CC CAC TAT TAC CTA TTG TCG C	552 bp	Detection	118
tet(L)	GGA TCG ATA GTA GCC ATG GG GTA TCC CAC CAA TGT AGC CG	516 bp	Detection	118
tet(M)	GAA CTC GAA CAA GAG GAA AGC ATG GAA GCC CAG AAA GGA T	741 bp	Detection	152
tet(O)	AAC TTA GGC ATT CTG GCT CAC TCC CAC TGT TCC ATA TCG TCA	519 bp	Detection	152
*tet*A(P)	CAC AGA TTG TAT GGG GAT TAG G CAT TTA TAG AAA GCA CAG TAG C	764 bp	Detection, sequencing	124
tet(Q)	ATT GCG GAA GTG GAG CGG AC GCC GGA CGG AGG ATT TGA GA	814 bp	Detection	151
tet(S)	CGC TAC ATT TGC GAG ACT CAG GGC TCT CAT ACT GAA TGC CAC	569 bp	Detection	118
tet(T)	CAG TGG GAA TAT AAG GAC ACG TC CAA GCC TTC TCT ACA GCA TC	644 bp	Detection	118
tet(V)	GAC AAC GGC ATG AAC GTT CGC GAG CAT GTT C	405 bp	Detection	60
tet(W)	GAG AGC CTG CTA TAT GCC AGC GGG CGT ATC CAC AAT GTT AAC	168 bp	Detection	8
tet(39)	CTC CTT CTC TAT TGT GGC TA CAC TAA TAC CTC TGG ACA TCA	711 bp	Detection	2
Trimethoprim				
dhfrVIII	CTA ACG GCG CTA TCT TCG TGA ACA ACG TAT GAA TTC TTC CAT GCC ATT CTG CTC GTA G	300 bp	Detection	218
dfr1	ACG GAT CCT GGC TGT TGG TTG GAC GC CGG AAT TCA CCT TCC GGC TCG ATG TC	254 bp	Detection	79
dfr9	ATG AAT TCC CGT GGC ATG AAC CAG AAG AT ATG GAT CCT TCA GTA ATG GTC GGG ACC TC	399 bp	Detection	79
dfrA	CCC TGC TAT TAA AGC ACC CAT GAC CAG ATA ACT C	262 bp	Detection, sequencing	52
dfrA1	GTG AAA CTA TCA CTA ATG G TTA ACC CTT TTG CCA GAT TT	474 bp	Detection, RFLP[o] analysis	143[p]
dfrA14	GAG CAG CTI[q] CTI TTI AAA GC TTA GCC CTT TII CCA ATT TT	393 bp	Detection	143[r]
dfrA7	TTG AAA ATT TCA TTG ATT T TTA GCC TTT TTT CCA AAT CT	474 bp	Detection	143[s]

(Continued on next page)

TABLE 1 *(Continued)*

Antimicrobial agent and gene	Primers (5′ → 3′)	Product size	Use	Reference(s)
dfrB1	GAT CAC GTG CGC AAG AAA TC AAG CGC AGC CAC AGG ATA AAT	141 bp	Detection	143[t]
dfrA12	GGT GS[u]G CAG AAG ATT TTT CGC TGG GAA GAA GGC GTC ACC CTC	309 bp	Detection	143[v]
Ethambutol				
embB (M. tuberculosis)	ACG CTG AAA CTG CTG GCG AT ACA GAC TGG CGT CGC TGA CA	400 bp	SSCP[l] assay	3
Pyrazinamide				
pncA (M. tuberculosis)	GCT GGT CAT GTT CGC GAT CG CAG GAG CTG CAA ACC AAC TCG	673 bp	Sequencing	212
Rifampin				
rpoB (M. tuberculosis)	GGG AGC GGA TGA CCA CCC A GCG GTA CGG CGT TTC GAT GAA C	350 bp	Sequencing	109
rpoB (Mycobacteria)	CCA CCC AGG ACG TGG AGG CGA TCA CAC AGT GCG ACG GGT GCA CGT CGC GGA CCT	224 bp	Sequencing	43
Streptomycin				
rpsL (M. tuberculosis)	GGC CGA CAA ACA GAA CGT GTT CAC CAA CTG GGT GAC	501 bp	Sequencing	211
rrs (M. tuberculosis)	TTG GCC ATG CTC TTG ATG CCC TGC ACA CAG GCC ACA AGG GA	1,140 bp	Sequencing	135
rrs (Mycobacteria)	GAT GAC GGC CTT CGG GTT GT TCT AGT CTG CCC GTA TCG CC	238 bp	Sequencing (530 loop)	94
rrs (Mycobacteria)	GTA GTC CAC GCC GTA AAC GG AGG CCA CAA GGG AAC GCC TA	238 bp	Sequencing (912–915 domain)	94
Isoniazid				
katG	GAA ACA GCG GCG CTG GAT CGT GTT GTC CCA TTT CGT CGG GG	209 bp	SSCP[l] assay	226
katG	TTT CGG CGC ATG GCC ATG A ACA GCC ACC GAG CAC GAC	894 bp	Sequencing, RFLP[o] analysis	90
inhA	TCG ACG GCC GGC ATG G CCG GTC CGC CGA ACG	905 bp	Sequencing	109
ahpC	ATG CAT TGT CCG CTT TGA TG TTC TAT ACT CAT TGA TT	588 bp	Sequencing	112

[a] This reference also describes primer sets for the detection of *aac*(6′)*-Ib*, *aac*(6′)*-Id*, *aac*(6′)*-If*, *aac*(6′)*-Ig*, and *aac*(6′)*-Ih*.
[b] This reference also describes primer sets for the detection of *aac*(6′)*-Id*, *aac*(6′)*-Ie*, *aac*(6′)*-Ig*, *aac*(6′)*-Ih*, *aac*(6′)*-Ii*, *aac*(6′)*-Ij*, *aac*(6′)*-Il*, and *aac*(6′)*-IIb*.
[c] This reference also describes primer sets for the detection of *aac*(3)*-IIa*, *aac*(3)*-IIIa*, *aac*(3)*-IVa*, *aad*(4′)*-Ia*, *aac*(6′)*-aph*(2″), and *aph*(3′)*-IIIa*.
[d] This primer set is used in this reference for the synthesis of an intragenic probe and not for direct detection.
[e] This reference also describes primer sets for the detection and sequencing of bla_{SHV} and the detection of bla_{TEM}.
[f] This primer set amplifies genes encoding PSE-1, PSE-4, and CARB-3.
[g] This primer set amplifies both the *nmcA* structural gene and its regulatory gene, *nmcR*.
[h] This primer set is one of six that comprise a multiplex PCR method for the detection of members of six families of plasmid-mediated *amp*C β-lactamase genes.
[i] This reference also describes primer sets for the detection and/or sequencing of bla_{FOX}, bla_{CMY}, and $bla_{ACT\text{-}1}$.
[j] This reference also describes primer sets for the detection of *vanB*, *vanC1*, and *vanC2*.
[k] This reference also describes primer sets for the DNA sequencing of *par*C and *parE* of *E. coli*.
[l] SSCP, single-strand conformational polymorphism.
[m] This reference also describes primer sets for the synthesis of probes for *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(H), and *tet*(M).
[n] This reference also describes primer sets for the synthesis of probes for *tet*(A), *tet*(B), *tet*(C), *tet*(D), and *tet*(G).
[o] RFLP, restriction fragment length polymorphism.
[p] This primer set also amplifies *dfrA5, dfrA15, dfrA15b, dfrA16*, and *dfrA16b*.
[q] I, inosine (International Union of Biochemistry [IUB] codes for DNA bases).
[r] This primer set also amplifies *dfrA1, dfrA5, dfrA6, dfrA15*, and *dfrA16*.
[s] This primer set also amplifies *dfrA17*.
[t] This primer set also amplifies *dfrB2 and dfrB3*.
[u] S, G, or C (International Union of Biochemistry [IUB] codes for DNA bases).
[v] This primer set also amplifies *dfrA13*.

TABLE 2 Multiplex PCR assays for detection of antimicrobial resistance genes

Antimicrobial agent organisms and genes	Product size (bp)	Reference
Aminoglycosides (enterococci)		
aac(6′)-Ie-aph(2″)-Ia	348	239
aph(2″)-Ib	867	
aph(2″)-Ic	444	
aph(2″)-Id	641	
aph(3′)-IIIa	523	
ant(4′)-Ia	294	
aac(6′)-Ii	410	116
aac(6′)-aph(2″)	675	
ant(4′)-Ia	266	
ant(6)-Ia	563	
ant(9)-Ia	476	
aph(2″)-Ic	837	
aph(3′)-IIIa	354	
β-Lactams *(Enterobacteriaceae)*		
$bla_{\text{MOX-1-2}}$, $bla_{\text{CMY-1}}$, $bla_{\text{CMY-8-11}}$	520	163[a]
$bla_{\text{LAT-1-4}}$, $bla_{\text{CMY-2-7}}$, $bla_{\text{BIL-1}}$	462	
$bla_{\text{DHA-1-2}}$	405	
bla_{ACC}	346	
$bla_{\text{MIR-1}}$, $bla_{\text{ACT-1}}$	302	
$bla_{\text{FOX-1–5b}}$	190	
Glycopeptides (enterococci and staphylococci)		
vanA	732	59
vanB	647	
*van*C1/2	815/827	
vanD	500	
vanE	430	
*van*G	941	
ddl (Enterococcus faecalis)	475	
ddl (E. faecium)	1,091	
nuc (S. aureus)	218	
Staphylococcus epidermidis gene fragment	125[b]	

[a] This reference describes primers for the detection of genes within six plasmid-mediated *amp*C-specific gene families in the *Enterobacteriaceae*.
[b] This PCR product is amplified with primers complementary to a chromosomal fragment specific for *S. epidermidis*.

setting. Conventional culture-based methods for detection of MRSA from nasal swabs frequently require ⩾48 h before a final report can be issued. Various studies have evaluated molecular methods that detect nasal carriers of MRSA more rapidly than conventional culture-based methods (96, 161, 247). For example, Warren and colleagues demonstrated the utility of a commercially available real-time PCR method for identifying MRSA directly from nasal swabs (247). This testing method anchors one primer in *orf X,* which is unique to the *S. aureus* chromosome, and the second in the staphylococcal chromosomal cassette carrying the *mec*A gene (SCCmec), which is adjacent to *orf X* (96). These investigators showed that this assay is as sensitive as a direct plating culture method; final results were available within 2 h versus 48 to 72 h for results from culture.

β-Lactam Resistance in Pneumococci

Resistance to penicillin, extended-spectrum cephalosporins, and other antimicrobial agents in pneumococci has become a global problem (123, 133). Resistance to β-lactams develops when pneumococcal PBPs are remodeled through the acquisition of chromosomal DNA from other pneumococci or other streptococcal species (46, 63). Although remodeling is not a random process, it has been difficult to develop PCR primers that accurately differentiate strains with low-level penicillin resistance from those with high-level resistance. Using primers for the *pbp2b* gene, Ubukata et al. (236) attempted to resolve this issue. In their assay, the lack of product in the presence of amplification controls suggests that the *pbp2b* gene had been remodeled and therefore mediates resistance. However, such an assay does not reliably indicate which strains could be treated with penicillin versus an extended-spectrum cephalosporin, as might be desirable for an assay to be used in a clinical laboratory, but may be used as a screening tool for analyzing large groups of strains for resistance. A study by du Plessis et al. (64) also attempted to identify penicillin-resistant pneumococci directly in cerebrospinal fluid by using PCR primers directed to *pbp2b* sequences. This method was modestly successful in identifying isolates with altered PBPs associated with penicillin resistance.

β-Lactamase Genes in Gram-Negative Organisms

A variety of PCR primer sets have been developed to detect the genes encoding the TEM, SHV, OXA, CTX-M, GES, and AmpC (e.g., CMY-2, DHA-1, and MIR-1) β-lactamases and the metallo-β-lactamases (e.g., VIM-1 and SPM-1) present in gram-negative organisms (Tables 1 and 2).

Health care-associated infections caused by *Klebsiella pneumoniae, K. oxytoca* (7, 72, 198, 214), and other *Enterobacteriaceae* that produce extended-spectrum β-lactamases (49, 129) and other enzymes capable of hydrolyzing cefotaxime, ceftriaxone, ceftazidime, cefepime, and aztreonam are increasing in the United States and around the world (7, 18, 23, 78, 103, 122, 160, 253). More important are the emerging KPC-type carbapenemases that mediate resistance to imipenem, meropenem, and ertapenem (24, 27, 95, 140, 256, 262, 263). Resistance mediated by these determinants may be missed by automated methods (26). Other carbapenemases, including NmcA, Sme-1, IMI-1, and GES-2 and IMP-, VIM-, and some class D OXA-type enzymes (142, 145, 175, 185, 200, 203, 246), present in *Enterobacteriaceae, Acinetobacter* species, and *Pseudomonas* species continue to mediate resistance to imipenem and, in most cases, meropenem. Phenotypically, it can be difficult to differentiate resistance caused by a carbapenemase from resistance mediated by an AmpC-type enzyme in a strain with decreased permeability to carbapenems due to down-regulation of one or more porins (25, 102, 169). Thus, PCR assays directed to AmpC β-lactamases (163) and carbapenemase genes (Table 1) can be useful in differentiating the two mechanisms. This differentiation may be facilitated by the findings in a recent report describing a multiplex PCR method for the detection of plasmid-mediated AmpC β-lactamases (Table 2) (163) and specific inhibition of these enzymes by boronic acid compounds (47, 259).

CHLORAMPHENICOL RESISTANCE

Genes encoding chloramphenicol acetyltransferases are present in both gram-negative and gram-positive organisms and mediate resistance to chloramphenicol (199, 202). PCR primers capable of detecting the *cat* genes present in streptococci and enterococci have been described previously (232). Primers to detect the *cat*A1 and *cml*A genes, both of which mediate chloramphenicol resistance in *Salmonella* species (87, 88), have also been reported previously.

RESISTANCE TO VANCOMYCIN AND OTHER GLYCOPEPTIDES

Acquired vancomycin resistance was first noted in enterococci (117, 187, 237) but has subsequently been documented in a variety of other pathogens (14, 15, 159, 176, 177, 215), including *S. aureus* (31, 33, 248). Glycopeptide resistance is mediated by several different determinants, including *van*A, *van*B, *van*C, *van*D, *van*E, and *van*G (11, 50, 57, 68, 134, 155, 164, 196). The *van*A determinant has spread via conjugation from enterococcal donors to five independent isolates of *S. aureus* in the United States (33, 230, 248; Centers for Disease Control and Prevention, unpublished data). Molecular analysis of the transposons involved has shown differences among the first four isolates examined (40; Centers for Disease Control and Prevention, unpublished data). The first VRSA isolate from Michigan and the fourth VRSA isolate (also from Michigan) demonstrate a classic Tn*1546* structure; the others contain insertion sequences but also show deletions at the 5′ end of the transposon (40). PCR assays can differentiate the three unique *vanB* genes, designated *vanB1, vanB2,* and *vanB3* (51, 120), and the three *van*C genes, *vanC1, vanC2,* and *vanC3* (39). Subtypes of *vanD,* including *vanD1, vanD2, vanD3,* and *vanD4,* also exist, but differentiation via PCR is difficult (21, 53). PCR has been used to detect *van*A and *vanB* in enterococci in fecal samples to aid infection control efforts (156, 197). Furthermore, a multiplex PCR method has been developed for the simultaneous detection of the *van* alphabet (*vanA, vanB, vanC, vanD, vanE*, and *van*G) and identification of the most clinically relevant enterococci and staphylococci at the species level (Table 2) (59). Commercial ASRs have become available for the direct and rapid detection of *van*A and *vanB* genes in perirectal swab samples by using real-time PCR. A recent report indicated enhanced sensitivity and a significant reduction in the time to results (3.5 versus ⩾72 h) for one ASR assay compared with those for vancomycin-containing agar plates (208). False-positive results may potentially occur with any assay intended to detect *vanB* genes in enterococci due to the occasional presence of these genes in anaerobic bacteria (14, 15, 62). However, as *van* genes from these organisms could potentially be transferred to enterococci, it may still be important from the infection control perspective to identify them, although this issue remains controversial. Genotypic detection of vancomycin-intermediate *S. aureus* strains has yet to be accomplished via molecular methods.

MACROLIDE, LINCOSAMIDE, AND STREPTOGRAMIN RESISTANCE

Resistance to macrolides, lincosamides (such as clindamycin), and streptogramins can be mediated by a variety of *erm* genes including *ermA* and *ermA* (subclass *ermTR*), *ermB*, and *ermC*. On the other hand, *msrA,* which is primarily in staphylococci, mediates resistance only to macrolides and streptogramins and the macrolide efflux gene *mefA*, found in streptococci and pneumococci, confers resistance to 14- and 15-membered macrolides only (36, 70, 154, 174, 192, 254, 255). Roberts et al. (192) proposed that *mefA* and *mefE,* which show high levels of DNA homology, be considered a single class. However, others suggest that, in spite of the high degree of relatedness of the genes, a distinction between them should be maintained because of significant differences that exist between them (114). A real-time PCR assay which can distinguish *mefA* and *mefE* has been described previously (115). Pneumococci containing both *ermB* and *mefA* are being identified with increasing frequency (67). Many community-associated MRSA isolates are erythromycin resistant and clindamycin susceptible and contain *msrA* (111). PCR assays can differentiate *msrA* from *erm* genes (113, 213). Mutations in 23S rRNA and ribosomal protein L4 mutations in pneumococci have also been shown to mediate macrolide resistance and can be detected through traditional PCR or pyrosequencing assays (58, 89, 223).

Mechanisms active against both the streptogramin A and streptogramin B components of quinupristin-dalfopristin are necessary to result in a resistance phenotype. Several of the loci previously classified as *sat* genes have been reclassified as *vat* genes (93, 192, 250, 251). For example, *vatD* is the former *satA* and *vatE* is the former *satG*. Resistance to the streptogramin B component is mediated by the lactonases *vgb* and *vgbB* or the *ermB* methylase. In staphylococci, in particular, many of these genes are clustered together.

MUPIROCIN RESISTANCE

Mupirocin is an antistaphylococcal agent that is used to reduce nasal carriage of staphylococci among infected patients and hospital personnel. A PCR assay that can detect high-level mupirocin resistance has been described previously (81). However, the practical value of the assay has not been assessed in a clinical laboratory setting.

OXAZOLIDINONE RESISTANCE

Resistance to the oxazolidinone, or linezolid, among staphylococci and enterococci has been described previously (13, 234, 252, 255). Mutations in 23S rRNA, usually at position 2576, are responsible for the resistance phenotype (178). Detection of the G2576T mutations in enterococci can be accomplished by using a simple PCR assay followed by restriction fragment length polymorphism analysis of the products. Alternatively, the mutation can be detected by using pyrosequencing (205, 257).

QUINOLONE RESISTANCE

There are two major mechanisms of quinolone resistance: (i) alteration of the target sites, the organism's gyrase (*gyrA* and *gyrB)*, and topoisomerase (*par*C and *parE)* (66, 100, 224, 225, 233) and (ii) active efflux of the drug out of the cell (56, 66, 100, 162), which limits access of the drug to the target site. Resistance is usually associated with point mutations in the *gyr* or *par* loci. Since DNA probes, in most cases, are not sufficiently sensitive to detect these changes, investigators have used PCR coupled with direct sequencing of the amplification products to identify changes in the nucleotide sequences of the *gyrA, gyrB, par*C, and *parE* genes (86, 104, 109, 244). The primers, however, appear to be species specific (Table 1). Low-level fluoroquinolone resistance mediated by the novel resistance genes of the *qnr* family (100, 106, 146, 172), which are frequently plasmid carried, can be detected via PCR (101, 126, 193).

SULFONAMIDE RESISTANCE

There are three major sulfonamide resistance genes, *sul*I, *sul*II, and *sul*III. All three have been cloned and sequenced, and PCR assays for each gene have been described (85, 98, 181). Interestingly, the *sul* genes are often associated with transposable DNA elements, such as Tn*21* and small, multicopy plasmids (84, 98), that can shuttle multiple resistance genes from organism to organism. Thus, the *sul* genes can serve as indicators of multidrug resistance in gram-negative organisms.

TETRACYCLINE RESISTANCE

Tetracycline resistance is widespread among the bacterial kingdom (35, 121, 190). PCR assays have been developed for the *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(F), *tet*(H), *tet*(K), *tet*(L), *tet*(M), *tet*(N), *tet*(O), *tet*(Q), *tet*(S), *tet*(U), and *tet*(V) determinants (34, 60, 61, 71, 73, 92, 127, 130, 189, 209, 210, 265). Multiple alleles of several of these determinants exist. For example, there are six *tet*(M) alleles present in *Streptococcus pneumoniae,* designated *tet*(M)1 through *tet*(M)6, that have been differentiated through restriction analysis of PCR fragments (61). Such epidemiologic studies using genotyping of resistance determinants contribute to our understanding of resistance transfer and evolution. Primers for the *tet*(M), *tet*(O), and *tet*(Q) determinants are useful for detecting tetracycline resistance genes directly in periodontal samples (151, 152), where the presence of resistant organisms may indicate those patients who are likely to fail therapy with tetracycline (127). Multiple types of the *tet*(M) gene also have been recognized through sequence analysis (153).

TRIMETHOPRIM RESISTANCE

The number of genes capable of mediating trimethoprim resistance in bacteria continues to expand (9, 97, 98, 119, 206). DNA probes have proven to be powerful tools for detecting and classifying novel trimethoprim resistance genes (called *dhfr*) (9, 105, 179). However, because consensus sequences common to all the *dhfr* genes have not been identified, PCR primers that could simplify the detection of this family of genes have not been developed, although PCR primers for some individual genes have been developed (52, 88, 119, 218).

GUIDELINES FOR USING GENETIC TESTS

PCR assays in which the products are visualized on agarose gels are rapidly being replaced by real-time PCR assays using fluorescent resonance energy transfer probes or molecular beacons for detection of amplification products. These closed systems are more efficient and less prone to overall contamination than traditional PCR assays. The critical issue with PCR assays of any sort, however, is the reliability of results. The need for quality control measures, including controls for the presence of inhibitory substances, cannot be stressed enough, especially for detection of genes directly in clinical specimens. The temperatures used in PCR assays optimized for use with purified DNA or DNA from bacterial isolates obtained in pure culture may not be stringent enough to avoid false-positive results when used with clinical samples, such as blood and cerebrospinal fluid, where considerably more nonspecific priming can occur (227). Using control reactions containing no template DNA to identify nonspecific products due to contamination of *Taq* polymerase with DNA, such as provided by cloning vector DNA, is critical (227, 235).

One should never assume that PCR primers reported in the literature have undergone rigorous testing. Rather, primer sets should be thoroughly tested for specificity, self-complementarity, and dimer formation before use. According to the Clinical Laboratory Improvement Amendments of 1988, validation of DNA probe and PCR tests by the clinical laboratory in which they are to be used is mandatory before they can be used for analysis of clinical specimens. Methods for validation are published by the Clinical and Laboratory Standards Institute (41).

Studies to determine the reservoirs of resistance genes and routes of resistance gene transfer in hospitals and community settings are still needed. Such studies would also help to determine the frequency with which organisms carry resistance genes that are not expressed. Although still considered experimental, many of the PCR methods for detecting resistance genes described herein are already having a positive effect on guiding therapy early in the course of infection and making the treatment of infectious diseases less empiric.

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The *Manual of Clinical Microbiology* is by its nature a continuously revised work which refines and extends the contributions of previous editions. Since its first edition in 1970, many eminent scientists have contributed to this important reference work. The American Society for Microbiology and its Publications Board gratefully acknowledge the contributions of all of these generous authors over the life of this Manual.

Preface

It seems that much of my professional career has revolved around the *Manual of Clinical Microbiology*, first as an author, then as a section editor, and finally for the last four editions as the editor in chief. I have learned a great deal from this experience, although I must confess that I have forgotten far more science than I have retained. My most valuable lesson has been to rely on my fellow editors. I have been privileged to have worked on all four editions with Ellen Jo Baron and Mike Pfaller, with Jim Jorgensen for the last two editions, and with Marie Landry, who ably filled Bob Yolken's shoes, for this edition. I have also relied on the help of 29 section editors, including 7 new editors for this edition, and hundreds of authors. Almost 40% of the authors of the ninth edition of the *Manual of Clinical Microbiology* (MCM9) are new. For each edition I have purposely selected new editors and authors with the conviction that they will bring a new perspective to their assignment. I have systematically added non-U.S. authors, representing almost 30% of the authors of this edition, so that this Manual will be truly international in scope. These changes have not come without difficulties. My fellow editors and I have frequently had to curtail our authors' "creativity" for the sake of consistency, taught them that eloquence can equate to brevity, and reminded them that deadlines are not the fantasies of publishers. Many unpopular decisions are made in the creation of any work as comprehensive as MCM9, so I must thank the editors and authors for their understanding. If I have offended anyone in the process, it was unintentional.

I am very proud of what we have accomplished with MCM9, and I hope that this feeling is shared by the entire editorial board and all the authors. Although the field of clinical microbiology is undergoing dramatic changes with the confluence of traditional techniques and exciting advances in genomics and proteomics, I believe that the *Manual of Clinical Microbiology* should form the road map for our understanding of this evolving scientific discipline. I hope that the readers share the opinion of the authors and editors that MCM9 successfully meets this goal. An additional feature for the ninth edition is a CD-ROM with close to 500 illustrations from the book. It is available for purchase through ASM Press.

I would be remiss if I did not acknowledge the wisdom and guidance provided by Susan Birch, the ASM Press Production Manager whose name should more appropriately be listed on the cover of MCM9, and Jeff Holtmeier, the director of ASM Press who makes everyone's job a little easier and more pleasant. The entire staff at ASM Press was very supportive and helpful during the process of preparing this edition of the Manual.

PATRICK R. MURRAY

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VIROLOGY

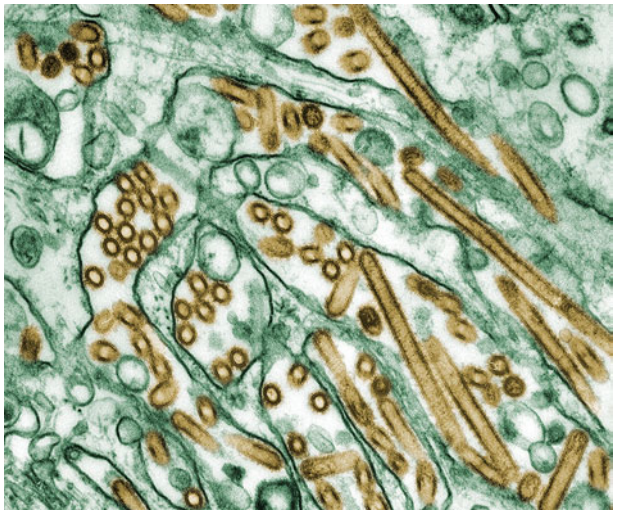
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GENERAL

Taxonomy and Classification of Viruses

CORNELIA BÜCHEN-OSMOND

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In 2007, taxonomists will celebrate the 300th anniversary of the birth of Carl von Linné, the founder of the universally accepted system of biological classification. Compared to other areas of biology, virus taxonomy is a relatively modern discipline that is still changing rapidly. Although the need for a taxonomy of viruses has been recognized for about 100 years, application of a strictly hierarchical system of classical Linnaean taxonomy, in which domains are divided into divisions, classes, orders, families, genera, and species, is difficult to apply in virology (14). Simply put, a virus is such a rapidly evolving biological entity that classification needs to be much more responsive to new information, ranging from molecular to clinical research data. Huge amounts of new DNA and RNA sequence data are reported daily on viruses associated with emerging diseases and perceived threats, reflecting viral evolution that is accelerated by horizontal gene transfer, homologous recombination, and other processes. Although viruses are a mere twig on the evolutionary tree, they seem certain to remain "the master explorers of evolutionary space" (13). Indeed, viruses have the most diverse and unusual sequence compositions of all living organisms and their genomes may represent an evolutionary shadow of ancient, long lost kingdoms of the tree of life. Moreover, the number of viruses that are pathogens or silent passengers of other organisms, from bacteria to dominant mammals, is very large. The list of viruses expands as we explore new niches for life and as detection techniques improve in sensitivity and specificity.

Today, the International Committee on Taxonomy of Viruses (ICTV) recognizes about 2,000 virus species, 650 of which infect humans and animals (10), but virologists in different fields of biology are tracking some 40,000 virus strains and isolates. The ICTV is the "international court" of experts that rules on names and relationships of all viruses but only to the level of species. Decision making in the specialist study groups of the ICTV provides the current consensus on higher-level relationships between viruses, and decisions are informed by all sorts of data derived from studies of isolates and lower-level taxa. The overarching framework of virus taxonomy that is continually updated by the ICTV thus sets an agreed-upon context for all aspects of higher-level virus nomenclature and phylogeny and plays an important role in clear communication in this sector of microbiology. An ICTV-approved virus species comprises a population of isolates and strains of closely related viruses. Individual isolates in this population may have identical or different genomic sequences, leading some researchers to subdivide such populations into "quasispecies," but this term has no taxonomic validity.

Although the taxonomy of viruses is a relatively modern discipline that is still changing rapidly under the persuasive influence of molecular biology and sequence data, the classical principles of independent differential diagnosis, based on all sorts of information, remain as important in virus taxonomy as in clinical practice. Recognizing this, the ICTV took early initiatives in electronic data management (1) to support the development of a universal virus database, ICTVdB. The database has been available on the Web since 1993 and was copyrighted in 2002. Originally designed as a tool for taxonomic research (5), ICTVdB has now evolved to serve two primary functions: (i) providing rapid access to contemporary decisions from ICTV study groups on nomenclature of higher-level virus taxa and (ii) serving as a repository for primary data at the level of isolates to facilitate decision making by ICTV study groups. These data are also made available, with linkages to sequence information, etc., as a continually updated reference source for clinical and other uses.

These functions, and the structural elements of the database, are summarized in Table 1 (http://phene.cpmc.columbia.edu/ICTVdB%20History/SFO2005.pdf). This chapter briefly outlines recent developments in virus taxonomy, describes the decimal code of classification that is the core of ICTVdB and that provides an unequivocal identifier of a virus at all taxonomic levels, and summarizes the taxonomy of viruses causing human diseases.

RECENT DEVELOPMENTS IN VIRUS TAXONOMY

The universal system of virus taxonomy devised by the ICTV now accepts Linnean-like classification at the levels of order, family, subfamily, genus, and species. The suffix *-virales* identifies an order. Families are identified by the suffix *-viridae,* subfamilies are identified by the suffix *-virinae,* and genera are identified by the suffix *-virus*. Over the last decade, the subgeneric classification of some viruses infecting humans has been drastically reorganized (9, 22). Few genera have escaped the attention of lumpers and splitters, primarily because sequence information has helped to refine earlier classifications. Recognizing the importance of distinguishing strains and isolates, the ICTV produced the hard-copy publication of its

TABLE 1 Functions and structural elements of ICTVdB[a]

Functions(s) or element(s)	Description
ICTVdB in DELTA format	Assigns unique decimal code identities to all virus taxa, clearly indicating taxonomic status and relationships (e.g., type species of 00.046.0.01, *Influenzavirus A*; 00.046.0.01.001, *Influenza A virus*); uses list of virus properties to generate descriptions of all taxa; includes 40,000-item code book to define 2,700 virus characteristics in natural-language descriptions
ICTVdB Web outputs	
Index of viruses	Catalogues ICTV approved virus taxonomy and nomenclature to species level plus additional data on isolates; linked to ICTVdB descriptions and Genbank
Virus descriptions	Describes all virus taxa down to type species, including about 1,100 plant viruses and some 2,000 isolates of human and animal viruses
Picture gallery	Presents classic EM images of virus species, reconstructions, and diagrams; linked to copyright owners and ICTVdB descriptions, including those of hosts and symptoms
Interactive data retrieval	Provides access to and interrogation of most recent ICTVdB data for virus identification via downloadable reader; linked to ICTVdB descriptions and images
To and from sequence databases	Offer linkages to NCBI, EMBL, and UniProt from ICTVdB via genome, sequence accession, and TaxID numbers (e.g., sequence V00603 and TaxID 11320); offer linkages from NCBI UniProt to ICTVdB via decimal codes (e.g., 00.046.0.01.001, *Influenza A virus*)
PC tools in DELTA	
DELTA database	World standard for data exchange in taxonomy; handles fully annotated biological information of all sorts, with data entry and handling facilitated by a network of dependencies
Natural-language translations	Provide automated translation of annotated data in ICTVdB available in rich text format (camera ready) and HTML (Web output)
Phylogenetic programs	Offer readily available tools for generating phylogenetic relationships from the DELTA data matrix of ICTVdB
Keys for virus identification	Intkey tool is an intelligent search engine that produces similarity, dissimilarity, or summary matrices on the fly for identifying and exploring relationships among virus taxa
To and from reference databases	Offer linkages to PubMed and NLM from ICTVdB; hard-copy references based on ICTVdB

[a] Abbreviations: EM, electron micrograph; NCBI, National Center for Biotechnology Information; PC, personal computer; NLM, National Library of Medicine.

Eighth Report (10) with lists of names of sequenced isolates included below the species name by using designations given by investigating scientists. These names follow laboratory conventions and do not require formal ICTV approval. Although these lower levels are not formally classified by the ICTV, they are readily accommodated in the decimal code of ICTVdB.

Criteria for Virus Classification

Historically, as electron microscopy became a more widely accessible tool in the last half of the 20th century, the morphology of the virus particle (Fig. 1) became a prime characteristic for virus classification. Although most viruses have been seen under an electron microscope, the virions (infectious particles) now tend to be better known by their chemical and genomic makeups, the complex disease symptoms in their hosts, and their vectors and geographic distributions. Virion stability (determined by varying of pHs and temperatures and exposure to lipid solvents and detergents, etc.) and virion antigenicity (determined by various serological methods) have also emerged as essential criteria for classification of viruses. Sequencing of the viral genome is now often done very early in identification protocols, even in developing countries. Although in most cases, sequencing places a virus in a specific taxonomic slot, virion morphology and serological test results remain important criteria for identifying an unknown virus (2, 6). In some recent cases (e.g., early in the identification of the virus causing severe acute respiratory syndrome), it has proven to be difficult to achieve unequivocal clinical disease diagnosis by using sequence data alone and serological tests were needed.

Characteristics Used To Describe Viruses

Today, the primary criteria used to differentiate virus orders, families, and genera are as follows:

- the type and organization of the viral genome
- the strategy of viral replication
- the structure of the virion

The species concept in viral taxonomy is predicated on the fact that viruses are "biological entities and not simply chemicals" (21), but the concept has been particularly fuzzy. Since 1991, the ICTV has accepted the definition that "a virus species is a polythetic class of viruses that constitute a replicating lineage and occupy a particular ecological niche." van Regenmortel (21) lists the following characters for discriminating among virus species:

- relatedness of genome sequence
- natural host range
- cell and tissue tropism
- pathogenicity and cytopathology
- mode of transmission
- physicochemical properties of virions
- antigenic properties of viral proteins

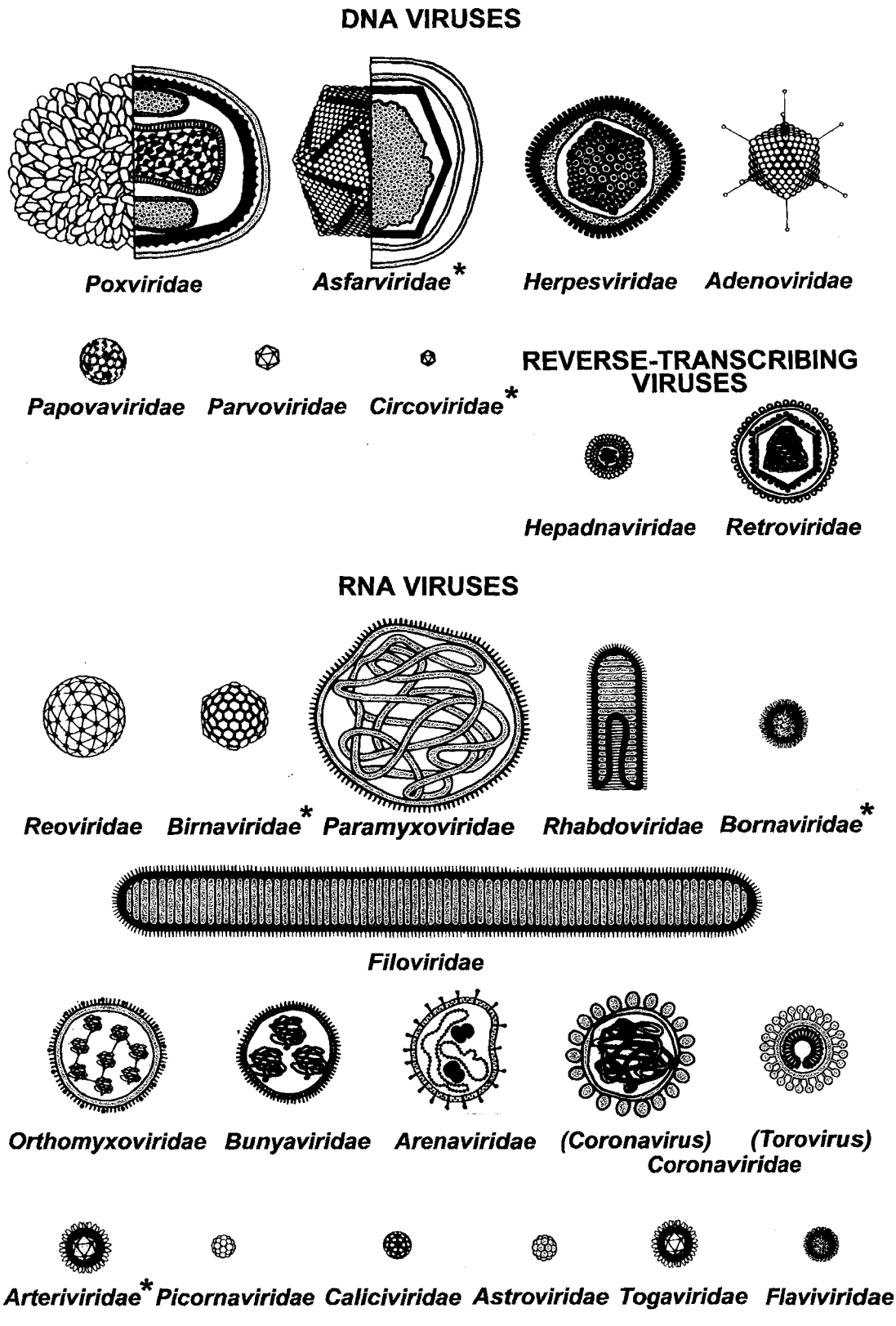


FIGURE 1 Shapes and sizes of viruses of families that include animal, zoonotic, and human pathogens. Names marked by an asterisk indicate viruses only known to infect animals.The virions are drawn to scale, but artistic license has been used in representing their structure. In some, the cross-sectional structures of capsid and envelope are shown, along with a representation of the genome; with the very small virions, only their size and symmetry are depicted.Viruses in families marked with an asterisk are not known to infect humans. (Reprinted from reference 15 with permission of Elsevier Science [USA].)

BANKING DIVERSE DATA IN ICTVdB

Developed at the Australian National University, and now based at Columbia University, ICTVdB owes much to the support of the U.S. National Science Foundation and sponsorship by the American Type Culture Collection. It was originally constructed to facilitate objective analysis of systematic and evolutionary relationships, but it can be used to generate character or distance matrices and to explore relationships by using all identified virus properties. ICTVdB has thus grown in concept and capability to become a major reference resource and research tool. Examples of this expanded functionality include the fact that the database is now up to date with the higher taxonomy in the ICTV *Eighth Report* (10) and also contains descriptions for 152 severe acute respiratory syndrome virus isolates.

Most databases deal with a relatively limited range of data attributes, such as nucleic acid or amino acid sequences, but the value of more complex (from a database perspective) text annotation is increasingly recognized. A huge range of diverse biological information has to be accommodated in ICTVdB, and all identified virus properties are covered in its character list, which uses a code to define virus characters (http://phene.cpmc.columbia.edu/chars.htm). Although only partly populated, ICTVdB already lists more than 4,000 virus descriptions (items; http://phene.cpmc.columbia.edu/ICTVdB/) constructed from about 3,000 characteristics, some of which (such as host range) have up to 2,000 options for selection (Table 2). By the time available data on virus isolates and strains are entered, the number of descriptions will be close to a million. A few of the data management issues resolved in ICTVdB are outlined here to assist user interaction with the database.

Structural Concepts in ICTVdB

The database uses the Description Language for Taxonomy (DELTA) system (8), developed at the Division of Entomology, Commonwealth Scientific and Industrial Research Organisation, Canberra, Australia, by Michael Dallwitz (7), which has now been adopted as a world standard for data exchange in taxonomy. A distinctive feature of DELTA is its capacity to store an extraordinary diversity of data and to translate these data into natural language for traditional reports and Web publication. On the input side, the capacity of DELTA to handle very large data sets one item at a time is ideally suited to a long list of virus properties (character list), often accompanied by extensive text comments and images.

Another advantage of ICTVdB is a user friendly, online data entry capability enabling peer review of new information, ranging from molecular properties of a virus to its geographic distribution and host range. Such diverse information, with intrinsic dependencies among genomic data and data on protein composition, particle structure, and infectivity, places particular demands on the flat-file system of DELTA. These have been met by building a dependency network in data specification files and by blending data from diverse sources (see below). For example, ICTVdB does not contain sequence data itself but has links to genomic and protein data banks. Conversion of ICTVdB data from DELTA into NEXUS format (an extensible file format for systematic information and construction of phylogenetic trees, commonly used by sequence databases; sequence data can be downloaded from all major sequence data banks in this format for tree analyses) was deemed essential for the comprehensive phylogenetic analyses that are widely used to monitor the evolution of viruses in relation to emerging diseases. If a database is to accept the latest data from all branches of virology and place these diverse data into contemporary taxonomic context, it will most commonly deal with information at the level of strains and isolates. Ideally, ICTVdB will serve virus taxonomy from the bottom up with primary data from researchers who describe their viruses by using rich and diverse semantics, reflecting geographic and linguistic factors. At the same time, the database must accept revisions and consolidations from the top down as the consensus in virus taxonomy reflects this new information.

Most new information in virology is generated at the molecular level and is deposited in sequence databases; however, significant events in virology tend to be associated with "host jumping," epidemics, and environmental disturbances. All of this information can be accommodated in and retrieved from ICTVdB. From the outset, DELTA was designed not only to generate identification keys but also to translate data into natural language for publication in hard copy, into hypertext markup language (HTML) format for publication on the Web, and into many other languages.

The Decimal Code in ICTVdB

The core infrastructure of ICTVdB is its distinctive table of contents, the *ICTV Index to Virus Classification,* a list of ICTV-approved and -unapproved virus names (http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/). Constructed as a taxonomic database from classifications that do not employ normal Linnean binomial nomenclature, ICTVdB has several distinctive features that are not usually used in systematics but were introduced of necessity (5). Chief among these is its decimal code (3, 4). Because virus names are changed frequently (11, 23), contain diverse linguistic and geographic elements, and are usually coupled to a disease or its symptoms, virus nomenclature (20) presents challenging semantic problems for a database.

The decimal code was introduced because the peculiar nomenclature used in virology defies direct and systematic interrogation in a database, and a decimal code (analogous to the code of enzyme nomenclature) seemed to offer a simple resolution to many problems. For example, the decimal code affords unequivocal identification of a virus to the level of strain or isolate and at the same time indicates its taxonomic context. Although the code was initially alphanumeric, new genera are now simply added numerically. The easy adaptability of the code is exemplified by its application

TABLE 2 Character list for ICTVdB[a]

Section heading	Corresponding nos.[b]
Isolate definition and isolation details	1–77
Classification	78–108
Virion properties	109–1158
Genome organization	1159–1620
Antigenicity	1621–1695
Biological properties	1696–2649
Taxonomic structure	2650–2662
Comments, references, and contributors	2663–2697

[a] Available at http://www.ncbi.nlm.nih.gov/ICTVdb/chars.htm.

[b] Characteristics are numbered in consecutive order of their logical appearance in the list. The character numbers are not unique identifiers but change when new characteristics are added to the list or the characteristics are reordered. In the example in the table, the designation of a virus isolate takes up 77 separate characters. The data defined in the characters 1 to 77 range from the isolation date (one character each for day, month, and year) to the location of the isolation to the isolation host and the person(s) and institution involved in the isolation.

TABLE 3 Decimal code applied to taxonomic revisions of *Poliovirus* (23, 24) and constructed to anticipate the explosion of lower-level data (serotypes, strains, and isolates)

Level	Extended decimal code
Order	00. = (not assigned to an order)
Family	00.05. = *Picornaviridae*
Subfamily	00.052.0. = (not assigned)
Genus	00.052.0.01. = *Enterovirus*
Serogroup	Superseded by new species concept
Species (type species)	00.052.0.01.001. = *Poliovirus*
Species	00.052.0.01.007. = *Poliovirus*
Subspecies	00.052.0.01.007.00. = (not assigned)
Serotype	00.052.0.01.007.00.001. = poliovirus 1
	00.052.0.01.007.00.002. = poliovirus 2
	00.052.0.01.007.00.003. = poliovirus 3
Strain, isolate	00.052.0.01.007.00.001.001. = PV-1[a] Mahoney
	00.052.0.01.007.00.002.001. = PV-2 Lansing
	00.052.0.01.007.00.003.001. = PV-3 Leon/37

[a] PV, poliovirus.

to the recently revised taxonomy of *Poliovirus,* in which the terms poliovirus 1, 2, and 3 now designate serotypes rather than species (Table 3) (17, 18).

Most importantly, the decimal code serves as a file name for database outputs as well as an accession number for external linkage to ICTVdB. It is used as a file name for transposing ICTVdB to the Web and also serves as a surrogate accession number used by sequence databases such as NCBI, EMBL, and UniProt to link to ICTVdB. The increasing focus on such lower-level taxonomic information and more frequent taxonomic revision requires a decimal code expanded to 19 digits that should cope with even the most ambitious splitters in the taxonomic community. Many virologists are finding the decimal code useful. The code has been adopted by *The Springer Index of Viruses* (19) and has been approved by the ICTV, but this invention is by no means universally accepted among virus taxonomists. Eventually, it is envisioned that the decimal code will be used in much the same way as sequence accession numbers and will need to be assigned and cited before publication of information leading to proposals for new taxa.

Operational Aspects of ICTVdB

One of the important operational considerations in ICTVdB is that binary statements at critical points in the character list, such as virus particle with or without envelope, are used to establish dependencies. Only the subsequently valid characters then need to be considered. These dependencies provide the internal linkage hierarchy in the data and direct the search path during interrogation; among other things, they reveal errors during data entry. The dependencies are very important for the decision-making process during identification and data comparison; some multistate characters in key positions (e.g., plant or animal virus) can control the applicability of up to 2,000 characters down the line.

Other important operational considerations in ICTVdB include the strategies developed to facilitate communication across semantic boundaries when one is dealing with data from diverse disciplines (bacteriology, agriculture, and veterinary and medical sciences), each of which has evolved a distinctive vocabulary. Although terms have been standardized within ICTVdB, these standards cannot be imposed on virologists in all disciplines and they cannot be imposed retrospectively on the literature. Therefore, semantic equivalents are given where needed and subsumed within dependencies. For example, the left-hand column of Table 4 lists character 120, which handles the semantic equivalence of tegument, inner lipid protein membrane and capsid, and head of a tailed phage. The right-hand columns of Table 4 show relevant dependencies so that character 120 option 4 or 5, for example, accesses only characters 681 to 725, handling the semantic equivalence of head and capsid. The dependencies build the internal hierarchy of the database.

Images of virus particles are used in several ways in ICTVdB. For example, text descriptions of key morphological characters become much more precise when they are linked in the character list to representative vignettes from electron micrographs. Thin-section electron micrographs of infected tissues are also used to illustrate virus infection cycles and host pathology. Descriptions of all viruses generated from ICTVdB will be enhanced by electron micrographs of the type species, irrespective of the presentation format selected. Not surprisingly, images of virus particles are among the most frequently accessed files in ICTVdB on the Web. A large image file is more instructive to users, but in the database it is functionally equivalent to numerous characters, like "virus 75–80 nm in diameter" in the case of *Rotavirus*. File size considerations and access paths dictate that image files be stored outside the main data set, in either local files or files accessed on the Internet.

The personal computer-based ICTVdB is presented as a natural-language translation on the Web by using HTML conversion for the DELTA-formatted data. This Web environment is essential for universal access, interactive data entry, interrogation, and interoperability with other databases. Currently, a plethora of accessories are available, many of which are standard components of DELTA (e.g., Web Intkey, an interactive identification program). Others, like the data entry forms, Java applets, and scripts used to display directory trees, have been developed specifically for use in ICTVdB. In the future, interoperability will be vastly improved by XML tagging. Just as it is certain that the flow of new information about viruses will not slow, it is also certain that new technology available to ICTVdB to handle these data will emerge.

TABLE 4 Semantic equivalence and dependencies among the major morphological properties of virus particles[a]

Virion or phage components	Dependent character blocks for protein annotations	
	Section heading	Available character nos.
1. An envelope <inner and outer envelope>/	Envelope	598–639
2. A surface membrane	Surface membrane	640–680
3. A tegument <inner lipid protein membrane>/	Tegument	897–918
4. A head <of phage treated as isometric capsid>/	Head	681–725
5. A capsid <including outer and inner capsid>/	Capsid (coat protein)	681–725
6. Spikes <on capsid structure>/	Spikes	726–736
7. A tail <of phage treated as elongated capsid>/	Tail	799–808
8. Fibers <at the tail>/	Fibers	820–830
9. A nucleocapsid/	Nucleocapsid	758–798
10. A core/	Core	831–875
11. A nucleoid <term for nucleocapsid in *Retroviridae*>/	Nucleoid	758–798
12. Lateral bodies/	Lateral bodies	881–904
13. A matrix/	Matrix	876–896

[a] Many viruses consist of a capsid only, whereas others are complex and are constructed from several components.

Incorporating the International Disease Code

The wisdom of retaining important "old-fashioned" taxonomic parameters, such as clinical and pathogenic properties, ecological properties, and transmission characteristics, in ICTVdB became evident during the incorporation of the International Code of Diseases, revision 10 (ICD-10), that is also based on these properties (16, 25). The importance of retaining such parameters is well illustrated by the example of viruses causing hepatitis (Table 5). If the virus infection is enteric and the primary tissue tropism of the infection targets the liver, leading to hepatitis, the disease is ranked according to severity. In ICD-10, the letters A and B are reserved for certain infectious and parasitic diseases. All viral hepatitis diseases in which the primary infection is restricted to the liver tissue are found in the section B15 to B19. For example, acute hepatitis A has been assigned the code B15, whereas B16 is reserved for acute forms of hepatitis B. B18 is used only for chronic forms of hepatitis. The digit after the decimal point is used to classify the form and severity of the disease. B15.0 and B16.0 are used to describe severe forms of hepatitis that have developed into a coma hepaticum; B15.9 and B16.9, on the other hand, refer to less severe forms of hepatitis, which are not accompanied by a coma hepaticum. If the virus infection is not enteric and the primary target tissue is not the liver, the disease is coded according to other criteria even though the infection may eventually result in a liver disease.

At the same time, the rapid growth in sequence information has to be woven into the above-described fabric. This becomes evident in comparisons of *Dengue fever virus* and *West Nile virus*, two clinically important species in the genus *Flavivirus*. The long-recognized *Dengue fever virus* has a single mosquito vector, infects only humans, has shown significant change in its geographical distribution, and shows only small sequence divergence in the isolate population that comprises the four serotypes of this species. However, these small differences in sequences among serotypes are critical determinants of the disease outcome (24). In evolutionary terms, the dengue virus may represent a "mature" virus species. On the other hand, the recent rapid reemergence and rapid extension of the range of *West Nile virus* in North America present a more complex situation. *West Nile virus* has several mosquito vector species and infects a large variety of avian and mammalian hosts. The sequences of isolates of this virus species show broad variability among and within vectors, but less sequence variability exists among isolates collected from birds or mammals. Thus far, it seems that the extensive sequence variability in this virus species is not clearly correlated with differential disease outcomes in vertebrate hosts (12). The challenges in virus taxonomy presented by this comparison are substantial and cannot be resolved by sequence comparisons alone. However, it is believed that the split functions of ICTVdB are adequate to accommodate information flow and decision-making processes in the future taxonomy of *West Nile virus*.

CURRENT TAXONOMY OF VIRUSES INFECTING HUMANS

The most recent taxonomic decisions (10, 11) have been incorporated into the *ICTVdB Index to Virus Classification* (http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/) and the nomenclature of the 23 families of viruses that infect humans is shown in Table 6. The table summarizes the important characteristics for differentiation of these viruses, listed according to the accepted taxonomic order based on the nature of the genome. As the designation of diseases, signs, and symptoms becomes more standardized throughout the world through the use of ICD-10, it will be a relatively simple matter to expand ICTVdB to include these data. Table 7 illustrates a comprehensive current reconciliation of data from ICTVdB and ICD-10 (16; ICD-10 Diagnosenthesaurus, version 3.0) for one virus family. The full list for other families is available within ICTVdB on the Web (http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/ICD-10.htm).

The construction, maintenance, and population of ICTVdB continues to owe much to support from members of the Executive Committee and Data Sub-Committee of the ICTV, currently and especially Andy Ball, Lois Blaine, John Mackenzie, and Tony Della-Porta. Eric Zurcher and Mithun Harkara have provided technical support for the infrastructure of ICTVdB. Aljoscha Brandt helped with the design of Table 1 and with comments on the manuscript.

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TABLE 5 Continuing importance of clinical properties, symptoms, and disease designations (as coded in ICD-10) in the taxonomy of viruses causing hepatitis

Virus name	Signs and symptoms	ICD-10 code	ICD disease description
Hepadnaviridae			
Orthohepadnavirus			
Hepatitis B virus (HBV)	Acute hepatitis which may progress to chronic hepatitis, liver cirrhosis, and primary hepatocellular carcinoma	B16.2	Acute hepatitis B without *Deltavirus* with hepatic coma
		B16.9	Acute hepatitis B without *Deltavirus* without hepatic coma
		B18.1	Chronic hepatitis B without *Deltavirus*
Hepatitis B virus and *Hepatitis delta virus*	Acute hepatitis which may progress to chronic hepatitis with superinfection with *Deltavirus*	B16.1	Acute hepatitis B with *Deltavirus* without hepatic coma
		B16.0	Acute hepatitis B with *Deltavirus* with hepatic coma
		B18.0	Chronic hepatitis B with *Deltavirus*
Picornaviridae	Acute hepatitis	B15.0	Hepatitis A with hepatic coma
Hepatovirus		B15.9	Hepatitis A without hepatic coma
Hepatitis A virus (HAV)			
Not assigned to a family	Acute hepatitis	B17.1	Acute hepatitis E
Hepevirus			
Hepatitis E virus (HEV)			
Flaviviridae	Acute hepatitis	B17.1	Acute hepatitis C
Hepacivirus		B18.2	Chronic viral hepatitis C
Hepatitis C virus			
GB virus B (unassigned hepacivirus)	Acute and chronic hepatitis	B17.8	Other acute viral hepatitis
		BB.8	Other chronic viral hepatitis
Unassigned flaviviruses	Acute and chronic hepatitis	B17.8	Other acute viral hepatitis
GB virus A (GB virus A-like agents)		B18.8	Other chronic viral hepatitis
GB virus C (Hepatitis G virus 1)	Acute and chronic hepatitis	B17.8	Other acute viral hepatitis
		B18.8	Other chronic viral hepatitis
Subviral agents unassigned to a virus family	Acute and chronic hepatitis	B16.0	Acute hepatitis B with *Deltavirus* (coinfection) with hepatic coma
Deltavirus			
Hepatitis delta virus		B16.1	Acute hepatitis B with *Deltavirus* (coinfection) without hepatic coma
		B17.0	Acute *Deltavirus* (super) infection of hepatitis B carrier
		B18.0	Chronic viral hepatitis B with *Deltavirus*
Herpesviridae	Cytomegalovirus mononucleosis, infectious mononucleosis	B25.0 (K77.0)	Pneumonitis
Betaherpesvirinae		B25.1 (J17.1)	Hepatitis
Human herpesvirus 5 (HHV-5)		B25.2 (K87.1)	Pancreatitis
		B25.8	Other cytomegalovirus diseases
		B25.9	Disease, unspecified
		B27.0	Gammaherpesvirus mononucleosis
		B27.1	Mononucleosis
Flaviviridae	Hepatitis, fever	A95.0	Sylvatic yellow fever
Flavivirus		A95.1	Urban yellow fever
Yellow fever virus (YFV)		A95.9	Yellow fever, unspecified

TABLE 6 Summary of important characteristics used to differentiate families (and some genera) of viruses infecting humans

Classification		Virion properties						
		Virion					Genome	
Code and group	Virus (disease[s])	Envelope	Shape	Size (nm)	Nucleocapsid symmetry	Nature[a]	Structure	Size (kb or kbp)
00.058. *Poxviridae*	Smallpox virus, monkeypox virus, molluscum contagiosum virus	±	Brick shaped or oval	250 × 200 × 200	Complex	dsDNA, linear	Monopartite, inverted terminal repeats, both ends covalently closed	130–375
00.031. *Herpesviridae*	Herpes simplex virus (chickenpox, shingles, zoster), cytomegalovirus (mononucleosis) Epstein-Barr virus(kissing disease), Kaposi's sarcoma-associated herpesvirus	+	Spherical	150	Icosahedral	dsDNA, linear	Monopartite, terminal and internal reiterated repeats, forming two covalently linked components, making two or four isomeric forms	125–235
00.001. *Adenoviridae*	Human adenovirus A to F (enteric infections, diarrhea, respiratory infections)	−	Isometric	70–90	Icosahedral	dsDNA, linear	Monopartite, inverted terminal repeats, ends covalently linked protein	28–45
00.099. *Papillomaviridae*	More than 100 human papillomaviruses (warts and tumors)	−	Isometric	55	Icosahedral	dsDNA, circular	Monopartite, strands circular supercoiled	6.8–8.4
00.047. *Polyomaviridae*		−	Isometric	40	Icosahedral	dsDNA, circular	Monopartite, strands circular supercoiled	4.7–5.3
00.050. *Parvoviridae*	B19 virus (exanthema in children) Human bocavirus (respiratory infections)	−	Isometric	25	Icosahedral	ssDNA, (−), linear	Monopartite, population mainly (−), same (+); sequence with palindromic ends that allow circularization during replication	5
00.030. *Hepadnaviridae*	Hepatitis B virus (hepatitis)	+	Spherical	30–34	Icosahedral	dsDNA, circular	Monopartite, circular dsDNA with regions of single-stranded DNA	3.2
00.061. *Retroviridae*	Human immunodeficiency virus 1 and 2 (AIDS)	+	Spherical	80–100	Icosahedral	ssRNA, (+), linear	Monopartite, diploid, each 5′ end H bonded, 5′ end capped, 3′ end poly(A)	7–11
00.060. *Reoviridae*	Reovirus (respiratory and enteric infections), rotavirus A and B (diarrhea, enteric infections)	−	Isometric	60–80	Icosahedral	dsRNA, linear	10–12 segments, depending on genus	16–27
01.025. *Filoviridae*	Ebola virus, Marburg virus (hemorrhagic fever)	+	Filamentous, pleomorphic	790–970 × 80	Helical	ssRNA, (−), linear	Monopartite	19.1
01.048. *Paramyxoviridae*	Parainfluenza virus, mumps virus, measles virus, human respiratory syncytial virus, hendravirus, human metapneumovirus	+	Pleomorphic	150–300	Helical	ssRNA, (−), linear	Monopartite	18–20
01.062. *Rhabdoviridae*	Rabies virus	+	Bullet shaped	180 × 75	Helical linear	ssRNA, (−),	Monopartite	13–16

00.046. *Orthomyxoviridae*	Influenza virus types A to C	+	Pleomorphic		Helical	ssRNA, (−), linear	6–8 segments, depending on genus	10–13.6
00.011. *Bunyaviridae*	California encephalitis virus, La Crosse virus, Hantaan virus, Sin Nombre virus, Crimean-Congo hemorrhagic fever virus	+	Spherical, pleomorphic	80–120	Helical	ssRNA, (−), linear	3 segments, negative- or ambisense with sticky ends that allow circularization during replication	11–12
00.003. *Arenaviridae*	Lassa virus, lymphocytic choriomeningitis virus, Guanarito virus, Junin virus, Machupo virus, Sabia virus	+	Spherical	110–130	Helical	ssRNA, (−), circular	2 segments, negative- or ambisense with sticky ends that allow circularization during replication	10–14
03.019. *Coronaviridae*	Human coronavirus (respiratory and gastrointestinal infections), severe acute respiratory syndrome coronavirus	+	Spherical, pleomorphic	80–220	Helical	ssRNA, (+), linear	Monopartite, nested set of transcription, 3′ end poly(A), 5′ end capped	20–30
00.052. *Picornaviridae*	Human enterovirus types A to D, poliovirus, rhinovirus types A to B, hepatitis A virus, parechovirus (human echovirus)	−	Isometric	28–30	Icosahedral	ssRNA, (+), linear	Monopartite, 3′ end poly(A), 5′ end covalently linked protein (VPg)	7.2–8.4
00.012. *Caliciviridae*	Norwalk virus, Sapporo virus	−	Isometric	35–39	Icosahedral	ssRNA, (+), linear	Monopartite, 3′ end poly(A), 5′ end covalently linked protein (VPg)	7.4–7.7
00.084.0.01. *Hepevirus*	Hepatitis E virus (gastroenteritis and hepatitus)	−	Isometric	27–34	Icosahedral	ssRNA, (+), linear	Monopartite, 3′ end poly(A), 5′ end covalently linked protein (VPg)	7.2
00.005. *Astroviridae*	Human astrovirus (gastroenteric and enteric infections)	−	Isometric	27–30	Icosahedral	ssRNA, (+), linear	Monopartite, 3′ end poly(A), 5′ end not known	7–8
00.073. *Togaviridae*	Ross River virus, Chikungunya virus, O'nyong-nyong virus, rubella virus	+	Spherical	70	Icosahedral	ssRNA, (+), linear	Monopartite, 3′ end poly(A), 5′ end capped	9.7–11.8
00.026. *Flaviviridae*	Tick-borne encephalitis virus, dengue virus, Japanese encephalitis virus, Murray Valley virus, St. Louis encephalitis virus, West Nile virus, hepatitis C virus, hepatitis G virus, hepatitis GB virus	+	Spherical	45–60	Icosahedral	ssRNA, (+), linear	Monopartite, *Flavivirus* 5′ end capped, *Pestivirus* and *Hepacivirus* 5′ ends unknown	9.5–12.5
00.022.0.01. *Deltavirus*	Hepatitis delta virus (aggravates hepatitis B virus infection)	+	Spherical	36–43	Helical?	ssRNA, (−), circular	Monopartite, transcription similar to viroids, requires *Hepadnaviridae* for replication (similar to satellite viruses)	1.7
90.001. Prions	(Creutzfeldt-Jakob disease, kuru, Gerstmann-Straussler-Schenker syndrome, fatal familial insomnia)	NA[b]	Rods	Protein PrP^{SC}	NA	NA	No nucleic acid, self-replicating infectious prion protein (PrP) with a molecular weight (M_r) of 33,000–35,000	NA

[a] dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; dsRNA, double-stranded RNA; ssRNA, single-stranded RNA; (+), positive stranded; (−), negative stranded.
[b] NA, not applicable.

TABLE 7 Reconciliation of comprehensive, current taxonomy from ICTVdB with transmission, symptom, and disease designations from ICD-10 and important fact sheets on the Web[a]

Virus code and name	Transmission	Signs and symptoms	ICD-10 code and URL to important disease fact sheets
01. Mononegavirales[b]			
01.025. *Filoviridae*	Biosafety level 4		http://www.cdc.gov/ncidod/dvrd/spb/mnpages/dispages/filoviruses.htm
01.025.0.01. *Marburg virus* (MARV)			http://www.cdc.gov/ncidod/dvrd/spb/mnpages/dispages/marburg.htm
01.025.0.01.001. *Lake Victoria Marburg virus*	Direct contact with blood or body fluids; droplet and aerosol infection may occur	Hemorrhagic fever	A98.3 Marburg virus disease
01.025.0.02. *Ebola virus*	As above		http://www.cdc.gov/ncidod/dvrd/spb/mnpages/dispages/ebola.htm
01.025.0.02.005. *Cote d'Ivoire Ebola virus* (CIEBOV)	Direct contact	Hemorrhagic fever	A98.4 Ebola virus disease
01.025.0.02.002. *Reston Ebola virus* (REBOV)	Direct contact	Hemorrhagic fever	A98.4 Ebola virus disease
01.025.0.02.003. *Sudan Ebola virus* (SEBOV)	Direct contact	Hemorrhagic fever	A98.4 Ebola virus disease
01.025.0.02.004. *Zaire Ebola virus* (ZEBOV)	Direct contact	Hemorrhagic fever	A98.4 Ebola virus disease

[a]The full length of Table 7 can be accessed at http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/fCD-10.htm.
[b]Order for viruses with a negative-stranded RNA genome, such as *Filoviridae*.

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Specimen Collection, Transport, and Processing: Virology

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In recent years, there have been many advances in diagnostic virology including improvements in cell culture and the development of viral antigen (AG) and nucleic acid (NA) detection assays. The increased sensitivity and decreased turnaround time of these macromolecule detection tests have greatly enhanced the clinical utility of viral diagnostics. Successful laboratory diagnosis of viral infections requires appropriate selection, collection, transport, and processing of a specimen, regardless of the method chosen. Maintenance of specimen quality throughout testing is essential for diagnosing viral infections and ensuring appropriate patient management. Specific laboratory policies must therefore be formulated and enforced. Here we briefly review methods currently employed to diagnose viral infections (synopsized in Table 1) and describe methods for procuring, transporting, and processing specimens to be tested in the clinical laboratory.

SPECIMEN SELECTION, COLLECTION, AND PROCESSING

General Guidelines

Detection of viral pathogens is highly dependent on three variables: (i) obtaining the appropriate specimens, (ii) the timing of specimen collection, and (iii) effective and timely processing. Diagnosis of a viral etiology of an active disease process usually requires collection of a specimen from the affected organ, since this is usually where the highest levels of virus replication occur. Clinical symptoms often identify the target organ(s) involved and help to determine the appropriate specimen(s) to collect. An understanding of viral pathogenesis is helpful in determining the specimen(s) most appropriate for testing. Table 2 lists clinical specimens from which viruses may be detected.

Specimen Collection

The timing of collection in relation to onset of symptoms is of utmost importance in detecting viral pathogens. Since viral titers decrease after the acute phase of illness, selecting specimens with as many viral particles, viral antigens, or viral nucleic acid molecules as possible will improve the laboratory's ability to make a diagnosis. For many virus infections, viral shedding begins before the onset of symptoms and disappears when symptoms resolve. However, the duration of viral shedding depends on the type of virus and the organ or system involved (21). In addition, the immunocompetence of the patient has a significant effect on the time and duration of virus shedding (22, 36). For optimal recovery of most viruses, specimens should be collected within 1 to 3 days of symptom onset. Specimen collection is guided by the test method that will be used to obtain a diagnosis (14). Methods of collection can have a profound effect on recovery rates. Variables such as swab composition and anticoagulants can affect virus recovery and NA detection (2, 23, 61). Specimens should therefore be collected only with validated devices and containers. The use of viral transport medium (VTM) during specimen collection is highly dependent on specimen source. A list of specimens that do and do not require VTM is shown in Table 3. Clinical and epidemiologic data on each patient should be provided when specimens are submitted to the laboratory. Information such as patient history, immune status, time of year, travel, age, and viruses suspected may help the laboratory choose the most suitable diagnostic assay(s) and may therefore improve virus detection (34).

Acceptance Criteria for Specimens

The virology laboratory is responsible for establishing criteria for acceptable specimens and guidelines for rejecting specimens that do not meet these criteria. Upon arrival in the laboratory, the specimen and requisition should be examined to ensure that all criteria for acceptance have been fulfilled. Many institutions use electronic order-entry systems at the site of specimen collection rather than requisitions. In these cases, data in the laboratory information system must be cross-checked for compliance with established guidelines when the specimen reaches the laboratory. Specimens should be rejected if (i) they are unlabeled or mislabeled, (ii) improper specimen collection devices or containers are utilized, (iii) specimen containers are broken and/or leaking, (iv) transport time has exceeded maximum limits, or (v) duplicate specimens have been collected within a short time period. Important information that should be provided with each specimen includes patient data (name, hospital number, sex, birth date, and location), ordering physician, the specific site(s) from which the specimen(s) were collected, specific viruses suspected, time and date of specimen collection, and specific diagnostic tests requested. The physician of record must be contacted immediately regarding rejected specimens or incomplete patient information. Although failure to detect

TABLE 1 Methods used for detection of viruses

Source	Disease	Viruses[a]	Detection method[b]
Cardiac	Myocarditis, pericarditis	Adenovirus	Culture, histology, NA
		Enteroviruses	Culture, NA
		Influenza viruses	Culture, serology
CNS	Encephalitis	Arboviruses	Serology, NA
		CMV	NA
		EBV	NA
		Hemorrhagic fever viruses[c]	Culture, EM, IA, serology
		HSV	NA
		HIV	Histology, NA
		Measles virus	NA
		Mumps virus	Culture, NA
		Rabies virus	Histology, IA
		VZV	NA
	Meningitis	Arboviruses	Serology, NA
		Enteroviruses	NA, culture
		HSV	NA
		LCMV	Serology
		Mumps virus	Culture, NA
	Progressive multifocal leukoencephalopathy	JCV	Histology, NA
Cutaneous	Maculopapular rash	Adenoviruses	Culture
		Enteroviruses	Culture, NA
		HHV-6	NA
		Measles virus	Culture, serology
		Parvovirus B19	NA, serology
		Rubella virus	Culture, serology
	Vesicular rash	Enteroviruses	Culture, NA
		HSV	Culture, IA, NA
		Poxviruses[c]	Culture, EM, histology
		VZV	IA, culture, NA
Fetal/newborn	Congenital/perinatal infections	CMV	Culture, NA
		HBV	NA, serology
		HIV	NA, serology
		Parvovirus B19	NA, serology
		Rubella virus	Culture, serology
Gastrointestinal	Diarrhea	Adenovirus 40, 41	IA, NA
		Astrovirus	NA
		CMV	Culture, histology, NA
		Norovirus	NA
		Rotavirus	IA
	Hepatitis	CMV	Culture, histology, NA
		EBV	NA, serology
		HAV	Serology
		HBV	IA, NA, serology
		HCV	NA, serology
		HDV	Serology
		HEV	Serology
		Parvovirus B19	Histology, NA, serology
Hematopoietic	Lymphoid disorders[d]	EBV	Histology, NA
		HIV	Serology, NA
		HTLV-1	Serology, NA

(Continued on next page)

TABLE 1 Methods used for detection of viruses *(Continued)*

Source	Disease	Viruses[a]	Detection method[b]
	Red blood cell disorders	Parvovirus B19	NA, serology
Ocular	Chorioretinitis	CMV	NA
		HSV	NA
		VZV	NA
	Conjunctivitis	Adenoviruses	Culture, IA, NA
		Enteroviruses	Culture, NA
	Keratoconjunctivitis	Adenoviruses	Culture, IA, NA
		HSV	Culture, IA, NA
		VZV	Culture, IA, NA
Respiratory	Bronchiolitis	Human coronaviruses	NA, serology
		Human metapneumovirus	NA, culture
		Influenza viruses	Culture, EA, IA, NA
		Parainfluenza viruses	Culture, IA, NA
		RSV	Culture, IA, NA
	Croup	Human metapneumovirus	NA, culture
		Parainfluenza viruses	Culture, IA, NA
		RSV	Culture, IA, NA
	Pharyngitis	Adenoviruses	Culture, IA, NA
		EBV	Serology
		Enteroviruses	Culture, NA
		HSV	Culture, NA
	Pneumonia	Adenoviruses	Culture, IA, NA
		CMV	Culture, histology, IA, NA
		Hantavirus	EM, NA, serology
		Human coronaviruses	NA, serology
		Human metapneumovirus	NA, culture
		Influenza viruses	Culture, EA, IA, NA
		Parainfluenza viruses	Culture, IA, NA
		RSV	Culture, IA, NA
		VZV	Culture
	Rhinitis, coryza	Adenoviruses	Culture, IA, NA
		Enteroviruses	Culture, NA
		Human coronaviruses	NA, serology
		Human metapneumovirus	NA, culture
		Influenza viruses	Culture, EA, IA, NA
		Parainfluenza viruses	Culture, IA, NA
		Rhinoviruses	Culture
		RSV	Culture, IA, NA
Urogenital	Hemorrhagic cystitis	Adenovirus (type 11)	Culture, NA
		BK virus	NA, cytology
	Urethritis, genital herpes	HSV	Culture, IA, NA
		VZV	Culture, IA, NA
	Genital warts, carcinoma	HPV	Histology, NA
	Molluscum contagiosum	Molluscum contagiosum virus	Histology
	Cervicitis	Adenoviruses	Culture
		HSV	Culture, NA

[a] Refer to specific chapters for details on individual viruses; only major viral pathogens, not including rare causes of disease, are listed. LCMV, lymphocytic choriomeningitis virus; HAV, hepatitis A virus; HDV, hepatitis D virus; HEV, hepatitis E virus.

[b] Most common detection methods used. NA assays include hybrid capture, nucleic acid sequence-based assay, and PCR; IA, immunoassay (includes immunofluorescent antibody, enzyme-linked immunosorbent assay, and immunochromatographic tests); EA, enzyme (neuraminidase) assay; EM, electron microscopy.

[c] Hemorrhagic fever and smallpox viruses processing requires BSL 4 facilities.

[d] Includes posttransplant lymphomas, immunoblastic B-cell lymphomas, and adult T-cell leukemia/lymphoma.

TABLE 2 Specimens for virus detection

Virus	Bone marrow	Blood[a]	CSF	Feces	Respiratory specimens[b]	Skin	Genital specimens	Saliva	Urine	Eye[c]	Tissue	Amniotic fluid	Pericardial fluid
Adenoviruses		+	+	+	+		+		+	+	+		
Arboviruses		+	+								+		
Arenaviruses		+	+		+				+				
Astrovirus				+									
Calicivirus				+									
Coronavirus		+		+	+						+		
Cytomegalovirus	+	+	+	+	+		+	+	+	+	+	+	
Enteroviruses		+	+	+	+	+			+	+	+		+
Epstein-Barr virus		+	+								+		
Filoviruses		+			+				+				
Hepatitis A virus		+		+									
Hepatitis B virus[d]		+											
Hepatitis C virus		+											
Hepatitis D virus		+											
Hepatitis E virus		+		+									
Herpes simplex virus		+	+	+[e]	+	+	+			+	+	+	
Human herpesvirus 6	+	+	+								+		
Human herpesvirus 8		+				+		+					
Human metapneumovirus					+						+		
Influenza viruses					+						+		
Measles virus		+			+				+		+		
Mumps virus			+					+	+		+		
Papillomaviruses							+				+		
Parainfluenza viruses					+						+		
Parvovirus B19	+	+									+	+	
Polyomaviruses			+						+		+		
Poxviruses						+		+					
Rabies virus		+	+			+		+			+		
Respiratory syncytial virus					+						+		
Retroviruses		+	+		+		+	+			+		
Rhinoviruses					+						+		
Rotaviruses				+									
Rubella virus		+		+[e]	+				+		+	+	
Varicella-zoster virus	+	+	+		+	+				+	+		

[a] Includes direct virus detection tests such as culture, antigen detection, and nucleic acid detection. Serology is considered separately (see Table 4).
[b] Includes throat and nasal swabs, nasal wash, nasopharyngeal aspirate, and bronchoalveolar lavage specimens.
[c] Includes conjunctiva, cornea, and aqueous and vitreous fluids.
[d] Similar specimen types for distantly related GBV-C (also referred to as "hepatitis G virus").
[e] Neonatal infections.

a virus may occur as a consequence of inappropriate specimen handling, there are instances when a given specimen must be processed even though quality has been compromised (if specimens were collected by surgical procedures, the patient is no longer accessible, or the sample is unique and cannot be re-collected).

Safety Issues during Processing

Specimens should be processed in a biological safety cabinet to protect laboratory personnel from laboratory-acquired infection, to protect the specimen from environmental contamination, and to prevent cross-contamination between samples. Most viruses encountered in the diagnostic virology laboratory are classified as biosafety level 2 (BSL 2) agents. This chapter does not discuss BSL 3 and BSL 4 viruses such as arboviruses, arenaviruses, filoviruses, smallpox, and rabies, which are associated with specific clinical histories and presentations and are diagnosed by laboratories specifically designed to work with these agents. However, at times laboratory workers may unknowingly work with specimens that contain BSL 3 and BSL 4 viruses. All specimens should be handled with Standard Precautions (previously termed Universal Precautions), and specific procedures should be in place when the laboratory is requested to handle BSL 3 and 4 level agents. Of note, NA extraction reagents TRIzol and AVL Buffer have been shown to render alphaviruses,

TABLE 3 VTM use for viral specimens

Specimens that should be collected in VTM	Specimens that should be collected in containers without VTM
Respiratory other than bronchoalveolar lavage and nasal wash fluids	Amniotic fluid
Swabs	Blood
Tissue	Bone marrow
	Bronchoalveolar lavage fluid
	CSF
	Ocular fluid (vitreous and aqueous)
	Pericardial fluid
	Pleural fluid
	Urine

flaviviruses, filoviruses, and a bunyavirus noninfectious (4). Poxviruses are inactivated by methods commonly used in the manufacture of plasma-derived products such as pasteurization at 60°C and solvent/detergent treatments (48). Many nucleic acid extraction methods employ both heat and detergents during lysis; however, studies to determine their effect on poxvirus infectivity have not been described.

Samples from patients with transmissible spongiform encephalopathies with the highest infectivity include brain, spinal cord, and eye. Cerebrospinal fluid (CSF) and other tissue may also be infectious. Standard precautions should be followed when working with these samples. Specific decontamination procedures for residual sample and waste must be followed (65).

General Processing Procedures

Upon receipt in the laboratory, the specimen should be vortexed to break up aggregates. Glass beads in the VTM help with this process (26). For virus isolation, grossly contaminated or potentially toxic specimens, such as minced or ground tissue, can be centrifuged, and the virus-containing supernatant can be used as inoculum. If the specimen volume is insufficient for all tests ordered, the ordering physician should be contacted to set priorities. The specimen should be diluted with VTM only if absolutely necessary. For specific test requests, additional specimens may be retrieved from other laboratories with the caveat that contamination from inappropriate handling can affect both culture and NA testing. Excess specimen should be saved at −70° in case additional tests are needed or repeat testing is required.

Collection and Processing of Selected Specimens

Blood

Viremia is often associated with the acute phase of a viral disease and may precede the appearance of symptoms. For example, parvovirus B19 is found in extremely high concentrations in the blood during acute infection and detection of cytomegalovirus (CMV) in blood is predictive of CMV disease in bone marrow and solid organ transplant recipients (43). Early detection of CMV in blood allows for preemptive treatment to prevent CMV disease (17). Detection of BK virus in plasma is predictive of nephropathy in renal transplant patients (41). Detection of adenovirus in blood serves as a marker of dissemination with multiorgan involvement (12). Viremia may also occur in chronic stages of diseases such as with human immunodeficiency virus (HIV), hepatitis B virus (HBV), and hepatitis C virus (HCV).

Blood samples are collected in a 3- to 5-ml vacuum tube collection system (BD Vacutainer tubes; BD, Franklin Lakes, N.J.). Anticoagulant tubes are required when detection of viruses in plasma or leukocyte fractions is required. The most commonly used anticoagulants are EDTA (purple-top), heparin (green-top), and acid-citrate-dextrose (ACD, yellow-top). Collection of blood specimens in EDTA may be preferable over heparin since the latter anticoagulant inhibits the PCR and the infectivity of HIV-1 (2, 23).

For serologic diagnosis, an acute-phase serum specimen should be obtained within the first few days of illness, and a convalescent-phase specimen should be obtained 2 to 4 weeks later. If a virus-specific immunoglobulin M (IgM) assay is available, an acute-phase specimen may be sufficient. The timing of serum collection and the type of antibody detected vary for specific viruses (Table 4).

Blood is a suitable specimen for many different assays in the virology laboratory. It should therefore be processed following the specific instructions dictated by the chosen assay method. A variety of commercial NA assays that detect and quantify viruses in blood are available. Whole blood used for NA amplification must be processed to remove inhibitors of DNA polymerases such as heme and metabolic precursors of heme. Many different extraction protocols are used in both commercial and laboratory-developed amplification tests to

TABLE 4 Serology as a primary diagnostic tool: relevant viruses and timing of serum collection[a]

Antibody testing at presentation	Antibody testing 4–10 days after presentation	Convalescent-phase antibody (2–4 weeks after presentation)[b]
EBV (IgM)	HEV (IgM)	Arboviruses (IgG)
Hantavirus (IgM, IgG)	Arboviruses (IgM)[c]	Human coronaviruses (SARS CoV)
HAV (IgM)		Measles
HBV (IgM, IgG)		Mumps
HCV (IgG)		Rubella
HDV (IgM, IgG)		
HIV (IgG)		
HTLV (IgG)		
LCMV (IgM)		
Parvovirus B19 (IgM)		

[a] HDV, hepatitis D virus; HTLV, human T-cell leukemia virus; LCMV, lymphocytic choriomeningitis virus; SARS CoV, severe acute respiratory syndrome coronavirus; HAV, hepatitis A virus.
[b] Includes IgG and total antibody (IgG and IgM).
[c] CSF IgM also useful.

remove inhibitors. Whole blood is used in the NA sequence-based Nuclisens assay (bioMerieux, Hazelwood, Mass.) for detection of CMV pp67 mRNA and in the CMV Hybrid Capture assay (Digene, Silver Spring, Md.). In these commercial assays, nucleic acids are isolated using proprietary lysis buffers and, in the case of the Nuclisens assay, are extracted by the Boom extraction method (6).

Plasma is used for qualitative and quantitative detection of viral NAs. Plasma is obtained by centrifuging blood collected in tubes containing anticoagulant (EDTA) at 1,500 × *g* for 20 min at 25°C. Generally, the plasma supernatant is decanted into a plastic tube or vial. A newer tube system (plasma partitioning tubes [PPT]; BD) has a gel barrier that results in physical separation of plasma and cellular constituents and obviates the need for decanting plasma. These tubes decrease the incidence of errors that occur when multiple specimens are being processed simultaneously (e.g., plasma is decanted into a tube labeled with another patient's name). PPTs have been shown to maintain stability of HIV during transport at 4°C (24). Freezing PPTs is not recommended since it leads to increased levels of RNA (20, 52). Therefore, routine testing of plasma from frozen PPTs should not be undertaken unless test performance has been verified with this processing method. Special care should be taken to adhere to blood collection and processing requirements specified by the manufacturer, since deviation may affect assay performance. Plasma for Versant (bDNA) HCV and HIV RNA assays and the Versant (TMA) HCV RNA qualitative assay (Bayer Diagnostics, Berkeley, Calif.) should be separated within 4 h of collection. Plasma to be tested with the Roche Amplicor CMV, HBV, HCV, and HIV Monitor tests, HBV and HCV Taqman tests, and the HCV qualitative test (Roche Molecular Systems, Inc., Pleasanton, Calif.) must be separated within 6 h and refrigerated (4°C) for up to 72 h or frozen (−70°C) for longer periods to prevent viral genome degradation. Repeated freeze-thaw cycles should be avoided for all assays.

Serum is used for serologic testing, for culture (enteroviruses and some arboviruses), and for NA assays to detect parvovirus B19, enteroviruses, HCV, HBV, and BKV. Serum specimens are separated from clotted blood by centrifugation at 1,000 × *g* for 10 min and refrigerated (4°C) for short-term storage (24 to 48 h) or frozen (<−20°C) for longer periods. Barrier tubes similar to PPTs are also available to separate serum from other blood constituents (SST; BD Diagnostic Systems).

Leukocytes can be fractionated from whole blood, and they are particularly useful in the diagnosis of CMV infection. Isolated leukocytes can be cocultured with human diploid fibroblast cells (conventional or shell vial culture [55]), directly stained with fluorescent-labeled antibody to detect the viral protein pp65 in leukocyte nuclei (the so-called antigenemia assay [59]), or extracted to detect CMV and Epstein-Barr virus (EBV) genomes by molecular techniques. Quantifying leukocyte input is essential for optimizing assay sensitivity (35). Leukocytes for antigenemia staining are separated from 3 to 5 ml of blood anticoagulated with EDTA or heparin by using direct red blood cell lysis with ammonium chloride. Leukocytes from a similar volume of blood for CMV culture are separated by gradient sedimentation. Several density gradient methods have been used for separation. Ficoll-Paque plus (Amersham Biosciences Corp., Piscataway, N.J.) and Lymphosep lymphocyte separation medium (MP Biomedicals, LLC., Irvine, Calif.) are used to separate lymphocytes. Mono-Poly resolving medium (MP Biomedicals, LLC., Irvine, Calif.) and Polymorphprep (Technoclone, Vienna, Austria) enable the resolution of both mononuclear and polymorphonuclear leucocytes into two distinct bands. HISTOPAQUE-1077 (Sigma Diagnostics, St. Louis, Mo.) is used for isolation of mononuclear cells, and ZEPTOGEL (ZeptoMetrix, Buffalo, N.Y.) is used for isolation of the total leukocyte fraction. Blood for CMV isolation should be fractionated and inoculated as soon as possible after collection. Processing delays may result in decreased CMV detection by culture. For the antigenemia assay, if processing is delayed, unfractionated blood should be stored at room temperature, with rocking. However, the number of CMV-positive cells quantified by the antigenemia assay is decreased for specimens held overnight (34).

Bone Marrow

CMV and parvovirus B19 are the most common viruses detected in bone marrow. PCR of bone marrow can be used to diagnose parvovirus B19-induced aplastic anemia, congenital anemia, and chronic red cell aplasia (66). Bone marrow can also be used to diagnose disseminated varicella-zoster virus (VZV) and human herpesvirus 6 (HHV-6) infections. Aspirated bone marrow is placed in tubes containing EDTA. The same processing considerations with respect to timing, anticoagulants, and removal of inhibitors that apply to blood also apply to bone marrow.

CSF

Rates of virus recovery from CSF are generally low. Enteroviruses are the most common viruses grown from CSF (with the exception of coxsackie A viruses, which are difficult to isolate in tissue culture). Herpes simplex viruses (HSV) and arboviruses are important causes of sporadic encephalitis in immunocompetent individuals. HSV can be isolated from CSF in neonatal herpes and in some cases of meningitis. Arboviruses are difficult to isolate from CSF, and IgM tests are now standardly used for diagnosis. CMV, VZV, EBV, and JC virus (JCV) are important central nervous system (CNS) pathogens in immunocompromised patients. CMV and VZV are also rarely cultured (8). Methods for EBV and JCV culture are not available in clinical laboratories. The application of molecular techniques has revolutionized the diagnosis of CNS disease caused by neurotropic viruses (47). In particular, NA amplification techniques are now the standard of care when a diagnosis of HSV encephalitis or meningitis is suspected (37). Reverse transcriptase PCR (RT-PCR) for the detection of enteroviruses increases sensitivity and decreases time to diagnosis. Enteroviruses, if cultivatable, typically take 3 to 14 days to isolate. With NA techniques, enteroviral RNAs are detectable within hours (18).

CSF is collected and placed into a sterile leak-proof tube. Dilution of CSF (or other sterile body fluids such as pericardial or pleural fluids) is contraindicated. Therefore, CSF specimens should not be collected in VTM. Susceptible cell lines can be inoculated directly without additional processing prior to culture. However, nucleic acids should be extracted from CSF prior to testing with molecular assays (particularly PCR) because CSF contains globulin and protein from high cell counts as well as uncharacterized substances that inhibit the activity of thermostable polymerases used in PCR (19, 46). Although it is theoretically optimal to quantify the nucleic acid content of a specimen prior to its use in molecular assays, the amount of recovered NA (from CSF or any other relatively acellular specimen) is likely to be below the limit of detection of standard quantitative techniques (e.g., spectrophotometry at an absorbance of 260 nm).

Therefore, in practice, NA is not quantified prior to performance of molecular assays.

Amniotic Fluid

Although rare, viruses can infect the fetus. The most common viruses that cause infections in utero are CMV, parvovirus B19, and HIV. HSV and VZV are less frequently observed. Congenital infections with rubella virus are rarely seen in the postvaccine era. Amniotic fluid is a useful specimen source for the diagnosis of congenital infections. CMV and rubella virus can be detected by culture. Nucleic acid tests can be used to diagnose parvovirus B19 (67), CMV (5), HSV (25, 30), VZV (38), and rubella virus (49). Amniotic fluid is collected by amniocentesis and sent to the laboratory in a sterile container without VTM.

Respiratory Specimens (Throat, Nasopharyngeal Swab, Nasopharyngeal Aspirate, Nasal Wash, and BAL Specimens)

Viruses that cause respiratory tract disease such as influenza viruses, parainfluenza viruses, respiratory syncytial virus (RSV), adenoviruses, and rhinoviruses are most frequently identified in samples of respiratory secretions. Many of these viruses infect the ciliated epithelium lining the posterior nasopharynx. They are often detected by direct AG detection methods such as enzyme immunoassay, immunochromatographic assays, and immunofluorescent-labeled antibody staining. Results of studies comparing nasopharyngeal swabs and aspirates are highly variable; however, nasopharyngeal swabs are generally collected from older children and adults; nasopharyngeal aspirates are the specimen of choice for infants and younger children (1, 32). The convenience of using a swab for specimen collection by medical personnel and the willingness of the older patient to allow collection with a swab (compared to an aspiration or wash) are important factors that explain their frequent use. Nasal wash specimens do not contain a large number of virus-infected cells; however, they are often collected from adults, particularly thrombocytopenic patients for whom nasopharyngeal aspiration is contraindicated. Bronchoalveolar lavage (BAL) and bronchial specimens are useful in the diagnosis of lower respiratory tract infections and offer a less invasive alternative to open lung biopsy.

Nasopharyngeal swab samples are collected by inserting a flexible, fine-shafted swab deep into the nares, past the point of resistance, and gently rubbing the posterior pharynx and the nasal turbinates. The swab is removed and placed in VTM. Aspirates are collected by passage of an appropriately sized tube or catheter into the nasopharynx and aspirating material with a syringe or a mechanical suction device. Aspirates are usually placed in VTM. Nasal wash specimens are obtained by instilling several milliliters of sterile saline into each nostril while the patient's head is tilted back slightly; the head is then brought forward, and the saline is allowed to flow into a small container held beneath the nose (7). BAL specimens are collected by inserting a fiber-optic bronchoscope into the involved segment of the lung, instilling saline, and applying suction to remove the lavage specimen.

All respiratory specimens are acceptable for culture (conventional or shell vial [1, 29, 39, 57, 58]). Respiratory specimens are often contaminated with upper respiratory tract microbial flora. These organisms have little effect on viral culture if they are susceptible to antibiotics in the transport and culture media. However, resistant organisms can overgrow cultures and prevent virus detection. Removal of most microbial contaminants by centrifugation ($3{,}000 \times g$ for 20 min) or filtration of the specimen (0.2-μm pore size) is advantageous; however, these procedures also remove virus-infected cells, resulting in reduced viral titers. AG and NA detection should be performed on the specimen prior to these manipulations since loss of host cells during processing may compromise the sensitivity of these assays. Mucus in respiratory specimens can significantly affect AG detection. In fluorescent-antibody tests, mucus can inhibit fixation of cells to slides and can cause nonspecific fluorescence. It also prevents penetration of the sample into membrane devices. To prevent these complications, mucous threads can be broken by repeated aspiration through a small bore pipette.

Urine

Urine is an important source for the detection of infections caused by CMV, enteroviruses, adenoviruses, BK virus, and HSV. Urine can be cultured (conventional or shell vial) or assayed using molecular techniques. Urine culture is the most common method for diagnosing CMV infection in neonates. In newborns, detection of CMV in urine within 21 days of birth confirms congenital acquisition of infection. Culture of enteroviruses from urine also establishes the diagnosis of enterovirus-induced disease. Mumps and rubella viruses were isolated from urine in the prevaccine era but are usually not seen today.

To collect urine specimens, the external genitalia are cleaned with a detergent-impregnated towelette. A volume of 5 to 10 ml of midstream-voided urine is collected in a sterile container. Collection in VTM is not recommended.

Urine must be processed for use in virus detection assays. Its acidic pH and the presence of microbial contaminants can inhibit virus growth. Urine also contains substances that inhibit PCR and other amplification techniques. To improve virus recovery, pH can be neutralized with sodium bicarbonate (7.5% solution) and bacteria can be removed by filtration (0.2-μm pore size) prior to culture. Attempts to improve virus recovery by culturing cell pellets versus supernatant after centrifugation have yielded variable results (3). For molecular assays, nucleic acids can be extracted from urine by a variety of manual or automated extraction methods.

Feces (Rectal Swab)

Many viruses that cause gastroenteritis such as enteric adenoviruses, caliciviruses (Norwalk group of viruses), astroviruses, and rotaviruses are noncultivatable in cell culture and require alternative methods for detection (9, 44, 60) (Table 2). Enteroviruses can be found readily by fecal culture or NA detection, but care must be taken in the interpretation of a positive result since asymptomatic shedding can occur in feces. Enveloped viruses are not commonly recovered in fecal specimens. One exception is CMV, which causes intestinal disease in immunosuppressed patients.

Fecal samples (2 to 4 g) are placed into a clean, dry, leakproof container, or samples are collected by inserting a swab 4 to 6 cm into the rectum and rotating the swab against mucosa. The swab is removed and placed in VTM. Fecal samples are preferred over rectal swabs since swabs often collect insufficient quantities of specimen.

Eye

HSV and adenovirus are the two viruses most often associated with keratitis and conjunctivitis. Diagnosis is made by conjunctival or corneal cultures. Enterovirus 70 and coxsackievirus A24 have been associated with hemorrhagic conjunctivitis and may be detected by PCR (54). Conjunctival swabs are collected from the lower conjunctiva

with a flexible, fine-shafted Dacron or rayon swab moistened with sterile saline and placed in VTM. Scrapings of the cornea or conjunctiva should be collected by an ophthalmologist or by other adequately trained personnel.

Viral causes of retinitis include CMV and VZV. These pathogens are detected in the aqueous and vitreous fluids by molecular techniques (amplification of viral genome) because they are not easily cultivated from these sites. These fluids have been shown to inhibit PCR (63); therefore, the original specimen must be extracted (see "CSF" above) to remove inhibitors. Vitreous and aqueous fluids are obtained from the eye during ocular surgery and placed in sterile containers for transport to the laboratory.

Tissue (Biopsy or Autopsy)

Many viruses can be isolated with ease from tissue. Lung is an ideal source for detecting lower respiratory tract infections due to viruses such as influenza, parainfluenza, adenovirus, HSV, and CMV. Other tissues that may yield positive viral results include liver, spleen, kidney, lymph node, and brain. In addition to culture, methods such as histology, AG detection (immunohistochemical stains), and NA detection can be used to detect viruses in tissue.

The major drawback of tissue specimens is the invasive nature of biopsy. However, for certain patients, biopsy is the only option. Tissue samples are obtained during surgery or autopsy and placed in VTM. An attempt should be made to excise the specimen from areas directly adjacent to the affected tissue. Tissue samples may also be obtained during endoscopy of the upper and lower gastrointestinal tract to detect viruses such as CMV, HSV, and adenoviruses.

Tissue specimens for culture are ground with a sterile mortar and pestle or a tissue grinder and resuspended in VTM. The tissue specimen is centrifuged (1,000 × g for 15 min at 4°C) to remove cell debris that may be toxic to cell culture, and the supernatant is inoculated onto cell monolayers. Fresh tissue specimens for NA detection are weighed (25 mg is optimal for most tissues), minced, treated with proteolytic enzymes, and extracted. NAs can also be obtained from formalin-fixed, paraffin-embedded tissues after deparaffinization and extraction.

Genital Specimens

The most common viral cause of external genital lesions in men and women is HSV-2. Although not as prevalent, HSV-1 is also sexually transmitted and can cause genital lesions (31). HSVs are easily cultured from these lesions, and growth usually requires only 24 to 48 h. HSV-1, HSV-2, and CMV are also frequently detected from the cervix, vagina, and urethra. Human papillomavirus (HPV) genotypes associated with a high risk of cervical cancer (genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) can be detected in cervical specimens by using molecular tests.

Genital specimens are collected with swabs that are then placed in VTM. Prior to collection of cervical specimens, mucus should be removed with a swab and discarded. Cervical specimens are collected by inserting a clean swab 1 cm into the cervical canal and rotating for 5 s. The swab is then removed and placed in VTM. Cells from the cervical os can also be obtained using a Cytobrush. Cervical samples collected in manufacturer-specific formats or as liquid-based cytology specimens are appropriate for HPV high-risk genotype detection (45). Cervical brush specimens in Standard Transport Medium for the hc2 High-Risk HPV DNA Test (Digene) may be stored for up to 2 weeks at room temperature, after which specimens can be stored for an additional week at 2 to 8°C. If testing is to be performed more than 3 weeks from collection, specimens should be placed at −20°C for up to 3 months. Cervical biopsy specimens must be placed into 1 ml of Standard Transport Medium and stored frozen at −20°C. Specimens in liquid-based cytology medium may be held for up to 3 weeks at temperatures between 4 and 37°C and cannot be frozen. To collect urethral specimens, exudates should be expressed and discarded. The patient should not have urinated for at least 1 h prior to collection. A flexible, fine-shafted swab is inserted 4 cm into the urethra, rotated 2 or 3 times, removed, and placed in VTM. Genital specimens are often contaminated with microbial flora. These specimens are usually centrifuged at 3,000 × *g* for 20 min to remove bacteria prior to culture.

Skin

Viruses can cause rashes with many different appearances including vesicular, maculopapular, and petechial. Vesicular lesions are commonly caused by HSV, VZV, and enteroviruses. Fresh vesicles should be sampled for optimal virus detection. Vesicles can rupture and ulcerate. Ulcers that have crusted may not contain viable virus or viral antigens. Samples are obtained by disrupting the surface of the lesion and collecting fluid with a swab or by aspirating with a 26- or 27-gauge needle attached to a tuberculin syringe (15). Specimens should be collected gently, without causing bleeding, since the presence of neutralizing antibody in blood can impair recovery. The base of the lesion should be scraped to collect cellular material for AG detection assays. Smears may be prepared at bedside. Swabs should be placed in VTM if the specimen is destined for NA detection, AG detection, and/or culture.

Viruses that cause maculopapular rashes include rubella, measles, adenoviruses, and enteroviruses. Viruses that cause petechial rashes include adenoviruses and enteroviruses. Recovery of viruses from these types of rashes requires a skin biopsy specimen, which is not routinely cultured by most clinical laboratories. Parvovirus B19 causes a maculopapular rash, but it is noncultivable in conventional tissue culture systems. Sources other than dermis are preferred for diagnosing these viral infections.

SPECIMEN TRANSPORT

Once a clinical specimen has been collected, it should be transported to the laboratory as soon as possible because virus viability decreases with time (some clinically important viruses are more labile than others). Overall diagnostic yield improves when specimens are expeditiously processed for viral culture. Viability is not required for antigen or NA detection techniques; therefore, transport time is not as much of an issue when these assays are to be performed. However, for timely diagnosis, delay should be avoided during transport of these specimens to the laboratory.

VTM should be used for all swabs and tissues, since they can dry during transport (Table 3). Specimens such as CSF, blood, urine, bronchoalveolar lavage fluid, vesicle fluid, amniotic fluid, and feces can be sent without transport medium (29).

Transport Conditions

Several steps may be taken to help preserve viruses during specimen transport to the laboratory, including the following: (i) transport of nonblood specimens at 4°C with wet ice or cold packs, especially if transit time is greater than 1 h; (ii) use

of VTM if appropriate, as described below; and (iii) avoiding freezing unless a delay of 24 h is anticipated (64). Specimens should be frozen at −60°C (dry ice). It is important to remember that recovery of RSV, CMV, and VZV may be seriously compromised by freezing.

Storage and transport conditions may vary somewhat based on the stability characteristics of specific viruses or viral NAs. A study to determine the feasibility of shipping adenovirus samples in M4RT showed that the virus is stable for up to 5 days at ambient temperatures (51). Severe acute respiratory syndrome coronavirus infectivity is maintained in serum, sputum, and feces for 96 h and in urine for 72 h (10a). CMV infectious titers in blood were decreased by up to 91% after storage at 4°C for 24 h (40). The data on CMV DNA quantification after storage are conflicting, with some studies reporting no decline in CMV DNA levels after 24 h (40) and 72 h (50) while another showed increased levels in blood stored at room temperature or at 4°C prior to plasma separation from lymphocytes (53). The increased levels of CMV DNA may be due to release of CMV from cells into plasma during storage. HSV DNA is stable in CSF specimens containing lymphocytes and monocytes for up to 30 days at temperatures ranging from 25 to −72°C (62). Temperature storage requirements for fluids, solid tissues, and extracted NA for diagnostic molecular testing have been described in a comprehensive review (13). RNAlater, an RNA stabilization buffer (Qiagen, Valencia, Calif.), has been shown to stabilize HCV and HIV RNA in plasma for up to 28 days at 37°C (33) as well as stabilizing virus infectivity of enveloped and nonenveloped viruses stored at room temperature for up to 50 days.

Transport Medium

The ideal transport medium maintains viability of a broad range of cultivatable viruses and is also compatible with the newer non-culture-based tests. VTM generally contains buffer to control pH, substances to maintain an appropriate osmotic environment, protein to stabilize the virus, and antibiotics to prevent growth of microbial contaminants. Many different transport media have been described and used successfully, including cell culture medium, bacteriologic broth-based media, buffered salt solutions with added protein, sucrose-based solutions, bentonite-containing media, and swab-tube combinations. Direct inoculation of cell monolayers and cell suspension at bedside has also been described (56). Some laboratories have reported that culturette swabs with Stuart's medium designed for collection and transport of bacteriology specimens are also acceptable for collection and transport of viral specimens. The swab-tube combination and liquid transport medium are two of the more common transport systems used today. VTM may be prepared in-house or purchased commercially. In most laboratories, commercial media have replaced "home-brew" transport solutions. Table 5 lists the different commercial media, as well as their sources, compositions, and storage conditions. Of note, CVM transport media contain no buffer to control pH, and the Viral Culturette contains no buffer, protein stabilizer, or antibiotics to control microbial contaminants. Therefore, delay in transport of viral specimens in these media should be avoided. Bartels ViraTrans contains fetal bovine serum, which may inhibit isolation of influenza viruses.

Unfortunately there have been no recent comprehensive comparisons of either commercially available or in-house prepared VTM. The most comprehensive reviews of transport systems for viruses appeared several years ago (27, 28), and many of these transport systems are no longer in use today. It is difficult to compare results of the few studies that have been performed because they were carried out in different laboratories under different conditions (with varying viral inocula and detection methods). Ideally, direct comparisons of several different transport media that support growth of multiple viruses should be performed in a clinical setting. However, the cost and logistics of performing these types of comparative studies remain problematic. One study comparing MicroTest M4 and Bartel's VTM for oral and genital specimens showed them to be equivalent for detection of HSV (42). A second study compared Copan VTM, BBL Viral Culturette, and Micro Test M4 (11). Copan VTM and Micro Test M4 were more effective than BBL Viral Culturette in maintaining low concentrations of viable virus that could be recovered in routine cell culture.

Manufacturers of commercial assays for NA or AG detection either supply transport media or make recommendations for transport systems that are compatible with their assays. The hybrid capture assay (Digene) for detection of high-risk HPV types is an example of the former, and Directigen assays (BD Diagnostic Systems) for influenza and RSV are examples of the latter. In addition to the Digene specimen transport medium and device, SurePath (TriPath Care Technologies, Burlington, N.C.) and ThinPrep (Cytyc, Marlborough, Mass.) liquid-based Pap tests have been evaluated for detection of high-risk HPV genotypes with favorable results (10, 45). Thus far, VTM has not been shown to inhibit amplification assays such as PCR (29) or interfere with AG detection tests (16).

Transportation Regulations

Few clinical virology laboratories can offer a complete battery of diagnostic tests for all human viruses. Therefore, shipment of samples to reference, specialty, state, or federal laboratories is common practice. A recent change in regulations has simplified the shipping process for most specimens. Currently, the majority of samples can be shipped as diagnostic or clinical specimens. Only specimens that have or may have a category A pathogen are classified as "Infectious Substance." Strict attention must be paid to specimen packaging and labeling for the transport of infectious substances. Shipping specimens through the mail or by commercial courier is regulated by federal and international agencies. Regulations are published by the U.S. Public Health Service and are also available from the U.S. Department of Transportation on the Internet (http://www.dot.gov). The International Civil Aviation Organization guidelines stipulate that training must be provided within 90 days of assuming a job involving the transport of dangerous goods. Retraining must take place every 24 months, and training records including time, place, instructor, and materials must be maintained. The International Air Transport Association 2005 dangerous goods regulations define infectious substances, containers, labels, and other packaging instructions required for shipping.

SUMMARY

Diagnosis of virus infection is based on likely etiologies, assay performance (sensitivity, specificity, positive predictive value, and negative predictive value), turnaround time, and cost. Viruses in and of themselves put limitations on test options (viruses that are noncultivatable must be identified directly in the specimen by electron microscopy, cytologic and histologic methods, AG detection, or NA detection or indirectly by serology). Regardless of the test method, the proper selection, collection, transport, and processing of specimens will

TABLE 5 Commercial sources and formulations of VTM

Medium	Composition	Source	Vol (ml)	Storage conditions
Bartels ViraTrans	Minimal essential medium with Hanks' salts Fetal bovine serum HEPES (pH 7.0–7.8) Sodium bicarbonate Gentamicin Streptomycin Penicillin Amphotericin B	Trinity Biotech Carlsbad, CA (800) 331-2291	2	2–8°C
Bartels FlexTrans	Minimal essential medium with L-glutamine Bovine serum albumin HEPES (pH 7.0–7.4) Sodium bicarbonate Sucrose Gentamicin Streptomycin	Trinity Biotech Carlsbad, CA (800) 331-2291	2	25°C or 2–8°C
Copan Universal Transport Medium	HBSS[a] Bovine serum albumin L-cysteine L-glutamic acid Gelatin Sucrose HEPES buffer Phenol red Vancomycin Amphotericin B Colistin	Copan Diagnostics Inc. Corona, CA (800) 216-4016	3	25°C
CVM Transport Media	HBSS (modified) Bovine serum albumin Sucrose Glutamic acid Gelatin Amphotericin B Colistin Vancomycin	Hardy Diagnostics Santa Maria, CA (805) 346-2766	2	2–8°C
Difco Viral Transport Medium	HBSS HEPES buffer Gelatin Gentamicin	BD Diagnostic Systems Cockeysville, MD (800) 675-0908	2	Below 30°C
Micro Test M4-3	HBSS (modified) Bovine serum albumin Gelatin HEPES (pH 7.3) Vancomycin Amphotericin B Colistin	Remel Lenexa, KS (800) 646-6678	3	−25–8°C
Micro Test M4RT	HBSS (modified) Bovine serum albumin Gelatin Sucrose L-glutamic acid HEPES (pH 7.3) Phenol red Gentamicin Amphotericin B	Remel Lenexa, KS (800) 646-6678	3	25°C

(Continued on next page)

TABLE 5 Commercial sources and formulations of VTM *(Continued)*

Medium	Composition	Source	Vol (ml)	Storage conditions
Multitrans Medium	Gelatin Sucrose Glutamic acid HEPES, sodium salt HBSS (modified) Sodium bicarbonate Bovine serum albumin Vancomycin Amphotericin B Colistin	Starplex Scientific Inc. Etobicoke, Ontario Canada (800) 665-0954	3	25°C
Viral Culturette	HBSS (modified) Rayon-tipped plastic swab	BD Diagnostic Systems Cockeysville, MD (800) 675-0908	0.5	25°C

[a] HBSS, Hanks' balanced salt solution.

always be of the utmost importance. In some instances, a common sample can be collected and shared across diverse testing platforms. However, many commercial assays have specific sample requirements that must be followed. All aspects of "user-defined" assays, including specimen type, collection device, transport, and processing, as well as any changes to package insert specifications for commercial assays, must be verified. On the surface, the initial steps of viral detection appear simple and virtually static. However, as the field of diagnostic virology evolves, it is likely that new collection devices, transport systems, and processing methods that enhance detection will continue to emerge.

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Reagents, Stains, Media, and Cell Lines: Virology*

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81

Viruses are intracellular parasites requiring living cells to replicate. Thus, the description by Enders et al. in 1949 (7) of a cultivated mammalian cell line to isolate poliovirus and the subsequent cytopathic effect (CPE) was a critical development that opened the door for the practice of clinical virology. Subsequent discovery of host-sensitive animal and human tissue cell lines, as well as identification of virus-specific monoclonal antibodies and fluorescent-antibody (FA) techniques, resulted in the ability to isolate and identify many viruses infecting humans in a clinically useful time frame. Despite viral culture becoming technically more challenging with the current workforce available to the laboratory (http://www.asm.org/Policy/index.asp?bid=39060) and rapid immunologic antigen screening assays becoming the mainstay of most virology laboratories, cell culture remains a vital function that needs to be retained for a number of reasons (20). This chapter describes reagents, stains, media, and cell lines unique to virology used in association with culture techniques. The reader is referred to documents from the Clinical and Laboratory Standards Institute (CLSI) that provide guidelines for viral cell culture (3) and Appendices 2 and 3 in the 3rd edition of the *Clinical Virology Manual* (15) for listings of state public health laboratory virology services and virology laboratories offering diagnostic services that perform more esoteric testing. In addition, multiple websites, including http://www.virology.net, are available that also include international laboratories (8, 9). Appendix 1 of this chapter includes manufacturers and suppliers mentioned throughout this text for virology media and reagents.

Specimens containing viruses have transport requirements different from those of specimens for which bacterial or fungal diagnoses are being entertained. Viral transport media are designed to provide an isotonic solution containing protein, antibiotics to control bacteria, and a buffer to control pH. The media come in polypropylene centrifuge tubes that contain approximately 2 to 3 ml of medium, 1-dram freezer vials with up to 2 ml of medium, or a tube and swab with a gel or sponge base. Most often laboratories are using transport systems that can be used for the culture of *Chlamydia* and viruses, as well as *Ureaplasma* and *Mycoplasma*. The antibiotics used in these dual-purpose media are not inhibitory to either bacterial pathogens or viruses. In addition, some transport systems are available to be stored and/or allow transfer of specimens at room temperature instead of refrigeration. The reader is referred to chapter 80.

Cell culture systems are live cell monolayers that support the growth of viruses. Most viruses are detected by their production of a CPE, characteristic and visible morphological alterations in a susceptible cell line due to viral replication. Use of cell cultures is the most sensitive method for detecting many viruses and permits the detection of a wide variety of viruses or new viruses from a few media, in contrast to single-method detection systems such as enzyme immunoassay. Additionally, viruses isolated in culture can be analyzed for epidemiological purposes or to assess pathogenesis. For example, annual characterization of influenza virus isolates can direct vaccine development for the next influenza season.

Cell cultures may be propagated and/or maintained within the laboratory; however, monolayer cell cultures are more commonly prepared commercially (Appendix 1) and shipped to the laboratory every 1 to 2 weeks depending on the laboratory needs. Cells are typically provided in 16- by 125-mm screw-cap tubes or in 1-dram shell vials. Cell cultures come with the cell monolayer on one side of the lower half of the tube. The monolayer is covered with nutrient medium, and the manufacturer's label appears on the opposite side of the monolayer to aid in orientation of the tube for positioning and viewing.

Selection of cell lines varies for each laboratory and is dependent on the patient population and the most likely viruses that will be isolated. The most sensitive host system for the suspected virus should be included (refer to the specific chapters for individual viruses). The experience of the technologist with specific cell lines, cost, and availability of a cell line are also considerations for cell line selection. The reader should be aware that some traditional cell lines are not readily available (fetal cell lines) and are beginning to be replaced with cell lines of animal origin.

There are a multitude of special conditions that must be considered when inoculating cell cultures and identifying viruses. Some of these include the lack of CPE by certain viruses and the need to perform subsequent testing, such as hemadsorption; pretreatment of specific specimens with inherent toxicity, such as urine and stool; pretreatment of a cell line with trypsin to facilitate infection by a particular virus; and filter sterilization or treatment with antibiotics for respiratory specimens to limit bacterial or fungal contamination (chapter

* This chapter contains information presented in chapter 79 by Kimberle C. Chapin and Fred W. Westenfeld in the eighth edition of this Manual.

80). In addition, it should be noted that culture of certain viruses requires biosafety level 3 (BSL-3) or BSL-4 facilities, and any suspect specimen with a virus in this category should be referred to the appropriate facility.

Viral susceptibility testing is performed by phenotypic or genotypic assays. Readers are referred to chapter 114 for a more detailed discussion. Phenotypic assays may be performed using several methods such as plaque reduction, dye uptake, enzyme immunoassay, inhibition assays, and yield reduction assays or DNA hybridization. All methods employ cell cultures infected with virus, to which various dilutions of antivirals are added. Any decrease in viral activity is then measured. An approved standard for the plaque reduction assay is available as a document from the CLSI and standardizes antiviral susceptibility testing for herpes simplex virus (1).

Quality control of cell culture media and cell lines is an important consideration for virus isolation. Documentation of cell culture records such as cell type, passage number, source, age, receipt date, and maintenance medium lot and date are appropriate (2, 3, 21). Some of these parameters are documented on the shipping receipt from the vendor. In addition, culture media and additives should be checked for sterility and absence of toxicity. Correct storage at 35 to 37°C and rotation of media, using the oldest cell cultures first, ensure consistency in inoculation of fresh cell lines. Examining cells to confirm use of healthy cell cultures before inoculation and inoculating within 8 to 10 days of seeding are best for optimal performance (16, 21). Uninoculated cells of each lot should be maintained as negative controls to monitor toxic and/or nonspecific changes associated with cell age, as well as bacterial or fungal contamination that may be confused with CPE. Use of a pH indicator in the maintenance medium allows detection of changes in the condition of the monolayer. Changes in the maintenance medium color could be due to such factors as virus proliferation, cellular toxicity, or bacterial contamination. Microscopic examination is necessary to identify the specific cause. Finally, laboratories should be aware of possible changes in sensitivity of specific viruses to host cells and decreased sensitivity in continuous cell lines that have undergone multiple serial passages or have come from other laboratories (15). Readers are referred to the CLSI document on viral culture, M41-P, for further information (3).

REAGENTS

■ HEPES

HEPES is a serum-free medium used as a buffer in virology culture media.

■ Formalin for cell culture preservation

A formalin solution may be used to preserve viral CPE in cell culture, allowing the user to demonstrate viral CPE indefinitely and produce a useful teaching set.

Minimal essential medium with Earle's balanced salt solution	81 ml
37 to 40% Formaldehyde	30 ml

Note that formaldehyde is normally purchased as a 37 to 40% HCHO solution. Mix the minimal essential medium with the formalin in a suitable glass container. Fill cell culture tubes exhibiting CPE with the above mixture and store at room temperature.

■ Hemadsorption test

The hemadsorption test is used to detect orthomyxoviruses and paramyxoviruses in cell culture. Viruses such as influenza, mumps, and parainfluenza viruses may not produce CPE. These viruses possess a surface hemagglutinin that is expressed in the plasma membrane of virus-infected cells. These proteins have an affinity for erythrocytes and cause clumping of guinea pig red blood cells when introduced into the culture tube (22).

Basic procedure

The following is a basic descriptive procedure. Slight variations exist in preparation and inoculation of the guinea pig red blood cell suspension.

A guinea pig red blood cell suspension is produced by placing guinea pig cells in cold phosphate-buffered saline (PBS). A 4 to 10% stock solution of suspended cells is made depending on the working dilution required. A series of two or three washes with PBS and centrifugation with removal of the supernatant between each wash is performed. Remove the saline after the final wash in order to achieve the desired percent concentration solution (if the result is 0.4 ml of packed red cells, remove supernatant until 4 ml remains to acquire a 10% solution). The red cell suspension should be stored at 4°C and can be used for 1 week. From the 10% suspension, prepare a 0.4% suspension of guinea pig red blood cells. Remove medium from cell culture tubes and replace with 1 ml of cold PBS. Place 0.2 ml of the 0.4% suspension into each tube. Incubate at 4°C for 30 min. Shake the tube and examine microscopically for red blood cells that are adhering to the monolayer and/or clumping in the fluid, indicating a positive test (14).

■ PBS for 1-liter volume (10)

Solution A

NaCl	8.0 g
KCl	0.2 g
$CaCl_2 \cdot 2H_2O$	0.132 g
$MgCl_2 \cdot 6H_2O$	0.1 g
Distilled water	800 ml

Solution B

Na_2HPO_4	1.15 g
KH_2PO_4	0.2 g
Distilled water	200 ml

Dissolve each salt in demineralized water in the order listed. Sterilize by autoclaving for 20 min or by filtration. Mix solutions A and B when cool and adjust pH to 7.0. Store refrigerated.

■ Density gradient media

Density gradient media are used to fractionate a patient's leukocytes. The cells are subsequently cultured or used for direct staining. The reader is referred to chapter 80 for a listing of manufacturers and specific uses of the various media available.

■ Dulbecco's PBS (4)

PBS with glucose and sodium pyruvate. An early described maintenance-type medium.

■ Saline

Saline is used as a diluent in a variety of procedures. Normal or physiological saline is 0.85%.

Sodium chloride	8.5 g
Distilled water	1,000 ml

Other concentrations (e.g., 0.45%) are also used.

■ 10× Gentamicin-amphotericin B solution

10× gentamicin-amphotericin B solution is added to clinical specimens during tissue culture inoculation to reduce bacterial and fungal contamination. Use at a ratio of 1:10 (0.1 ml of 10× gentamicin-amphotericin B for every 1.0 ml of specimen) before centrifugation of the specimen. The supernatant is then inoculated to the appropriate cell culture line.

Eagle's minimal essential medium	89 ml
Gentamicin (50 mg/ml)	1 ml
Amphotericin B (250 μg/ml)	10 ml

Add all three ingredients and store frozen at –20 to –70°C in suitable working aliquots.

■ Trypsin solutions

Trypsin releases cells from the monolayer of a culture. Release of cells allows for subsequent passage of the cells or antibody-specific identification of cell-associated viruses such as varicella-zoster virus (VZV), adenovirus, and cytomegalovirus (CMV). Trypsin can be used instead of scraping cells from the culture vessel. Solution is made with either 2.5% PBS or Versene solution, which contains EDTA. Both are available commercially. See Appendix 1.

VIROLOGY STAINS

A rapid viral diagnosis may be made by directly examining stained clinical specimens. Specimens can be prepared in a number of ways, including direct application of mucous membrane scrapings onto a slide, concentration of bronchoalveolar lavage fluid by cytocentrifugation, or touch preps from unfixed tissue onto a slide. Papanicolaou (Pap), hematoxylin and eosin, and Wright-Giemsa stains are used to examine these preparations. A preliminary identification can be made when characteristic cell morphologies are observed (e.g., "smudge cells" or large basophilic inclusions consistent with adenovirus, or "owl's eye" nuclear inclusions consistent with CMV); however, a specific diagnosis is generally not possible unless virus-specific antibody staining methods are used (18, 20). For example, intranuclear inclusions and multinucleated cells are seen in both VZV and herpes simplex virus infections. Smears fixed in 95% alcohol or a cytological fixative are stable indefinitely at room temperature and can easily be transported in cardboard or plastic slide carriers to the laboratory (18).

Immunostaining

Specific immunostaining techniques using FA stains and horseradish peroxidase-labeled antibodies have become the most commonly used staining techniques for the detection of viruses (20). Viral antigen for the specific virus is sought using a monoclonal antibody specific for the virus. Fluorescein isothiocyanate (FITC)-labeled mouse monoclonal antibodies with Evans blue counterstain and tetramethylrhodamine isothiocyanate (TMIT) are the most commonly used markers. FITC yields an apple-green fluorescence and TMIT yields a reddish orange fluorescence when excited by the proper wavelength (13). FA staining is either direct or indirect depending on the antibody sandwiching technique used. Readers are referred to chapter 18 for specific details about the methodology. Immunostaining can be performed on slides obtained by direct preparation from a clinical specimen (such as with a VZV lesion scraping) or from virus-infected cells from a cell culture or spin amplification coverslip. Direct examinations using cytocentrifuged clinical specimens and leukocyte preparations are also common for respiratory viruses and CMV, respectively.

MEDIA AND CELL LINES

Cell culture media for viruses serve three general purposes: (i) short-term survival of cells such as during specimen transport, (ii) long-term survival such as when cell lines are passaged over an extended period, and (iii) rapid cell proliferation. The basic component of all of these media is a balanced salt solution with a CO_2-bicarbonate buffering system for maintaining pH within acceptable ranges. The solution contains inorganic ions, glucose, and a buffer (HEPES) with a phenol red indicator. The components allow maintenance of the osmotic pressure and the pH within the range of 6.8 to 7.6, which is optimal for mammalian cells, as well as aid in the detection of pH changes in the media. A carbohydrate source and inorganic ions are necessary for the regulation of osmotic pressure, pH, and the cells' enzymatic and metabolic activities. The addition of serum aids in the attachment and spreading of cells on the culture vessel. Amino acids and vitamins must be added to any media that will be used for other than short-term survival. There are 12 amino acids that are necessary for the development of protein and synthesis of nucleic acids. Amino acids also act as buffers. Vitamins, particularly the B vitamins, can act as vitamin supplements or as coenzymes for metabolism. Fat-soluble vitamins, such as vitamin A, are often added to media without serum. Other additives, such as antibiotics and hormones, commonly supplement the basic media. Most media are commercially available in liquid form. Serum, glutamine, and antibiotic solutions are filter sterilized (0.2-μm-pore-size filter) or purchased filter sterilized and typically added before use.

■ Balanced salt solutions (Hanks' and Earle's)

Balanced salt solutions are used alone for short-term survival, the transport of specimens, and the processing of viruses. Earle's and Hanks' salt solutions are the two most commonly used balanced salt solutions and have similar formulations. Earle's balanced salt solution has a better buffering capacity with CO_2, and Hanks' balanced salt solution has a better buffering capacity with air.

■ Basal medium Eagle cryoprotective medium (5)

Eagle's minimal essential medium with Hanks' balanced salt solution and 15% dimethyl sulfoxide (DMSO) is the original medium described for preserving virus. DMSO acts as the cryoprotective agent.

■ Buffered glycerol saline

Buffered glycerol saline is a multipurpose transport medium. The transport medium is used for the isolation of bacteria as well as viruses. Glycerol-containing media may also be used for long-term storage of isolates and transport or storage of biopsy specimens.

■ Growth medium with 10% fetal bovine serum

Growth medium is medium used to enhance the growth and multiplication of cell numbers. This medium is necessary in virology laboratories where cell cultures are prepared and transferred. Serum is added at a much greater concentration (8 to 20%) than that used in maintenance media. The serum component aids in promoting cell attachment and spreading on the surface of the culture vessel. Yeast extract and peptones also help cell proliferation. The colony sizes of the cells are larger in growth medium than in maintenance medium, but serum may contain inhibitors, toxins, infectious agents, or antibodies that interfere with cell growth. Typically, fetal bovine serum is preferred since it is less likely to contain these substances. Heat inactivation of serum may reduce some of its growth-promoting properties. Antibiotics and antifungal agents are typically added to reduce bacterial and fungal contamination. Fetal bovine serum (50 ml), L-glutamine (5 ml), gentamicin (0.5 ml; 50 mg/ml), and amphotericin B (5 ml; 250 μg/ml) are added to 500 ml of Eagle's minimal essential medium. The 10% growth medium is used when seeding a young monolayer or when greater confluency of the cell monolayer is desired.

■ HH medium

HH medium is a viral transport medium containing Hanks' balanced salt solution with 0.88% sodium bicarbonate, 0.2% bovine serum, 30 mM HEPES buffer, and 50 μg of gentamicin per ml. The pH should be adjusted to 7.2 by adding 1 N NaOH. HH medium was found to be an excellent transport medium for many viruses (11, 12).

■ Maintenance medium with 2% fetal bovine serum (6)

Eagle's medium is a maintenance medium suitable for growth and survival of a variety of cell lines for days to weeks at a low metabolic state. It contains the 12 essential amino acids, including glutamine, and vitamins in Earle's balanced salt solution. Antibiotics and antifungal agents are often added to the basic formula to control bacterial and fungal contamination. Fetal bovine serum is added at less than or equal to 2%. Serum supports life of the cell line but may contain inhibitors, toxins, infectious agents, or antibodies that interfere with cell maintenance. Typically, fetal bovine serum is preferred because it is less likely to contain these substances. Modifications include the addition of components to fortify the basic formulation for better support of cell lines. Fetal bovine serum (10 ml), L-glutamine (5 ml), gentamicin (0.5 ml; 50 mg/ml), and amphotericin B (5 ml; 250 μg/ml) are added to 500 ml of Eagle's minimal essential medium.

■ RPMI 1640 medium (15)

RPMI 1640 medium is an enriched medium used for the cultivation of cells in tissue culture. The medium is commonly used for the culture of human immunodeficiency virus and antiviral susceptibility testing. The base consists of glucose; essential amino acids, including glutathione (reduced); vitamins; and HEPES buffer. Additional enrichment is provided with incorporation of other amino acids. Antibiotics (e.g., penicillin and streptomycin) and a phenol red indicator are incorporated as well.

■ Sucrose-phosphate-glutamate medium

Sucrose-phosphate-glutamate transport medium is used for the maintenance and transport of *Chlamydia* species and viruses. Sucrose and two potassium phosphate buffer solutions are the base. Bovine serum and glutamic acid are additives. Glutamic acid is a stabilizing agent that is especially useful for enveloped viruses. The antibiotic combination may be the same as or slightly different from that in 2-sucrose-phosphate. Most commonly the antibiotic combination is vancomycin, streptomycin, and nystatin, which inhibit both gram-positive and gram-negative organisms, as well as yeasts.

■ 2-Sucrose-phosphate transport medium

2-Sucrose-phosphate medium is used for the transport of specimens for the purposes of culturing *Chlamydia trachomatis*. Sucrose (0.2 M) and two potassium phosphate buffers are the base. Fetal bovine serum allows the *Chlamydia* to maintain infectivity, and the antibiotics nystatin and gentamicin are added to inhibit yeasts and bacteria.

■ Transport medium (viral and universal)

Many viral transport media are available for the collection of viruses. The reader is referred to chapter 80 for specific company products. Most media are made for the isolation of *Chlamydia, Mycoplasma,* and *Ureaplasma* and are often called universal transport media. However, some are specific for viral isolation. In addition, some are able to be stored and shipped at room temperature, come with a variety of swab types, and contain from 1 to 5 ml of medium. The user should note each of these specific differences for any transport system being considered to optimize the most needed functions. The main components for these media are minimal essential medium with Hanks' balanced salts, bovine serum albumin, and sucrose with a phenol red pH indicator (pH 7.0 to 7.4). Microbial growth is inhibited by the incorporation of antibiotics, such as vancomycin, colistin, and amphotericin B, that inhibit gram-positive and gram-negative organisms as well as fungi.

CELL CULTURES

Sensitivity of the suspected viral pathogen for the host cell system and rate of CPE formation are two critical factors for virus isolation and identification. Thus, it may be appropriate to inoculate a specimen into a variety of cell systems to maximize recovery of a virus and/or recognize the characteristic CPE. Three general cell systems exist: primary, diploid, and continuous. Primary cell lines are those derived directly from human or animal tissues and are only subcultured once or twice. An example is rhesus monkey kidney (RhMK) cells. Subsequent subculture reduces the sensitivity to virus infection. Diploid cell lines are those which may support viral infection for up to 20 to 50 passages and are typically derived from fetal or newborn cells. An example is human diploid fibroblasts. Continuous cell lines are those typically derived from human or animal cancers or cells transformed in vitro; thus, the continuous cell lines have a heteroploid karyotype and can be subcultured indefinitely without loss of sensitivity to virus infection. An example is human epidermoid carcinoma of the larynx (HEp-2) (17). Decreased availability of fetal cell lines and contamination of monkey cell lines with endogenous virus have resulted in the use of more novel cell lines.

Cell lines may be used in conventional cell culture tubes and examined for CPE or grown on round coverslips in the bottoms of shell vials that are used in a spin-amplification technique.

Genetically altered cell lines and mixed cell lines have also been developed, as have lymphocyte preparations for the isolation of retroviruses. Unusual cell culture lines not listed may be available from vendors specializing in cell culture products. See Appendix 1.

■ A549 cells

A549 cell cultures originated from a human lung carcinoma. They are a continuous epithelial cell line. They are most commonly used to isolate adenovirus, herpes simplex virus, and VZV. Some have found A549 shell vials to be effective for diagnosing measles virus infections (13). This cell line is easily maintained in a laboratory with cell culture experience.

■ B95a or B95 cells

B95 or B95a is a lymphoblastoid cell line transformed by Epstein-Barr virus. The cell line is sensitive for measles and mumps virus isolation.

■ BGMK cells

BGMK cells are derived from Buffalo African green monkey *(Cercopithecus aethiops)* kidney cells. They are a continuous cell line most useful in isolating enteroviruses, specifically coxsackie B virus and polio virus, as well as *C. trachomatis*. Their range is quite narrow, making their regular use impractical for many clinical laboratories.

■ C6/36 and AP61 cells

C6/36 and AP61 are cell lines derived from mosquito cells for the isolation of arboviruses.

■ Genetically altered cell lines (ELVIS)

Genetically modified cell lines have been constructed to detect viruses by means other than CPE. Genetic elements derived from viral, bacterial, or cellular sources are introduced into a cell line where the end product (typically an enzymatic reaction) is dependent on presence of a specific virus. The original detector cell line was described by Stabell and Olivo (19). A baby hamster kidney cell line serves as the detector cell line and contains the genetic element of the *Escherichia coli* β-galactosidase gene. The gene is under the control of a promoter gene from herpes simplex virus and is activated only by the presence of herpes simplex virus proteins and thus viral replication. If the detector cells are exposed to the herpes simplex virus proteins, the promoter will be activated and cause production of the β-galactosidase enzyme. This, in turn, is recognized by a simple histochemical reaction or color reaction in the cells that is visible by light microscopy. The technology is described by the acronym ELVIS, which stands for enzyme-linked virus-inducible system. Cultures are typically evaluated within 16 to 24 h, making the assay rapid, simple, and able to be read by someone unfamiliar with CPE. Genetically altered cell lines have been developed for detection of both RNA and DNA viruses, as well as for antiviral susceptibility testing (15).

■ HEK cells

HEK cells are a primary cell culture line from human embryonic kidney. Human epithelial cell lines are optimal for isolation of adenoviruses. They are also used for BK virus. Embryonic cell lines are now less commonly available. Most enteroviruses, rhinovirus, measles virus, mumps virus, and herpes simplex virus will also grow in HEK cells.

■ HEK 293 or Graham 293 cells

HEK 293 cells are human embryonic kidney cells transformed with type 5 adenovirus. They are used only in specialized situations to isolate some of the more difficult-to-isolate enteric adenoviruses.

■ HeLa cells

HeLa cells are a continuous cell line derived from a cervical epithelioid carcinoma. HeLa cells may be used to isolate adenovirus, coxsackie B virus, herpes simplex virus, poliovirus, CMV, vesicular stomatitis virus, and VZV.

■ HeLa 229 cells

HeLa 229 cells are a HeLa variant used for isolation of *Chlamydophila pneumoniae.*

■ HELF cells (see WI-38 cells and MRC-5 cells)

Human embryonic lung fibroblasts (HELF cells) are diploid cell lines used especially for the isolation of rhinovirus and adenovirus.

■ HEp-2 cells

HEp-2 cells are commonly used in many laboratories because they support a broad range of viruses. A continuous human epidermoid carcinoma of the larynx cell line, it is used most for isolation of respiratory viruses such as respiratory syncytial virus and adenovirus. It may also grow herpes simplex virus, some enteroviruses, vesicular stomatitis virus, arboviruses, measles virus, and *C. pneumoniae*.

■ HFF cells

Human newborn foreskin cells are a diploid fibroblast line. Similar to other fibroblast lines, HFF cells are susceptible to herpes simplex virus and CMV.

■ HNK cells

HNK is a fibroblast line (human neonatal kidney) used for the isolation of VZV, herpes simplex virus, and adenovirus.

■ LLC-MK_2 cells

LLC-MK_2 is a serially propagated, continuous culture line of rhesus monkey kidney *(Macaca mulatta)* cells (RhMK cells). This cell line is most susceptible to enteroviruses, including strains of coxsackie A and B viruses, echovirus, and poliovirus, as well as influenza viruses A and B, mumps virus, and parainfluenza virus.

■ McCoy cells

McCoy cells are derived from a mouse fibroblast line and are used to isolate *C. trachomatis*. Fifteen serotypes of *Chlamydia* with at least 43 passages have shown satisfactory isolation.

■ MDCK cells

MDCK (Madin-Darby canine kidney) is a continuous polarized cell line. MDCK cells are used primarily for isolating influenza A, B, and C viruses. Exogenous protease must be added to the maintenance medium to cleave the viral hemagglutinin and produce infectious viral progeny. Some investigators have found MDCK cells to be superior to RhMK cells for isolating influenza virus (13). Coxsackie B-5 virus, adenoviruses, and reoviruses also are supported by this cell line.

■ Mink lung (Mv1Lu) cells

Mink lung cells are a continuous cell line used for isolation of herpes simplex virus, CMV, and influenza viruses A and B. Advantages include sensitivity equivalent to those of MRC-5 and WI-38 cells but with the ability for extended propagation in the laboratory and decreased effects from toxicity.

■ Mixed-cell cultures

A combination of cell lines within a single culture vessel is used to enhance recovery of viruses with fewer cell cultures and has been described by a number of investigators to be equivalent to and more cost-effective than routine single-cell-line tubes. Mixed-cell cultures have been developed for detection of respiratory viruses, herpesviruses, and enteroviruses by using a combination of cell lines that are sensitive to these specific viruses. Both conventional tube culture and the shell vial format have been described (15). Currently available mixed cell lines include the following: R-Mix (Diagnostic Hybrids), which includes A549 plus mink lung cells; H and V mix, which is African green monkey and MRC-5 cells; and E Mix, which contains BGMK and A549 cells. See individual cell line descriptions for permissiveness for specific viruses.

■ MRC-5 cells

MRC-5 cells are a diploid human fibroblast line propagated from embryonic lung. They are susceptible to a wide range of human viruses. The cell line is especially useful for growing CMV and VZV. Other viruses that may be isolated in MRC-5 cells include adenovirus, coxsackie A virus, echovirus, poliovirus, rhinovirus, respiratory syncytial virus, and herpes simplex virus. This cell line is also used for cytotoxicity testing for *Clostridium difficile*.

■ Rabbit kidney cells

Rabbit kidney cells (New Zealand White rabbits) are a primary cell line used for isolation of herpes simplex virus. CPE does not occur with VZV, which helps to differentiate VZV from herpes simplex virus. The cell line can also be used for the isolation of paramyxoviruses.

■ RD cells

RD is a continuous cell line derived from a human embryonic rhabdomyosarcoma cell line. RD cells are used to detect coxsackie A virus, herpes simplex virus, and poliovirus.

■ Rhesus monkey kidney (RhMK) cells

RhMK cells are an integral part of many diagnostic laboratories. RhMK cells are a primary cell line derived from the rhesus monkey *(M. mulatta)*. Although sometimes contaminated with simian viruses, RhMK cells are a general cell line used for primary isolation of enteroviruses, influenza virus, and parainfluenza virus. They will support the growth of many strains of coxsackie A and B viruses, echovirus, poliovirus, influenza A and B viruses, parainfluenza viruses 1, 2, and 3, measles virus, and mumps virus. Isolation of VZV may be enhanced by using both human diploid fibroblasts and RhMK cells (22).

■ SF cells

SF is a diploid cell line derived from human foreskin (at birth) by ViroMed Laboratories and is similar to the fibroblast lines MRC-5 and WI-38. SF supports the growth of a wide range of viruses, including adenovirus, coxsackie A virus, CMV, echovirus, herpes simplex virus, poliovirus, and VZV. This cell line is also used for *C. difficile* cytotoxicity testing.

■ Vero cells

Vero cells are a serially propagated continuous cell line derived from African green monkey kidney *(C. aethiops)*. Coxsackie B virus, herpes simplex virus, poliovirus, and mumps virus may be isolated in Vero cells. This cell line has also been successful in isolation of Marburg virus, Ebola virus, and lymphocytic choriomeningitis virus. Variant Vero line E-6 has been used to isolate hantavirus.

■ WI-38 cells

WI-38 cells are similar to other fibroblast lines. They are a diploid cell line derived from human embryonic lung fibroblasts. WI-38 cells support growth of a wide range of viruses and are useful for isolating enteroviruses such as coxsackie A virus, echovirus, and poliovirus. Respiratory viruses, such as rhinovirus, respiratory syncytial virus, and adenovirus, as well as herpes simplex virus, CMV, and VZV, are also supported. This cell line is also used for cytotoxicity testing.

APPENDIX 1

Virology Media and Reagents

1. American Type Culture Collection
   Manassas, VA 20110
   http://www.atcc.org
2. BD Biosciences
   Franklin Lakes, NJ 07417
   http://www.bdbiosciences.com
3. Cambrex Corporation (Biowhittaker Cell Products)
   http://www.cambrex.com
4. Chemicon International, Inc.
   Temecula, CA 92590
   http://www.chemicon.com
5. Dakocytomation
   Glostrup, Denmark
   http://www.dako.com
6. Diagnostic Hybrids, Inc.
   Athens, OH 45701
   http://www.dhiusa.com
7. Hardy Diagnostics
   Santa Maria, CA 93455
   http://www.hardydiagnostics.com
8. Invitrogen (Gibco)
   Carlsbad, CA 92008
   http://www.invitrogen.com
9. Medical Wire and Equipment-MWE
   Corsham, Wiltshire, United Kingdom
   http://www.mwe.co.uk
10. Remel (Fisher Scientific is the distributor)
    Lenexa, KS 66215
    http://www.remelinc.com

11. Trinity Biotech (Bartels, MicroTrak)
Carlsbad, CA 92008
http://www.trinity
12. ViroMed Laboratories, Inc.
Minnetonka, MN 55343
http://www.viromed.com

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Algorithms for Detection and Identification of Viruses

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82

Virology is a dynamic field that in recent decades has moved from the periphery to the mainstream of clinical laboratory practice. When the first edition of the *Manual of Clinical Microbiology* was published in 1970, diagnostic virology was practiced in a limited number of laboratories, primarily in public health, research, and academic settings. Methods focused on conventional cell cultures, classical serologic techniques, and light and electron microscopy. Time to result was slow, and it was often said that the patient was dead or better by the time the result was received. Over the intervening years, diagnostic advances have transformed the field by allowing accurate results in a clinically useful time frame. These include enzyme immunoassays for antigen and antibody detection, immunoglobulin M (IgM) assays, monoclonal antibodies for rapid identification of culture isolates, and direct detection in clinical specimens by immunofluorescence, shell vial centrifugation cultures, and nucleic acid amplification techniques.

Since the last edition of this Manual, the greatest growth has been in molecular methods, which are both rapid and sensitive, can be largely automated, and can detect viruses not amenable to routine culture. The greater variety of instruments for nucleic acid extraction and real-time amplification and detection and the increasing availability of commercial reagents and kits have facilitated this trend. Some molecular assays have now become standard of care. Furthermore, quantitative monitoring of viral load in blood has become more widely applied due to implementation of real-time PCR techniques. In addition to the advantages of molecular testing, however, some pitfalls are also becoming apparent as the tests are more widely used. For example, sensitivity and specificity can vary significantly between laboratories. In addition, despite targeting conserved regions of the genome, strain variability and mutations can lead to underquantification of viral load or even falsely negative results. Furthermore, as tests become more sensitive, low levels of clinically irrelevant viruses may be detected and can be misleading to clinicians. Requirements for staff training, test validation, and quality control can be challenging. At the other end of the spectrum has been the proliferation of rapid tests requiring minimal or no equipment or reagent additions. These tests often employ immunochromatographic detection for viral antigen or antibody and can be used at the point-of-care for immediate impact on clinical decisions.

Thus, with progress have come new challenges. Laboratories need to choose which tests to offer. Performance characteristics, staff expertise, and cost impact that choice. Laboratories must recognize the uses and also the limitations of each test in order to guide clinicians in interpreting the results. This Manual should serve as a key resource for accomplishing these tasks. Selecting the appropriate test depends on the virus(es) sought, sample site, clinical presentation, clinical purpose (e.g., screening, confirmation, diagnosis, or monitoring), patient characteristics, and disease prevalence. The methods available for each virus differ and continue to evolve. Table 1 provides a concise overview for each virus group; however, the reader is referred to the specific chapters for more detailed discussions.

While it can be daunting for laboratories to acquire and maintain expertise in the variety of test methods now available, it is extremely gratifying to witness the impact of state-of-the-art testing on patient care. As we move forward, it is critical that laboratorians communicate with each other to address problems, including the optimization and standardization of methods, and in addition encourage input and feedback from clinicians. Due to the speed of methodological change and the continuing discovery of new viruses and new therapies, keeping abreast of the most recent literature is strongly recommended.

TABLE 1 Methods for detection and identification of viruses[a]

Virus	Applicability[b] of:					Comments
	Virus isolation	Antigen detection	Nucleic acid detection	Electron microscopy	Antibody detection	
Adenoviruses	A	A	B	C	C	IFA and culture widely used for respiratory specimens. Rapid antigen assays used for enteric adenoviruses. NAAT used to monitor viral load in compromised hosts.
Arboviruses	C	D	B	C	A	Serology is primary diagnostic method. Most arboviruses are readily cultured; isolation of some agents may require BSL-3 or BSL-4 facilities.
Coronaviruses	C	C	A	C	A	Positive SARS CoV NAAT and antibody results require confirmation by a reference laboratory. NAAT becoming more widely used for non-SARS CoV.
Cytomegalovirus	A	A	A	C	B	Shell vial culture rapid and sensitive for nonblood specimens. Quantitative NAAT and pp65 antigenemia used to assess risk of disease and response to therapy. Serology primarily used to determine prior infection.
Enteroviruses	A	D	A	D	D	Enterovirus RNA detection preferred for CNS infection. Parechovirus detected primarily through virus isolation.
Epstein-Barr virus	D	B	A	D	A	Serology test of choice for routine diagnosis. NAAT useful for virus-related tumors. Quantitative NAAT useful for monitoring viral load in blood of transplant recipients. IHC or ISH can be used on tumor biopsy specimens.
Filoviruses and arenaviruses	A	A	A	C	B	Testing confined to specialized laboratories. Antigen and NAAT key to rapid diagnosis. BSL-4 facility needed for culture, except for LCM. Patients with severe disease may die without developing antibody. LCM diagnosed primarily by serology.
Hantaviruses	C	B	A	C	A	Testing confined to specialized laboratories. Serology and NAAT equally useful for diagnosis. IHC used in fatal cases. BSL-4 facility needed for culture. Isolation difficult.
Hendra and Nipah viruses	B	B	A	C	A	Testing confined to specialized laboratories. Serology and NAAT equally useful for diagnosis. IHC used in fatal cases. BSL-4 facility needed for culture. Patients with severe disease may die without developing antibody.
Hepatitis A virus	D	C	C	D	A	Serology is the standard diagnostic test. False-positive IgM problematic in low-prevalence areas.
Hepatitis B virus	D	A	A	C	A	Detection of specific viral antigens and antibodies allows for diagnosis and for monitoring the course of infection. NAAT used to monitor therapy.
Hepatitis C and G viruses	D	D	A	D	A	Serology is used for diagnosis. Qualitative NAAT used to confirm active infection. Quantitative NAAT used to monitor response to therapy. Genotyping helps determine duration of therapy.
Hepatitis D virus	D	B	B	D	A	Testing confined to reference laboratories. Diagnosis is relevant only in the presence of hepatitis B infection. IHC of biopsy tissue useful for diagnosis.
Hepatitis E virus	D	D	B	D	A	Serology is the standard diagnostic test. False-positive IgM problematic in low-prevalence areas. NAAT is specific for acute infection but lacks sensitivity.

(Continued on next page)

TABLE 1 Methods for detection and identification of viruses[a] *(Continued)*

Virus	Applicability[b] of:					Comments
	Virus isolation	Antigen detection	Nucleic acid detection	Electron microscopy	Antibody detection	
Herpes simplex virus	A	A	A	C	B	Shell vial used for rapid determination of viral replication. IFA used for rapid detection in skin or mucous membrane lesions or tissue specimens. NAAT is test of choice for CNS infection. Serology used to determine prior infection.
Herpesviruses 6 and 7	C	C	A	C	B	NAAT test of choice for diagnosis. MAbs available to differentiate virus isolates and for IHC. Serology can document primary infection in children.
Herpesvirus 8	D	B	B	C	A	Serology used to identify infected persons; sensitivity and specificity hampered by difficulty in setting cutoff values. NAAT of blood may be useful for monitoring KS risk. IHC more specific than NAAT for KS.
Human immunodeficiency virus	C	C	A	D	A	Serology is primary diagnostic method; rapid antibody testing becoming more widely available. Proviral DNA and plasma RNA tests used to diagnose neonatal infection. Quantitative RNA tests used to guide therapy and monitor response.
Human metapneumovirus	B	C	A	C	D	NAAT is the test of choice for diagnosis. Shell vial culture and antigen detection reagents not yet widely available. Conventional culture difficult.
Human T-cell lymphotropic virus	C	D	B	D	A	Serology is primary diagnostic method. NAAT useful for virus identification in HTLV Western blot-positive but untypeable specimens.
Influenza viruses	A	A	A	D	B	Rapid antigen tests widely used but suboptimal in sensitivity and specificity; IFA and rapid culture more sensitive. NAAT most sensitive but not yet widely used. Serology useful for epidemiological studies or retrospective diagnosis.
Measles viruses	B	B	C	C	A	Serology most useful for diagnosis and determination of immunity. Isolation useful only if attempted early (prodromal period to 4 days postrash).
Mumps virus	A	C	C	C	A	Serology used most commonly for diagnosis and determination of immunity.
Noroviruses	D	C	B	B	D	NAAT promising but challenging due to strain variability. EM useful in suitably equipped laboratories. Reagents for antigen detection not commercially available.
Parainfluenza viruses	A	A	B	D	C	IFA most common rapid detection method. NAAT more sensitive than isolation but not yet widely adopted.
Papillomaviruses	D	D	A	C	D	NAAT test of choice for detection and genotype differentiation. Cytopathology useful for diagnosis. Serologic diagnosis of exposure not available.
Parvoviruses	D	C	A	C	A	Serology used to diagnose B19 in immunocompetent individuals. NAAT test of choice for exposed fetuses and immunocompromised hosts. NAAT can be used to detect human bocavirus.
Polyomaviruses	C	B	A	B	C	JC virus DNA detection in cerebrospinal fluid useful for presumptive diagnosis of PML. BK virus DNA quantification in plasma or urine used for preemptive diagnosis of PVAN. IHC and EM useful for biopsy tissues.
Poxviruses	A	C	A	A	A	EM is the most useful routine test. NAAT allows virus inactivation and rapid detection. Smallpox isolation requires BSL-3 or BSL-4 and should be attempted only in WHO Collaborating Centers. Vaccinia virus requires BSL-2 and grows readily in cell culture.

Rabies virus	B	A	B	C	B	IFA performed on fresh and frozen tissue at the CDC. Serology used to confirm immunization status and confirm exposure in absence of RIG. NAAT most useful for non-CNS samples when fresh CNS tissue not available.
Respiratory syncytial virus	A	A	A	C	C	Rapid antigen tests, especially IFA, more sensitive than culture. NAAT most sensitive but not yet widely used. Serology useful only for epidemiological studies.
Rhinoviruses	A	C	B	D	D	Culture still most common test in clinical laboratories. NAAT more sensitive and most common test in research and epidemiological studies; cross-reaction with enteroviruses can occur.
Rotaviruses	D	A	B	C	D	Direct antigen detection is test of choice for diagnosis. EM useful in suitably equipped laboratories. NAAT is promising.
Rubella virus	A	D	B	D	A	Serology most useful for diagnosis and determination of immunity. Isolation useful for postnatal rubella if attempted early (prodromal period to 4 days postrash). In CRS, virus can be isolated for weeks to months after birth.
Transmissible spongiform encephalopathy agents	D	C	B	C	D	Histology most useful diagnostic test. Surrogate markers popular but lack specificity. Western blot for PrP performed in specialized laboratories. Human genome sequencing useful for diagnosis of genetic disorders.
Varicella-zoster virus	A	A	A	C	B	IFA and NAAT are most commonly used rapid tests. Culture less sensitive than IFA. Serology most useful for determination of immunity.

[a]Virus isolation includes conventional cell culture with detection of viral growth by cytopathic effects or hemadsorption; shell vial centrifugation culture uses immunostaining for detection of viral antigens. Viral antigens can be detected by a variety of immunoassays such as enzyme-linked immunosorbent assays, agglutination assays, immunofluorescence or immunoperoxidase techniques, and immunochromatography. Viral nucleic acids (DNA or RNA) can be detected and quantified by direct hybridization or by the performance of amplification methods such as PCR. Electron microscopy involves the visualization of viral particles by negative staining or immunoelectron microscopy or by thin-section techniques. Antibody detection involves measurement of total or class-specific immunoglobulins directed at specific viral antigens. Abbreviations: IFA, immunofluorescence assay; NAAT, nucleic acid amplification test; BSL, biosafety level; EM, electron microscopy; SARS, severe acute respiratory syndrome; CoV, coronavirus; CNS, central nervous system; LCM, lymphocytic choriomeningitis virus; IHC, immunohistochemistry; ISH, in situ hybridization; MAb, monoclonal antibody; KS, Kaposi's sarcoma; HTLV, human T-cell lymphotropic virus; PML, progressive multifocal leukoencephalopathy; PVAN, polyomavirus-associated nephropathy; RIG, rabies immune globulin; CRS, congenital rubella syndrome; PrP, prion protein.

[b]A, test is generally useful for the indicated diagnosis; B, test is useful under certain circumstances or for the diagnosis of specific forms of infection, as delineated in the right-hand column and in the text of the individual chapters; C, test is seldom useful for general diagnostic purposes but may be useful for epidemiological studies or for the diagnosis of unusual conditions; D, test is generally not used for laboratory diagnosis of infection.

Human Immunodeficiency Viruses*

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83

TAXONOMY

Origin and Historical Perspective

The human immunodeficiency virus (HIV) is the etiologic agent of AIDS. AIDS was first recognized clinically in 1981 (93), although the first known case of AIDS has been traced to 1959 (136). HIV exists as two major viral species, HIV type 1 (HIV-1) and HIV-2. The more virulent of the two HIVs, HIV-1, is responsible for the worldwide AIDS pandemic, with over 39 million people living with HIV/AIDS as of the end of 2004. HIV-2, on the other hand, is less pathogenic and has a more limited geographic distribution.

HIV-1 was initially discovered in 1983 (3) and was confirmed the following year to be the cause of AIDS (55, 83, 123). Clavel et al. first isolated HIV-2 in 1986 (22). Both HIV-1 and HIV-2 are related to simian immunodeficiency viruses (SIV) (38, 69). Simian immunodeficiency virus strains have been found in over 25 species of nonhuman African primates (69). Chimpanzees and a number of species of Old World monkeys are the natural hosts for these primate lentiviruses, and it has been suggested that both the HIV-1 and HIV-2 epidemics are the result of simian-to-human transmissions. The HIV-1 epidemic appears to be the result of zoonotic virus transmissions from chimpanzee to human (56), while the sooty mangabey apparently is the source of HIV-2 infections in humans (136).

HIV Groups and Subgroups

Both types of HIV are members of the genus *Lentivirus* within the family of *Retroviridae*. Because of the mutation and recombination capabilities of HIV, various genetic clades have evolved (94, 119), and the genetic diversity of HIV-1 plays a significant role in the design and interpretation of HIV diagnostic assays. HIV-1 is subdivided into three genetic groups designated M (for major), O (for outlier), and N (for non-M, non-O), based on sequence diversity within the HIV-1 *gag* and *env* genes. HIV-1 group M is divided into nine subtypes (A through D, F through H, J, and K). Viruses belonging to different M subtypes have been isolated in distinct regions of the world. The initial epidemic in the early 1980s in North America and Europe was due to HIV-1 subtype B. Currently, subtype C dominates the global epidemic, although subtype B continues to be the major subtype in the United States, Europe, and Australia. Certain HIV-1 viral isolates appear to be recombinant, containing sequences from more than one subtype. Group O viruses are only rarely isolated and found mostly in persons from Cameroon, Gabon, and Equatorial Guinea. Group N viruses have been recovered from Cameroon. Seven subtypes of HIV-2 (A through G) have been defined, although HIV-2 groups C through G have each been recovered only from a single individual.

DESCRIPTION OF THE AGENTS

Structure and Genomic Organization

HIV viruses are enveloped plus-stranded RNA viruses. The HIV genome is organized similarly to other retroviruses. It contains the *gag, pol,* and *env* genes which encode structural proteins, viral enzymes, and envelope glycoproteins, respectively. These are flanked by long terminal repeats. In addition, the genome comprises open reading frames for *trans*-acting transcriptional activator (Tat), the regulator of viral expression (Rev), and several other proteins such as Vif, Vpr, and Nef.

Mature viral particles measure 100 to 150 nm in diameter and have a conical core surrounded by a lipid envelope (Fig. 1). The core contains two identical copies of single-stranded RNA, approximately 10 kb in length, which are surrounded by structural proteins that form the nucleocapsid and the matrix shell as well as by products of the *pol* gene. The lipid envelope is acquired as the virus buds from infected cells. The glycoprotein gp120/41 forms spikes protruding from this envelope. The glycoprotein, a product of the *env* gene, is synthesized as a precursor, gp160, which is cleaved into the heavily glycosylated gp120, forming the majority of the external portion and the transmembrane gp41 portion, which contains the membrane spanning domain. The major structural proteins that are encoded by the *gag* gene include p17, p24, p7, and p9. Products of the *pol* gene include the protease, reverse transcriptase, RNase, integrase, and ribonuclease.

HIV-2 is structurally analogous to HIV-1, but some of its protein components differ, most notably the outer envelope and transmembrane glycoproteins gp125 and gp36, as well as the core proteins p16 and p26. The major gene products that are of significance for the diagnosis of HIV-1 and HIV-2 are listed in Table 1.

* This chapter contains information presented in chapter 80 by Jörg Schüpbach in the eighth edition of this Manual.

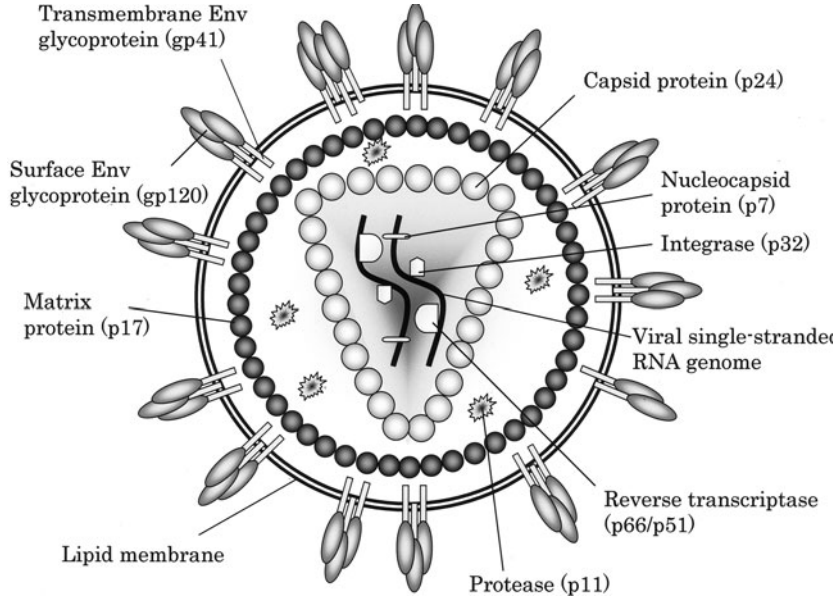


FIGURE 1 Schematic representation of a mature HIV virion showing the localization of viral proteins.

Replication Cycle

The replication cycle of HIV-1 is accomplished in vivo in approximately 24 h (120). Replication begins with the attachment of virus to the target cell via the interaction of gp120 and the cellular receptor CD4 (32). This binding results in gp120 conformational changes which allow the virus to interact with other cellular coreceptor sites, CXCR4 or CCR5. The interaction with CXCR4 occurs primarily with T-cell-tropic, syncytium-inducing viruses (51). In contrast, the β-chemokine receptor CCR5 is involved in macrophage-tropic non-syncytium-inducing HIVs (21).

Following fusion with the host cells, HIV enters the cell and RNA is released. HIV particles contain reverse transcriptase (RT), an enzyme that plays a crucial role in the replication process. RT possesses three distinct functions: RNA-dependent DNA polymerase, which serves to synthesize cDNA; RNase H, which degrades RNA from the cDNA-RNA complex; and a DNA-dependent DNA polymerase, which duplicates the cDNA strand. The reverse-transcribed genome is associated with several viral proteins and transported into the nucleus. The DNA copy becomes integrated into the genome of the infected cell by the virally encoded integrase; this integrated retroviral DNA genome is called the provirus. The cDNA then serves as a template for viral RNA. Activation of HIV transcription and gene expression is modulated by cellular transcription factors and by viral regulatory proteins, including Tat, Rev, Nef, and Vrp. The regulatory genes *tat* and *rev* greatly influence the rate of viral replication. The Tat protein increases transcription from the long terminal repeat. The Rev protein facilitates export of unspliced or partially spliced RNAs encoding the viral structural proteins. At the end of the replication cycle, the virion assembles and buds through the plasma membrane. Due to the infidelity of the RT, many errors are made during the replication cycle; thus, in an infected individual the virus exists as quasispecies of closely related viral genomes.

EPIDEMIOLOGY AND TRANSMISSION

Epidemiology

The AIDS pandemic has had an enormous toll throughout the world, and HIV-1 has been reported in virtually every country on earth. In contrast, HIV-2 has remained limited

TABLE 1 Major HIV-1 and HIV-2 gene products

Gene	Description of gene products	Gene products of:	
		HIV-1	HIV-2
Core *(gag)*	Precursor of Gag protein	p55	p53–55
	Capsid protein	p24	p26
	Matrix protein	p17	p16
Polymerase *(pol)*	RT component	p66	p68
	RT component	p51	
	Endonuclease component	p32	p33
Envelope *(env)*	Precursor of Env glycoprotein	gp160	gp125
	Outer Env glycoprotein	gp120	gp105
	Transmembrane Env glycoprotein	gp41	gp36

primarily to West Africa and to European immigrants from West Africa. Two African countries which are not in Western Africa, Angola and Mozambique, also have high HIV-2 seroprevalence rates, and additional spread of HIV-2 has been documented in Portugal, France, and India. Group M viruses are responsible for most HIV-1 infections, with a predominance of subtype C. Subtype B is most prevalent in the United States, Europe, and Australia but is rarely found in Africa. Subtypes A, C, and D predominate in Africa.

The HIV epidemic continues to grow and change (50). In 2003, an estimated 4.8 million people became newly infected with HIV, bringing to 37.8 million the number of individuals living with HIV. In addition, the AIDS epidemic killed 2.9 million people in 2003 and has killed over 20 million people since the first cases of AIDS were identified in 1981. Over 60% of HIV-infected individuals live in sub-Saharan Africa. Globally, the overall proportion of HIV-positive women has increased steadily in recent years; and women represented 50% of people living with HIV in 2002 (151). This trend is most obvious in sub-Saharan Africa and the Caribbean.

In the United States, the cumulative estimated number of AIDS cases diagnosed through 2003 is 929,985 and the cumulative estimated number of AIDS-related deaths is 524,060 (15). The demographic characteristics of the epidemic have changed significantly since the 1980s. In the early days of the epidemic, populations most affected in the United States included mostly homosexual and bisexual men (50). During the past decade, an estimated 40,000 people have been infected with HIV every year, but the epidemic now affects a large proportion of African-Americans and greater numbers of women (15), with approximately one-half of new infections reported among African-Americans. Most HIV infections in men occur during injecting-drug use or sex between men. The main risk factor for women is heterosexual transmission from a male partner with undisclosed risk behaviors. AIDS-related deaths have steadily decreased since the introduction of antiretroviral therapy in 1995–1996. However, African-Americans have the poorest survival rates among individuals diagnosed with AIDS in the United States.

Transmission

HIV-1 and HIV-2 have the same modes of transmission. The most common mode of HIV infection is sexual transmission at the genital mucosa through direct contact with infected blood fluids, including blood, semen, and vaginal secretions (74, 130, 157). Infection may also occur through inoculation of infected blood, via transfusion of infected blood products, transplantation of infected tissues, or reuse of contaminated needles. The risk of HIV-1 infection following occupational percutaneous exposure to HIV-1-infected blood has been estimated to be 0.3% (57).

The vast majority of HIV transmission from mother to child occurs in resource-poor countries. Maternal HIV-1 RNA in blood and genital fluids has been documented to correlate strongly with mother-to-child transmission (102). HIV transmission can occur in utero, during labor and delivery, and during breast-feeding. In the absence of therapeutic intervention, the risk of mother-to-child transmission can range from 15 to 30% and is further increased in the setting of breast-feeding. However, the risk can be significantly reduced to below 2% if antiretroviral therapy is administered to women during pregnancy and labor (25). Antiretroviral treatment of the mother and infant after birth can also significantly decrease the risk of HIV infection in the newborn.

CLINICAL SIGNIFICANCE

Virological Parameters during the Course of HIV Infection

The natural history of HIV-1 infection can be divided into three phases: a transient acute retroviral syndrome, a period of clinical latency, and finally progression to AIDS. Each of these stages is associated with specific changes in virologic and immunologic parameters. Following HIV infection, HIV-specific markers appear in the blood in the following chronological order: HIV RNA, p24 antigen, and HIV antibody (Fig. 2). The exact time when each of these three markers can be detected depends on a number of factors including the type of test used, characteristics of the infecting virus, and host immune responses of the individual. During the first week after infection, HIV RNA, antigen, and antibody are generally not detectable, although viral replication is occurring. Within 1 to 4 weeks after infection, viral RNA increases rapidly and viral antigen is detectable, but HIV antibody is usually not detectable. The time period when HIV antibody is not detectable is referred to as the window period. In most infected persons, HIV antibody is detected within 1 to 2 months, but in a small percentage of individuals, seroconversion may require up to 6 months. Following the initial HIV RNA peak, viremia generally decreases to a lower comparatively stable level, which reflects ongoing viral replication and immune system damage. The HIV RNA pattern in perinatally infected infants differs from that in infected adults, since high HIV RNA levels may persist in HIV-infected children for prolonged time periods.

Acute Retroviral Syndrome

Acute HIV-1 infection is a transient symptomatic illness which is associated with high levels of HIV-1 replication and a developing virus-specific immune response. The diagnosis of acute HIV-1 infection is often missed because symptoms tend to be nonspecific and HIV-1-specific antibodies are not detectable. Symptoms present in 40 to 80% of the cases within 3 to 6 weeks after primary infection and usually last 7 to 14 days. The clinical symptoms of acute HIV-1 infection were first described as a mononucleosis-like syndrome in 1985 (29). Most commonly, fever, maculopapular rash, oral ulcers, lymphadenopathy, malaise, weight loss, arthralgia, pharyngitis, and/or night sweats are seen (31, 70).

During acute HIV-1 infection, there is an initial rise in plasma viremia, reaching levels of up to 100 million copies of HIV-1 RNA per ml of plasma (79). In addition, destruction of HIV-1-specific $CD4^+$ T lymphocytes as well as widespread dissemination of the virus, with seeding of lymphoid organs and other tissue reservoirs, occurs. During resolution of primary infection, $CD4^+$ T-cell counts rebound, and viremia declines before reaching a steady level, which has been shown to be an important predictor of disease progression (96, 97).

Clinical Latency

The asymptomatic period, which usually lasts several years, is the period between primary infection and the development of clinical immunodeficiency. During that period, the number of $CD4^+$ lymphocytes declines slowly, and the virus continues to replicate, albeit at a relatively stable level. During this clinical latency period, high titers of virus can be found in lymphoid and other tissue compartments (116). The length of the clinical latency period can vary considerably but is on average 10 years. In rapid progressors, AIDS develops within 1 to 2 years, but the clinical latency period

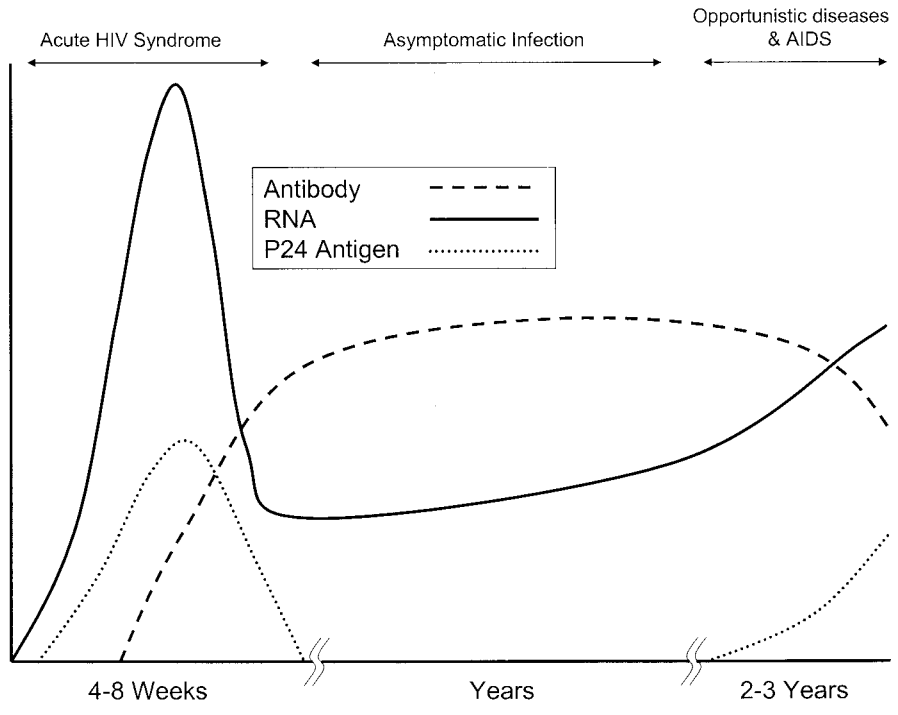


FIGURE 2 Time course of appearance of markers of HIV infection during the course of HIV infection.

can be short, as in the recent well-publicized New York case, where the infection was reported to result in symptomatic AIDS in 4 to 20 months (91). In contrast, 5 to 10% of HIV-infected individuals are long-term nonprogressors and remain symptom-free for longer than 20 years (104). Several factors influence the steady-state level of viral replication and the rate of disease progression, including host immune responses, host genetic factors, and the fitness of the infecting virus. One of the most important host genetic factors reported to affect virus replication rates is the deletion of CCR5; most individuals homozygous for a 32-bp deletion in CCR5 are resistant to HIV-1 infection (133) and showed slower HIV-1 replication (122). In addition to mutations in chemokine receptor genes, a number of HLA class I alleles have been found to be associated with lower steady-state viral levels and slow disease progression (1), and it has been suggested that rates of disease progression may also be influenced by other concurrent infections (143).

Disease Progression to AIDS

The continuous replication of HIV-1 in productively infected cells together with the elimination of host cells and chronic immune activation results over the years in deterioration of the immune system. Studies of HIV-1 replication dynamics have shown that, in productively infected lymphocytes, the interval between infection, virus production, and cell death is very short. The half-life of HIV-1 in plasma is thought to be 6 h, the length of the replication cycle in $CD4^+$ lymphocytes has been estimated at 2.6 days, and 35 million $CD4^+$ cells are thought to be lost daily (73). As the ability of the host to eliminate productively infected cells declines, $CD4^+$ lymphocytes with integrated provirus accumulate. These resting memory $CD4^+$ lymphocytes serve as a long-lived reservoir for HIV-1. The mean half-life of this latent reservoir has been reported to be as long as 40 months for many patients (52).

Clinical manifestations can affect nearly every organ system. In addition, destruction of the immune system is clinically manifested by the occurrence of opportunistic infections and tumors, and central nervous system involvement can be seen, most commonly the HIV-associated dementia complex (124). Conditions listed in the 1993 AIDS surveillance case definition include a CD4 T-cell count of <200; opportunistic infections caused by viruses, bacteria, fungi, and protozoa; neoplastic disease; HIV encephalopathy; wasting syndrome; and progressive multifocal leukoencephalopathy. These conditions complicate the clinical course of HIV-1 infections and are often the cause of death.

Several public health organizations, including the CDC and the WHO, have published HIV-1 disease classification systems for both public health purposes and HIV disease staging. HIV-1 disease staging classifies disease according to prognosis and is useful in guiding treatment decisions. The current CDC classification system for HIV-infected adolescents (>13 years) and adults combines three categories of CD4 counts (categories 1, 2, and 3, corresponding to >500, 200 to 499, and <200 cells/mm^3, respectively) with three symptom categories (categories A, B, and C) (12).

The course of HIV infection and the clinical characteristics of AIDS in children differ from those in adults, and the CDC has developed a separate classification system to describe the spectrum of HIV-1 disease in children less than 13 years of age born to HIV-infected mothers (13). The disease progresses rapidly in infants with vertically acquired HIV infection. The most common AIDS defining conditions include *Pneumocystis jirovecii* pneumonia, lymphoid interstitial pneumonia/pulmonary lymphoid hyperplasia, and recurrent bacterial infections.

Therapy and Vaccination

Significant scientific advances in the development of effective antiretroviral therapy have occurred in the past 20 years. The first effective drug against HIV-1, zidovudine (AZT), was approved by the FDA in March 1987. Currently, a total of 24 antiretroviral drugs or combinations of antiviral drugs have been approved by the FDA for the treatment of HIV infections (Table 2). Approved antiviral agents used to treat HIV-1 fall into four classes: nucleoside RT inhibitors (NRTIs), nonnucleoside RT inhibitors (NNRTIs), protease inhibitors (PIs), and fusion inhibitors. The combination of three or more of the anti-HIV drugs, also referred to as highly active antiretroviral therapy (HAART), is the recommended treatment for HIV-1 infection. Guidelines for the use of antiretroviral agents in adults and in children evolve rapidly; these are updated on a regular basis, and the most recent guidelines can be obtained from the AIDS information website (http://AIDSinfo.nih.gov). Not all drugs used to treat HIV-1 infection are effective against HIV-2 infection. HIV-2 is resistant to NNRTIs and thought to be less susceptible to zidovudine and some of the protease inhibitors.

Although currently available therapies have been shown to slow down progression to AIDS, the activity of these powerful combination therapies can be altered by a number of factors including adherence, drug toxicity, and the development of antiviral resistance. Therefore, proper management of HIV-infected patients must include systematic monitoring of the efficacy of antiviral treatment through use of sensitive and accurate tests to measure viral load changes during therapy and to detect the emergence of antiviral resistance.

Several obstacles have slowed down the progress towards the development of effective vaccines against HIV (38, 105). These include the inability of HIV-1 to produce neutralizing antibodies, the lack of understanding of correlates of protective immunity, the genetic diversity of the virus, and the limitation of animal models. A variety of vaccine strategies have been investigated, including live attenuated subunit, recombinant vector, and DNA vaccines (132). Various adjuvants have been tried, and alternate sites of delivery have been explored. Although results of trials of subunit vaccines showed no protection against infection and did not lower viral loads, newer strategies combining DNA vaccines and recombinant vector vaccines are under investigation.

COLLECTION, STORAGE, AND TRANSPORT OF SPECIMENS

Serum samples are used routinely for standard HIV antibody and antigen determinations, although plasma samples are also acceptable. The serum/plasma should be promptly separated from the clot/cellular elements and refrigerated at 2 to 8°C. If testing is not to be performed within 7 days, the serum specimens should be frozen at −20°C or lower. Serum/plasma specimens can be transported either refrigerated (if transport takes places within 7 days of collection) or frozen in screw-capped plastic vials.

Dried blood spots, as well as noninvasive specimens such as urine or oral fluid, are approved for use in some of the HIV antibody kits. The Orasure HIV-1 specimen collection device (Epitope Inc., Beaverton, Oreg.), which increases the flow of mucosal transudate across the mucosal surfaces onto an absorbent cotton pad, is available for collection of oral fluids. Rapid, point-of-care HIV assays usually require finger stick or venipuncture whole blood, although one of the assays is approved for use with oral fluid, and some of the assays have been validated for use with serum samples.

TABLE 2 Antiretroviral formulations approved for use by the FDA (as of March 2006)

Type of antiretroviral agent	Generic name (approval yr)	Other name(s)
NRTIs	Zidovudine (1987)	Retrovir, AZT, ZDV
	Didanosine (1991)	Videx, ddI
	Zalcitabine (1992)	HIVID, ddC
	Stavudine (1994)	Zerit, d4T
	Lamivudine (1995)	Epivir, 3TC
	Abacavir (1998)	Ziagen, ABC
	Tenofovir (2001)	Viread, TDF
	Emtricitabine (2003)	Emtriva, FT
NRTI coformulations	Zidovudine-lamivudine (1997)	Combivir
	Zidovudine-lamivudine-abacavir (2000)	Trizivir
	Tenofovir-emtricitabine (2004)	Truvada
	Abacavir-lamivudine (2004)	Epzicom
NNRTIs	Nevirapine (1996)	Viramune, NVP
	Delavirdine (1997)	Rescriptor, DLV
	Efavirenz (1998)	Sustiva, EFV
PIs	Saquinavir (1995)	Invirase, Fortonase, SQV
	Ritonavir (1996)	Norvir, RTV
	Indinavir (1996)	Crixivan, IDV
	Nelfinavir (1997)	Viracept, NFV
	Atazanavir (2003)	Reyataz, ATV
	Fosamprenavir (2003)	Lexiva, FPV
	Tripranavir (2005)	Aptivus
PI coformulation	Lopinavir-ritonavir (2000)	Kaletra, LPV/r
Fusion inhibitors	Enfuvirtide (2003)	Fuzeon, T-20

TABLE 3 Comparison of HIV-1 viral load assay characteristics

Assay characteristics	Amplicor HIV-1 Monitor Test Version 1.5		VERSANT HIV-1 RNA 3.0 bDNA assay	NucliSens HIV-1 QT assay
	Standard	Ultrasensitive		
Specimen requirements				
Anticoagulant	EDTA, ACD	EDTA, ACD	EDTA, ACD	EDTA, ACD, heparin
Plasma vol	0.2 ml	0.5 ml	1.0 ml	50 μl-2 ml
Kit characteristics				
RNA isolation method	Precipitation	Ultracentrifugation, precipitation	Ultracentrifugation	Binding to silica particles
Amplification method	RT-PCR	RT-PCR	bDNA	Isothermal
Amplified product	DNA	DNA	Signal	RNA
Detection method	ELISA	ELISA	Chemiluminescence	Electrochemiluminescence
Standards and controls	1 internal QS,[a] 3 external controls	1 internal QS,[a] 3 external controls	6 external standards, 3 external controls	3 internal calibrators
Dynamic range (copies/ml)	400–750,000	50–100,000	75–500,000	176–3,400,000[b]

[a] Quantitation standard RNA.
[b] Use of a 2 ml input volume instead of 1 ml improves the lower limit of sensitivity to 80 copies/ml.

HIV RNA viral load assays are most commonly performed with plasma specimens. To ensure accurate HIV RNA quantitation in plasma, proper collection, processing, storage, and transport of plasma specimens are essential to avoid RNA degradation. The same collection system and procedures should be used to follow up patients over time, because different collection systems may produce discrepant values. In addition, if a change in the collection system or technology is planned, baseline HIV values should be determined with two specimens obtained less than 2 weeks apart. Each of the three FDA-approved kits available for determination of HIV viral load in plasma has specific volume and collection tube requirements (Table 3). These assays have also been used to measure HIV viral loads in specimens other than plasma, including dried blood spots, cerebrospinal fluid, cervical secretions, seminal plasma/semen, and serum. The RNA content in serum has been found to be lower than in the paired plasma sample (66). In addition, acid citrate dextrose (ACD) anticoagulated specimens have also been shown to yield results that are approximately 15% lower than results obtained with EDTA plasma (61, 84). For all assays, plasma must be separated from blood cells within 6 h of collection, because delays in the separation of plasma from cellular elements can result in RNA degradation. HIV RNA is generally stable in cell-free plasma at refrigerated temperature for several days and remains stable for at least three freeze/thaw cycles (66). For long-term storage, plasma samples should be stored at −70°C. Plasma preparation tubes (PPT) (BD Vacutainer PPT tubes; Becton Dickinson, Franklin Lakes, N.J.), which contain dried EDTA and a gel separator that after centrifugation forms a barrier between the plasma and cellular elements, have become available in recent years. PPT tubes provide a closed collection system for the preparation and transport of plasma specimens; plasma samples do not need to be poured off from PPT tubes after centrifugation, and plasma transfer and relabeling steps are eliminated. This improves convenience, decreases risks of errors, and improves safety. Initial recommendations from the manufacturer allowed for either refrigerated or frozen transport of centrifuged PPT tubes. However, recently, minor discrepancies have been reported to occur in plasma samples that were frozen in situ (64, 142). The differences were noted only for samples with viral loads close to the limits of detection; a large proportion of samples with undetectable viral load in the standard EDTA aliquot were found to have detectable HIV RNA in the corresponding PPT tube. As a result, the manufacturer has recently recommended that plasma in PPT tubes should be stored without freezing and that plasma from PPT tubes should be transferred to a secondary tube if plasma samples need to be stored frozen.

For HIV resistance assays, genotypic and phenotypic, plasma collected from EDTA, ACD, or PPT/EDTA tubes can be used. The plasma must be separated from the cellular elements within 6 h of collection and frozen immediately. Plasma specimens should be transported frozen. For HIV DNA and viral culture assays, whole blood is commonly used. It should be collected on EDTA, ACD, or cell preparation tubes. HIV can be cultured from plasma or from peripheral blood mononuclear cells (PBMCs), but HIV DNA assays require whole blood or PBMCs. For preparation of PBMCs, the blood specimen should not be refrigerated or frozen, but instead should be kept at ambient temperature for no longer than 4 days.

DIRECT DETECTION

p24 Antigen Assays

In the early days of the AIDS epidemic, p24 antigen testing played an important role as a tool for the diagnosis, prognosis, and evaluation of antiretroviral activity and for monitoring of HIV-1-infected cultures. In 1996, the FDA approved the Coulter HIV-1 p24 Ag assay (Coulter Co., Miami, Fla.) for screening blood products. This test was used for screening blood and plasma donors in the United States for a few years until it was replaced with more sensitive nucleic acid tests in 1999. At present, use of this assay is very limited in the United States, and only one kit (Coulter HIV-1 p24 antigen assay) is commercially available, for research use only. HIV-1 antigen assays are utilized more commonly in areas with limited resources for the detection of early HIV-1 infection and for the diagnosis of HIV-1 infection in the newborn, as well as for monitoring antiretroviral therapy, because of its reduced cost and ease of performance.

The test is of diagnostic value during early infection. p24 antigen is usually not detectable during the first week after infection, but it is detectable thereafter during acute infection before HIV-1 antibody (Fig. 2). After HIV-1 antibody

seroconversion, p24 antigen forms immune complexes with HIV-1 antibody and becomes undetectable. Therefore, p24 antigen is found in the blood either in a free form or in a complex with HIV-1 antibody. The presence of HIV-1 p24 antigen can be detected in plasma or serum by antigen capture enzyme-immunosorbent assay (EIA). Because p24 antigen tests can produce false-positive reactions due to interfering substances, positive samples need to be confirmed by a neutralization procedure.

Standard EIA procedures for HIV-1 antigen determination are insensitive, and modifications to the HIV-1 antigen test have been made in recent years to boost the sensitivity of the assay including acid or heat treatment for dissociation of antigen antibody complexes (107, 134), and increased sensitivity has been achieved by the addition of signal amplification (6, 82, 106, 118, 125, 137). These variants of HIV-1 antigen assays are of interest because they provide highly sensitive yet inexpensive and easy to use tools for use in settings with limited resources. The enzyme-linked immunosorbent assay (ELISA) amplification system tyramide (ELAST; Perkin-Elmer) is designed to amplify the signal generated by horseradish peroxidase when applied to solid-phase EIA, and Perkin-Elmer has developed a kit, the ultrasensitive p24 antigen assay (Up24), that combines heat denaturation and signal amplification-boosted detection of HIV-1 antigen (125). These assays have been evaluated for their use in pediatric diagnosis (106, 137) and therapeutic monitoring (7) and appear to be able to detect non-B as well as B subtypes (121). Heat denaturation combined with tyramide signal amplification has been reported to increase the sensitivity of p24 antigen detection to levels comparable to detection of viral RNA by PCR and has been found to be effective for the monitoring of responses to antiretroviral therapy (6, 82, 118, 125, 135) as well as for pediatric diagnosis (30).

Recently, real-time immuno-PCR (IPCR), a procedure combining EIA and real-time PCR, was reported to be as sensitive for the detection of HIV-1 antigen as other available viral load methods and allowed for the detection of ultralow levels of HIV-1 p24 antigen (2). In the IPCR procedure, anti-HIV-1 p24 antibody adsorbed to the microwell plate is used to capture the antigen. Streptavidin-horseradish peroxidase serves as a bridge between a biotinylated detector anti-HIV-1 p24 antibody and biotinylated reporter DNA. The reporter DNA is amplified by PCR using a fluorescent probe for real-time analysis. In their study, Barletta et al. found that IPCR detected 42% of patient samples that had HIV-1 RNA viral loads of <50 copies/ml, although the specificity of the assay has not been adequately assessed (2).

HIV-1 DNA Qualitative Assays

Detection of HIV-1 DNA assays can be used to supplement or substitute antibody testing for the diagnosis of HIV-1 infection in special situations such as primary infection during the diagnostic window period or in newborns of infected mothers. HIV-1 DNA qualitative assays make use of PCR to amplify conserved regions of the HIV-1 genome to detect proviral HIV-1 DNA in blood mononuclear cells. Only one qualitative HIV-1 DNA PCR assay is commercially available (Roche Amplicor HIV DNA assay, version 1.5; Roche Diagnostics, Indianapolis, Ind.), but it is not approved by the FDA. Several laboratories have also developed home brew assays including nested as well as real-time PCR procedures (36, 54, 87).

The sensitivity and specificity of standard HIV-1 DNA PCR assays have been reported to be 96 and 99%, respectively, at 1 month of age for HIV-1 subtype B (43). The potential utility of qualitative DNA PCR assays in the diagnosis of vertical HIV-1 transmission has been greatly increased with the development of simple and sensitive procedures using dried blood spots (54, 87); a sensitivity of 98.1% and a specificity of 100% were reported with use of real-time PCR assays (87). False-negative HIV-1 DNA results have been reported for infants with non-B subtypes (81), although the latest 1.5 version of the Amplicor assay appears to allow for the detection of most HIV-1 subtypes.

HIV-1 RNA Viral Load Assays

Viral load assays which measure the quantity of HIV-1 RNA in plasma are used as prognostic markers, to monitor therapy, and to determine infectiousness. HIV-1 plasma viral load is a strong predictor of the rate of disease progression (96, 110). But HIV-1 RNA load assays are most commonly used to guide HIV-1 treatment decisions (77, 131). In the United States, three commercial assays are FDA approved for the quantification of HIV-1 RNA in plasma. A comparison of the plasma specimen requirements and characteristics of the assays is shown in Table 3. These assays are not reliable for use in HIV-2 and HIV-1 type O individuals because they have not been optimized for these viruses and will likely underestimate HIV RNA levels.

The Amplicor HIV-1 Monitor Assay (Roche Diagnostics) is based on reverse transcription of the target HIV-1 RNA and on PCR amplification of the resulting cDNA (103). After the viral RNA extraction step, reverse transcription and PCR amplification occur in a single step using the thermostable enzyme *Thermus thermophilus* DNA polymerase. With repeated cycles of heating and cooling in the presence of biotinylated oligonucleotide primers specific for a conserved region of the HIV-1 *gag*, segments of the viral cDNA are amplified exponentially to generate high numbers of amplicons. These amplicons are denatured into single-stranded DNA which are then bound to a solid phase coated with HIV-specific oligonucleotide probes. Avidin-horseradish peroxidase conjugate is added, and the amount of bound amplicon is measured after a colorimetric substrate is added. An internal quantitation standard RNA, consisting of a known concentration of RNA identical in size to the target amplicon, is added to each sample before the RNA extraction step. It allows for the quantification of HIV-1 RNA and compensates for plasma inhibitors that may affect extraction and amplification. The HIV-1 RNA copy number is calculated based on the input copy number of the quantitation standard RNA. The version of the assay currently in use (version 1.5) can adequately quantitate all group M subtypes of HIV-1, in contrast to older versions of the assay. Version 1.5 differs from version 1.0 in test primers, thermocycling conditions, and composition of the RT PCR buffer (100). One disadvantage of the Amplicor HIV-1 Monitor assay is that it has a limited dynamic range, requiring performance of both the standard and ultrasensitive assays for samples that fall outside the dynamic range. One advantage of the assay is that there is minimal risk of amplicon contamination because the kit includes UTP and uracil-*N*-glycosylase. Two FDA-approved versions of this assay exist, both of which are needed to cover the clinically relevant range of viral load values. The standard assay has a dynamic range of 400 to 750,000 copies/ml, and the ultrasensitive assay has a dynamic range of 50 to 100,000 copies/ml. Modifications that are included in the ultrasensitive assay include an increase in the initial input volume (500 μl), an ultracentrifugation step, and a lower resuspension volume (148). Two variations of these assays exist: the Amplicor HIV-1 Monitor

assay, which is performed in microwell plates, and the Cobas Amplicor HIV-1 Monitor assay, in which amplicons are captured on magnetic beads, and the Cobas analyzer is used to automate most of the assay.

The VERSANT HIV-1 RNA 3.0 Assay (Bayer Nucleic Acid Diagnostics, Norwood, Mass.) is a branched DNA (bDNA) method which quantifies HIV-1 RNA by signal amplification instead of target nucleic acid amplification (40). In this assay, virions are pelletted by centrifugation and disrupted by detergent and proteinase K to release the RNA. HIV-1 RNA is captured on microwells by specific synthetic oligonucleotide probes containing capture extenders. Target probes, which have numerous target extenders, hybridize to both the viral RNA and preamplifier probes. These then bind to the amplifier probe to form bDNA complexes. Multiple copies of alkaline phosphatase-labeled probe are then hybridized to the bDNA complexes. Detection is accomplished by chemiluminescence. Quantitation is determined from an external standard curve run on the same plate; six kit standards of know viral concentration are used. When compared to other viral load assays, the reproducibility of the bDNA assay has been reported to be superior, particularly at the low end of the dynamic range (85, 108). The VERSANT HIV-1 RNA 3.0 bDNA assay offers the advantages of high throughput; in the current version, 84 specimens can be run on one plate. In addition, the assay can reliably quantify all subtypes of HIV-1. Disadvantages of the bDNA assay include the requirement for a large volume of plasma and the absence of an internal quantitation standard for each sample tested. The Bayer System 340 bDNA Analyzer provides semiautomation of the assay.

The NucliSens HIV-1 RNA QT assay (bioMerieux, Inc., Durham, N.C.) uses nucleic acid sequence-based amplification (155). In a first step, HIV RNA is isolated from the patient's plasma sample, together with three internal calibrators (Qa, Qb, and Qc), by binding to a solid phase consisting of silicon dioxin particles. The three synthetic RNA calibrators differ from the wild-type RNA sequence by only a small number of bases; this allows for independent quantification and detection of the three calibrators and the patient's RNA. Second, coamplification of the patient and calibrator RNAs is performed in the presence of avian myeloblastosis virus RT, RNase H, and T7 RNA polymerase under isothermal conditions for 90 min. Finally, to detect the amplification products (HIV-1 RNA from the patient, Qa, Qb, and Qc) aliquots of the amplified sample are added to four hybridization solutions, each containing a probe specific for one of the four products. The amplified products are hybridized to an oligonucleotide fixed to a magnetic bead and a ruthenium labeled probe. The magnetic beads are captured on the surface of an electrode by a magnet, and the light emitted by the ruthenium labeled probe is quantified by electrochemiluminescence. The second-generation assay currently in use cannot reliably quantify subtypes A and G (59). Advantages of the NucliSens HIV-1 QT assay include a wide dynamic range (176 to 3,400,000 copies/ml) when a 1-ml input plasma volume is used. In addition, the assay can be used for measuring viral loads at other body sites because the RNA extraction procedure consistently generates RNA products that are free of interfering substances (53).

Numerous other types of HIV RNA viral load assays are in use including a number of real-time assays (58, 113, 129), some of which can be utilized for HIV-2 and HIV-1 group O viruses (33, 67). The commercial development of real-time HIV-1 assays is ongoing. In these assays, amplification and detection are performed in a single closed tube; this eliminates the risk of amplicon carryover contamination. The next generation of assays for measuring HIV viral load such as TaqMan or molecular beacon technology is likely to soon replace current assays (9).

The high cost and complex technical requirements of nucleic acid-based testing strategies have resulted in the development of other types of viral load assays for use in settings with limited resources. Two of these assays have received recent attention: the heat-denatured signal-boosted p24 antigen assays (Perkin-Elmer, Wellesley, Mass.) and the RT assay (Exa Vir Load assay; Cavidi Tech AB, Uppsala, Sweden) (7, 144). Although both methods remain less sensitive and reproducible than the nucleic acid-based assays, they are affordable alternatives to HIV RNA quantitation. Furthermore, an automated, low-cost real-time reverse transcription-PCR test is routinely used for diagnosis and monitoring of HIV-1 infection in a West African setting where resources are limited (129).

ISOLATION PROCEDURES

Because HIV can be isolated from the blood of the majority of HIV-infected individuals, HIV culture was frequently utilized in the early years of the epidemic as a diagnostic or prognostic marker or for assessing the efficacy of antiviral therapy (75, 96). Although a positive culture provides direct evidence of HIV infection, HIV culture is no longer used for routine diagnosis but is utilized primarily in research laboratories. The procedure for HIV culture is elaborate and time-consuming (63, 75). To isolate HIV, the patient specimen is first cultured by mixing patient cells with cells from healthy donors stimulated with phytohemagglutinin and interleukin-2; fresh stimulated donor cells must be added weekly, because HIV-1 produces cell death. In the second step, the presence of RT or p24 antigen released in the culture supernatants is assayed periodically.

Viral culture assays based on recombinant technology are now used in resistance assays and to determine the viral replicative capacity (RC assay) of HIV-1. The RC assay, also referred to as viral fitness assay, has been developed by Monogram Biosciences (formerly Virologic, South San Francisco, Calif.). It measures the ability of HIV-1 from a patient undergoing antiretroviral treatment to replicate in vitro compared to that of a wild-type reference virus. The patient RC value is expressed as a percentage of the replication capacity of the wild-type reference standard. The assay uses a retroviral vector constructed from an infectious clone of HIV-1. The vector contains a luciferase expression cassette inserted within a deleted region of the envelope gene (121). HIV-1 protease and RT sequences are amplified from the patient plasma samples and inserted into the vector. The amount of luciferase produced by patient-derived viruses is then compared to the amount of luciferase produced by well-characterized wild-type reference virus. RC has been suggested as an additional parameter for making decisions regarding antiretroviral therapy. Patients who do not experience increases in viral loads despite the accumulation of multiple resistance mutations have been shown to harbor virus with decreased RC (35). In addition, certain drug resistance mutations have been shown to reduce RC (39, 91), and low replicative capacity has been correlated with a slower rate of CD4 cell loss in individuals who have experienced viral rebound on treatment (147).

TABLE 4 Major FDA-approved HIV-1/HIV-2 standard EIAs[a]

Test name	Antigens used for detection	Antibodies detected
Abbott HIV AB HIV-1/HIV-2[b]	Recombinant gp41, recombinant gp120, recombinant gp36	IgG and IgM
Bio-Rad Genetic Systems HIV-1/HIV-2[c]	Synthetic gp41, synthetic p32, recombinant gp36	IgG
Bio-Rad Genetic Systems HIV-1/HIV-2 plus O[c]	Recombinant p24, recombinant gp160, recombinant gp36, synthetic group O peptide	IgG and IgM
bioMerieux Vironostika HIV-1[d]	HIV-1 viral lysate	IgG and IgM
bioMerieux Vironostika HIV-1 plus O[d]	HIV-1 viral lysate, purified viral env proteins, synthetic group O peptide	IgG and IgM

[a] Data from reference 155.
[b] Abbott Diagnostics, Abbott Park, Ill.
[c] Bio-Rad, Hercules, Calif.
[d] bioMerieux, Marcy l'Etoile, France.

SEROLOGIC TESTS

The diagnosis of HIV infection is commonly accomplished via detection of HIV antibody by using a screening test and then confirmation using a subsequent confirmatory or supplementary test. Screening tests are highly sensitive and easy to perform; confirmatory tests are more specific (and generally, less sensitive than screening tests) and are more complex and difficult to perform and interpret (27). Because HIV causes a chronic infection which typically lasts for years and the initial infection may be minimally symptomatic, most patients who are identified with HIV are identified by antibody detection. In recent years, rapid assays for detection of HIV antibody have played an increasingly important role in situations in which rapid provision of antiretroviral therapy may prevent HIV, including labor and delivery (24) and employee exposures, for which current guidelines call for post exposure prophylaxis within 2 h of exposure.

Serological testing for HIV antibody is used for various purposes, including primary diagnosis, screening of blood products, management of untested persons in labor and delivery, evaluation of occupational exposures to blood/body fluid, and epidemiological surveillance. Screening of blood products for HIV and other viral transfusion-transmitted diseases has nearly eliminated a major source of HIV transmission in parts of the world where such screening has been implemented (145, 146).

Antibody Screening Tests: Standard EIAs

Considerable progress has been made over the past 3 decades in the development of HIV antibody screening tests. The first generation of HIV antibody assays relied on the detection of antibody to HIV viral protein lysates. The second generation assays utilized HIV recombinant antigens as the source of antigen bound to the solid phase and included recombinant antigens for detection of HIV-2. Relative to first-generation tests, second-generation methods provided fewer false-positive results and the ability to detect HIV-2. Third-generation assays utilize solid phases coated with recombinant antigens and peptides, and HIV antibody in the patient's sample is detected with labeled recombinant antigens. Relative to second-generation assays, third-generation methods provide earlier detection during seroconversion, since these antigen-antigen sandwich assays efficiently detect immunoglobulin M (IgM) and other non-IgG antibodies. Fourth-generation assays combine antibody and antigen detection, further decreasing the time to detection during seroconversion (88, 89, 90). At present, most of the commonly used assays provide for the simultaneous detection of antibodies to both HIV-1 and HIV-2 (Table 4). In addition, numerous standard EIA kits are also available for the detection of HIV-1 antibody only, and two kits are approved for the detection of HIV-2 antibody.

The shortage of trained medical laboratory workers and increasing fiscal pressures on laboratories have driven increasing automation in clinical laboratories. Modern random-access immunochemistry platforms provide substantial labor savings over automated EIA approaches. Methods for performing HIV antibody testing are being developed on at least three major platforms, but none is as yet FDA approved. The Abbott Axsym uses a fluorescent EIA method and combines antigen-antibody detection (138); the Bayer Advia-Centaur (45, 156) and the Ortho Vitros-ECi use chemiluminescent assay formats. All have the potential to make laboratory-based HIV testing simpler, faster, and more widely available.

Antibody Screening Tests: Rapid Assays

HIV prevalence continues to increase in the United States, and many at-risk persons are unaware that they are infected (15). CDC goals for HIV prevention include making HIV testing a routine part of medical care, and recent publications suggest that expanded screening for HIV is a cost-effective health intervention (5, 115, 127). Serving hard-to-reach populations has been limited by the logistics of conventional HIV testing, which require a follow-up visit before results of testing are available, even for seronegative patients. In addition, an immediate result is medically desirable in certain settings, for example, in assessment of the source patient in occupational blood and body fluid exposures, and in labor and delivery settings with women of unknown HIV serostatus.

To address these problems, rapid HIV tests have been under development and in active use for over a decade (78, 141). Performed both in clinical laboratories and at the point of care, rapid HIV tests have a role when rapid results are necessary and when HIV testing is provided to previously inaccessible populations and situations. Rapid HIV tests fall mostly into two categories; membrane EIAs and immunochromatographic assays. Membrane EIAs typically involve a series of steps, including addition of sample, wash, addition of conjugate, wash, addition of substrate, and stop reagent. The result is read as a colored spot or triangle on a solid surface. Most of these tests include a built-in control.

If the test differentiates two analytes, such as HIV-1 and HIV-2, two controls are included. Immunochromatographic or lateral-flow assays require the addition of only one or no reagent and thus are extremely simple to perform. They are especially well-suited for testing outside traditional laboratory settings. These tests utilize antibodies spotted onto nitrocellulose membranes with lateral or vertical flow of sample or reagents to interact with immobilized antibody. The sample is added at one end and moves along the membrane by capillary action to reach the immobilized antibody stripes.

Four rapid HIV antibody tests were available in the United States as of March 2006 (Table 5). The Abbott Oraquick and Trinity Uni-gold Recombigen both have waived status, while MedMira Reveal and Bio-Rad Multispot HIV-1/HIV-2 are nonwaived. Waived tests are simple methods approved for use in settings outside dedicated clinical laboratories. Laboratories operating under a "certificate of waiver" are subject to minimal regulations, and waived tests are required to be simple and have minimal chance of error. Uniquely, the Multispot allows discrimination between HIV-1 and HIV-2 reactivity. Many other tests are in development and in use outside the United States; and an older kit, Abbott's Single Use Diagnostic System HIV-1 (SUDS), has been removed from the market, but not before extensive experience was gained with its use.

Disadvantages of rapid assays include subjective interpretation, lack of automation, and possible errors if the reader has vision problems such as color-blindness. Although these kits are easy to use, lack of attention to technique and less-skilled personnel can lead to errors (62). Samples must be dispensed within specified time limits, and pipettes must be held vertically for correct delivery of reagent volumes. These formats are useful primarily for small-volume testing. Accurate timing of steps can be adversely affected when multiple samples are tested, and conventional EIAs and similar methods scale up to larger sample volumes more efficiently.

Numerous published studies support the manufacturer's validation data suggesting that rapid tests perform similarly to laboratory-based standard EIA methods when performed by skilled staff. In the largest such study, the MIRIAD trial, HIV testing with Oraquick in point-of-care settings had equal sensitivity to a laboratory-based EIA and had fewer false positives (8). In this trial, testing was performed at the point of care by labor and delivery nurses, midwives, and obstetrics and gynecology residents. This represents the most extensive trial to date documenting the accuracy of the rapid test when used by nonlaboratorians. Unfortunately, the procedures used for training, certifying, and assessing the testing personnel were not specified in the publication. The Oraquick was also found to perform as well as a laboratory-based EIA in a region with transmission of multiple HIV subtypes (126). Other rapid tests have also been evaluated for patients with non-B subtypes (76), but the existing data are limited relative to the large number of tests and HIV subtypes. No data are available on the accuracy or impact of rapid tests in a home environment, although the FDA is considering approval of one rapid HIV kit as a home test.

Other Types of Antibody Screening Tests

Alternative specimens for HIV antibody testing include oral fluid and urine. Either may be useful in testing of patients who are reluctant to undergo phlebotomy or who have poor vascular access, as well as in mass screening settings and in locations in which phlebotomy is impossible. Since there are no sharps involved, collection is also safer. Oral fluid has also been used for home testing, whereby a patient collects the specimen and sends it for analysis; counseling is provided when the result is communicated to the patient. Fluids in the oral cavity come from several sources, from the secretions of multiple different glands, and also from transudated plasma derived from the capillaries of the gum. The former contains primarily secretory IgA, which may have a role in protection against infection but is not a reliable target for diagnostic testing. In contrast, the transudated gingival fluid contains primarily blood-derived IgG. Collection devices such as the Orasure specimen collection device are specifically targeted to enhance collection of this component of the oral fluid. Oral-fluid-based HIV EIA, Western blot, and waived rapid tests are FDA approved; indeterminate Western blots appear to be more common from oral fluid than from serum samples (27, 140, 149). IgG is found, usually in small quantities relative to serum, in the urine, and FDA-licensed EIAs and Western blots are available for urine (153).

Detuned EIAs allow for the distinction between recent and distant HIV infections. This type of assay is used to estimate the incidence of new infections by assessing the titer or affinity of HIV antibody. This is done by comparing the results of a standard, sensitive HIV antibody test with one whose sensitivity has been intentionally decreased—a "detuned" assay. Patients positive for both the standard and detuned test are considered to have long-standing infections; patients positive on the standard test but negative on the detuned tests are considered to have recent infection. Variations have been seen in the performances of these methods in different geographic regions and with different HIV subtypes. Approaches to detuning HIV tests include sample dilution, shortened incubation times, and chaotropic agents (41, 117).

TABLE 5 Rapid and point-of-care HIV tests approved for use in the United States

Test	Antigens used for detection of:		Approved for detection of HIV type:
	HIV-1	HIV-2	
Abbott/Orasure Oraquick Advance Rapid HIV 1/2[a]	gp41	gp36	HIV-1 and HIV-2
Bio-Rad Multispot HIV-1/HIV-2 Rapid[b]	Recombinant and synthetic gp41	gp36	HIV-1 and HIV-2 (separate result for each)
Trinity Unigold Recombigen HIV[c]	gp41, gp120		HIV-1
MedMira Reveal G2 Rapid[d]	gp41, gp120		HIV-1

[a] Abbott Diagnostics, Abbott Park, Ill.
[b] Bio-Rad, Hercules, Calif.
[c] Trinity Biotech, Bray, County Wicklow, Ireland.
[d] MedMira, Halifax, Nova Scotia, Canada.

HIV Antibody Confirmation Assays

Both standard and rapid HIV screening tests require confirmation if the initial test result is positive (see Fig. 4 and 5). Screening HIV tests are optimized to provide very high sensitivity, at the expense of specificity. HIV testing algorithms therefore incorporate highly specific, but less sensitive, confirmatory tests. The most commonly used confirmation test is the Western blot, but other confirmation methods and strategies are available, including IFA (indirect fluorescent antibody) and LIA (line immunoassay). In addition to the traditional EIA screening/Western blot confirmation algorithm, a number of alternative algorithms have been proposed which include the use of a second, different EIA or rapid test as the confirmatory method. Such a scheme has been modeled for cost-effectiveness, even recommended, but has not been extensively studied in practice (47, 162, 163). Results of pilot projects using varying strategies for accelerated confirmatory testing have been encouraging (98, 128, 152).

The Western blot is considered by many to be the "gold standard" for the validation of HIV antibody initially reactive samples. Commercially available Western blots are supplied in kit form with HIV antigens separated by molecular weight by electrophoresis and blotted onto a membrane which is then cut into strips. The testing laboratory incubates the strips with patient serum and washes and then develops the reaction with an enzyme-labeled anti-human antibody, which detects patient antibodies bound to the specific viral proteins. Antibodies to the following HIV-1-associated antigens can be detected: gp 160, gp 120, p66, p55, p51, gp41, p31, p24, p17, and p15. Western blots are specific for HIV species and often subspecies. Therefore, it is necessary to use separate Western blots for HIV-1 and HIV-2, and results with subtypes other than M have been variable (19, 28). HIV-1 Western blot kits approved for use in the United States can be purchased either from Biorad/Genetic Systems (Hercules, Calif.) or from Calypte/Cambridge Biotech (Lake Oswego, Oreg.). Western blots are prepared from HIV grown on cell culture; therefore, nonviral proteins may be present on the nitrocellulose strip and lead to nonspecific reactions.

An indirect fluorescence assay (Fluorognost; Sanothemia, Westport, Conn.) has also been used as both a screening and a confirmatory test for HIV-1 infection. HIV-infected and -uninfected lymphocytes are fixed on a slide. The slide is incubated first with patient serum and then with a fluorescent-labeled anti-human antibody. For interpretation, patterns of fluorescence in infected and uninfected cells are compared for each patient sample. LIAs employ a strategy similar to that of the Western blot. Recombinant or synthetic antigens are placed on a strip rather than transferred from an electrophoresed gel. This approach has the advantage of placing only viral antigens in the reaction, eliminating the background from cross-reactivity with nonspecific cell proteins. The manufacturer has control over the quantity and type of antigens represented and can include HIV-2 and type O antigens to confirm these infections in a single test (19, 20, 101). The INNO-LIA HIV1/11 Score test (Innogenetics, Alpharetta, Ga.) is widely available outside the United States and can be used for confirmation of HIV-1, HIV-2, and HIV-1 type O infections.

Similar to the LIA, rapid confirmatory tests have been developed which incorporate multiple, separate diagnostic antigens in a rapid test format. Methods include the Quix HIV-1 Rapid Antibody Confirmation Test (Universal Healthwatch, Guardian Scientific Africa, Monmouth Beach, N.J.), the Bionor HIV-1&2 Confirmatory Rapid EIA (Bionor A/S, Skien, Norway), and the HIV 1 & 2 CombFirm (Orgenics, Inverness Medical Innovations, Waltham, Mass.). These tests may provide a robust confirmatory testing capability to settings where rapid screening tests are valuable. This would eliminate the need for collecting a specimen for off-site confirmation and awaiting the result prior to initiating treatment (26, 139).

ANTIVIRAL SUSCEPTIBILITIES

Resistance testing has become a crucial element in the management of antiretroviral-treatment-experienced patients, because it is useful for the selection of the antiretroviral treatment in patients who are failing their current regimen due to the development of antiviral resistance. In addition, given the high rates of transmission of resistant viruses, resistance testing is also taking an increasingly important role in the choice of the initial antiretroviral therapy (86). The clinical utility of HIV-1 resistance testing has been evaluated in a number of prospective randomized clinical trials (4, 23, 44, 99, 150). Although not all of the trials demonstrated a clear benefit for using resistance testing (99), several of the studies showed that patients for whom the antiretroviral treatment was based on the results of resistance testing had greater decreases in viral load than did patients in whom the antiretroviral regimen was based on prior antiretroviral usage (4, 23, 44, 150). One of these studies showed that expert advice further improved the benefit of using genotyping resistance testing (150). In addition, use of resistance testing to guide antiretroviral treatment has been reported to be cost-effective (159). Two types of methods are available to assay for HIV-1 resistance. Genotyping tests examine the population of viral genomes in the patient sample for the presence of mutations known to confer decreased sensitivity to antiretroviral drugs. Phenotypic assays measure viral replication of the patient's virus in the presence of antiretroviral drugs.

Genotyping Assays

Genotyping assays determine the sequence of nucleotides in the viral genome and compare the sequence to that of wild-type virus. Mutations that appear during the course of antiretroviral therapy are considered to cause drug resistance if in vitro studies confirm that the presence of these mutations decreases the susceptibility of HIV to a specific drug.

The initial steps of genotypic assays include extraction of viral RNA from plasma and RT-PCR to amplify viral RNA sequences which code for the patient RT and protease, the targets of most available antiretroviral drugs. Next, the nucleotide sequence of amplified sequences is determined and examined for the presence of known resistance mutations. This can be accomplished most commonly in the clinical laboratory setting by using automated sequencing technology. In this technology, the nucleotide sequence of the gene of interest is obtained and translated into the predicted amino acid sequence in order to assess for the presence of specific mutations. The process requires alignment and editing of the sequence, a comparison to the wild-type sequence, and final interpretation linking the mutations to specific antiretroviral drugs. Two FDA-approved kits, containing sequencing reagents as well as the software programs required for sequence alignment and interpretation, are commercially available. The two assays, i.e., the Trugene HIV-1 Genotyping Kit and OpenGene DNA Sequencing System (Bayer Diagnostics Co., Tarrytown, N.Y.) and theViroSeq HIV-1 Genotyping System (Abbott Diagnostics,

Abbott Park, Ill.), have been found to perform in an equivalent manner (49).

One limitation of both assays is that they are able to detect only mutants comprising major fractions of patient's virus; resistant variants must constitute at least 25 to 30% of the virus population (68). In addition, the assays do not detect the ENV gene mutations resulting from use of the fusion inhibitor T-20. Although the clinical significance of resistant mutations present at low levels remains to be fully elucidated, there is evidence that minor mutations which are missed by standard genotyping assays can lead to failure of subsequent treatments (18, 95). Because of the lack of sensitivity of standard genotyping assays for detection of low-frequency drug resistance mutations, efforts are under way to increase the sensitivity of detection of mutations through single-genome sequencing (114). Single-genome sequencing involves serially diluting the cDNA until there is a single copy present for amplification and sequencing. This is not presently a practical clinical tool as the process is expensive and labor-intensive.

Another genotypic assay, the HIV PRT GeneChip assay (Affymetrix, Santa Clara, Calif.), is used mostly in research settings. In this assay, the HIV sequencing is accomplished by DNA hybridization of the amplified viral genes to oligonucleotide probes on high-density microarrays followed by automated base calling and mutation detection (160). The HIV LiPA line probe assay is no longer available commercially. This reverse hybridization-based method used probes immobilized on a carrier surface to identify selected drug resistance mutations and provided limited information on the presence or absence of specific mutations at particular positions.

Phenotyping Assays

In phenotyping assays, the ability of HIV-1 to grow in the presence of various concentrations of a given antiretroviral agent is measured. The amount of drug required to inhibit virus replication by 50 or by 90% is determined and given as a 50 or a 90% inhibitory concentration (IC_{50} or IC_{90}), respectively. The IC_{50} or IC_{90} obtained with the patient sample is compared to a control wild-type virus, and the result is reported as a "fold" difference. Early phenotypic resistance assays were labor-intensive because they necessitated the isolation and culture of HIV-1 from the patient's specimen. Currently, commercially available methods use HIV-1 RNA amplified from plasma and are based on recombinant DNA technology. These phenotypic assays are automated but remain labor-intensive and technically complex. They have not been developed in a kit format and are performed at only two commercial laboratories: Virco (Mechelem, Belgium) and Monogram Biosciences, formerly ViroLogic (San Francisco, Calif.). The first step involves extraction of HIV-1 RNA from plasma. Reverse transcription and PCR amplification of the protease and RT genes follow. The amplified genes from the patient's specimen are then inserted into vectors. The PhenoSense assay (Monogram BioSciences) uses an HIV-1 vector with a luciferase reporter gene replacing the viral envelope gene. In the AntiVirogram assay (Virco), patient and HIV-1 vector sequences are combined by recombination in vitro, and viral replication is measured using a reporter gene system. The virtual phenotype, an approach developed by Virco, can be used as an alternative to phenotypic testing, although it is really a genotyping resistance interpreted with the aid of a large database of samples with paired genotypic and phenotypic data. The virtual phenotype is determined by entering the genotype into the database and by finding the closest matching phenotyping results.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Use and Interpretation of Antibody Assays

Currently available standard EIAs are exquisitely sensitive and specific. Causes of false-negative and false-positive screening tests are listed in Table 6. The currently approved rapid methods appear to have sensitivity comparable to that of conventional EIA methods, although occasional false-positive and false-negative results were seen in large panels, but never in numbers sufficient to discriminate between different kits in a significant manner (154, 154a–154c).

In the rapid HIV antibody tests, only one or occasionally two antigens are used for detection of HIV-1 and HIV-2 antibody. In theory, this should limit sensitivity in some patients. A report of a series of patients who were treated

TABLE 6 Causes of false-positive EIA, false-negative EIA and indeterminate Western blot HIV antibody results

Result	Nonspecific causes	HIV-specific causes
False-negative EIA	Hypogammaglobulinemia, immunosuppressive therapies	Acute infection during the window period, seroreversion[a], advanced AIDS, HIV-2 (if EIA kit detects only HIV-1), HIV-1 subtype O (if EIA kit does not detect HIV-1 subtype O), other HIV strain variations
False-positive EIA	Multiple transfusions, hypergammaglobulinemia, recent vaccinations, hemodialysis, antibodies to HLA antigens, autoantibodies associated with autoimmune disease, cross-reactivity with vector proteins	Experimental HIV vaccination
Indeterminate HIV-1 Western blot	Multiple transfusions, hypergammaglobulinemia, recent vaccinations, hemodialysis, antibodies to HLA antigens, autoantibodies associated with autoimmune disease, idiopathic	Early seroconversion, incomplete HIV-1 antibody evolution[a], cross-reactivity with HIV-2 proteins, cross-reactivity with HIV-1 subtype O proteins, experimental HIV vaccination

[a] In acutely infected individuals treated with antiretroviral therapy.

with HAART early after a known HIV exposure has been published. These patients developed HIV infection with low viral loads and a declining gp41 antibody response which was not detected by the Oraquick method (111). While this is not a patient population for which rapid testing would be appropriate currently, this report points to a potential sensitivity issue with rapid tests, particularly if used for two-stage confirmatory testing. The quality of testing is of concern at all times, but particularly for rapid HIV assays when testing is performed outside standard laboratory settings and by personnel whose chief expertise is not performing laboratory tests. The CDC has issued extensive performance and quality assurance guidelines for use of rapid HIV tests (16). In addition, it should be recognized that no laboratory test is 100% accurate, and results inconsistent with clinical findings should be repeated or assessed with different tests (27, 28).

Two types of criteria are used worldwide for the interpretation of HIV-1 Western blots. Criteria from the WHO require the detection of at least two envelope products (161). In the United States, HIV-1 Western blots are usually interpreted following the CDC guidelines, which require detection of at least two of three antigens: p24, gp41, or gp120/160 (10, 11). The absence of all bands is a negative result. HIV-associated bands that are present but do not meet the criteria for positivity are an indeterminate pattern (Fig. 3). Causes of indeterminate HIV-1 Western blots are listed in Table 6. p24 reactivity is most commonly seen with indeterminate HIV-1 Western blots; this is often due to nonspecific reactivity, but it is important to note that p24 is also the first positive band seen during HIV-1 seroconversion. Indeterminate HIV-1 Western blots should be followed with a repeat screening test and Western blot after 2 weeks to 6 months. HIV-2-infected

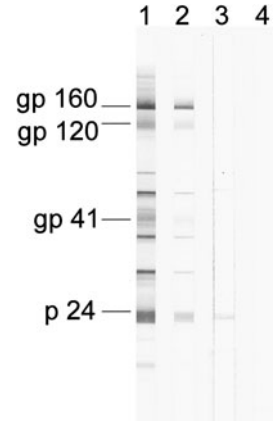


FIGURE 3 Examples of HIV-1 Western blot results interpreted according to CDC guidelines. High positive, low positive, indeterminate, and negative results are shown in lanes 1, 2, 3, and 4, respectively.

patients sometimes exhibit unusual indeterminate patterns on HIV-1 Western blot, consisting of reactivity to *gag* (p55, p24, or p17) and *pol* (p66, p51, or p32) gene products, in the absence of reactivity to envelope proteins. In addition, reactivity to p65, p51, p31, and gp160 can be seen on HIV-1 Western blots with samples from patients infected with HIV-1 type O. Samples from patients with HIV-2 or HIV-1 type O epidemiological risk factors should be further tested when the screening HIV-1/HIV-2 plus O EIA is positive and the confirmatory test is negative or indeterminate (Fig. 4). RNA or DNA testing may be used to further assess cases in which the

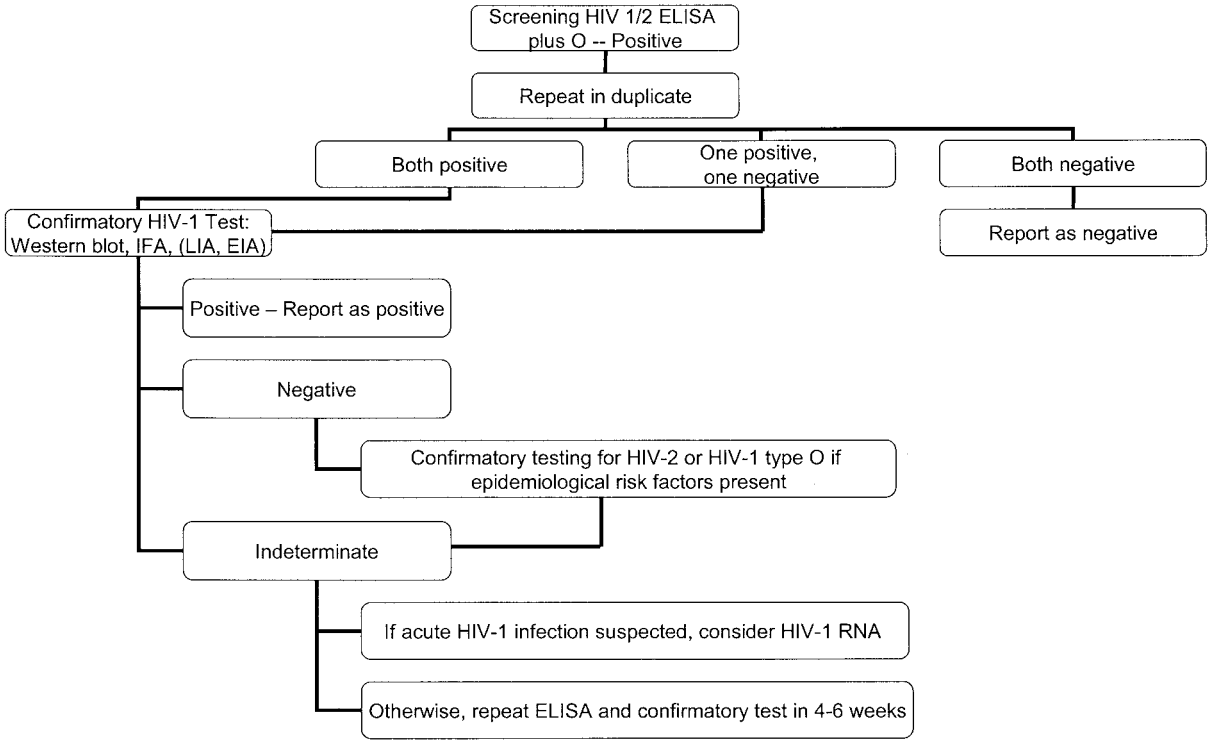


FIGURE 4 HIV antibody testing algorithm for samples initially reactive in a standard HIV-1/HIV-2 PLUS O assay showing the sequence of suggested follow-up confirmatory testing.

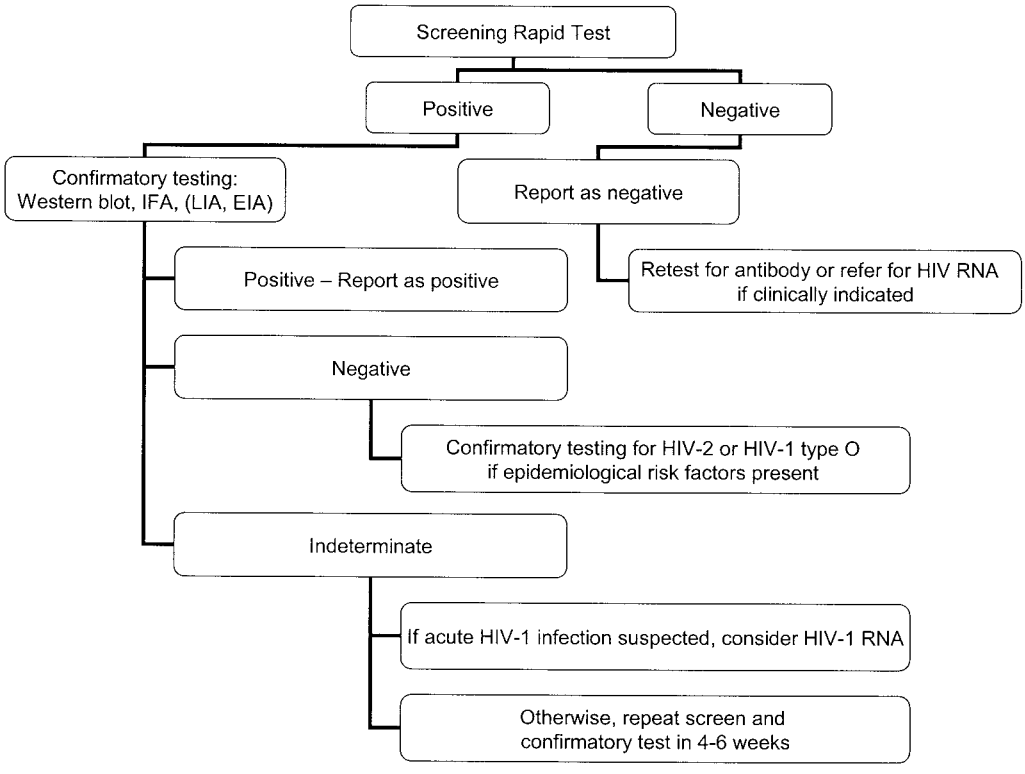


FIGURE 5 HIV antibody testing algorithm for samples tested with rapid HIV assays. Note that for rapid tests, as opposed to conventional screening assays, there is no recommendation to repeat an initial positive in duplicate prior to performing confirmatory testing. It is *not* recommended to perform a second EIA on a positive rapid test result prior to confirmatory testing; if a second screening test is used, confirmatory testing should be performed even when the result is negative.

HIV-1 serological results are inconclusive, although these tests are not approved for diagnosis of HIV-1 infection.

The algorithm for confirmation of rapid HIV tests is shown in Fig. 5. All reactive rapid HIV test results require confirmatory testing, regardless of EIA results. Samples reactive by rapid tests and negative by standard EIA have been subsequently confirmed as HIV positive. Therefore, retesting of samples by standard EIA prior to Western blot testing is not recommended (16, 17).

Use and Interpretation of HIV-1 DNA Qualitative Assays

The clinical usefulness of HIV-1 DNA testing in adults is very limited. However, HIV-1 DNA PCR is the preferred method in the United States for determining an exposed infant's HIV-1 infection status. Indeed, serological tests are not useful for the detection of HIV-1 infection in infants since maternal antibodies can be present in children until 18 months of age. The DNA test can be performed on whole blood or on dried blood spots, but the specimen collected at birth must be a neonatal and not a cord blood sample; cord blood samples yield a high rate of false-positive results (80). To exclude HIV-1 infection in an exposed infant, HIV-1 DNA PCR testing should be performed at birth, at weeks 4 to 7, and at weeks 8 to 16 (80). The sensitivity of the assay is 38% at 48 h of life and 99% at 4 weeks of age (43). Several studies have shown that HIV-1 RNA assays are at least as sensitive as HIV-1 DNA assays, especially for the detection of non-B subtypes (30, 164). It should be noted, however, that the sensitivity of the HIV-1 DNA and RNA assays may be affected by the use of prophylactic antiviral regimens and that HIV-1 RNA assays have not been approved for the diagnosis of HIV.

Use and Interpretation of Viral Load Assays

The clinical utility of HIV-1 viral load testing has been well established (14), and details can be found on line at http://www.hivatis.org and http://www.iasusa.org. Indications for use of HIV-1 RNA testing are listed in Table 7. Viral load assays are used widely to monitor changes in plasma viremia during antiretroviral therapy because they are useful for predicting time to progression to AIDS, determining when to initiate therapy, and monitoring responses to therapy. The magnitude of the decrease in viral load is dependent on the effectiveness of the antiretroviral therapy, and the optimum goal of therapy is to achieve viral loads below the detection limit of the assay. Baseline testing as well as repeated levels of HIV-1 RNA should be obtained before initiating or changing antiretroviral therapy regimens.

Guidelines for the proper use and interpretation of HIV-1 RNA load assays are listed in Table 8. Biological variation of HIV-1 RNA among clinically stable patients has been estimated to be approximately 0.3 $\log_{10}$ (34). In addition, commercial tests show assay reproducibility ranges from 0.2 to 0.5 $\log_{10}$ depending on the region of the assay's dynamic range. Generally, a change in viral load greater than 0.5 (threefold)

TABLE 7 Indications for use of HIV-1 RNA load assays

Prediction of HIV disease progression
To offer better prognostic information
To identify patients whose disease is becoming more active
To decide when to initiate or change antiretroviral therapy
Measure of response to antiretroviral therapy
To find out whether a particular drug combination is working well in an individual patient
To determine whether therapy should be changed
Aid in establishing diagnosis (when HIV antibody test is negative or indeterminate)
Syndrome consistent with acute HIV infection[a]
Infants from HIV-infected mothers[a]
Screening of blood products

[a] HIV-1 RNA assays are not FDA approved for diagnosis; standard antibody methods should first be used to confirm diagnosis.

log is considered to be significant. Viral load changes for samples close to the 50-copies/ml low detection limit of assays should be interpreted with particular caution because at the low end of the dynamic range, assay variability has a greater impact on assay interpretation. For borderline viral load values in patients with no prior data, it may be indicated to verify patient status, scrutinize laboratory results, and consider repeat testing.

Numerous studies comparing the performances of the three FDA-approved assays have been done (46, 48, 60, 71). Overall, high correlations between assays have been shown, and net changes in plasma RNA levels after antiretroviral therapy are similar. The current-generation assays have been standardized so that the differences in viral load are narrowing among assays. However, values do differ, and for a given sample, the viral loads obtained with the Bayer Versant 3.0 assay are consistently lower than the viral load values generated by the other two assays. It is therefore optimal for patients to be monitored over time with a single assay.

HIV-1 RNA load testing is sometimes requested to resolve equivocal serologic findings or to facilitate the diagnosis of HIV-1 infection during the acute phase or in a pediatric setting. In these settings, it is important to keep in mind that these molecular methods are not approved by the FDA for the diagnosis of HIV-1 infection. In addition, false-positive results can be obtained because of contamination during specimen processing, carryover of amplified products, or incorrect thresholds. With the bDNA assay, nonspecific hybridization can also lead to false-positive results. In CDC proficiency surveys, false-positive results as high as 5,000 copies/ml have been reported. Furthermore, herpes simplex virus infections, acute respiratory infections, and vaccinations can cause transient increases in viral load; HIV-1 RNA viral loads usually return to baseline within 1 month (42, 109).

TABLE 8 Guidelines for use and interpretation of HIV-1 RNA load assays

The same assay and the same laboratory should be used to monitor viral load changes.
A change in viral load is significant only if there is a threefold or 0.5 log increase or decrease *at a minimum*.
Changes in viral loads for samples with viral load at the low end of the dynamic range should be interpreted with extreme caution.
Infections and immunizations may produce transient nonspecific changes in viral load level.
To establish a baseline, viral load must be measured in two separate samples collected 1 to 2 weeks apart.
Patients should be tested 2 to 8 weeks after initiation of therapy for initial assessment of drug efficacy.
To evaluate the durability of antiretroviral efficacy, viral load assays should be performed every 3 to 4 months for patients on antiretroviral therapy.

Use and Interpretation of Resistance Assays

Clinical guidelines for the use of HIV-1 resistance testing in adults are published and updated on a regular basis (72), and a list of general suggestions for the use and interpretation of resistance assays is provided in Table 9. In contrast to phenotypic tests, genotyping assays are widely available, technically easier to perform, faster, and less expensive. In some cases, a resistance mutation may be detectable before a change in the phenotype has occurred, and therefore, genotypic and phenotypic results are not always correlated. One limitation of genotyping assays originates from the complexity of data generated by the genotyping assays. In the face of the rapid development in new drugs and new information of HIV-1 resistance, it remains a challenge to keep updated on which mutations are associated with each specific drug combination. Another problem with resistance testing is that drugs are used in combination but are not tested in combination. Phenotyping assays offer the advantage that they provide results in a format that is more familiar to clinicians. In addition, there appears to be less need for expert interpretation because susceptibility is measured directly. However, clinically significant cutoffs for distinguishing sensitive from resistant isolates have not been clearly defined for all of the available drugs.

Both types of assays will yield results only if the plasma used for testing contains at least 500 to 1,000 HIV-1 RNA copies per ml. In addition, for both assays, the resistant viral mutant must constitute at least 20 to 30% of the viral quasispecies to be detected. Also, cross-contamination can occur with both resistance assays, because both procedures rely on an RT-PCR step to amplify the HIV-1 gene sequences. In general, genotyping assays appear to show more evidence of clinical efficacy than phenotyping assays. Phenotyping assays, on the other hand, can help in defining the significance of

TABLE 9 Guidelines for use and interpretation of resistance testing

Resistance testing is recommended:
For patients failing therapy (to aid in the selection of salvage therapy)
For patients with recent infection, before initiation of therapy (to determine if a resistant virus was recently acquired)
For pregnant women (to optimize therapy)
Results of test are most reliable if test is performed when patient is taking antiviral therapy.
Genotype and phenotype results are not always correlated.
Genotype and phenotype assays require 500 to 1,000 HIV-1 RNA copies/ml in plasma.
Genotype and phenotype assays are insensitive to minor variants (resistant mutant must make up at least 25 to 30% of the virus population).

newly recognized resistance mutations and in elucidating the effects of complex mutation interactions.

The interpretation of HIV-1 genotyping results is complex. It requires knowledge of the identity of mutations associated with each specific drug, of the interactions of resistance mutations, and of the genetics of cross-resistance. This problem has been addressed by the development of several systems for facilitating the interpretation of HIV genotypes. Most systems use a rules-based approach; a group of experts establish interpretation algorithms based on the type of mutations or combination of mutations that are associated with resistance to specific drugs. As new information becomes available, the algorithms must be updated. In the Trugene and ViroSeq assays, these algorithms are used to generate automated HIV-1 genotyping reports. Depending on the mutations detected, the report indicates, for each drug in each of the antiviral categories, whether virus contained in the sample shows no evidence of resistance, resistance, or possible resistance, or if there is no sufficient evidence to place the virus in one of the three other categories. The Virtual Phenotype circumvents the complexity of HIV-1 genotyping data but provides only an estimate of the probable phenotype. Further, the correlation between actual and virtual phenotype is likely to be lower when there are few matches in the database as well as for newer drugs.

For HIV-1 phenotyping, thresholds to distinguish sensitive from resistant isolates can be defined as either biological cutoffs or clinical cutoffs. Biological cutoffs are based on biological variations measured in treatment-naïve patients, and the standard interpretation is that the isolate is susceptible if it is within the mean plus 2 standard deviations of the cutoff. Clinical cutoffs are based on the distinction between diminished and full clinical responses. These cutoffs vary from drug to drug and with the two different assays.

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Human T-Cell Lymphotropic Virus Types 1 and 2

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TAXONOMY

Human T-cell lymphotropic virus types 1 and 2 (HTLV-1 and HTLV-2) are members of the *Delta retrovirus* genus, different from the *Lentivirus* genus that includes human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2).

DESCRIPTION OF THE AGENT

HTLV is a complex retrovirus with regulatory genes in addition to the structural and enzymatic *gag, pol,* and *env* genes found in all classical retroviruses. HTLV-1 and HTLV-2 appear to use the ubiquitous glucose transporter, GLUT-1, for cellular entry (26). HTLV has made numerous cross-species jumps from nonhuman primates into humans and between nonhuman primate species (5, 8, 44, 61).

HTLV-1 and HTLV-2 are typical C-type retroviruses; they contain an electron-dense, centrally located nuclear core and bud from the cell surface. Within the core are two positive-sense single-stranded RNA genomes. Once a cell is infected, the RNA genome is converted by reverse transcriptase to DNA and integrates into the host genome. HTLV-1 and HTLV-2 have similar genomic organization: *gag, pro/pol, env, pX,* flanked by the long terminal repeats (LTRs) (Fig. 1). Further, they share approximately 60% of their nucleotide sequences. The *gag* gene encodes the structural proteins, p19, p24, and p15. The *pro/pol* genes encode the protease and the reverse transcriptase, respectively. The *env* gene encodes the transmembrane and external envelope glycoproteins, gp21 and gp46. HTLV-1 and HTLV-2 use alternative splicing and internal initiation codons to produce several regulatory and accessory proteins encoded by four open reading frames (ORFs) located in the pX region of the viral genome between *env* and the 3′ *LTR.* The *pX* gene encodes the spliced, regulatory proteins Tax and Rex and several additional ORFs (Fig. 1).

EPIDEMIOLOGY AND TRANSMISSION

HTLV-1 infects 15 to 20 million people worldwide, with foci of endemicity in southern Japan, the Caribbean, large parts of sub-Saharan Africa and Central and South America, and some rare areas in Melanesia and the Middle East (northern part of Iran) (13, 28). The seroprevalence rate in adults ranges from 1 to 5% in the Caribbean islands to approximately 5 to 25% in southern Japan (13). HTLV-2 is endemic in Amerindian tribes throughout the Americas, as well as in a few Pygmy tribes in Central Africa with seroprevalence ranging from 1 to 30% in adults (13, 37). In all areas of endemicity, HTLV-1/2 seroprevalence increases with age, especially in women. In the United States and Europe, the seroprevalence for both HTLV-1 and HTLV-2 among low-risk populations is less than 1% (28, 48). However, high-risk populations such as intravenous drug users (IDUs), in which HTLV-2 infection predominates over HTLV-1 infection, are reported to have a seroprevalence of up to 20% (13, 37).

HTLV-1 and HTLV-2 infections are spread through sexual (mainly male-to-female), vertical (mother-to-child by prolonged breast-feeding), and parenteral (drug use and blood transfusion) transmission (13, 28, 48). IDU and sex with IDUs are the most important risk factors for HTLV-2 transmission (13, 37). Both cross-sectional and prospective studies support sexual transmission, and there was a strong concordance of seropositivity between spouses in an area where HTLV-2 is endemic (38). A study has indicated that the mode of transmission can impact the rate of viral evolution (42). Lineages of the HTLV-2 virus evolve at a rate 150 to 350 times faster when the virus is transmitted between IDUs than when it is propagated by mother-to-infant transmission. Salemi et al. (42) postulated that the increase in viral transmission rate between IDUs, which can be many transmissions per year, accounted for the increase in the HTLV-2 evolution rate, whereas vertical transmission would be expected to occur just once every 14 to 30 years for a given viral lineage. This change in the mode of virus spread creates a possible risk for emergence of HTLV-2 strains with higher virulence.

Highly effective screening programs for volunteer blood donors have been implemented in Japan, Australia, the United States, Canada, and several Caribbean and European countries to reduce the risk of transfusion-related HTLV-1 and HTLV-2 transmission (11, 16, 43, 48, 50). In the United States, the incidence rate of seroconversion associated with HTLV in blood donors is estimated to be 1.59 per 100,000 persons per year (16), and the residual risk of transmitting HTLV infection by transfusing screened blood is estimated to be 1 in 641,000 (43).

Both HTLV-1 and HTLV-2 are the result of zoonotic transmission of simian retrovirus from simian to human hosts. The phylogeny of primate T-lymphotropic viruses (PTLV) indicates that both HTLV-1 and HTLV-2 emerged from respective primate lineages, PTLV-1 and PTLV-2, but their subsequent

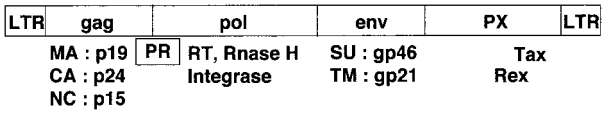


FIGURE 1 Organization of the HTLV-1 (9,046 bp) and -2 (8,952 bp) proviral genomes. MA, matrix; CA, capsid; NC, nucleocapsid; SU, surface; TM, transmembrane; RT, reverse transcriptase; PR, protease. Regions I to V correspond to ORFs of the pX region, located between the *env* gene and the LTR region of the HTLV-1/2 proviruses. ORF 1 and II encode P12, P13, and P30 for HTLV-1, and P10, P11, and P28 are for HTLV-2. ORF III and IV encode Rex and Tax regulatory proteins for HTLV-1 and -2. The gag and env proteins are most immunogenic, and antibodies to these proteins are commonly detected by serological tests (EIA and Western blotting). PCR assays are designed to detect regions within the LTR region and *gag*, *pol*, *env*, and/or *tax* genes.

evolution continued in different patterns (44). HTLV-1 includes at least four main subtypes, which evolved from PTLV-1 through several independent interspecies transmissions between simian and human hosts at different geographic locations (44, 56, 60). HTLV-2 includes three subtypes, which appear to originate from a common human ancestor virus that emerged possibly from only one simian-to-human transmission (5, 44). The possibility of additional zoonotic transfer of primate retroviruses from simian to human hosts and the impact that can result from such primate zoonotic transmission events remain a public health challenge (5, 44).

Molecular epidemiologic studies have shown HTLV proviruses to be remarkably stable genetically (34, 60). The different rates of evolution of HIV-1 and HTLV-1 are due to clonal expansion of HTLV-infected cells versus active replication of HIV-1 (58). While the overall genome of HTLV is highly conserved, nucleotide divergence in the LTR and the *gp21 env* gene has been exploited to genotypically subtype both HTLV-1 and HTLV-2 (44). The impact of diversity and emerging variants continues to challenge retroviral serology. An understanding of phylogenetic relationships is therefore useful (Fig. 2). There are at least four major geographic HTLV-1 subtypes: cosmopolitan (subtype A), Central African (subtype B), Australo-Melanesian (subtype C), and Central African (subtype D, mainly among Pygmy tribes) (44). Likewise, HTLV-2 has three subtypes: 2a, 2b, and 2d (5, 37, 44). Subtype 2a is commonly found among IDUs worldwide, whereas subtype 2b is found primarily among Amerindians and in some Pygmies from Cameroon. Subtype 2d has been found in only one Pygmy, from Zaire. Earlier studies had identified a molecular subtype 2c; however, subsequent analysis confirmed this to be subtype 2b (54). This genetic heterogeneity within HTLV-1 and HTLV-2 has provided valuable information on geographic clustering, movement of ancient populations, and viral transmission (37, 44).

CLINICAL SIGNIFICANCE

HTLV-1 has been associated with two major diseases: adult T-cell leukemia (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (13, 24, 36, 47). ATL is a severe malignant lymphoproliferation of CD4-positive cells diagnosed biologically by seropositivity for HTLV-1, the presence of morphologically distinct $CD3^+/4^+/25^+$ lymphocytes with cleaved nuclei (flower cells), and clonal integration of HTLV-1 provirus(es) in the tumor cells, as detected by Southern blotting or inverse PCR (24, 36).

The clinical features of the chronic neuromyelopathy HAM/TSP are muscle weakness in the legs, hyperreflexia, clonus, extensor plantar responses, sensory disturbances, urinary incontinence, impotence, and low-back pain (47). Biological diagnosis includes the presence of high titers of HTLV-specific antibodies in the serum and cerebrospinal fluid. The estimated lifetime risk of developing HAM/TSP among HTLV-1 infected persons is less than 2% (13, 28) but can vary according to the geographical area. Other uncommon inflammatory disease associations include infective dermatitis, uveitis, myositis, and HTLV-associated arthropathy.

HTLV-2 has not been associated with malignancies; however, it has been associated rarely with a neurological disease resembling HAM/TSP (4, 37). The estimated lifetime risk of disease development for HTLV-2-infected persons is unknown but appears to be less than that estimated for persons with HTLV-1 infection. While the majority of HTLV-2-infected persons remain asymptomatic (>95%), recent studies report an increased incidence of infectious diseases (such as bronchitis and kidney and/or bladder infections) in HTLV-1- and HTLV-2-infected persons (4, 37).

COLLECTION AND STORAGE OF SPECIMENS

Serum or plasma is suitable for use in serologic assays for HTLV-1 and HTLV-2 detection. These specimens can be drawn at the time of presentation. Seronegative patients who are suspected of having HTLV-1 or HTLV-2 or those with seroindeterminate Western blot patterns should be redrawn at 6 months and retested to capture persons during the seroconversion window period. Serum and plasma specimens can be stored at 4°C or frozen for later use. Package inserts should be consulted for storage limitations and limitations on the number of freeze-thaw cycles permitted. Peripheral blood mononuclear cells (PBMCs) are appropriate specimens for nucleic acid detection and virus isolation since HTLV-1 and HTLV-2 are cell-associated viruses. Whole blood is collected, and PBMCs are typically isolated on a Ficoll-Hypaque gradient (discussed in chapter 80). DNA is prepared using standard methods, and it can be stored at −20°C until needed.

DIRECT DETECTION

Nucleic Acid Detection

Amplification of proviral DNA by PCR is the preferred method for determining infection status, testing the validity of serologic assays, distinguishing between HTLV-1 and HTLV-2, and studying tissue distribution in vivo. Two qualitative PCR procedures, utilizing primers in the *pol* or *tax* gene regions, have been used to confirm and differentiate between HTLV-1 and HTLV-2 infections (Table 1) (17, 23). The first uses HTLV consensus primers that allow amplification of both viruses; typing is achieved either by hybridizing the product to an HTLV-1-specific or HTLV-2-specific probe or by specific restriction digestion patterns (17, 23). A second approach employs type-specific primers and probes in separate amplifications. The PCR products can be detected with labeled internal probes by Southern blot hybridization (17). Most of these assays are performed for research use only.

Viral load assays are based on proviral DNA amplification; RNA viral loads are not feasible in the plasma for

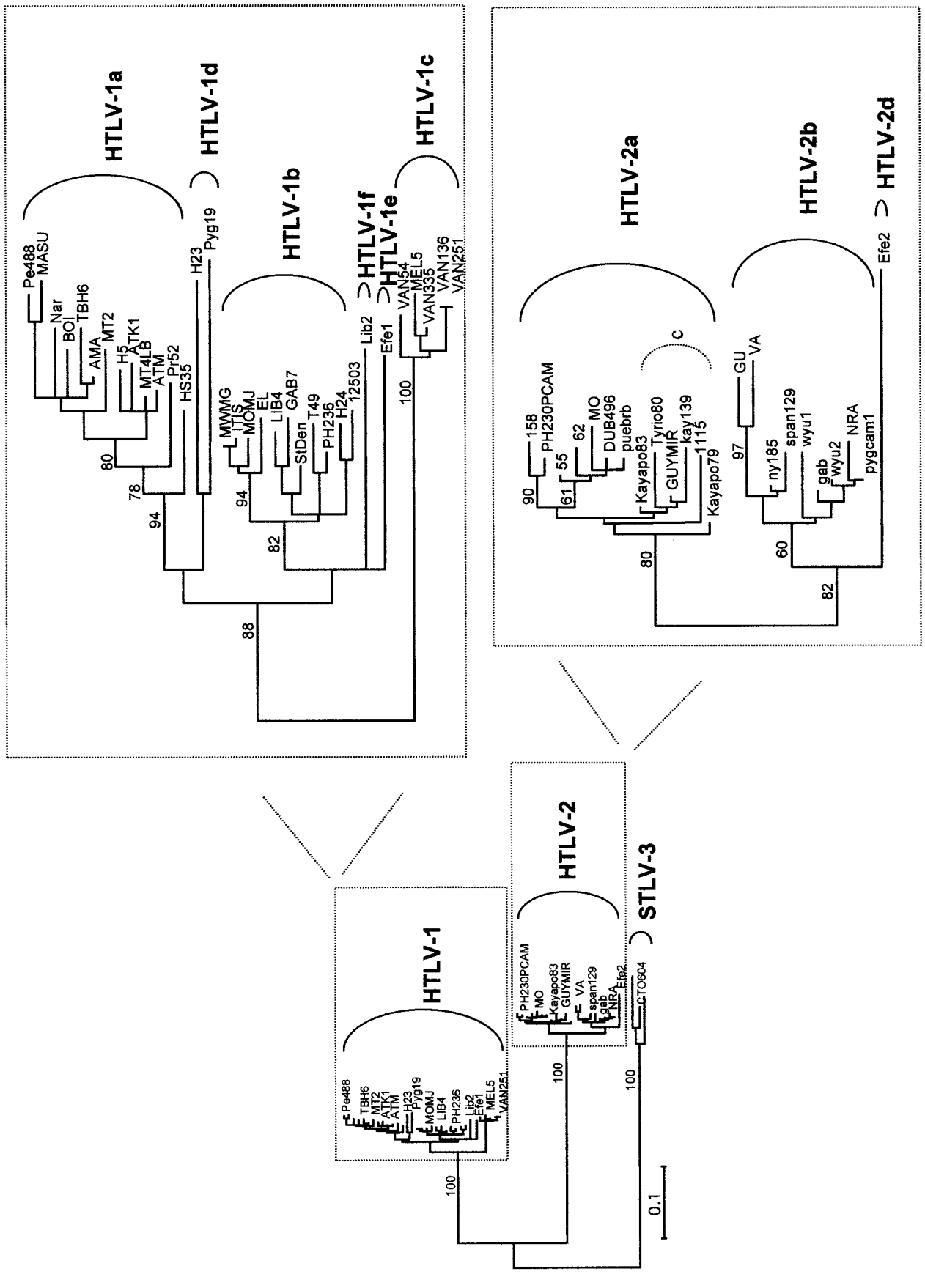

HTLV-1a
HTLV-1d
HTLV-1b
HTLV-1f
HTLV-1e
HTLV-1c
Pe488
MASU
Nar
BOI
TBH6
AMA
MT2
H5
ATK1
MT4LB
ATM
Pr52
HS35
H23
Pyg19
MMMG
ITIS
MOMJ
EL
LIB4
GAB7
StDen
T49
PH236
H24
12503
Lib2
Efe1
VAN54
MEL5
VAN335
VAN136
VAN251
HTLV-2a
HTLV-2b
HTLV-2d
158
PH230PCAM
55
62
MO
DUB496
puebrb
Kayapo83
Tyrio80
GUYMIR
kay139
1115
Kayapo79
c
GU
VA
97
ny185
span129
wyu1
gab
wyu2
NRA
pygcam1
Efe2
90
61
80
60
82
HTLV-1
HTLV-2
STLV-3
CT0604
100
94
78
88
0.1

TABLE 1 Summary of serological and supplemental confirmatory tests for HTLV-1 and HTLV-2 infections

Detection method	Description	Reference(s)	Availability in United States[a]
Serological screening tests			
EIA	HTLV-1- and HTLV-2-infected cell lysates and/or recombinant antigens used to detect HTLV antibodies by colorimetric readout. Excellent sensitivity and specificity.	2, 23, 35	Two FDA-licensed tests
ChLIA	Similar to EIA but with chemiluminescent readout.		Research use only
Particle agglutination	Viral lysate coats gelatin or latex particles. Antigen-specific antibodies bind, which results in agglutination of particles. Visual readout by operator.		Research use only
Serological supplementary tests			
WB	HTLV-1 viral lysates ± recombinant antigens used to detect HTLV-1- and -2-specific antibodies. May contain recombinant antigens to differentiate antibodies to HTLV-1 and HTLV-2.	57	Research use only
LIA	Recombinant or peptide HTLV-1 and HTLV-2 antigens painted onto membrane. Antibodies to individual viral proteins are visualized.	40	Research use only
IFA	Antibodies bind to HTLV-1- and HTLV-2-infected cells. Detected by secondary anti-human antibody with fluorescent label. Differentiates between antibodies to HTLV-1 and HTLV-2.	15	Research use only
Nucleic acid detection tests			
Qualitative PCR	Distinguishes between HTLV-1 and HTLV-2 either by using HTLV consensus primers with typing done by HTLV-1- or HTLV-2-specific primers and probes in separate tests, or by differential patterns using restriction enzyme fragment length polymorphisms.	17, 44	Research use only
Quantitative PCR	Determines proviral DNA copy number by limiting dilution, quantitative competitive PCR, or real-time PCR using gene- and type-specific primers.	1, 18, 31, 52	Research use only

[a] FDA licensed: may be used for blood donor screening and as an aid in clinical diagnosis of HTLV-1 or HTLV-2 infection and related diseases. Research use only: not for clinical use.

HTLV due to the cell-associated nature of these viruses (12). Quantitative nucleic acid amplification tests have been developed to determine HTLV-1 and HTLV-2 proviral DNA load using quantitative competitive PCR (1) or real-time PCR (18, 21, 31, 52). The real-time noncommercial PCR assay has excellent sensitivity and a broad dynamic range, from 10 to 10^6 copies/reaction (21), and has been used to study the relationship between proviral load and pathogenesis. Studies using these methods demonstrate that the proviral load is higher among HAM/TSP patients than among asymptomatic carriers (28, 47), suggesting that proviral load may be an indicator for future disease development (47). Follow-up of the proviral load or of the presence of a specific clone can also be done during ATL treatment (6, 36). Further, recent studies suggest that sexual transmission and mother-to-child transmission are associated with higher HTLV proviral load in the index case (22, 32, 38). Thus, quantification of HTLV proviral load is providing a better understanding of correlates of transmission and disease progression (32, 38, 47).

PCR also has been used to genetically subtype HTLV-1 and HTLV-2 based on the LTR region. Earlier studies used restriction endonuclease digestion of PCR products; separation through an agarose gel with visualization of the bands by ethidium bromide allows for subtype determination (44). However, most recent studies now perform direct sequencing of the PCR products (44, 56). Likewise, sequence and phylogenetic analyses of *env* and LTR regions have resulted

FIGURE 2 Unrooted phylogenetic tree generated by the neighbor-joining method using a fragment of the LTR region (622 bp) of representative sequences of the different subtypes of HTLV-1 and HTLV-2 strains. Bootstrap support (1,000 replicates) is noted on the branches of the tree. The branch lengths are drawn to scale, with the bar indicating 0.1 nucleotide replacement per site. The four main HTLV-1 subtypes (a, b, c, and d) are represented as well as the three main subtypes of HTLV-2 (a, b, and d). HTLV-1e and HTLV-1f represent the two other rare HTLV-1 subtypes, which have so far been found in few inhabitants of Central Africa.

in elucidation of the molecular epidemiology and the geographical distribution of virus-carrying populations (5, 44).

VIRUS ISOLATION AND IDENTIFICATION

Viral isolation of HTLV-1 or HTLV-2 has been difficult due to the cell-associated nature of this virus (12). Nevertheless, cocultivation of HTLV-1- and HTLV-2-infected PBMCs with activated, allogeneic HTLV-negative PBMCs or cord blood cells is used to obtain viral isolates. The culture supernatants are collected weekly for up to 4 weeks, and the presence of p19 gag antigen in the supernatant is tested using a research use antigen capture assay (Zeptometrix Corp., Buffalo, N.Y.). Because of the time required and labor- intensive nature of this method, virus isolation and antigen detection are generally not done for diagnosis of HTLV infection but rather serve as a research tool.

SEROLOGIC TESTS

Testing for antibodies to HTLV-1 and HTLV-2 should be performed for all blood donors and any patients presenting with relevant clinical signs and symptoms. In the United States, all blood donors have been screened for antibodies to both HTLV-1 and HTLV-2, using assays that include both HTLV-1 and HTLV-2 antigens (14; http://www.fda.gov/cber/gdlns/htlv-ii.pdf) since 1997. Testing for HTLV-1 and HTLV-2 should also be offered to persons who are from areas where HTLV is endemic, who engage in high-risk behaviors such as needle sharing, and who have had sexual contact with persons from either group. Currently, HTLV-1 and HTLV-2 testing is not routinely performed for fertility or pregnancy testing in the United States. This is different in some European countries, including some islands in the West Indies, where recommendations have been made to test pregnant and breast-feeding women originating from areas of HTLV-1 endemicity (48, 50).

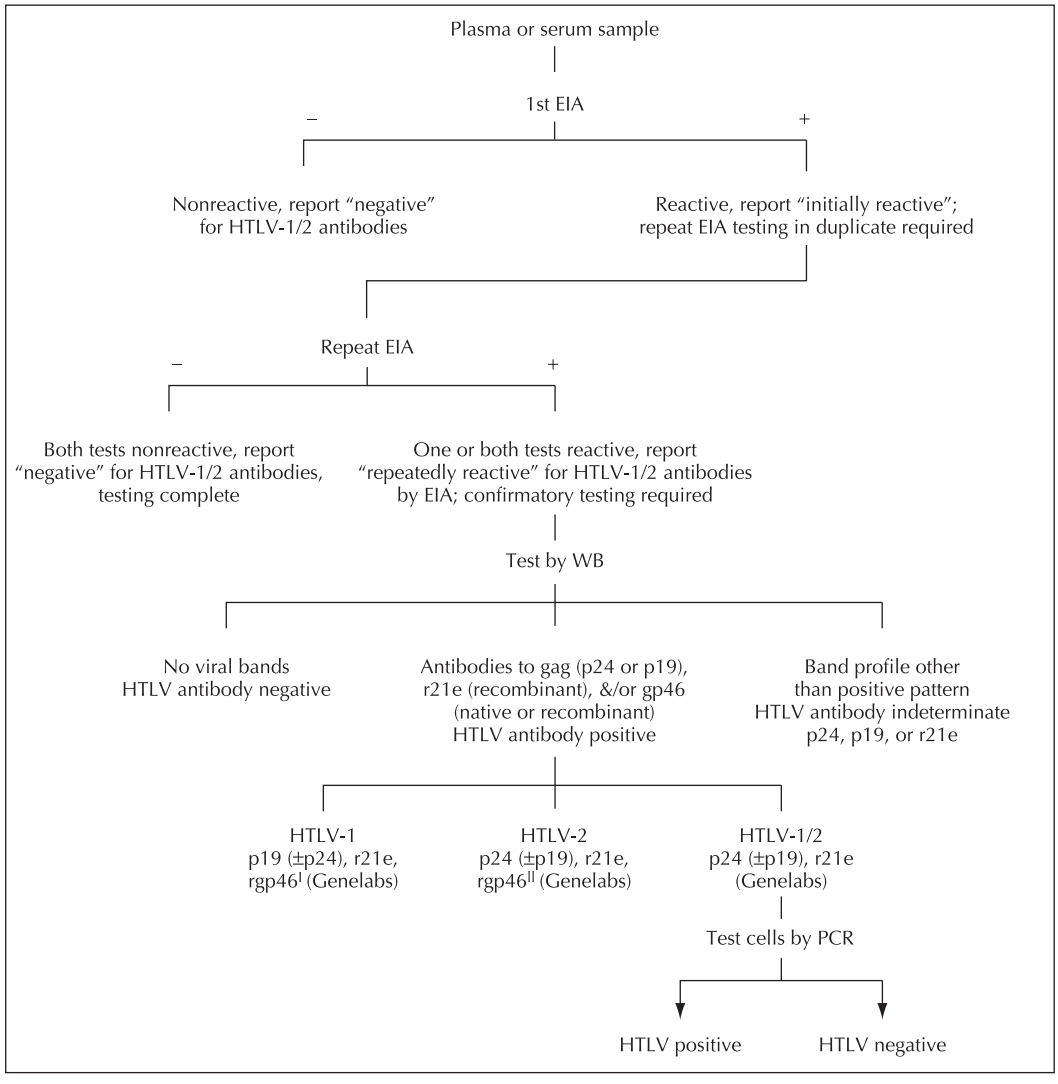


FIGURE 3 Serologic testing algorithm for the detection and confirmation of HTLV-1 and -2 infection. If the initial screening EIA is reactive, a repeat assay with the same specimen is performed in duplicate. If one or both of the repeat tests are reactive, the specimen is classified as repeatedly reactive and supplemental testing is done for the purpose of confirmation. Western blot criteria shown are those used by the manufacturer and not the PHS working group. In some cases, further follow-up is done using PCR.

The most common screening assays detect antibodies in serum or plasma (11, 14, 51). The immunodominant regions of structural and regulatory proteins are well characterized (20) and are used in diagnostic assays that detect and differentiate HTLV-1 and HTLV-2. The major tests for HTLV-1 and HTLV-2 are described in Table 1. The serologic testing algorithm is comprised of a primary screening assay followed by testing for confirmation and identification of HTLV type (Fig. 3).

Primary screening assays include enzyme immunoassay (EIA), particle agglutination (Fujirebio America, Fairfield, N.J.), and immunofluorescence assays (Table 1). The EIA is a sensitive and simple colorimetric test that uses purified infected cell lysates and/or recombinant antigens or synthetic peptides (2, 11, 51). The addition of HTLV-2 antigens to screening test kits significantly improved the detection of antibodies to HTLV-2, compared with results using kits that contained only HTLV-1 antigens (2, 23, 35). The EIA and next-generation versions that use chemiluminescent technology (chemiluminescent immunoassays [ChLIAs]) can be automated and performed on a large scale. While this test is not yet licensed in the United States, it is being routinely used outside the United States. However, neither EIAs nor ChLIAs can differentiate between HTLV-1 and HTLV-2 infections because of the significant homology in structural proteins between the two viruses; therefore, these screening assays are referred to as tests for HTLV-1/2 (2, 11, 51).

Comparative analysis of various commercial screening assays containing both HTLV-1 and HTLV-2 antigens indicates that sensitivity ranges from 98.9 to 100% for confirmed HTLV-1-positive specimens and 91.5 to 100% for confirmed HTLV-2-positive specimens. Specificity ranges from 90.2 to 100% (2, 11, 23, 35, 51). Currently, two HTLV-1/2 EIAs are FDA licensed for use in the United States (Vironostika HTLV-I/II Microelisa System [BioMerieux, Durham, N.C.] and Abbott HTLV-I/HTLV-II EIA [Abbott Laboratories, Abbott Park, Ill.), with reports of overall sensitivity of 99.76 to 100% and specificity of 99.63 to 99.98%. These ranges reflect differences in the source of antigen and assay configuration among the various tests, as well as the source of the specimens (2). These licensed assays are used both for blood donor screening and as an aid in clinical diagnosis of HTLV-1 and HTLV-2 infection and related diseases. Further attempts have been made to develop a dual EIA algorithm to increase the predictive values of HTLV tests (46). Pooling of samples for seroepidemiologic studies has been proposed; however, pooled testing is not recommended for blood donor testing (3).

While most screening assays are highly sensitive, specimens containing low titers of antibodies to HTLV-1 and HTLV-2 from certain areas of HTLV-2 endemicity or specimens from early seroconverters can be missed by current EIAs (27, 35). Nucleic acid assays can be used to detect infected individuals either with low antibody titers or within their window period prior to the development of an antibody response (median window period, 51 days; range, 36 to 72) (27). However, these assays are used mainly for research and are not in routine clinical use.

Supplemental confirmatory or differentiation tests for HTLV-1 and HTLV-2 infection include Western blots (WB) containing the viral lysates supplemented with recombinant proteins (57), line immunoassays (LIAs), (Innogenetics, Ghent, Belgium) (40), and immunofluorescence assays (IFAs) (15) (Table 1). Although WB assays using purified viral lysates are highly sensitive for detecting p24 and p19 gag antibodies, they do not always detect antibodies to native envelope glycoproteins. An alternative approach used in a second-generation confirmatory assay is a modified WB that contains type-specific gp46env recombinant proteins from both viruses and a truncated form of r21e that reduces nonspecific reactivity, improves performance (specificity and sensitivity), and allows differentiation between HTLV-1 and HTLV-2 (Genelabs WB 2.4; Genelabs Diagnostics/ZeptoMetrix Corp.) (Fig. 4) (23, 57).

Another supplementary test is the LIA, consisting of recombinant and synthetic HTLV-1 and HTLV-2 antigens painted onto strips (Innogenetics) (40). Some of these antigens are specific for HTLV-1 or HTLV-2; therefore, this assay can differentiate between HTLV-1 and HTLV-2 infections. IFAs that detect binding of antibodies from a specimen to HTLV-1- or HTLV-2-infected cells can be used to discriminate HTLV-1/2 infections and to determine antibody titer (15). No assay has been approved or licensed as a confirmatory test for clinical use by the U.S. Food and Drug Administration; however, WB and LIA have been certified for use in Europe and other areas of endemicity (Brazil, Argentina, West Indies, Iran, and Japan) to confirm any EIA repeatedly reactive sample (48, 50).

ANTIVIRAL SUSCEPTIBILITIES

There are limited treatment options for HTLV-associated diseases. Although zidovudine (AZT) and alpha interferon (IFN-α) yield some responses and improve ATL prognosis, alternative therapies are needed. New drugs such as arsenic trioxide, proteasome inhibitors, retinoids, and angiogenesis inhibitors, as well as cellular immunotherapy, are under investigation (6). Antiretroviral treatment such as lamivudine and high-dose IFN-α and IFN-β have recently showed some clinical benefit among HAM/TSP patients (33, 41, 49). However, no randomized trials have been done to compare the effectiveness of these therapies for ATL or HAM/TSP treatment. For uveitis, topical and systemic corticosteroids appear to improve sight whereas infective dermatitis responds well to antibiotic treatment.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

A typical algorithm for HTLV testing for diagnostic purposes is outlined in Fig. 3. If the initial screening EIA is reactive, a repeat assay on the same specimen is performed in duplicate. If one or both of the repeat tests are reactive, the specimen is classified as repeatedly reactive. The repeatedly reactive specimens are subjected to confirmatory supplemental testing that is typically done by WB, even though these tests have not been licensed by the FDA (Fig. 4 and Table 1). Based on the data available in 1992, the U.S. Public Health Service (PHS) recommended that the diagnostic criteria for confirmation of HTLV-1 or HTLV-2 seropositivity by supplemental tests include demonstration of antibodies to p24gag and to native gp46env and/or r21e (9). Subsequent data with more-sensitive assays suggest that alternative patterns of WB reactivity may be considered. The second-generation modified WB is the most commonly used WB assay worldwide (Genelabs WB 2.4). Specimens with reactivity to p19gag (with or without p24gag), r21e, and rgp46^{I} are referred to as HTLV-1 (Fig. 4A). Sera with reactivity to p24gag (with or without p19), r21e, and rgp46^{II} are referred to as HTLV-2 (Fig. 4B). Specimens with no immunoreactivity to any bands are considered negative for antibodies to HTLV-1 and HTLV-2 (false-positive EIA specimens). Specimens reacting with p24gag (with or without p19gag) and r21e, but with no reactivity to either rgp46^{I} or rgp46^{II}, are referred to as untypeable and are considered to be

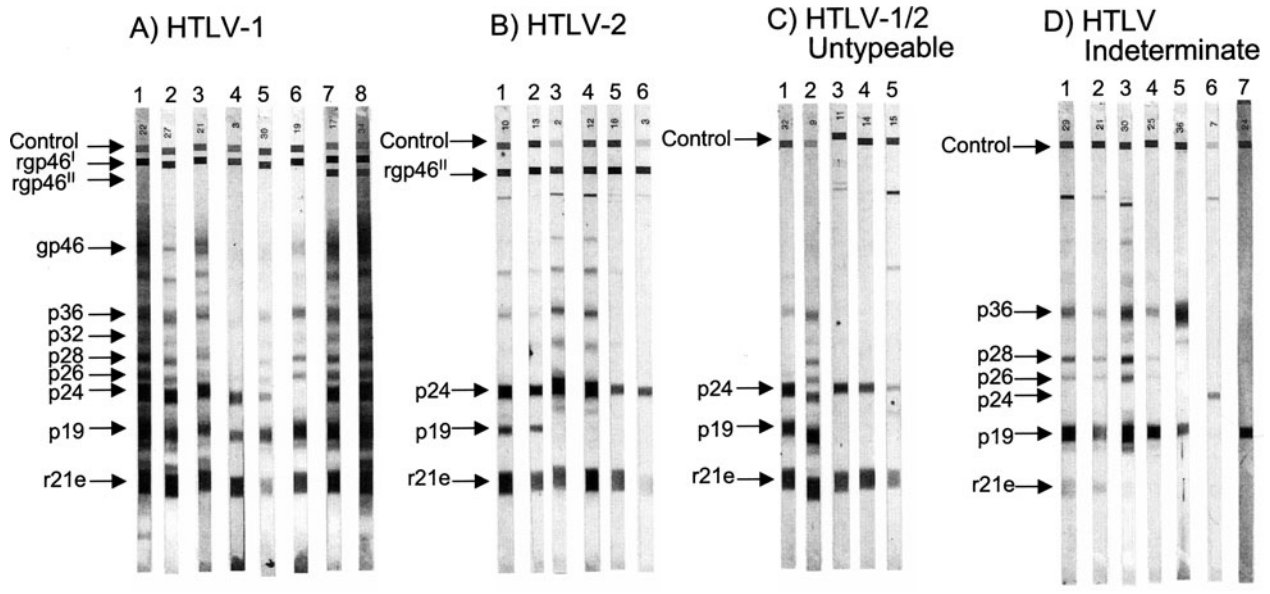


FIGURE 4 Western blot analysis of representative plasma or serum specimens from persons infected with HTLV-1 (A), HTLV-2 (B), and HTLV-1/2 untypeable (C). Representative seroreactivity patterns are shown for Western blots from Genelabs Diagnostics (HTLV-2.4 version), which contain HTLV-1 antigens spiked with recombinant r21e (common to HTLV-1 and HTLV-2) and two external envelope recombinant proteins specific for HTLV-1 (rgp46^{I}) and HTLV-2 (rgp46^{II}). Panel A shows typical patterns for HTLV-1 reactivity (lanes 1 to 5), atypical reactivity lacking p24 response (lane 6), and specimens with high antibody titers showing dual reactivity to both rgp46 proteins (lanes 7 and 8; titration of sera results in reactivity only to rgp46^{I}). Panel B shows typical patterns for HTLV-2 reactivity (lanes 1 to 6; note that the p24 band is stronger than p19 reactivity, which is usually absent from HTLV-2-infected sera). Panel C shows HTLV-1/2-positive but untypeable specimens, with reactivity to gag (p24, with or without p19) and r21e but not gp46^{I} or gp46^{II}. Lanes 1 and 2: characteristic patterns of specimens that are usually found to contain HTLV-1 after additional testing; lanes 3 to 5: characteristic patterns of specimens that are usually found to contain HTLV-2 after additional testing. Panel D demonstrates typical patterns from HTLV indeterminate specimens. Shown are typical HTLV gag indeterminate profiles frequently found in plasma or sera from individuals originating from tropical regions (Central Africa, Papua New Guinea, etc.) (lanes 1 to 4) and those from low-risk populations (lanes 5 to 7). In the great majority of the cases, neither HTLV-1 nor HTLV-2 infection could be demonstrated in samples with such seroreactivity.

HTLV positives (Fig. 4C). PCR analysis of these untypeable specimens has identified the presence of HTLV-specific sequences (55). Similar criteria are used for LIA (see manufacturer's instructions).

Both the WB assays and LIA can give indeterminate results (immunoreactivity to a single HTLV gene product or multiple bands, conditions that do not meet the criteria for seropositivity [Fig. 4D]) (51, 57). Antibody to only gag proteins (p24, p19) is the most common indeterminate pattern that is observed in EIA-reactive specimens and is an area of intense investigation (7, 10, 19, 25, 29, 30, 39, 53, 55). Extensive PCR analyses using primers to detect multiple gene regions have failed to detect HTLV-1 or HTLV-2 proviral sequences in low-risk seroindeterminate persons, thus indicating that these individuals are not likely to be infected with HTLV-1 or HTLV-2 (7, 10, 19, 30, 39, 55). The possibility that such indeterminate WB results may represent a novel retrovirus with partial homology to HTLV has been explored; however, no DNA amplification was observed using generic PCR primers that would detect HTLV-related viruses (7, 55), with the exception of HTLV-3 and HTLV-4 (8, 61), two novel human retroviruses closely related to HTLV-1 and HTLV-2 isolated from individuals living in Central Africa. Limited studies have established that individuals with indeterminate WB profiles generally do not have risk factors for HTLV infection (10, 29, 30, 39). Such indeterminate WB results among low-risk persons appear to represent antibodies to different viral and cellular antigens that cross-react with HTLV proteins (25).

In rare instances, specimens with confirmed HTLV infection have an indeterminate WB pattern of reactivity to p19gag (in the absence of p24gag) and r21e (10, 39). Likewise, in some instances, antibody to r21e may represent an early antibody response during seroconversion (28). Individuals with such reactivity should be retested in 6 months by EIA and/or PCR.

Recent data suggest that a very small number of patients with chronic progressive neurological disease from the United States with HTLV-indeterminate WB patterns may be infected with a defective HTLV or have HTLV-1 in low copy numbers (45, 59). Persons who have clinical neurological symptoms or are from high-prevalence areas (e.g., African, Asian, South American, and Caribbean countries) with HTLV-1/2-indeterminate reactivity should be further investigated for possible retroviral infection, including infection with the recently identified viruses HTLV-3 and HTLV-4.

Further testing could include PCR in conjunction with serologic testing.

In the United States, decisions to accept blood donations and defer blood donors are currently based on only screening test (EIA) results because of the lack of FDA-licensed supplemental tests. Persons who are ultimately confirmed to be antibody positive to HTLV-1 or HTLV-2 (i.e., when licensed supplemental testing becomes available) are permanently deferred from donating blood. Blood donors with plasma specimens that are repeatedly reactive on screening but not confirmed as seropositive for HTLV-1 or HTLV-2 (including false EIA-reactive and supplemental test indeterminate specimens and specimens for which supplemental testing was not performed) should be notified and deferred if the same test result is obtained on two separate donations or if the same donation repeatedly tests reactive on screening tests from two different manufacturers (14). Screening tests are weighted toward better sensitivity than specificity because of the public health implications of false-negative results. Therefore, in the absence of known risk factors for a given blood donor, repeatedly reactive results from a single-test kit or discrepant results between multiple test kits most likely represent false-positive reactions. Reactivity in a dual-screening test algorithm increases the probability of infection (46).

Persons with HTLV-1- or HTLV-2-positive or -indeterminate test results are counseled according to the guidelines established by the PHS Working Group (CDC). These guidelines state that persons should be informed that HTLV is not the AIDS virus and that their risk of developing HTLV-related diseases is low. HTLV-1- or HTLV-2-infected persons are asked not to donate blood, semen, organs, or other tissues and not to share needles or syringes. To prevent transmission of HTLV, the infected person is counseled to use protective measures during sexual relations and women are counseled to refrain from breast-feeding. Persons who have indeterminate results on two separate occasions at least 3 months apart should be advised that their specimens were reactive in screening for HTLV-1/2, but these results could not be confirmed by more-specific tests. Further, they should be reassured that indeterminate results are very rarely caused by HTLV-1 or HTLV-2 infection. Repeat testing should be offered.

The authors employed by the U.S. Government are writing as individual experts whose views should not be taken to represent those of the U.S. Government agencies by which they are employed.

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Influenza Viruses*

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TAXONOMY

The influenza viruses are members of the family *Orthomyxoviridae*. Antigenic differences in two major structural proteins, the matrix protein (M) and the nucleoprotein (NP), are used to separate the influenza viruses into three genera within the family: *Influenzavirus A*, *Influenzavirus B*, and *Influenzavirus C*. Members of these three genera are also referred to as influenza type A, B, and C viruses, respectively. The influenza A viruses are further classified into subtypes based upon characteristics of the two major surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). Subtypes are recognized by the lack of cross-reactivity in double immunodiffusion assays with animal hyperimmune sera corresponding to each antigen (58). Sixteen HA subtypes and nine NA subtypes are now recognized (23). The following information is used in the naming of individual virus strains: type, species of origin (if nonhuman), geographic location of isolation strain or laboratory identification number, year of isolation and subtype (influenza A viruses only). Thus, a human strain of influenza is A/California/7/2004 (H3N2), while an avian strain isolated in the current Asian epizootic is A/quail/Vietnam/36/2004 (H5N1).

DESCRIPTION OF THE AGENTS

Orthomyxoviruses are enveloped, single-stranded-RNA viruses with segmented genomes of negative sense. Influenza A and B viruses have eight RNA segments, and influenza C viruses have only seven segments. Each segment ranges from ~800 to ~2,500 nucleotides in length, and the entire genome ranges from 10 to 14.6 kb. Viruses are spherical and pleomorphic, with diameters of 80 to 120 nm after serial passage in culture. Filamentous forms also occur and may be up to several micrometers in size. The lipid envelope is derived from the host cell membrane through which maturing virus particles bud, and HA and NA form characteristic rod-like spikes (HA) and spikes with globular heads (NA) on the virus surface. As its name implies, the HA can agglutinate red blood cells from both mammalian (e.g., human [type O] and guinea pig) and avian (e.g., chicken and turkey) species by binding to sialic acid residues, and this characteristic is used for virus identification. The lipid envelope surrounds the nucleocapsid which has helical symmetry and consists of the genomic RNA segments, several copies of the polymerase proteins, and the NP. The matrix-1 (M1) protein is present between the nucleocapsid and the envelope, and the matrix-2 (M2) protein forms an ion channel across the envelope in influenza A viruses.

EPIDEMIOLOGY AND TRANSMISSION

Influenza viruses cause annual epidemics in areas with temperate climates; in tropical climates, seasonality is less apparent and influenza viruses can be isolated throughout the year. In the temperate regions of the Northern Hemisphere, epidemics generally occur between December and March, and in the Southern Hemisphere, the epidemic period is usually between May and August. Epidemics are characterized by a sudden increase in febrile respiratory illnesses and absenteeism from school and work, and within a community the epidemic period usually lasts from 3 to 8 weeks. A single subtype (A) or type (B) of influenza virus usually predominates, but epidemics have occurred in which both A and B viruses or two influenza A virus subtypes were isolated (13). Global epidemics, or pandemics, occur less frequently and are seen only with influenza A viruses. Pandemics occur following the emergence of an influenza A virus that carries a novel HA and that can be readily transmitted from person to person. The pandemic strain may develop due to gene reassortment following coinfection of a susceptible host with human and avian influenza viruses or through gradual adaptation of an avian strain to mammalian hosts.

Influenza viruses are transmitted from person to person primarily via droplets generated by sneezing, coughing, and speaking. Direct or indirect (fomite) contact with contaminated secretions and small-particle aerosols is another potential route of transmission that has been noted. The relative importance of these different routes has not been determined for influenza viruses (6). For avian strains of influenza virus, direct contact with infected birds has been the most common route of transmission, but direct inoculation into the pharynx or gastrointestinal tract may also lead to infection (3).

Concern is increasing about the pandemic potential of avian strains of influenza A/H5N1 viruses. In 1997, several human cases occurred in Hong Kong in association with a large poultry outbreak. This outbreak was controlled by

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slaughtering all poultry in Hong Kong, but H5N1 viruses again caused outbreaks in poultry in China in 2003 and in several southeast Asian countries in 2004 and 2005. By late 2005 and early 2006, the virus had spread to poultry in other parts of Asia, as well as to parts of Europe, Africa, and the Middle East. More than 150 human infections have been documented; most cases have been in southeast Asia, but several cases have also occurred in Turkey and Iraq. Most human cases have been due to direct contact with infected birds, but limited human-to-human transmission has also occurred (3). The virus continues to evolve, raising the possibility that it may acquire the ability to spread efficiently among humans (49).

CLINICAL SIGNIFICANCE

Influenza A and B virus infections typically cause a febrile respiratory illness characterized by fever, cough, upper respiratory tract symptoms (including sore throat, rhinorrhea, and nasal congestion), and systemic symptoms (including headache, myalgia, and malaise). This constellation of symptoms is called influenza, although other clinical presentations, from asymptomatic infection to viral pneumonia, also occur. Illness begins abruptly after a 1- to 5-day incubation period (average, 2 days). Fever generally lasts for 3 to 5 days, but symptoms of dry cough and malaise may persist for several weeks. Complications include otitis media in children, sinusitis, viral pneumonia, secondary bacterial pneumonia, exacerbation of underlying cardiac or pulmonary disease, myositis (including rhabdomyolysis), neurologic problems (seizures, acute encephalitis, and postinfectious encephalopathy), Reye's syndrome (associated with aspirin use), myopericarditis, and death (27, 53). In contrast, influenza C viruses cause mild respiratory illnesses that clinically are not distinguishable from common colds.

Influenza A/H5N1 virus causes a similar clinical syndrome, with fever (>38°C) and lower respiratory tract symptoms. Upper respiratory tract symptoms may be absent, and gastrointestinal symptoms (watery diarrhea, vomiting, and abdominal pain) occur in some patients (3). Acute encephalitis may occur (14). Infection is associated with a high mortality (~50%), with most patients dying of progressive pneumonia. Viral replication is prolonged, and levels of several inflammatory mediators (e.g., interleukin-6, interleukin-8, and interleukin-1beta) in plasma have been higher in fatal than in nonfatal cases. Surviving patients develop measurable serum antibody responses 10 to 14 days after symptom onset (3).

Influenza A and B virus infections spread rapidly through the community, with clinical attack rates having been documented to be as high as 70% following a common source exposure in an enclosed space (42). Epidemic disease is associated with an increase in hospitalization rates, especially in young children and in the elderly, and an increase in mortality rates in the elderly. Mortality rates have been higher in epidemics caused by influenza A/H3N2 viruses than in those caused by A/H1N1 or B viruses in the past 20 years. Additional information on the clinical presentation, manifestations, and complications of the diseases can be found in clinical textbooks (27, 53).

There are four licensed antiviral medications available for the treatment of influenza virus infection. Treatment with any of these medications must be initiated within 2 days of symptom onset to have demonstrable clinical benefit. Amantadine and rimantadine block the M2 ion channel and are active against only influenza A viruses, and zanamavir and oseltamivir are NA inhibitors and are active against both influenza A and B viruses. Clinically significant resistance has been seen following treatment of children or immunocompromised patients with amantadine and rimantadine, and the prevalence of resistance is increasing among all influenza A/H3N2 virus isolates (7). These drugs have also been used for prophylaxis, but annual immunization with a trivalent influenza vaccine is the primary means of prevention of influenza.

Both vaccines using inactivated virus and those using live, attenuated virus are available (25). Influenza vaccines used in the United States are derived from viruses grown in embryonated chicken eggs. For inactivated-virus vaccines, virus is harvested and then inactivated. Viral proteins are partially purified and standardized to contain 15 μg of HA. The vaccine is trivalent, containing HAs of influenza A/H1N1, A/H3N2, and B virus strains. The strains to be included in the vaccine are selected in January and February to make a vaccine for use in September. The vaccine with live, attenuated virus is also trivalent, and strain selection occurs at the same time as that for inactivated-virus vaccines. A reassortant vaccine virus for each strain to be included is derived to contain six internal genes from a parental attenuated influenza (A or B) virus and the HA and NA from the strain selected in January or February. The vaccine is licensed for use in persons 5 to 49 years of age (25). It is given topically into the nose, and virus replicates in the upper respiratory tract. Vaccines derived from viruses grown in mammalian cells and those containing higher amounts of HA or additional viral proteins are currently under evaluation to improve the efficacy of current influenza vaccines.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Influenza viruses infect the respiratory epithelium and can be found in respiratory secretions of all types. The level of virus shedding parallels the severity of clinical symptoms in uncomplicated influenza and is maximal in the first several days of illness. Samples should be collected during this time (first 2 to 3 days) to maximize the likelihood of virus detection. A variety of upper respiratory tract samples alone or in combination are routinely used for virus identification, including nasal aspirates, nasal wash fluids, nasal or nasopharyngeal swabs, throat swabs, and throat wash fluids. Virus titers tend to be lower in samples collected from the throat, so assays of these samples alone tend to be less sensitive. However, recent reports of human infection with H5N1 strains suggest that throat samples may have better diagnostic yields than samples collected from the nose (3). Lower respiratory tract samples, including sputa, tracheal aspirates, and bronchoalveolar lavage fluids, also may yield virus and can be assayed when indicated. Virus can occasionally be identified in nonrespiratory clinical samples (3, 36).

Once collected, the clinical samples should be placed in viral transport medium. A number of transport media are suitable for influenza viruses, including veal infusion broth, Hanks balanced salt solution, tryptose phosphate broth, sucrose phosphate buffer, and commercially available cell culture medium. All of these media are supplemented with 0.5% bovine serum albumin or 0.1% gelatin to stabilize the virus and antimicrobials (antibiotics and antifungals) to inhibit the growth of other respiratory flora. However, the use of transport medium may interfere with the test performance for certain commercially available virus detection assays; the package inserts of these assays should be consulted if they are to be used for diagnosis (Tables 1 and 2). Influenza virus

TABLE 1 Commercially available kits for detection of influenza A and B viruses by FA staining[a]

Test (manufacturer)	Assay format	Acceptable clinical sample(s) for direct detection[b]	Comment	Assay sensitivities (%)/ specificities (%) for direct detection of virus types[c]	Assay sensitivities (%)/ specificities (%) for identification of type A and B isolates[c]	Other virus(es) detected
Bartels respiratory viral detection kit (Trinity Biotech)	IFA	NA, NPA, NPS, NW, TS	Also available as individual components for influenza A or B virus	A, 86/99; B, 65/98	A, 100/99.9; B, 100/100	Adenovirus, P1, P2, P3, RSV
D^3 DFA respiratory virus screening and identification kit (Diagnostic Hybrids, Inc.)	DFA	NA, NPA, NW	Also available as individual components for influenza A or B virus	A, 96.6–100/100; B, 100/100	A, 100/99.5–100; B, 100/99.5–100	Adenovirus, P1, P2, P3, RSV
Imagen influenza virus A and B (DakoCytomation)	DFA	NPA		A, 96.2/100; B, 86.7/99.5	A, 100/100; B, 100/100	None
PathoDx respiratory virus panel (Remel)	DFA	Cell culture only	Not approved for direct use on clinical specimens	Not applicable	A, 100/100; B, 100/100	Adenovirus, P1, P2, P3, RSV
Light Diagnostics Simulfluor viral diagnostic screen (Chemicon)	DFA	NPA, NPS, NW, TS	Also available as flu A/flu B kit	A, 80/98.6; B, 50/100	A, 97.8/100; B, 100/100	Adenovirus, P1, P2, P3, RSV

[a] Abbreviations: NA, nasal aspirate; NPA, nasopharyngeal aspirate; NPS, nasopharyngeal swab; NW, nasal wash fluid; TS, throat swab; RSV, respiratory syncytial virus. All kits listed can be used to detect influenza virus types A and B.
[b] All kits can be used to identify virus type from positive cell cultures.
[c] Sensitivities and specificities are per the manufacturers' brochures.

infectivity is maintained for up to 5 days when samples are placed in transport media and maintained at 4°C (2). The clinical sample should be transported to the diagnostic laboratory as rapidly as possible after collection under these conditions. If a sample cannot be cultured during this time frame, it should be stored immediately at −70°C; storage at higher temperatures (e.g., −20°C) leads to the loss of virus viability. Immediate transport and processing of samples after collection are necessary for immunofluorescence detection of virus antigen in exfoliated epithelial cells.

DIRECT DETECTION

Microscopy

Influenza viruses have been detected in clinical specimens by direct and indirect visualization of their typical morphological appearance by electron microscopy (EM). Immune EM has been the most sensitive EM method and allows differentiation of viruses as to type and subtype when specific hyperimmune sera are used in the assay (44). However, large numbers of viruses ($>10^5$ to 10^6 per ml) must be present in the clinical sample for successful detection using this diagnostic approach. Because of the need for an experienced microscopist and access to an electron microscope, the relatively high costs of assay performance, and the greater sensitivity of other diagnostic approaches, EM is not routinely used for the diagnosis of influenza virus infection.

Antigen Detection

Antigen detection assays are used in a variety of formats to rapidly detect influenza viruses in clinical specimens and to confirm the identity of isolates grown in culture. These assays are based upon detecting the interaction of viral proteins with specific antibodies. A variety of different formats is used and include direct and indirect fluorescent-antibody (FA) staining, radioimmunoassay, enzyme immunoassay, immunochromatographic assay, and fluoroimmunoassay.

FA assays identify viral antigens present on infected, exfoliated epithelial cells present in respiratory secretions. Cells are collected on swabs or in aspirates or wash fluids and are washed in cold buffer to remove mucus before being applied and fixed to a microscope slide. Use of cytocentrifugation for application of the cells to slides can improve the number and morphology of cells for evaluation and enhance the accuracy of interpretation (33). Virus-specific antibodies are applied to the fixed cells; monoclonal antibodies directed against viral proteins that are conserved and expressed in large quantities (e.g., M and NP) are used because of their greater specificity compared to polyclonal sera and are available from a number of manufacturers (63). A fluorochrome is conjugated to the virus-specific antibody in direct FA (DFA) assays, and it is conjugated to a second antibody that reacts with the virus-specific antibody in indirect FA (IFA) assays. Antibody staining of cells is detected with a fluorescent microscope. Contaminating mucus can cause nonspecific fluorescence that can be reduced by treating the samples with

N-acetylcysteine or dithiothreitol and by centrifuging cells through Percoll (38, 54). DFA and IFA assays take 2 to 4 h to perform, although some diagnostic laboratories batch samples and do not perform tests as soon as the sample is received, delaying the availability of results. In theory, IFA assays should be more sensitive and less specific than DFA assays, but there is significant overlap in the sensitivities (50 to 90%) and specificities (generally >90%) of these assays noted in published reports (55). An advantage of FA assays is that sample quality can be determined by observing whether an adequate number of epithelial cells are present. In addition, kits are available to screen for other respiratory viruses (e.g., respiratory syncytial virus, parainfluenza viruses, and adenovirus) as well as for influenza A and B viruses (Table 1). These multiplex assays allow for efficient screening for other viral causes of febrile respiratory disease. Disadvantages include the need for specialized equipment (a fluorescent microscope) and the impact of technician expertise on assay performance characteristics (i.e., sensitivity and specificity). Each laboratory should establish its own performance characteristics compared to those of cell culture.

A variety of immunoassays that utilize different reporter formats (isotopic, colorimetric, fluorometric, and chromatographic) have been developed for the detection of influenza virus antigen in clinical specimens. Many of these assays take at least 2 h to perform and have 50 to 80% sensitivity compared to culture methods (63). A number of commercially available kits utilize the immunoassay format for rapid (≤30-min) detection of influenza A and B viruses in clinical specimens (Table 2). The kits utilize monoclonal antibodies to detect the presence of the influenza A or B NP by enzyme immunoassay or chromatographic immunoassay. All of the kits provide results within 30 min, and some of them can be used as point-of-care tests (i.e., those classified by the Clinical Laboratory Improvement Amendments [CLIA] as waived). The types of specimens that are appropriate for testing vary among the kits, and specific instructions for sample collection and processing must be followed for optimal results. Assay performance characteristics (sensitivity and specificity) in clinical settings often differ from those described in the manufacturers' brochures (Table 2) (55, 61).

NA Activity Assay

The determination of the three-dimensional structure of the influenza virus NA by Peter Colman and colleagues in the 1980s led to the development of specific substrates that can block or be catalyzed in the enzyme's catalytic site (12). An influenza virus-specific diagnostic assay was developed using a small-molecule derivative of sialic acid coupled to a reporter chromogen detectable only after it is cleaved from the sialic acid substrate (ZStatFlu; ZymeTx). The presence of influenza A or B virus NA in the clinical sample (throat swab) leads to the enzymatic cleavage and release of the reporter that is then identified visually. The assay does not distinguish influenza A virus from B virus strains, and it does not identify influenza C viruses, which lack NA (Table 1). The NAs of other viruses (e.g., paramyxoviruses) and bacteria do not recognize the sialic acid derivative-reporter complex as a substrate and thus do not catalyze the release of the reporter molecule. A modified version of the test, in which the reporter gives a chemiluminescent rather than a colorimetric signal, is under development (50).

Nucleic Acid Analyses

Molecular methods are increasingly being used both for the detection and for the characterization (see below) of influenza viruses. The most commonly used molecular method is reverse transcription-PCR (RT-PCR). Viral nucleic acids are first extracted from clinical samples. The use of guanidinium thiocyanate with silica particles or commercial kits based upon this approach reliably removes inhibitors of the enzymatic amplification that are often present in clinical specimens (5, 20). cDNA is then synthesized by using random hexamers or a virus-specific oligonucleotide. The cDNA is amplified by using virus-specific oligonucleotides as primers and a heat-stable DNA polymerase. Resulting amplicons are identified as virus specific by using a variety of different methods (identification by size, hybridization, restriction enzyme mapping, and sequencing).

A large number of different RT-PCR assays have been developed since the initial description in 1991 of an RT-PCR method to detect and distinguish influenza A, B, and C viruses (65). Assays that identify and distinguish different influenza virus types have targeted conserved genes, such as the M gene, and subtype-specific assays have amplified a portion of the HA gene (15, 17). RT-PCR assays can be used in a multiplex format to simultaneously detect multiple strains or to identify different genes (e.g., those for HA and NA) (62). A disadvantage of multiplex assays is that the limit of detection for each substrate is often increased, which may lead to false negatives when small amounts of viral RNA are present. Nested PCR assays have been developed to improve assay sensitivity, but the inherent problem of carryover contamination associated with the use of this assay format limits its utility for most diagnostic laboratories. When a nested PCR format has been used, assay sensitivity has been better than that achieved with cell culture (64). Real-time RT-PCR assays can directly and rapidly detect influenza viruses in clinical specimens with a sensitivity approaching or exceeding that of culture (56). A real-time RT-PCR for the Asian lineage avian H5 virus causing the panzootic and human cases has recently been approved by the Food and Drug Administration for distribution by the Centers for Disease Control and Prevention (CDC) to the Laboratory Response Network-designated laboratories (11).

Another molecular method for direct detection of influenza viruses in clinical samples is nucleic acid sequence-based amplification (NASBA). NASBA is an isothermal method that amplifies a portion of the RNA genome by using avian myeloblastosis reverse transcriptase, T7 RNA polymerase, and RNase H. RNA amplicons complementary to the genomic RNA are generated and are detected by using a virus-specific probe, and when the probe is a molecular beacon, results can be obtained in real time. NASBA can be more sensitive than culture or immunofluorescent-antibody staining for the diagnosis of influenza virus infection, but additional experience with this method is needed (41).

ISOLATION PROCEDURES

Influenza virus isolation procedures should be performed under biosafety level 2 (BSL-2) conditions. When the clinical sample comes from a patient suspected to be infected with a highly pathogenic avian influenza (HPAI) virus strain, attempts at virus isolation should be performed under BSL-3 or higher conditions (9). Human clinical samples should be processed in separate laboratories and by staff members other than those handling clinical material from swine or birds (59).

TABLE 2 Commercially available kits for rapid (≤30-min) detection of influenza A and B viruses[a]

Test (manufacturer)	Assay format (viral protein detected)	Acceptable clinical sample(s)	Sample collection restrictions	Virus type(s) detected (differentiation of A and B)	Assay sensitivity(ies) (%)/ specificity(ies) (%)[b]	Assay performance time (min)	Assay complexity[c]
Directigen flu A (Becton-Dickinson)	Immunochromatographic membrane assay (NP)	NA, NPS, NW, TS	For swab samples, use polyester- or rayon-tipped swabs with aluminum wire; do not use calcium alginate	A only	67–96/92–100	15	Moderate
Directigen flu A + B (Becton-Dickinson)	Immunochromatographic membrane assay (NP)	BAL, NA, NPS, NS, NW, TS	For swab samples, use polyester- or rayon-tipped swabs with aluminum wire; do not use calcium alginate	A and B (yes)	A, 86/91; B, 81/99	15	Moderate
Directigen flu A + B/EZ (Becton-Dickinson)	Immunochromatographic membrane assay (NP)	NA, NW, TS	For swab samples, use polyurethane foam swabs	A and B (yes)	A, 77–86/86–99; B, 69–80/99–100	15	Moderate
FLU optical immunoassay (Thermo Electron)	Optical immunoassay (NP)	NA, NPS, sputum, TS	NPS, use polyester swab; TS, use cotton swab; do not use transport media prior to testing	A and B (no)	Combined, 62–88/52–80	15	Moderate
FLU optical immunoassay A/B (Thermo Electron)	Optical immunoassay (NP)	NA, NPS, sputum, TS	NPS, use polyester swab; TS, use cotton swab; do not use transport media prior to testing	A and B (yes)	Equivalent to those of FLU optical immunoassay	15	Moderate
NOW influenza A (Binax)	Immunochromatographic membrane assay (NP)	NA, NPS, NW	For swab samples, use cotton, rayon, foam, or polyester flexible-shaft swabs; do not use calcium alginate	A only	78–82/92–94	15	Waived
NOW influenza B (Binax)	Immunochromatographic membrane assay (NP)	NA, NPS, NW	For swab samples, use cotton, rayon, foam, or polyester flexible-shaft swabs; do not use calcium alginate	B only	58–71/97	15	Waived
NOW influenza A and B (Binax)	Immunochromatographic membrane assay (NP)	NA, NPS, NW	For swab samples, use cotton, rayon, foam, or polyester swabs; do not use calcium alginate	A and B (yes)	Equivalent to those of individual NOW influenza A and NOW influenza B tests	15	Waived
QuickVue influenza test (Quidel)	Immunochromatographic membrane assay (NP)	NA, NS, NW	Do not use any kind of transport medium to store or transport sample	A and B (no)	Combined, 73–81/96–99	10	Waived
QuickVue influenza A + B test (Quidel)	Immunochromatographic membrane assay (NP)	NA, NS, NW	Do not use any kind of transport medium to store or transport sample	A and B (yes)	A, 72–77/96–99; B, 73–82/96–99	10	Waived
Influenza A test (SA Scientific, Inc.)	Immunochromatographic membrane assay (NP)	NA, NW		A only	76/98	30	Waived
Influenza B test (SA Scientific, Inc.)	Immunochromatographic membrane assay (NP)	NA, NW		B only	91/100	30	Waived

Wampole Clearview influenza A/B (Fisher)	Immunochromatographic membrane assay (NP)	NS, NW, TS	For swab samples, use cotton, rayon, foam, or polyester swabs; do not use calcium alginate	A and B (yes)	Equivalent to those of individual NOW influenza A and NOW influenza B tests	15	Moderate
Xpect flu A and B (Remel)	Immunochromatographic membrane assay (NP)	NS, NW, TS	For swab samples, use rayon- or Dacron-tipped swabs with aluminum or plastic shafts; do not use calcium alginate	A and B (yes)	A, 89–100/100; B, 83–100/100	15	Moderate
ZstatFlu (ZymeTx)	Colorimetric detection of viral NA activity	TS	Use foam swab included in kit for TS collection	A and B (no)	Combined, 62/99	30	Waived

[a] Data are from references 25, 55, and 61. Abbreviations: BAL, bronchoalveolar lavage fluid; NA, nasal aspirate; NPS, nasopharyngeal swab; NW, nasal wash fluid; NS, nasal swab; TS, throat swab.
[b] Sensitivities and specificities are per the manufacturers' brochures.
[c] CLIA-waived laboratory assays employ methodologies that are so simple and accurate as to render the likelihood of erroneous results negligible. CLIA moderate-complexity assays require some knowledge, training, reagent preparation, processing, proficiency, ability to troubleshoot, or interpretation and judgment in the performance of the tests.

Cell Culture

Influenza viruses can be grown in a number of different cell lines, including primary monkey kidney cells, Vero cells, human diploid lung fibroblasts, and Madin-Darby canine kidney (MDCK) cells (24, 46). Although some variability can be seen from season to season, MDCK and primary monkey kidney cell lines have similar isolation frequencies (24), and MDCK cells are more sensitive than Vero or diploid lung fibroblast cells (46). Thus, MDCK cells (CCL 34; American Type Culture Collection, Manassas, Va.), a continuous polarized cell line, are the most common cell line used for isolation of influenza viruses and will support the growth of type A, B, and C strains. Continuous cell lines do not produce proteases that will cleave the viral HA, a step necessary to produce infectious viral progeny, so exogenous protease must be added to the maintenance medium. L-(Tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin at a concentration of 1 to 2 μg/ml provides the necessary proteolytic activity and is the recommended protease for virus isolation. Chymotrypsin cleavage of the HA prevents the trypsin-mediated enhancement of viral infectivity, and TPCK treatment inactivates chymotrypsin activity which may contaminate pancreatic extracts of trypsin (32, 34).

MDCK cells are propagated in growth medium that contains 5 to 10% fetal calf serum (FCS). FCS contains inhibitors that prevent the production of infectious virus, so the FCS must be removed prior to inoculation of the clinical sample (16, 63). The inhibitory effects of FCS can be prevented by washing the cell sheet with Hanks buffer or serum-free medium sufficiently to remove the protein-containing growth medium and then adding serum-free medium to cover the cell sheet. The clinical sample is then inoculated into the medium. After a 2-h incubation, the inoculum-medium mixture is removed and replaced with serum-free medium supplemented with TPCK-treated trypsin. Alternatively, the sample can be inoculated directly onto cells with serum-free medium supplemented with TPCK-treated trypsin and incubated overnight prior to changing of the medium the next day. The cultures are maintained at 33 to 34°C and monitored for virus growth.

The replication of influenza viruses typically leads to cytopathic effects (CPE) and destruction of the cell sheet within a week following inoculation. CPE may be inapparent or absent in the presence of viral replication, but viral replication can be identified by the ability of the viral HA to bind to sialic residues on the erythrocytes of different animal species. Cultures should be screened every 2 to 3 days by hemadsorption (binding of erythrocytes to the viral HA of infected cells) or hemagglutination (cross-linking of erythrocytes by virus in the culture medium) for evidence of viral replication. To evaluate hemadsorption of cells grown in a tissue culture tube, CPE are first determined by microscopic examination (Fig. 1A), and then the medium is removed and stored. The cell sheet is rinsed three times with 1 ml of 0.05% guinea pig red blood cells. One milliliter of 0.5% guinea pig red blood cells is then added, and the tube is stored at 4°C for 20 min, with the red blood cell suspension covering the cells. The tube is then shaken, and adherence of red blood cells to the cell sheet is determined microscopically (Fig. 1B). If cytopathic changes are scored as less than 4+ (i.e., less than 75% of cell sheet with CPE), the tissue culture tubes are rinsed with phosphate-buffered saline and refed with culture medium. The media collected initially from tubes with 4+ cytopathic changes can be used for further characterization. All procedures are performed in a BSL-2 safety cabinet, and care must be taken to prevent

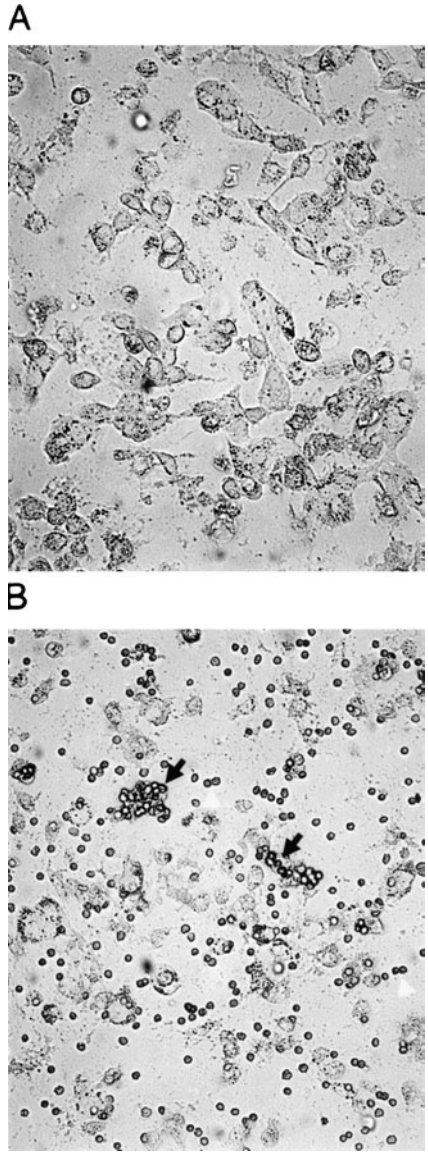


FIGURE 1 Influenza virus-infected MDCK cells. (A) Cytopathic changes. (B) Hemadsorption with guinea pig red blood cells. Red blood cells adsorb to both infected cells (black arrows) and the plastic previously occupied by infected cells (white arrowheads).

cross-contamination between cultures. Guinea pig red blood cells are more sensitive for detection of influenza virus than are avian cells, but influenza C virus does not agglutinate guinea pig red blood cells. Chicken red blood cells can be used in agglutination assays to identify influenza C viruses. Although most isolates will be identified within 1 week after inoculation, additional strains can be detected by maintaining the culture for 10 to 14 days and by blind passaging of negative cultures. Presumptive isolates are characterized further, as outlined below.

A disadvantage of traditional cell culture methods is the time needed to obtain a positive result (average, 4 to 5 days). More rapid methods have been developed by inoculating samples onto cell culture monolayers maintained in shell vials or multiwell plates. This approach can utilize either cell lines employed in traditional cell culture for identification of influenza virus (e.g., MDCK cells) or cell mixtures (A549 cells plus mink lung cells) to screen for multiple respiratory viruses (R-Mix FreshCells; Diagnostic Hybrids, Athens, Ohio) (19, 21, 39). The cells are fixed after 24 to 72 h, and type-specific monoclonal antibodies are used to detect viral antigen. Sensitivity is generally lower than that achieved by using standard isolation methods (56 to 100%), and this approach has the disadvantage of not producing virus for additional studies (e.g., antigenic characterization) (63). Screening for viral antigen by immunofluorescence also can be used at the end of the 10- to 14-day incubation period for standard culture prior to discarding of cells (57). This step is usually not necessary if screening by hemadsorption is being performed, but it may detect virus in the absence of cytopathic changes if other strategies for virus detection are not utilized.

Isolation from Embryonated Chicken Eggs

The amniotic and allantoic cavities of 10- to 11-day-old embryonated chicken eggs are inoculated with the clinical sample for isolation of influenza A and B viruses. Seven- to eight-day-old eggs are used for isolation of influenza C viruses, although these viruses are also isolated by using 10- to 11-day-old-eggs. Embryonated eggs have endogenous proteases that are able to cleave the viral HA to yield infectious virus, so exogenous administration of proteases is not necessary. Inoculated eggs are incubated at 33 to 34°C for 2 to 3 days (5 days for influenza C viruses), and then both amniotic and allantoic fluids are collected and assayed for hemagglutination activity. Influenza A and B viruses can grow both in cells lining the allantoic cavities and in those lining the amniotic cavities, whereas influenza C virus grows only in cells lining the amniotic cavities of embryonated eggs. If no hemagglutination activity is detected, influenza viruses may still be recovered by performing one or two blind passages. A pool containing equal volumes of the amniotic and allantoic fluids is inoculated into eggs as described above (16).

IDENTIFICATION AND TYPING SYSTEMS

A variety of methods are used to identify and characterize influenza virus isolates. The most common are shown in Table 3 and are based upon immunologic or molecular approaches. The initial step is to identify the isolate as an influenza virus and to distinguish it from other respiratory viruses that have the ability to agglutinate or adsorb red blood cells (e.g., parainfluenza viruses and mumps virus). In many instances, it is sufficient to identify the virus by type, and this may be accomplished by using immunofluorescent or immunoperoxidase stains or an enzyme-linked immunosorbent assay (ELISA) using commercially available, type-specific antibodies targeting the viral NP or M. These assays are particularly useful for working with cell culture isolates. The rapid immunochromatographic assays described in Table 2 may be able to identify isolates and type them, but there are limited data on the use of these assays for this purpose, and these assays are not approved for this use. Importantly, the immunochromatographic assays may give false-negative results when the quantity of virus in a cell culture harvest is low.

Hemagglutination inhibition (HAI) assays have been performed for more than 60 years and are still used for identification (28, 59). HAI assays can be type, subtype, or strain specific, and they are particularly useful for examining antigenic relationships among strains of the same subtype. Immune sera are usually produced in ferrets, sheep, or chickens (63). The hemagglutination activity of the virus is quan-

TABLE 3 Methods to identify and characterize influenza virus isolates

Assay	Advantage(s)	Limitation(s)
Assays using type- or subtype-specific antisera		
ELISA	Standard assay with known performance characteristics; most labs experienced with assay format	For subtyping of influenza A virus strains, need to update sera periodically to detect circulating strain
HAI	Standard assay with known performance characteristics; no special equipment needed	For subtyping of influenza A virus strains, need to update sera periodically to detect circulating strain; many clinical labs not experienced with this method
Immunofluorescence or immunoperoxidase staining of infected cells	Standard assay with known performance characteristics; most labs experienced with assay format	For subtyping of influenza A virus strains, need to update sera periodically to detect circulating strain
Molecular methods		
RT-PCR	Very sensitive	Potential for carryover contamination; need for stringent laboratory controls
Identification by amplicon size	Ease of performance	Potential for false-positive results due to nonspecific amplification
Hybridization	Most commonly used approach for confirmation of PCR results	Depending on hybridization format used, may add time to performance of assay
Restriction analysis	Ease of performance	Need to know specific sequence; increased handling of post-PCR samples relative to that in other methods
Sequencing	Sequence data obtained may be used in other studies	Need for specialized equipment; increased cost relative to those of other methods
Microarray analysis	Potential to analyze multiple genetic sequences simultaneously	Investigational; limited experience

titated, and a standard amount of viral HA (4 HA units) is mixed with serial dilutions of the immune serum. A fourfold or greater difference in HAI activities between the isolate and the reference strain is an indication that the isolate may be an antigenic variant (Table 4). Because the HA undergoes antigenic change over time, subtype-specific antisera for interpandemic strains must be prepared and standardized every few years (1). Thus, subtype identification is usually performed only as part of surveillance activities or investigation of a case in which there is a strong epidemiologic suspicion of infection with a nonhuman strain.

Molecular assays are increasingly being used for virus identification and characterization. The same RT-PCR assays used for detection of viruses in clinical samples also can be used to identify clinical isolates. An advantage that molecular assays have over immunology-based assays is that the molecular assays can identify influenza A virus subtypes even after significant antigenic variation has occurred because there are well-conserved regions of the HA gene that serve as targets for the primers and probes used for identification (1). Multiplex assays can also be used to distinguish influenza A and B viruses or to identify HA and NA subtypes (52, 62). Results are determined by identification of amplicon size, by hybridization to type- or subtype-specific probes, and by direct sequencing of the amplicons. If the sequences of different variants are known, it may be possible to identify restriction fragment length polymorphisms unique to each strain. For example, this method was used to distinguish two H3N2 variants that cocirculated during a single season (43). Influenza A/H3N2/Wuhan/359/95 virus-like variants generated amplicons that could be digested with the BstF5I restriction enzyme, whereas amplicons from influenza A/H3N2/Sydney/05/97 virus-like variants could be digested by HindIII. Direct sequencing of amplicons, or even entire genes, is a more common way to track and characterize specific strains.

DNA microarrays are being used increasingly in diagnostics for identification of specific pathogens. Oligonucleotide probes are arrayed on a chip or membrane, and hybridization of virus-specific sequences is then detected. The viral sequences can be generated by cDNA synthesis from viral

TABLE 4 Antigenic variation of influenza A/H3N2 virus strains detected by HAI assays using guinea pig red blood cells[a]

Reference antigen	Value[b] for reference ferret antiserum raised against:	
	A/Wyoming/03/2003	A/California/07/2004
A/Wyoming/03/2003	**640**	80
A/California/07/2004	80	**320**

[a] Data are from the CDC (10).

[b] Values are reciprocals of the highest serum dilutions that inhibit hemagglutination. Boldface indicates interaction of the virus with its homologous antiserum. The differences led to a recommendation for a change in the A/H3N2 virus used for the 2005–2006 influenza virus vaccine (10, 25).

genomic RNA or by amplification of fragments of genomic RNA by RT-PCR. Microarray analysis strategies have been developed that distinguish influenza virus types (A versus B) and subtypes (H1, H3, H5, N1, and N2) (30, 35) but at the present time are too costly for most individual laboratories to develop. Nevertheless, this technology has the promise of being able to more fully characterize strains in surveillance studies and to provide rapid and accurate identification of influenza virus strains.

SEROLOGIC TESTS

Influenza virus infections are also identified by using serologic methods. Most persons have been infected previously with influenza viruses, so detection of virus-specific immunoglobulin M or other immunoglobulin subclasses has not been particularly useful (47). An exception may be detection of immunoglobulin M responses to novel HAs from avian strains (29). Instead, paired acute- and convalescent-phase serum samples collected at least 10 days apart are needed to detect a significant (fourfold or greater) increase in serum antibody levels. The requirement for paired sera to identify infection makes serology an impractical method for identification of influenza virus infection in the acutely ill individual. Instead, serology is used primarily in surveillance and in epidemiologic studies. The most widely used assay formats include complement fixation, HAI, neutralization, and enzyme immunoassay. Complement fixation identifies type-specific antibodies to the NP, but it is not as sensitive as the other commonly used serologic assays in detecting significant rises in antibody levels. HAI and neutralization antibodies in serum are functionally significant in that higher serum antibody levels correlate with protection from infection and illness, and these antibody levels are used to measure responses to vaccination as well as to identify infection (13). HAI antibodies block the binding of the viral HA to sialic acid residues on red blood cells and thus inhibit hemagglutination. Each of the components in the HAI assay may affect the outcome of the test. Human and animal sera may contain nonspecific inhibitors of hemagglutination, but methods to remove these inhibitors have been developed (16). The source of the viral antigen can affect results in that virus initially isolated in cell culture may detect a greater frequency of antibody rises than egg-grown virus (45). The species from which the red blood cells are derived can affect assay results. Chicken and turkey red blood cells are commonly used to measure HAI antibody to human strains of influenza viruses, but they may fail to detect HAI antibodies to avian strains (such as H5N1). Substitution of horse red blood cells can improve HAI assay sensitivity for detection of antibodies to avian influenza virus strains (51). Neutralizing antibodies block viral infectivity and provide a more sensitive assay for detection of antibodies to influenza A and B viruses (60). Although neutralization assays have been available for more than 35 years, they are less standardized than are HAI antibody assays (48, 59). Nevertheless, neutralization assays are the preferred method for detection of antibody to HPAI virus strains (48). Because these assays require the use of live virus, their use with HPAI virus strains is restricted to those laboratories with BSL-3 or higher facilities (63). Enzyme immunoassays are also used for detection of antibody responses to whole virus antigen or to specific viral proteins. The conjugate and the antigen used in the assay are factors that affect the performance characteristics (sensitivity and specificity) of these assays. Enzyme immunoassays are used to measure specific immunoglobulin responses in a variety of clinical specimens (serum samples and respiratory secretions) (8).

ANTIVIRAL SUSCEPTIBILITIES

Plaque inhibition assays are the "gold standard" for measuring susceptibility to amantadine and rimantadine, but the assays are cumbersome and time-consuming to perform (26). ELISA methods have also been used to measure decreases in the expression of viral antigens in the presence of these drugs. These assays can be used in combination with genotypic characterization of the M2 gene since in vitro and in vivo resistance to these drugs is associated with specific M2 gene mutations (4). RT-PCR amplification followed by restriction fragment length polymorphism analysis or direct sequencing of amplicons is a genotypic method used to identify resistant viruses (18, 31).

Cell culture assays do not reliably identify antiviral susceptibility to the NA inhibitors, zanamivir and oseltamivir. Instead, NA enzyme inhibition assays with chemiluminescent or fluorescent substrates are used to identify resistance (37). The results of these assays also correlate with mutations in the NA gene that can be identified by sequencing. Mutations in the HA gene may also lead to a resistance phenotype through decreased binding affinity of HA to cell surface receptors and decreased reliance on NA function to release budding viruses from infected cells. No reliable cell culture system currently exists for identifying HA resistance mutations, so identification relies upon sequencing of the receptor binding site of the HA gene.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

The results of a diagnostic test must be considered in the context of the overall setting in which the test is ordered. Clinicians play a critical role in assessing the plausibility of a test result, but the laboratory also can contribute to this appraisal. Seasonal, epidemiologic, and clinical factors are elements that must be evaluated in addition to the type of assay used. Unexpected laboratory results can be recognized by the laboratory as well as by the clinician. For example, a positive influenza test result when influenza is not recognized to be circulating in the community should prompt an assessment as to whether epidemiologic (e.g., travel history) or clinical (e.g., immunocompromised host) factors support the diagnosis of influenza virus infection. Close interactions between the laboratory and clinician are a vital component of a quality control program.

No diagnostic assay has 100% sensitivity and specificity, so false-negative and false-positive results can be expected to occur. Many factors that contribute to lowered sensitivity and specificity are known and can be addressed in ongoing quality control programs. False-negative results may be due to poor quality or inappropriate clinical sample collection, delays in sample transportation or processing, inadequate sample storage (e.g., wrong temperature or transport medium), the time of sample collection during the clinical illness (e.g., later in the illness than recommended, when viral shedding has decreased), and the performance characteristics of the diagnostic assay (i.e., lower sensitivity). False-positive results may also be due to other characteristics of the diagnostic assay (i.e., nonspecific reactions), as well as to cross-contamination within the laboratory, mislabeling of specimens, and microbial contamination. Standard operating procedures in the collection, transportation, and pro-

cessing of clinical samples should be established and followed to minimize the occurrence of the inaccurate test results. Reagents should be standardized, and periodic assessments of assay performance should be performed with known positive and negative controls. The timing of these assessments will be based upon the type and number of tests being performed and the sources of reagents.

Each laboratory must decide upon the goals of its influenza virus diagnostic program in selecting the diagnostic assays to be performed. Rapid and sensitive assays can favorably impact patient management by allowing the prescription of targeted antiviral therapy and the institution of appropriate infection control isolation procedures. Positive test results may form the basis for offering prophylactic therapy to close contacts of infected patients, especially those contacts with high-risk medical conditions. Early and rapid laboratory diagnosis also can be important for evaluating influenza-like illnesses in the setting of a nosocomial outbreak, at the beginning of the influenza season (before influenza is recognized to be circulating in the community), and in persons with a history of contact with pigs or birds or travel to an area where influenza virus is circulating. The laboratory's expertise, staffing, and available equipment also will influence test selection. For example, a fluorescent microscope and an experienced technician are necessary for the performance of immunofluorescence assays, and a thermal cycler along with other equipment is needed for RT-PCR assays. If the clinical specimen being tested comes from a patient who may be infected with an HPAI virus strain (e.g., H5N1), non-culture-based assays are currently recommended for laboratories that do not meet the BSL-3 or higher conditions recommended for growth of these strains (9). Commercially available antigen detection assays or the more sensitive H5-specific RT-PCR assays may be performed by using BSL-2 work practices. In the United States, influenza A virus-positive samples from patients meeting the clinical (febrile [>38°C] respiratory illness [cough, sore throat, or dyspnea]) and epidemiologic (contact with poultry or domestic birds or with a patient with known or suspected H5N1 virus infection in an H5N1-affected country) parameters for suspected A/H5N1 virus infection are referred to the CDC for further evaluation. Selected negative samples may also be sent to the CDC for analysis in consultation with the local public health department (9).

As new strains of influenza virus emerge, the sensitivities of established methods to detect these strains may change. For example, cell lines may have diminished sensitivity to new strains, or the ability to detect influenza virus antigen in infected tissue culture cells (e.g., by hemadsorption) may decrease (57). Thus, it is prudent to reevaluate periodically the performance characteristics of established methods, especially if results do not correlate with those expected based upon clinical and epidemiologic criteria.

Influenza diagnosis is also performed for reasons other than patient management. On the local level, knowledge that influenza is circulating in a community allows diagnosis of influenza based upon clinical symptoms (febrile respiratory illness with cough) with a sensitivity (60 to 80%) similar to that of many rapid antigen tests (40, 63). Influenza viruses isolated in national and global surveillance systems are characterized antigenically and genetically to identify variants. Information gained from these surveillance activities is used in the annual selection of strains for inclusion in updated trivalent influenza vaccines. Surveillance and characterization of isolates also allow the identification of infection with novel subtypes, as has occurred with influenza A/H5N1 viruses in southeast Asia and A/H7N7 strains in The Netherlands (22).

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Parainfluenza and Mumps Viruses*

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TAXONOMY AND DESCRIPTION OF AGENTS

The human parainfluenza viruses (HPIVs) and mumps virus are members of the *Paramyxoviridae* family. This family includes the *Paravirinae* subfamily, of which both HPIV and mumps virus are members. However, within this subfamily, HPIV type 1 (HPIV-1), -2, -3, and -4 and mumps virus are classified into different genera. The *Respirovirus* genus is home for HPIV-1 and HPIV-3, and the *Rubulavirus* genus is home for HPIV-2, HPIV-4, and mumps virus. The *Paravirinae* subfamily includes two additional genera: *Morbillivirus,* of which measles virus is a member, and *Megamyxovirus,* of which Hendra and Nipah viruses are members. The *Pneumovirinae* subfamily completes the *Paramyxoviridae* family. Within this subfamily are the *Pneumovirus* genus, which includes respiratory syncytial virus (RSV), and the *Metapneumovirus* genus, which includes human metapneumovirus. The *Paramyxoviridae* family of viruses produces significant human and veterinary disease, with its effects noted among virus families as "one of the most costly in terms of disease burden and economic impact to our planet" (18).

DESCRIPTION OF THE AGENTS

Although some of the HPIV types and mumps virus are classified into different genera, they are all pleomorphic, enveloped, medium-sized helical viruses, usually ranging from 150 to 250 nm in diameter. All have single-stranded, nonsegmented RNA with negative polarity. The genome encodes six common structural proteins. The largest, L, is a polymerase. L, along with the phosphoprotein (P) and the nucleocapsid protein (N, or NP), is associated with the viral RNA to make up the viral nucleocapsid. The surface glycoproteins, hemagglutinin neuraminidase (HN) and fusion protein (F), project from the viral envelope and can be seen with the electron microscope. The sixth structural protein is a membrane (M) protein. In viral replication, N protein binds to viral RNA, L and P function in transcription and replication, and the HN and F surface glycoproteins interact with the M protein, which attracts completed nucleocapsids to areas of infected membrane that will become the envelope of the new virions during budding. The HN surface glycoproteins also function in virus-host cell attachment via sialic acid receptors, and the F proteins function in virus-host cell membrane fusion that allows the viral nucleocapsid to enter and infect a host cell.

Four serotypes of HPIV have been identified: HPIV-1, -2, -3, and -4. HPIV-4 can be subdivided further into HPIV-4A and HPIV-4B; because these are so closely related, they are referred to as HPIV-4 for the remainder of this discussion. Only one antigenic type of mumps virus has been identified, although strains show differences in expression of a small hydrophobic (SH) gene; 12 genotypes have been identified and designated A to L. HPIV and mumps virus are inactivated by temperatures above 50°C, organic solvents, UV irradiation, formalin treatment, low pH (3.0 to 3.4), drying, and desiccation. Preservation by freezing at –70°C or colder is effective, and the addition of 0.5% bovine serum albumin, skim milk, 5% dimethyl sulfoxide, or 2% chicken serum prior to freezing prolongs survival.

Although similar in structure and antigenic composition, HPIV and mumps virus currently present very different pictures in disease production in the United States. The HPIVs are common agents of respiratory infections in children and adults in the United States, and virology laboratories routinely test for HPIV with both direct antigen testing and virus isolation. In contrast, mumps virus, which was considered one of the common diseases of childhood prior to the introduction of an effective vaccine in 1968, is now relatively uncommon, and most virology laboratories no longer focus on its isolation and identification. Because of these differences, the HPIV and mumps viruses are discussed separately.

PARAINFLUENZA VIRUSES

Epidemiology and Transmission

HPIV is thought to be transmitted by large-droplet aerosolization and by contact with contaminated surfaces. The viruses have been shown to survive for up to 10 h on porous surfaces; however, HPIV-3 experimentally placed on fingers has been shown to lose more than 90% of its infectivity in the first 10 min (1). Currently HPIV-1, -2, -3, and -4 represent approximately 5% of all viruses isolated in routine hospital diagnostic laboratories (26) and 13% of viruses isolated from respiratory specimens (3). HPIV-1 occurs most often in the autumn and biennially. The incidence of HPIV-2 is generally less than that

* This chapter contains information presented in chapter 88 by Joseph L. Waner and Ella M. Swierkosz in the eighth edition of this Manual.

of HPIV-1 and HPIV-3. HPIV-2 may occur biennially with HPIV-1, in alternate years from HPIV-1, or yearly. HPIV-3 occurs yearly in the spring and summer. HPIV-4 is seldom isolated.

Clinical Significance

HPIV-1, -2, and -3 are associated with upper respiratory infections in infants, children, and adults. These are typically fairly mild and self-limited (30). Reinfection is common. HPIV accounts for the majority of cases of viral croup, which is the most common cause of upper airway obstruction in children 6 months to 6 years of age. Croup is characterized by inspiratory stridor, barking cough, and hoarseness (28). As many as 7% of all hospitalizations for viral acute respiratory illness in children younger than 5 years have been shown to be due to HPIV infection (21). HPIV-1, -2, and -3 have also been found in as many as one-third of lower respiratory infections in children younger than 5 years in the United States and are second only to RSV as a cause of hospitalization for viral lower respiratory infections (16). Although associated with infections in children, HPIVs have the potential for serious pulmonary infections in adults, with HPIV-1 and HPIV-3 among the four most common pathogens detected in adults requiring hospitalization for community-acquired pneumonia (31).

HPIVs also produce lower respiratory infections in the elderly and in those with chronic diseases, such as heart and lung disease and asthma. HPIV is increasingly recognized as a source of severe morbidity and mortality in immunocompromised patients, especially in those with congenital immunodeficiencies, and is capable of infecting tissues in the gastrointestinal and urinary systems in these individuals (30). In immunocompromised children, HPIV causes more than 50% of the respiratory infections in bone marrow transplant recipients, 24% in those with hematologic malignancies, and 19% in solid-organ transplant recipients (19). HPIVs are important causes of nosocomial respiratory infections (20).

HPIV-1 is associated with up to 50% of cases of croup reported in the United States, with the majority of cases occurring in children 7 to 36 months. HPIV-2 causes typical lower respiratory syndromes in otherwise healthy children, with about 60% of infections occurring in children younger than 5 years; peak incidence is in children between 1 and 2 years of age. HPIV-2 is associated with croup in immunocompromised or chronically ill children. HPIV-3 is more frequent in infants younger than 6 months, with 40% of infections occurring during the first year of life. Only RSV causes more lower respiratory infections in neonates and young infants than HPIV-3. HPIV-2 and -3 can cause acute parotitis in children. Although HPIV-4 is rarely isolated, it can cause all of the different respiratory syndromes (16).

There are currently no U.S. Food and Drug Administration (FDA)-approved antivirals for treatment of HPIV infection. Ribavirin has been used to treat HPIV infections in immunocompromised patients, but results have varied. Elizaga et al. (11) reported no efficacy for this therapy. In contrast, Chakrabarti et al. (6) reported effective ribavirin therapy when high doses were used with early intervention. Management of symptoms through administration of corticosteroids has been recommended (28). The diagnosis of HPIV may be based primarily on clinical signs and symptoms, and laboratory diagnostic studies may not be needed in all patients. However, laboratory assays to confirm HPIV infection, thus differentiating it from the many other viral infections that present with respiratory tract signs and symptoms, are widely available, and confirmation of infection may improve patient management and decrease costs (17, 18).

Collection, Transport, and Storage of Specimens

Testing to confirm HPIV infection may include virus isolation, antigen or nucleic acid detection, or antibody detection (Table 1). Specimen collection, transport, and storage guidelines are provided in chapters 5 and 80 of this Manual. Because detection of HPIV often involves immunofluorescence assays performed directly on the clinical material, care should be taken to include cellular material in samples collected from respiratory sites. The nasopharynx and oropharynx are primary locations of initial HPIV replication, and children shed virus from 3 to 4 days prior to the onset of clinical symptoms until approximately 10 days past. Virus recovery in adults is much more difficult than in children, although immunocompromised patients and adults with chronic diseases, especially lung disease, have been shown to persistently shed HPIV for many months (16). Throat swabs, nasopharyngeal swabs, nasal washes, and nasal aspirates have all been used successfully to recover HPIV. The highest rates of virus recovery have been shown for nasal washes or nasal aspirates. Specimens should be collected and placed in viral transport medium and kept at 4°C until cell culture inoculation. Inoculation within 24 h of collection is recommended (16).

Peripheral blood samples for use in HPIV antibody assays should be collected in tubes without anticoagulant, and serum should be separated from the clot as soon as possible to ensure sample integrity. Serum samples may be stored at 4°C if testing will be performed within 24 to 48 h but should be frozen at –20°C or lower if testing is delayed.

Direct Examination

Microscopy

Electron microscopy can easily demonstrate HPIV, but HPIV and the other paramyxoviruses appear the same. Lung, other bronchial tissues, pancreas, kidney, and bladder, when examined with light microscopy, exhibit the typical appearance of giant-cell formation when infected with HPIV (30).

Antigen Detection

HPIV-1, -2, and -3 antigens are routinely detected in clinical specimens through the use of immunofluorescence techniques that employ monoclonal antibodies. Cells from nasopharyngeal washes, aspirates, and swabs are fixed on the microscope slide, usually in several cell spots or dots or by cytocentrifugation (25). At least 20 columnar epithelial cells must be present if the assay is to be valid. Many laboratories use a pool of monoclonal antibodies that includes antibodies against seven common respiratory pathogens; these are adenovirus; influenza A and B viruses; HPIV-1, -2, and -3; and RSV. These antibodies are applied in either a direct (DFA) or an indirect (IFA) fluorescent-antibody assay staining protocol that detects antigens of all seven viruses. When a positive result is seen, individual monoclonals to the seven viruses must be applied to the sample to determine which of the seven is present. Sensitivity of HPIV antigen detection compared to virus isolation in cell culture varies from laboratory to laboratory and with the various DFA and IFA staining methods and reagents but has been reported to range from 70% (26) to 83% (25). Specificity is very high.

Pooled and individual monoclonal antibodies for respiratory viruses, in DFA or IFA formats, are commercially marketed in the United States and approved for use in direct

TABLE 1 Diagnostic methods for parainfluenza and mumps viruses

Virus	Virus isolation	Antigen detection	Molecular methods	Antibody detection
HPIV-1–HPIV-3	Widely available. Replicate in PMK cells in 4–8 days. HAD positive.	IF[a] only. Widely available as single tests or as part of respiratory virus panel. Sensitivity, 80–95% compared to culture.	Available only at larger laboratories. Most sensitive method.	May be available at reference laboratories. Cross-reactivity with other *Paramyxoviridae*
HPIV-4	Replicates in PMK cells in 4–8 days. HAD stronger at room temperature.	IF only. Testing not widely available.	May be available at reference laboratories	Not available at most laboratories
Mumps virus	Widely available. Replicates in PMK cells in 6–8 days. HAD positive.	IF only. Testing not widely available.	May be available at reference laboratories	Immune status (IgG) testing widely available. Seldom used to confirm acute infection due to cross-reactivity with other *Paramyxoviridae.*

[a] IF, immunofluorescence.

specimen testing. Distributors of these reagents include, but are not limited to, Chemicon International, Temecula, Calif.; DakoCytomation USA, Carpinteria, Calif.; Diagnostic Hybrids, Inc., Athens, Ohio; and Trinity Biotech, Carlsbad, Calif. A brief overview of the protocols used for one DFA and one IFA method for respiratory virus screening follows.

DFA (D^3 Respiratory Virus Screening and ID Kit; Diagnostic Hybrids, Inc.)

Detailed instructions provided by the manufacturer should be followed. Briefly, smears from clinical specimens or of cells scraped from an infected cell culture monolayer are prepared on microscope slides. Shell vial monolayers may also be stained. All cells must be acetone fixed (10 min) and dried prior to staining. Fixed smears may be stored for several days at 2 to 8°C or frozen at –20°C for up to a year when stored in an airtight container. For smears on microscope slides, 1 or 2 drops of the fluorescein-labeled monoclonal antibody preparation are added. For shell vial monolayers, 3 or 4 drops of antibodies are added. The preparations are incubated for 15 min at 37°C in a humidified environment. The smears are washed with diluted phosphate-buffered saline (PBS)–detergent solution (supplied in the kit) and then air dried. Mounting fluid and coverslips are added, and the smears are examined microscopically at ×200 or higher magnification using a fluorescein isothiocyanate (FITC) filter system. Infected cells demonstrate bright apple-green fluorescence that is primarily cytoplasmic and often punctate, with irregular inclusions. Uninfected cells appear brick red, due to Evans blue counterstaining, and show no bright green fluorescence. The same protocol is followed both for the seven-virus screening assay and for subsequent staining of positive samples with the individual monoclonal antibodies.

IFA (Bartels VRK Viral Respiratory Kit; Trinity Biotech)

Detailed instructions concerning all aspects of specimen handling, smear preparation, and staining/interpreting results are provided by the manufacturer and should be followed. The IFA is appropriate for the same types of samples as listed above for the DFA, with the same smear preparation and fixation guidelines. In IFA staining, briefly, sufficient unlabeled antiviral antibody reagent is added to each fixed preparation to cover the cells. The samples are incubated in a humidified environment at 35 to 37°C for 30 min. Smears are then washed in PBS for 5 to 10 min and air dried. Sufficient anti-mouse FITC antibodies are added to cover the cells, and the samples are again incubated at 35 to 37°C for 30 min. Following a 5-min wash in PBS and drying, buffered glycerol mounting medium and coverslips are added, and the smears are observed microscopically at ×200 or higher magnification using an FITC filter system. Infected and uninfected cells appear as described above for the DFA procedure. The same protocol is followed both for the seven-virus screening assay and for subsequent staining of positive samples with the individual monoclonal antibodies. Cell culture confirmation is recommended for samples that produce a negative result.

Monoclonal antibody pools that screen for the seven respiratory viruses have also been marketed in a format that allows definitive identification of more than one virus at a time through the use of different fluorescent dyes with overlapping spectra (Light Diagnostics SimulFluor reagents; Chemicon International). The reagents are approved by the FDA for direct specimen testing and for culture confirmation. When stained preparations are examined with a fluorescence microscope with an FITC filter set, one antibody will produce apple-green fluorescence and the second will appear gold or golden orange. SimulFluor reagents that are useful in HPIV antigen detection include a respiratory screen reagent in which RSV appears golden and the other six respiratory viruses, including HPIV-1, -2, and -3, appear green; an RSV/HPIV-3 reagent in which RSV appears green and HPIV-3 appears golden; an HPIV-1, -2, and -3/adenovirus reagent in which HPIV-1, -2, and -3 appear green and adenovirus appears golden; and an HPIV-1 and -2/HPIV-3 reagent in which HPIV-1 and HPIV-2 appear green and HPIV-3 appears golden. The SimulFluor reagents have shown excellent sensitivity and specificity, comparable to those of individual stains for the respiratory viruses (25).

HPIV-4, which is the least frequently encountered of the HPIV types, is not detected by most respiratory virus immunofluorescence screening reagent pools; only HPIV-1, -2, and -3 are detected. Although monoclonal antibodies are available for immunofluorescent staining of HPIV-4 cell culture isolates, these are not approved for use in HPIV-4 antigen detection directly in clinical specimens.

Nucleic Acid Detection Techniques

Several different approaches for detection of HPIV RNAs have been described; all methods, compared to virus isolation in traditional cell cultures, detect more, sometimes double the number (15), of HPIV-positive samples. A reverse transcriptase PCR (RT-PCR) assay involving enzyme hybridization (EH) has been shown to be more sensitive than cell culture in detection of HPIV-1 in nasal wash specimens (12). This RT-PCR–EH technology has been converted into a multiplex assay (Hexaplex; Prodesse, Inc., Milwaukee, Wis.) that rapidly detects RNA of RSV A and B; influenza A and B viruses; and HPIV-1, -2, and -3 in a single test (13). In testing nasal wash samples and other respiratory samples, Hexaplex was 100% sensitive compared to cell culture and detected viral RNA in low copy numbers in several culture-negative samples (13, 22). Hexaplex has been used with various respiratory samples and in detecting HPIV-1 and -3 in the middle ear fluids of children with acute otitis media (7).

Although the Hexaplex assay is not marketed as a "kit," the reagents are commercially available in the United States. The manufacturer's instructions should be followed. Briefly, viral genomic RNA is extracted from 280 μl of specimen, and cDNA is synthesized. The cDNA undergoes PCR amplification in a mixture of DNA polymerase and Supermix (Prodesse, Inc.). AmpliTaq Gold DNA polymerase (Perkin-Elmer Cetus, Foster City, Calif.) may be used (22). The Supermix is the proprietary mixture that contains six and one-half pairs of primers: a pair of primers to the HN genes of HPIV-1, -2, and -3; a pair of primers to the M1 (matrix) gene of influenza A virus; a pair of primers to the nonstructural gene of influenza B virus; and primers to the 1B and nucleocapsid genes of RSV A and B. The PCR mixture also contains Tris-HCl, 2.5 mM $MgCl_2$, a 0.2 mM concentration of each of the four deoxynucleoside triphosphates, and 0.5 μM concentration of the primers. Denaturation is at 95°C for 2 min. Amplification is by two cycles at 95°C for 1 min, 55°C for 45 s, and 72°C for 45 s and 38 cycles at 94°C for 1 min, 60°C for 45 s, and 72°C for 45 s. Reactions are then held at 72°C for 7 min. The PCR product is purified and added to a neutravidin-coated microtiter plate. Proprietary peroxidase-labeled probe solutions 1 to 7 (Prodesse, Inc.), representing highly conserved regions of the previously described genes, are added to each well. After the capture and hybridization reaction is complete, a substrate solution is added, followed after 10 min by addition of a stop solution. The optical density of each well is measured at 450 nm on a spectrophotometer. Cutoff values are established by comparison with negative control values. The assay requires approximately 7 h to complete.

Other molecular methods are being developed to detect respiratory viruses. A rapid real-time multiplex PCR assay using a two-tube multiplex reaction employing molecular beacons to discriminate the pathogens was developed to detect influenza A and B viruses; RSV; and HPIV-1, -2, -3, and -4 in clinical samples. Within 6 h, the PCR assay detected 100% of the respiratory pathogens that were eventually isolated in traditional cell cultures, as well as several additional viruses of each type that failed to grow in traditional cell cultures (39).

Isolation and Identification

Many commonly used cell lines support the growth of HPIV, but the best growth is seen in primary monkey kidney (PMK) cells, including rhesus, cynomolgus, and African green monkey cells (16). LLC-MK2 is also acceptable for primary isolation when 2 to 3 μg of trypsin per ml is added to the cell culture medium (16). Madin-Darby canine kidney (MDCK), HeLa, Vero, and HEp-2 cells may be used for transferring isolates but are not recommended for primary isolation (40). At this writing, commonly used cell lines are available commercially from two suppliers in the United States: Diagnostic Hybrids, Inc., and Viromed Laboratories, Minnetonka, Minn.

In preparation for cell culture inoculation of specimens collected on swabs and transported in a viral transport medium, the transport medium tube is extensively mixed on a vortex mixer, and excess fluid is expressed from the swab by pressing the swab against the side of the tube; the swab is then discarded. The transport medium is then centrifuged at 1,500 × *g* for 10 min. The medium is decanted from cell culture tubes that are to be inoculated, and the supernatant from the centrifuged transport medium, usually 0.2 ml for each tube, is applied to each cell culture monolayer. The inoculated tubes are incubated in a horizontal position in a 35 to 37°C incubator for 1 h before excess inoculum is discarded and fresh cell culture medium is added. Inoculated cell culture tubes are incubated in rotating racks at 35 to 37°C and examined microscopically on alternate days for 14 days.

In PMK cells HPIV may produce some rounding of cells and syncytia in 4 to 8 days. However, many HPIVs will produce little, if any, cytopathogenic effect (CPE) in traditional cell culture tubes. Fortunately, HPIVs produce hemagglutinin proteins that are inserted into the membranes of infected cells. These proteins have an affinity for erythrocytes, a phenomenon called hemadsorption (HAD), and this property can be used as another approach for detection of HPIVs in cell cultures. In HAD testing, culture medium is replaced with a dilute suspension of guinea pig erythrocytes, and the cell culture tubes are refrigerated at 4°C for 30 min; then the tubes are examined microscopically (26). When a hemadsorbing virus is present, erythrocytes adhere to the infected cell monolayer. If hemadsorbing virus is absent, erythrocytes will not adhere and will float free when the tube is tilted or tapped. Uninfected and infected control tubes should be included in HAD testing. HAD testing may be performed at the end of the typical incubation period of 14 days or earlier, after 3 to 7 days of incubation (33). Although an HAD-positive result indicates that the infecting virus has hemagglutinin proteins, this result alone does not identify the virus as HPIV. HPIV-4 hemadsorbs weakly at 4°C and more strongly at room temperature. Other members of the *Paramyxoviridae* family, including mumps viruses, and other viruses such as influenza virus also give positive HAD test results. Whether the presence of HPIV is suspected based on the appearance of typical CPE or by a positive HAD test result, confirmatory testing must be done to definitively identify the virus.

Confirmatory testing is routinely completed by immunofluorescence techniques involving the use of HPIV monoclonal antibodies in DFAs or IFAs as described for HPIV antigen detection in clinical samples. Antibodies against HPIV-1, -2, and -3 are readily available in the various respiratory virus antibody testing kits. However, HPIV-4 antibodies are not included in the testing kits and must be purchased separately. Infected cells are scraped from the cell culture monolayer, applied to a microscope slide, fixed, and stained. The presence of apple-green fluorescence when the smear is viewed with a fluorescence microscope confirms the identification of HPIV. Confirmatory testing may also be accomplished by many other methods, including hemagglutination inhibition, complement fixation, and neutralization.

These techniques are laborious and time-consuming compared to immunofluorescence and are seldom used.

Cell cultures grown on coverslips in shell vials or in microwell plates provide an alternative to traditional tube cell cultures for isolation of HPIVs. Centrifugation of the inoculated vials and plates, usually at 700 × *g* for 1 h, is an important feature of the inoculation process. Inoculated vials or plates are incubated for 24 to 48 h or as long as 5 days. Detection of viral proliferation depends on pre-CPE detection of viral antigens by application of HPIV monoclonal antibodies to the monolayers in a typical DFA or IFA staining procedure. Use of shell vial cultures with centrifugation-enhanced inoculation and pre-CPE detection by immunofluorescence staining has been shown to be very useful in HPIV detection.

Cocultivated mink lung (Mv1Lu) and A549 cells, marketed as R-Mix (Diagnostic Hybrids, Inc.), are used with centrifugation-enhanced inoculation and pre-CPE detection with a DFA staining technique. Staining involves pooled monoclonal antibodies to seven respiratory viruses (adenovirus; influenza A and B viruses; HPIV-1, -2, and -3; and RSV). This system has been shown to effectively detect HPIV-1, -2, and -3 (Fig. 1). In a recent study comparing detection of respiratory viruses in 3,800 clinical samples in R-Mix and in shell vials of PMK, A459, and MRC-5 cells, 33 of 38 (87%) HPIV-positive samples were detected after overnight incubation in the R-Mix cells (10). This included 26 of 30 HPIV-1 samples, 2 of 2 HPIV-2 samples, and 5 of 6 HPIV-3 samples.

An overview of the recommended protocol from the manufacturer of R-Mix (Diagnostic Hybrids, Inc.) is shown here; however, this may be adjusted to meet the needs of the laboratory. Briefly, the processed clinical specimen for respiratory virus detection is inoculated into three vials containing R-Mix cells, and the vials are spun in the centrifuge (700 × *g* for 1 h). At 16 to 24 h, one vial is stained with respiratory virus screening reagent (tests for seven respiratory viruses). If fluorescence is observed, the cells are scraped from one of the remaining shell vials, rinsed in PBS, and spotted onto an eight-well slide; seven of the wells are stained with individual monoclonal antibodies against the seven respiratory viruses, and the eighth well is used as a control well. If no fluorescence is observed when the first vial is stained, the second vial is stained after an additional 16 to 24 h of incubation. If this vial is positive, the cells are scraped from the remaining vial and stained as described above. If the second vial is negative, the culture may be considered negative for the seven respiratory viruses (i.e., the remaining vial is discarded and the culture is terminated), or the third vial may be observed for CPE for up to 7 days.

Serologic Tests

Most children are born with HPIV-neutralizing antibodies, but these diminish by 6 months of age, allowing more than two-thirds of children to be infected by HPIV during the first year of life. Most children have early serologic evidence of HPIV-3 infection, with HPIV-1 and -2 antibodies developing later, at age 2 to 3 years. Antibodies to HPIV-4 peak in school-age children (16). Immunoglobulin M (IgM) is produced in most primary HPIV infections of children, so IgM detection in a single sample supports current infection. Likewise, measurement of a significant increase in IgG antibody level, which is an increase of fourfold or more in assays that test serial twofold dilutions, in serum samples collected 2 weeks apart supports current

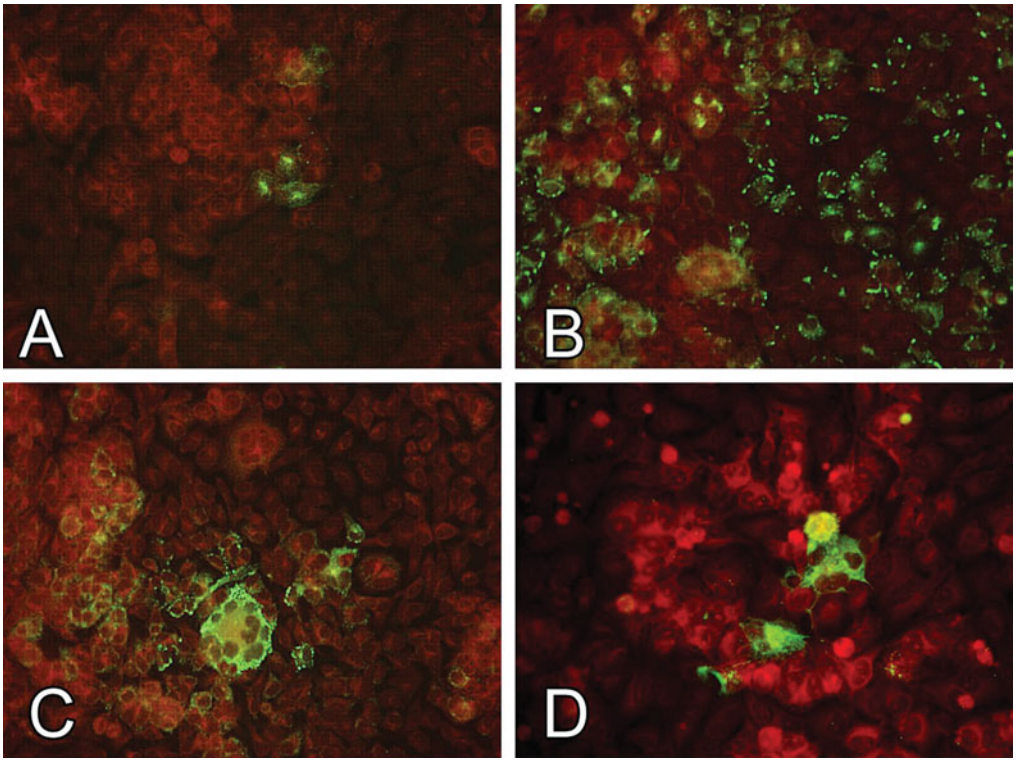


FIGURE 1 Immunofluorescence staining of infected R-Mix cells. (A) HPIV-1; (B) HPIV-2; (C) HPIV-3; (D) mumps virus. Magnification, ×200 (all). Photos courtesy of Diagnostic Hybrids, Inc.

infection. Cross-reactivity among the various HPIV types and other paramyxoviruses such as mumps virus makes serologic diagnoses more difficult. Serologic differentiation of the HPIV types is unreliable by all methods; this determination can be made only through virus isolation and antigen typing with monoclonal antibodies or identification of the viral RNAs.

Antibodies to the HN protein may appear early, but both HN and F protein antibodies must be present for protection. Immune status determinations are not useful for HPIV. Serology, in general, is seldom used as a diagnostic approach for HPIV infection because of the wide availability of virus isolation and viral antigen and nucleic acid detection methods.

Principles of various serologic assays are described in detail in chapter 18 of this Manual. HPIV antibodies can be detected by many types of assays, including hemagglutination inhibition, complement fixation, and neutralization; all of these detect total antibody (IgG and IgM), and most laboratories will not offer these methods due to their cumbersome nature. A detailed procedure for hemagglutination inhibition testing for HPIV antibodies was published previously (40). HPIV antibody detection can be accomplished by enzyme immunoassay (EIA), but commercial kits are not available. Complement fixation is the least sensitive method for antibody detection, and EIA is the most sensitive.

Evaluation, Interpretation, and Reporting of Results

Isolation of HPIV-1, -2, -3, and -4 in traditional or shell vial or microwell cell cultures and detection of HPIV RNA in clinical samples are the best evidence for current or very recent infection because HPIV is seldom present in the absence of infection. These methods are the most sensitive and specific. Rapid reporting of these positive results is important (40). Although the sensitivity of HPIV antigen detection by immunofluorescence is lower than that of virus isolation in cell culture or molecular detection, the rapid availability of results make such testing useful in patient management. Because these tests are widely available and have high specificity, they should be considered an important diagnostic tool. Confirmation of HPIV infection via the serologic route and HPIV immune status determinations are seldom employed due to the difficulties with cross-reactions among the paramyxoviruses.

MUMPS VIRUS

Epidemiology and Transmission

Most mumps cases occur in children. More than 150,000 cases of mumps were expected each year in the United States before the aggressive vaccination programs implemented in the late 1960s. The mumps vaccine was combined with the measles and rubella vaccines to make a trivalent vaccine (MMR). In the United States the initial dose of MMR is administered to children between 12 and 15 months of age. Vaccination produced a dramatic decline in the incidence of mumps, resulting in fewer than 3,000 cases in the United States by 1985. There was a brief resurgence of mumps in 1986 and 1987 that peaked at 8,000 to 12,000 cases. Following the resurgence, a requirement for a second dose of the MMR vaccine was implemented. This dose is administered to children between ages 4 and 6 or 11 and 13 years (4) and should confer lifelong immunity. The number of mumps cases continues to decline in the United States. Only 231 mumps cases were reported in the United States in 2003 (5), although sporadic and imported cases continue to occur. Mumps remains common throughout much of the world.

The virus is transmitted from person to person through respiratory droplets and is highly contagious, with approximately 85% of susceptible contacts becoming infected when first exposed. Humans are the only known host and reservoir for the virus. The virus replicates initially in epithelial cells in the upper respiratory tract and in regional lymph nodes. Initial replication is followed by viremia, which results in infection of salivary glands and other sites. The period of communicability can be from 7 days before salivary gland involvement until 9 days after. The virus is excreted in urine for as long as 14 days after the onset of illness.

Clinical Significance

The average incubation period for mumps is 16 to 18 days. Mumps virus infection is clinically inapparent in 25 to 30% of cases and is associated with nonspecific or respiratory symptoms in 50% of cases. Symptoms are typically mild and characterized by slightly elevated temperature and enlargement of one or both parotid glands in 30 to 40% of cases. Complications of mumps include meningoencephalitis (up to 15% of cases) and, in postpubertal individuals, orchitis in males (20 to 30%) and oophoritis in females (7%). Polyarthritis and pancreatitis have also been associated with mumps.

When mumps was a common disease of childhood, the diagnosis was made largely on clinical grounds alone. With the decrease in incidence of mumps, many physicians no longer readily recognize the symptoms. In addition, typical clinical signs and symptoms may be absent in underimmunized or immunocompromised individuals. Parotitis, the hallmark of clinical diagnosis, is now known to be present in other viral and nonviral diseases or conditions. Mumps-like symptoms in acutely ill children who previously received the MMR vaccine have been associated with Epstein-Barr virus, HPIV, adenovirus, and human herpesvirus type 6 (9). Therefore, laboratory confirmation of mumps virus infection is now more important in making the diagnosis.

Collection, Transport, and Storage of Specimens

The accepted laboratory criteria for the diagnosis of mumps infection are isolation of the virus from clinical specimens, a significant rise between acute- and convalescent-phase antibody titers in serum, or a positive IgM result for mumps virus. However, collection of samples to allow molecular analysis should be considered (Table 1). Guidelines for specimen collection, transport, and storage of specimens are presented in chapters 5 and 80 of this Manual.

Specimens for mumps virus isolation in cell culture include the following: saliva, blood, urine, and cerebrospinal fluid (CSF). Virus can be isolated from saliva 9 days before and up to 8 days after the onset of parotitis, recovered from urine for up to 2 weeks after onset of symptoms, isolated from CSF during meningitis, and detected rarely in peripheral blood. Throat swabs and urine samples have been shown to be of similar usefulness as sources of mumps virus (38). It is always advisable to collect samples for virus isolation early in the course of the infection, when the viral titer is the highest.

The virus is stable for several days at 4°C, although inoculation of susceptible cell cultures within a few hours of specimen collection is recommended for optimal virus isolation. The virus may survive for months or longer when frozen at –70°C or lower.

Blood samples for use in mumps virus antibody testing should be collected and stored as described in this chapter in the "Parainfluenza Virus" section under "Collection, Transport, and Storage of Specimens." An alternative to serum collected by venipuncture is whole blood obtained by

fingerstick or heel prick and spotted onto filter paper and dried. Venipuncture blood may also be spotted onto filter paper and dried. Following elution and dilution of the dried blood samples, commercial antibody assays may be used to test the sample for mumps virus antibody. Results of EIA for mumps virus IgG and IgM using samples collected as blood spots have shown excellent correlation with those obtained in testing of fresh serum. The dried blood spots may be stored for 6 to 24 months without significant change in antibody testing results (8).

In order to compare IgG or total antibody levels, two samples should be collected, the first (acute) as soon as possible after onset of symptoms and the second (convalescent) within 2 to 3 weeks following collection of the acute sample. A single serum sample collected within 10 days of onset is all that is required for IgM-specific antibody testing. If the purpose of the serologic testing is simply to determine the patient's mumps immune status, a single serum sample collected at random is sufficient.

Direct Examination

Microscopy

Diagnosis of mumps does not typically involve examination of biopsy samples. However, microscopic examination of affected salivary glands reveals edematous interstitium, diffusely infiltrated by macrophages, lymphocytes, and plasma cells, which compress acini and ducts. Neutrophils and necrotic debris may fill the ductal lumen, causing focal damage to the ductal epithelium (32).

Antigen Detection

Mumps virus antigen detection by immunofluorescence in cells from CSF and salivary glands was described in the early 1970s (2, 29). More recent studies of immunofluorescence staining of throat swab specimens for mumps virus antigen have shown sensitivity as high as 98 to 100% compared to mumps virus isolation in cell culture (38). Although mumps virus monoclonal antibodies are available commercially, most are not approved for use in direct detection of mumps virus antigens in tissues and other clinical samples, and most virology laboratories do not offer direct antigen analysis for mumps virus.

Nucleic Acid Detection Techniques

Various molecular approaches have been developed to aid in mumps diagnosis. Mumps RNA has been detected by RT-PCR in oral fluid, CSF, saliva/throat, and urine specimens (36). A nested RT-PCR, targeting a short fragment of the SH gene, was successfully applied directly to CSF and for identification of mumps virus isolates from cell culture (35). A loop-mediated isothermal amplification method has also been used to amplify and detect mumps virus RNA in clinical samples; this method detected more mumps virus-positive samples than cell culture (34). A TaqMan-based one-step real-time RT-PCR that uses oligonucleotides targeting the matrix protein gene was shown to accurately detect and quantify mumps virus strains (24). Although these methods hold great promise in dealing with mumps virus detection and quantification, they are not well standardized, approved reagents are not commercially available at this writing, and testing is available only in specialized laboratories. In routine laboratories, virus isolation remains the standard method for diagnosing mumps virus infection.

Isolation and Identification

Mumps virus proliferates in traditional cell cultures of several cell lines commonly used in viral diagnostic laboratories. These include PMK, human neonatal kidney, HeLa, and Vero. Recently, a marmoset lymphoblastic cell line, B95a, was shown to be equally as sensitive for mumps virus isolation as PMK cells (23). Directions for cell culture inoculation for swab samples in viral transport medium are the same as those described in the "Parainfluenza Virus" section under "Isolation and Identification."

In cells infected with mumps virus, CPE characterized by rounding of cells and multinucleated giant cells is typical, usually appearing after 6 to 8 days of incubation at 35 to 37°C. However, this characteristic CPE may be very subtle, may not appear at all, or may be confused with a similar CPE produced by endogenous contaminant viruses that sometimes infect PMK cells.

Mumps virus, like the HPIVs, inserts hemagglutinin proteins into the membranes of infected cells, so mumps virus-infected cells can be demonstrated through HAD testing. The HAD testing protocol is described in the "Parainfluenza Virus" section under "Isolation and Identification." Whether mumps virus is detected by CPE production or by a positive HAD result, confirmatory testing must be performed. Confirmatory testing for mumps is routinely performed by IFA. The staining protocol for a typical IFA is provided in the "Parainfluenza Viruses" section under "Direct Examination."

Many laboratories currently use centrifugation-enhanced inoculation and pre-CPE detection with monoclonal antibodies to detect mumps virus within 24 to 48 h after inoculation. Various cell lines can be used in either shell vial or microwell plate formats for this purpose. The recommended centrifugation speeds and times may vary for inoculation, ranging from 700 × *g* for 45 min (38) to 3,000 × *g* for 20 min (10). Up to 66% of mumps virus-positive samples were identified within 2 days of inoculation in the shell vial system; after 5 days of incubation, the shell vial system was 96% sensitive compared to mumps virus isolation in traditional cell cultures (14). When various cell lines were compared for efficacy of mumps virus isolation in shell vials, Vero and LLC-MK2 cells were found to be most sensitive (100%), followed by MDCK (78%), MRC-5 (44%), and HEp-2 (22%) (37). Mumps can also be isolated in R-Mix cells (Fig. 1). An overview of the recommended protocol from the manufacturer of R-Mix (Diagnostic Hybrids, Inc.) is discussed in the "Parainfluenza Viruses" section under "Isolation and Identification."

Serologic Tests

In mumps virus infection, IgM is detectable initially within 3 to 4 days of the appearance of clinical symptoms and persists for 8 to 12 weeks. IgG is detectable within 7 to 10 days of the onset of symptoms, is maintained at high levels for years, and remains detectable for life. Traditional serologic diagnosis of mumps virus is based on the detection of virus-specific IgM in a single sample or measurement of a significant increase in titer of IgG or total antibody, equal to or greater than fourfold in methods that use serial twofold dilutions, between two specimens collected 2 weeks apart. Antibodies produced in mumps virus infection often cross-react with related viruses, which complicates interpretation of results. Assays for mumps immune status indicate whether mumps virus IgG is present in detectable levels or is absent or undetectable. With the rarity of mumps cases in the United States at present, most laboratories focus mumps serology on immune status testing.

Basic principles of serologic assays are described in chapter 18 of this Manual. Mumps virus antibodies can be detected by many types of assays, including hemagglutination inhibition, complement fixation, and neutralization; all of these detect total antibody (IgG and IgM). These methods are usually laborious and are available only at reference or specialty laboratories. A detailed procedure for mumps antibody hemagglutination inhibition testing was published previously (27). The complement fixation technique can be used with two different mumps virus antigens, V and S. Antibodies against the S antigen appear earlier and rise quickly, in contrast to antibodies to the V antigen, which appear later. The presence of both V and S antibodies is thought to signal a recent past infection, while V antibodies alone signal a long-past infection (40).

Most diagnostic laboratories use commercially supplied mumps virus antibody EIA kits, in either manual or automated formats, or IFA testing systems for mumps virus antibody determinations. Most of these measure only mumps virus IgG. Antibodies are produced against various mumps virus proteins, but protection is most closely correlated with antibodies to the mumps HN protein. Most assays use whole-virus or viral extract antigens that detect HN antibody effectively. Written protocols, along with the proper reagents and controls, are included with each commercial product, and each manufacturer's guidelines must be followed if assays are to yield high-quality results. The procedural steps for performance of antibody testing are not published here due to the need for strict adherence to manufacturers' guidelines. No specific recommendation on which specific mumps virus antibody test is most useful can be made at this time.

Evaluation, Interpretation, and Reporting of Results

Isolation of mumps virus in traditional or shell vial or microwell cell cultures is evidence for current or very recent infection. The same can be said for the detection of mumps virus RNA in clinical samples. Rapid antigen detection may also confirm the presence of mumps virus. However, neither molecular methods nor rapid antigen assays are routinely available in U.S. laboratories, so mumps virus isolation in culture remains the most sensitive approach. Confirmation of mumps virus infection via the serologic route is less straightforward. Mumps virus IgM may be detected, along with significant increases in IgG, in patients infected with related viruses, including the other paramyxoviruses, such as HPIV-1, -2, -3, and -4. In general, cross-reactivity with related viruses can be ruled out by testing for antibodies to the related viruses in parallel with mumps virus antibody testing. The greatest increase in antibody level should identify the true infection. Virus isolation in culture and detection of mumps virus RNA in clinical samples are the most effective ways to confirm true infection.

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Respiratory Syncytial Virus and Human Metapneumovirus*

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Respiratory syncytial virus (RSV) and human metapneumovirus (hMPV) are two cousins with a 50-year difference in their discovery "age" in the list of viral pathogens causing respiratory tract infections. RSV was first identified as the agent that caused chimpanzee coryza in 1956 (14). After its subsequent isolation from children with pulmonary disease in Baltimore (25), RSV has been described as the single most important virus causing acute respiratory-tract infections in children. hMPV was not recognized until the year 2001 in The Netherlands (135). While diagnostic procedures for detecting and identifying hMPV are emerging, diagnostic procedures for RSV have improved significantly over the past decade thanks to technological revolutions in the field. Improvements include the introduction of more-sensitive mixed cell lines for virus culture and an early detection by shell vials, the application of monoclonal antibodies for rapid detection of viral antigens directly in clinical specimens, and the detection of viral RNA in clinical specimens by nucleic acid amplification techniques.

RESPIRATORY SYNCYTIAL VIRUS

Taxonomy

RSV belongs to the *Pneumovirus* genus of the *Paramyxoviridae* family. Within this same genus are the morphologically and biologically similar pneumonia virus of mice, bovine RSV, ovine RSV, and caprine RSV. Distinctive features of RSV include the number and order of genes and lack of hemagglutinin and neuraminidase activity. This virus derives its name from the characteristic formation of multinucleated giant cells (syncytia) in monolayer cell cultures of nonpolarized epithelial cells (28).

Description of the Agent

RSV is an enveloped virus that contains a single-stranded, negative-sense, nonsegmented RNA genome with 10 genes that are transcribed into 11 monocistronic polyadenylated mRNAs, each of which encodes a major polypeptide chain (Fig. 1). Three of the proteins are associated with the nucleocapsid: the N (nucleoprotein) protein serves as the major structural protein for the nucleocapsid, while P (phosphoprotein) and L (polymerase or large) are involved in transcription and replication (28). The envelope contains five viral proteins: nonglycosylated M (matrix), M2, and the surface glycoproteins F (fusion), G (glycosylated), and SH (short hydrophobic). The SH protein accumulates within lipid raft structures of the Golgi complex during RSV infection (113), the M protein is present in detergent-solubilized cores, and M2 is expressed on the surfaces of infected cells. Between the two glycosylated surface proteins, the F protein initiates viral penetration by fusing viral and cellular membranes and promotes viral spread by melding infected to adjacent uninfected cells; and the G protein mediates attachment of the virus to the host cells (28). Two subgroups of RSV (A and B) are recognized primarily based on variations in the structure of their G protein (22, 128). Comparisons of the G ectodomains showed a 94% homology within subgroup A and a 47% divergence between subgroups A and B RSV viruses. In contrast, the F protein is highly conserved, and antibodies against it cross-react with both subgroups A and B (28). The other two proteins, NS1 and NS2, are nonstructural proteins of the virion that inhibit the induction of alpha and beta interferon in A549 cells and human macrophages (124).

RSV is one of the most vulnerable pathogens to environmental changes. Only 10% of RSV remained alive after exposure to 55°C for 5 min. At room temperature, 10% infectivity was present after 48 h, and at 4°C, 1% of the infectivity remained after 7 days. The RSV infectivity titer fell by approximately 90% after each freeze-thaw cycle. RSV also withstands an acid medium poorly, and the optimal pH is 7.5 (64). The virus is inactivated quickly by ether, chloroform, and a variety of detergents such as 0.1% sodium deoxycholate, sodium dodecyl sulfate, and Triton X-100. Storage of RSV can be enhanced by flash freezing in an alcohol and dry ice bath and by adding stabilizing agents such as glycerin or sucrose. At room temperature, RSV in the secretions of patients may survive on nonporous surfaces such as countertops for 3 to 30 h, depending on the humidity. The survival of RSV in the environment depends in part on drying time as well as humidity (28, 62).

Epidemiology and Transmission

RSV is the most common cause of bronchiolitis and pneumonia among infants and young children worldwide. In the United States, early reports estimated that 100,000 hospitalizations and 4,500 deaths are related to RSV infection annually with expenses in excess of 300 million dollars

* This chapter contains information presented in chapter 89 by Debra A. Tristram in the eighth edition of this Manual.

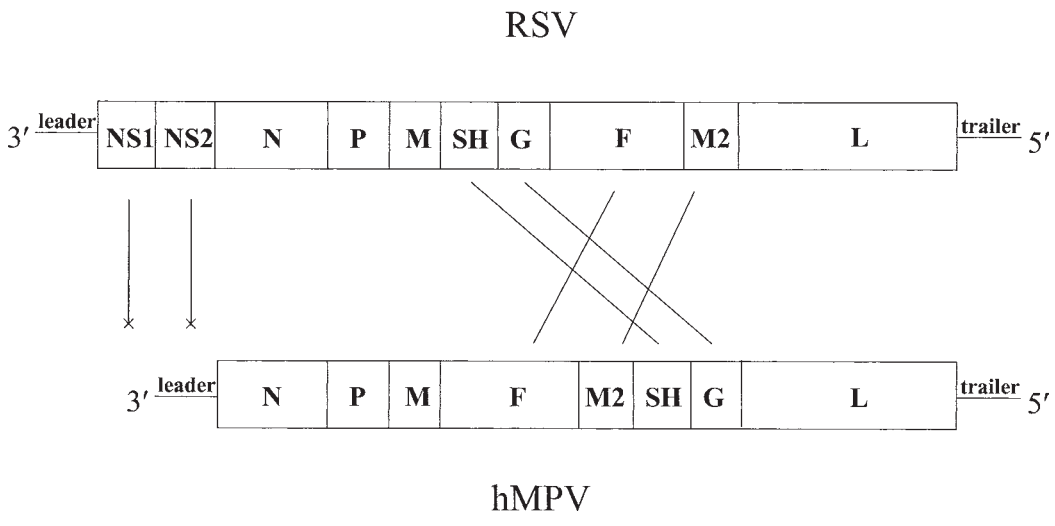


FIGURE 1 A schematic of RSV and hMPV genomic RNAs. The order of the genes from 3′ to 5′ is depicted. The virus-specific proteins encoded by these genes are shown.

per year (33, 62). A recent study revealed that high altitude, above 2,500 m, is a modest predictor for RSV-associated hospitalization (26). A large-scale epidemiology study suggested that medical care has reduced the mortality in children to about 100 cases a year, while over 17,000 deaths a year occur among the elderly (133). Virtually all children are infected by the time they reach 3 years of age. Repeated infections with RSV are common, and no age group appears protected. In older children and immunocompetent adults, RSV infections are seldom severe and are usually manifested as upper respiratory infection or tracheobronchitis. Individuals at high risk for severe RSV infection include premature infants, young infants, immunocompromised individuals, and the elderly (62, 121). RSV activity follows a seasonal pattern in temperate zones of the world. In urban centers, annual outbreaks occur during the fall, winter, and early spring. In the northern hemisphere most outbreaks peak in February or March. In tropical or subtropical areas, epidemics peak during the rainy season (121). Climate change may be shortening the RSV season (34).

Crowding increases the attack rate of RSV. Schools or day care centers provide ideal settings for the spread of RSV to susceptible individuals. Passive exposure to tobacco and cigarette smoke is associated with the development of more severe illness (19). Infants who have not been breast-fed appear to be at greater risk for developing severe RSV infections in some studies, but a strong effect has not always been noted (84). Children with underlying heart disease, in particular those conditions complicated by pulmonary hypertension, or chronic lung disease (i.e., bronchopulmonary dysplasia), as well as prematurity, are at increased risk for acquiring more severe RSV infections, as evidenced by their increased rates of intensive care unit admissions and need for mechanical ventilation. RSV infection is an important illness in elderly and high-risk adults, with a disease burden similar to that of nonpandemic influenza A in a population in which the prevalence of vaccination for influenza is high (45, 133). The mean hospital length of stay was 11 days for RSV-infected children with congenital heart disease or chronic lung disease, second only to the group with immunodeficiencies (74). Both subgroups A and B can cocirculate each year, and the predominating subgroup may shift with the season (80, 117).

Clinical Significance

Primary infections with RSV may be manifested as lower respiratory tract diseases, pneumonia, bronchiolitis, tracheobronchitis, or upper respiratory tract illness, often accompanied by a low-grade fever. Otitis media has also been associated with RSV infection (62, 121). The incubation period ranges from 2 to 8 days. Illness begins most frequently with rhinorrhea, sneezing, and cough. Most children experience recovery from illness after 8 to 15 days. During their first RSV infection, 25 to 40% of infected infants and young children have signs or symptoms of bronchiolitis or pneumonia and 0.5 to 2% require hospitalization. In contrast, hospitalization is required in up to 25% of high-risk children such as those with a history of prematurity or chronic lung disease. RSV bronchiolitis or pneumonia should be suspected when an infant or young child presents with progressive cough, wheezing, and dyspnea during an RSV season. Among immunocompetent adults, RSV infection usually manifests with rhinorrhea, pharyngitis, cough, bronchitis, headache, fatigue, and fever (62, 121). In older persons, particularly the institutionalized elderly and those with chronic cardiopulmonary illnesses, severe pneumonia may occur, leading to respiratory failure (33, 45, 121). Among immunocompromised individuals, RSV infection typically begins as an upper respiratory tract infection and may evolve into lower respiratory tract disease in up to 50% of cases (62, 121). The clinical significance of subgroup differentiation has been suggested by some studies. Subgroup A RSV may be more virulent than subgroup B, and its infection may result in greater disease severity among hospitalized infants (89, 92, 141). Subgroup B viruses grow less efficiently in conventional cell culture, so in some studies a detection bias may have occurred.

RSV causes hazardous nosocomial infection and produces outbreaks each year with widespread infection in both children and adults, including medical personnel, who may have a mild enough illness as to not cause absences from work. Furthermore, the spread of the virus is facilitated by

the number of young infants admitted during an outbreak who tend to shed high titers of virus for prolonged periods (62). Outbreaks producing lower respiratory tract disease in nursing homes and institutions have been reported (48). In a classical experiment, volunteers in close physical contact with RSV-infected infants were more readily infected than those who remained 6 feet away, suggesting that small-particle aerosol is less important in the spread of RSV than direct contact with infectious secretions via fomites or large-particle aerosols (61). A targeted infection control intervention has been demonstrated to be cost-effective in reducing the rate of RSV nosocomial infections (84). A contact isolation procedure is recommended for RSV-infected patients when they are hospitalized.

For the majority of infants with mild disease, no specific treatment is necessary other than supportive care. Children with severe disease may require hospitalization. Aerosolized ribavirin (Virasole) was approved in 1986 for the treatment of infants and children with severe RSV disease (97); however, a meta-analysis of clinical trials in infants with RSV lower respiratory tract infection showed that there was no evidence of a significant benefit and that studies lacked the power to detect reductions in mortality (111). In most hospitals, the use of ribavirin is now infrequent and restricted to immunocompromised patients or to those who are severely ill. Palivizumab (Synagis) is a humanized monoclonal antibody directed to the fusion (F) protein that was licensed in the United States for prophylaxis against RSV disease in high-risk infants. Administration of palivizumab to infants with chronic lung disease and/or extremely premature birth reduced the rate of hospitalization by 40 to 50%; however, the intramuscular treatment must be delivered monthly and its impact on mortality remains controversial (72). Most children admitted to the hospital with severe RSV are otherwise healthy infants and thus were not candidates for prophylaxis. Palivizumab has not shown convincing evidence of therapeutic efficacy after infection is established. An experimental combination treatment of ribavirin and palivizumab has been reported to be well tolerated and to improve the outcome of severe RSV infection among bone marrow transplant recipients (146). Delay of immunosuppressive chemotherapy should be considered when severely immunosuppressed individuals develop acute RSV infection (33, 121).

Collection, Transport, and Storage of Specimens

To detect the presence of RSV in clinical specimens, it is important to select the appropriate specimen. Appropriate respiratory specimens include nasal secretions, nasal swabs, nasopharyngeal swabs, and nasopharyngeal aspirates. Among them, secretions obtained by either wash or suction contain the infectious virus necessary for identification by culture and exfoliated nasopharyngeal cells if viral antigen detection methods are used (2, 90). Endotracheal tube aspirate or bronchoalveolar lavage (BAL) fluid collection is more sensitive than the above-mentioned sample types for rapid antigen testing for immunocompromised adults who are being mechanically ventilated (38). The use of nasal or nasopharyngeal swabs is an alternative method for sample collection; cotton-tipped swabs without calcium alginate are recommended (52). However, it has been shown that the use of nasopharyngeal swabs yields the worst results (62). After specimen collection, the swab should be introduced into a vial containing the viral transport medium, with the shaft cut to close the top of the vial, and transported to the laboratory promptly.

While limited studies indicate that there is no significant difference in the RSV antigen detection rates obtained by the nasal wash and by the nasopharyngeal wash/aspiration methods (20), nasal washing should be the recommended method for specimen collection, being the least traumatic for infants and young children (62, 85). A recent study demonstrated that the quantity of virus in the upper respiratory tract (nasal secretions) is nearly equivalent to that found in the lower respiratory tract by using both quantitative culture and real-time PCR (21, 107). To correctly collect nasal washes, the patient should be held from behind in an upright position and the head should be stabilized. A tapered bulb syringe containing 5 to 7 ml of sterile, prewarmed saline solution is introduced into one nostril, and the nasal wash is collected immediately into a medicine cup as it runs out of the other nostril. Residual mucus from both nares is suctioned into the bulb syringe and mixed with nasal wash. The nasal wash specimen is poured into viral transport medium, labeled, and placed on ice for proper transport to the laboratory. The prompt collection of a mucus sample for diagnosis of RSV infection is critical and enhances the rapid detection by viral cultures (62).

All general viral specimen collection rules, such as collecting during the acute phase of infection, keeping intact cells, delivering the specimen promptly, refrigerating specimens if stored temporarily, and sealing well in an O-ring-sealed cryovial if stored on dry ice, apply to specimen collection for RSV detection and culture. In addition, RSV is an extremely labile virus and does not tolerate changes in temperature or prolonged storage during transit to the laboratory (62, 64). Heating or freezing the specimen results in a decreased number of infectious virions in the sample. To effectively isolate RSV, specimen containers should be transported to the laboratory in wet ice as quickly and expeditiously as possible. If specimens cannot be processed rapidly and inoculated into cell culture within 2 h of sample collection, they should be kept refrigerated. Samples should never be frozen without first consulting the virology laboratory.

Direct Detection

Microscopy

Although RSV can be visualized by electron microscopy, its application has been limited to research laboratories. Conventional bright-field microscopy has been used for identifying RSV by visualizing the characteristic syncytial formation cytopathic effect (CPE), which is usually observed 4 to 6 days after inoculation on HEp-2 cell monolayer cultures (Fig. 2).

Antigen

The development of rapid diagnostic techniques for viral detection has received much attention over the last 2 decades. The use of rapid tests in diagnosis of RSV infection allows implementation of appropriate infection control measures, thus reducing nosocomial spread (74, 78, 84), and may be useful for consideration of timely treatment with aerosolized ribavirin in severe cases. It was reported that a reduction in the duration of hospitalization and use of additional laboratory investigations for patients with respiratory infections was achieved after rapid viral diagnosis became available at a hospital (149). The clinical and financial benefits of rapid detection of RSV in respiratory specimens have been demonstrated in several studies (7, 74, 78, 84, 149), indicating a direct correlation between rapid turnaround time and decreased mortality, length of stay, overall costs, and better antibiotic stewardship. There are several rapid RSV antigen tests currently available utilizing four major

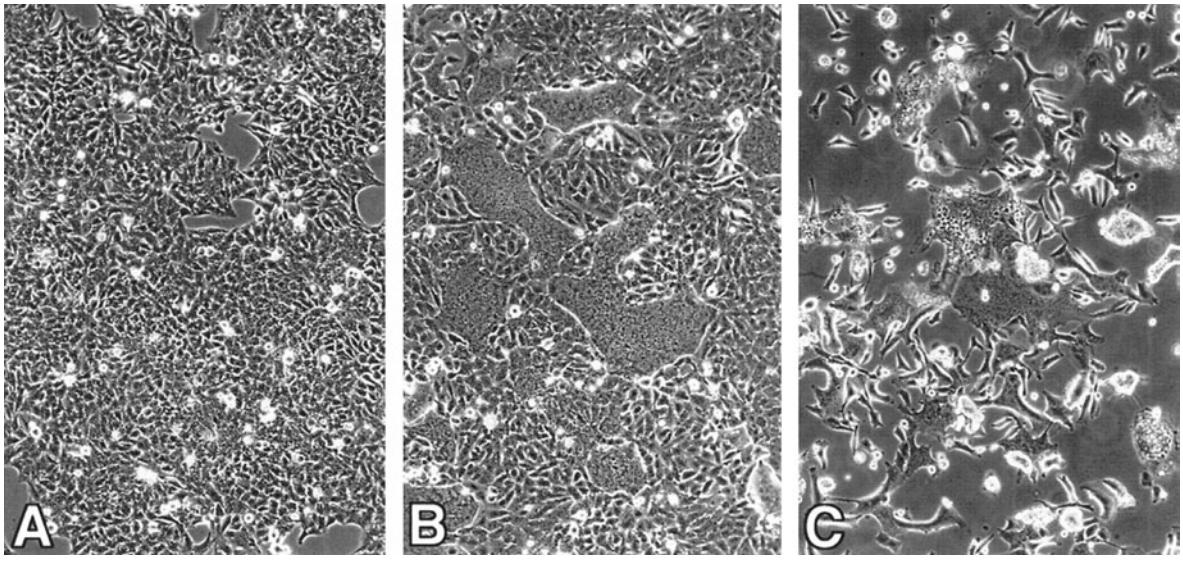


FIGURE 2 RSV-infected respiratory epithelial cells. Respiratory epithelial cells (HEp-2, laryngeal carcinoma cell line) on day 1 (A), day 3 (B), and day 5 (C) following infection with RSV. Magnification, ×400. After 1 day, the cells continue to appear healthy. After 3 days, many of the cells have fused, forming large syncytia. After 5 days, most of the syncytia have detached from the surface and the majority of the cells are dead. Adapted from reference 33.

platforms, i.e., immunofluorescent-antibody assay (IFA), optical immunoassay, enzyme immunoassay (EIA), and chromatographic immunoassay (CIA). Each platform offers a wide range of performance, specimen choices, and limitations for drug interference. Key factors that influence choice include test accuracy, turnaround time, interference with commonly used medications, choice of specimen type, choice of transport products, and reliable supply.

IFA tests have been used widely for rapid diagnosis of RSV infection including the detection of RSV from clinical specimens and confirmation and subgroup differentiation on isolates. Reagents needed for performing the IFA RSV assay are commercially available from a number of companies (Table 1). According to its basic procedure, IFA can be divided into direct and indirect formats. Direct IFA (D-IFA), which uses a fluorophore-conjugated RSV-specific antibody to minimize the test background and enhance the whole test turnaround time, has demonstrated its comparability with culture (81, 110). Indirect IFA (I-IFA), on the other hand, involves two antibodies: one RSV-specific antibody and one species-specific antibody that is preconjugated with a fluorophore. The two-step procedure consumes more time but generally enhances test sensitivity. The use of monoclonal antibodies in these assays has greatly improved the sensitivity and specificity of the test, both of which are more than 85% in most studies (76). An early review discussed several studies in which either D-IFA or I-IFA was used for the detection of RSV infection. With either technique, the sensitivity of the IFA in comparison to culture was usually above 80% and could be as high as 97%. The specificity was generally greater than 90% (76). In general, when an IFA is correctly performed, the sensitivity is higher than that of other rapid antigen tests (81, 110). "Multiplex" D-IFA reagents are available commercially to detect a panel of common respiratory viruses simultaneously (81).

However, IFA testing requires an adequate number of respiratory epithelial cells and a skilled technologist for accurate interpretation (91). Results are usually subjective; background fluorescence and nonspecific staining create difficulties in distinguishing infected cells. The specimen type and collection technique are important factors for the detection of RSV (96). Among respiratory specimens submitted for IFA, approximately 5% were inadequate for IFA interpretation, which requires a sufficient number and quality of cells (146). The high specificity of the IFA and availability of results in 45 min to 3 h have made the IFA a useful diagnostic technique. However, test turnaround time could be significantly longer if larger numbers of specimens are tested in one run, in comparison to the EIA procedure run in a microtiter format (60, 63).

In addition to IFA, other rapid antigen detection immunoassays, with or without enzyme involved, are commercially available (Table 2). These assays offer the advantages of ease of performance, objective reading of results, and very rapid results (15 to 30 min). Another advantage is that they do not require the presence of viable virus to detect an infection, and specimens obtained later in the course of the illness or those with suboptimal handling may yield a positive result. Several membrane flowthrough EIAs are commercially available for the rapid diagnosis of RSV infection. One membrane flowthrough EIA, the TestPack RSV (Abbott Laboratories, Chicago, Ill.), was well received by clinical laboratories until it was recalled by the U.S. Food and Drug Administration (FDA) recently due to its false-positive results (1, 16, 115). Currently available EIA kits include the Directigen RSV EIA (Becton Dickinson). The sensitivity and specificity relative to culture isolation for the second-version Directigen EIA ranged from 61 to 86.1% and from 69 to 95%, respectively (24, 100, 112, 115). Sensitivity and specificity of the rapid RSV EIA assays vary among different kits in different reports.

Another testing format, lateral-flow CIA, has recently been introduced and has been well received for the rapid detection of RSV-specific antigens (Table 2). Since late 2002,

TABLE 1 Commercial RSV IFA reagents and their performances

Format and product(s) (company)	Performance claimed by manufacturer[a]	Application	Remarks (reference[s])
Direct			
Bartels RSV DFA (Bartels, a Trinity Biotech Company, Carlsbad, Calif.)	Sensitivity, 88–100%; specificity, 100%	Direct specimen detection and culture confirmation	Single- or dual-reagent D-IFA available. Acquired recently by Trinity Biotech (63, 110).
Light Diagnostics RSV and Respiratory Virus Panel Screen and/or ID (Chemicon International, Temecula, Calif.)	Sensitivity, 100%; specificity, 86%	Direct specimen detection and culture confirmation	In either single RSV or panel format. The panel covers RSV, influenza A/B virus, adenovirus, and parainfluenza virus 1, 2, and 3 (80).
SimulFluor Respiratory Screen, RSV/flu A or RSV/Para 3 (Chemicon International, Temecula, Calif.)	Sensitivity, 83.3–100%; specificity, 83.8–100%	For screening clinical specimens and culture	Three formats available. Covers RSV, influenza A/B virus, adenovirus, and parainfluenza virus 1, 2, and 3 (81).
Merifluor RSV, Meridian Bioscience Inc., Cincinnati, Ohio)	NA[b]	For culture confirmation	Subgroup differentiation available.
Imagen RSV (Dakocytomation Ltd., United Kingdom)	Sensitivity, 93%; specificity, 98%	Detection of identification in human clinical specimens	Mixture of 3 monoclonal antibodies (17, 54, 59, 132).
PathoDx RSV and Respiratory Virus Panel (Remel Inc., Lenexa, Kans.)	Sensitivity, 77.6–100%; specificity, 98–100%	For clinical specimen detection and culture confirmation	The respiratory panel covers RSV, influenza A/B virus, adenovirus, and parainfluenza virus 1, 2, and 3.
Indirect			
Light Diagnostics Respiratory Virus Panel Screen and/or ID (Chemicon International, Temecula, Calif.)	Sensitivity, 100%; specificity, 100%	For culture confirmation	In a panel format, which covers RSV, influenza A/B virus, adenovirus, and parainfluenza virus 1, 2, and 3.
Bartels Respiratory Virus Antibody Pool (Bartels, a Trinity Biotech Company, Carlsbad, Calif.)	Sensitivity, 91%	For viral respiratory screening and identification	Covers RSV, influenza A/B virus, adenovirus, and parainfluenza virus 1, 2, and 3. Acquired recently by Trinity Biotech.
Imagen Respiratory Screen (DakoCytomation Ltd., United Kingdom)	Sensitivity, 97.8%; specificity, 99.1%	Rapid screen clinical samples or infected cell cultures	Covers RSV, influenza A/B virus, adenovirus, and parainfluenza virus 1, 2, and 3.

[a] Compared to cell culture unless specifically noted. In many cases, the culture used for comparison and validation was suboptimal, making the rapid tests look artificially better. It should be recognized that performances in various laboratories may be different.
[b] NA, not applicable.

the FDA has approved two new antigen detection tests under this category, the Now RSV test by Binax (Portland, Maine) and the Directigen EZ RSV by Becton Dickinson. Several recent parallel validations among Now RSV, Directigen EZ RSV, and Directigen RSV demonstrated that the three antigen assays performed similarly well. Hands-on processing for the two CIA tests (Binax Now and BD Directigen EZ) was significantly reduced, making them more convenient for rapid screening (87, 100, 154). A similar result was achieved when D-IFA and culture were used as the validation standard (5). Another new commercial one-step CIA test, originally manufactured by SA Scientific (San Antonio, Tex.), has been available commercially from SA Scientific (SAS), Remel (Xpect), Fisher Scientific (Sure-Vue), Meridian Bioscience (ImmunoCard), and Wampole (Clearview). The sensitivity and specificity of the kit were 82.5 and 93.3%, respectively, and were equivalent to those yielded by Directigen EZ RSV when a D-IFA and/or a reverse transcriptase (RT)-PCR was used as the "gold standard" (17, 79, 122). In comparison to D-IFA and EIA procedures, these lateral-flow CIA-based procedures require extremely short hands-on time and test turnaround time; some of them require a hands-on time of only 1 to 2 min and only one incubation and are very easy to read.

Some of these rapid detection formats have been considered to be used as point-of-care (POC) tests within clinical laboratories and clinicians' offices by personnel with little formal training in virology techniques (127). There are many such tests commercially available with varying degrees of sensitivity and specificity. One recent study compared the accuracy of the Thermo Electron RSV OIA with D-IFA and viral culture. The sensitivity and specificity were 87.9 and 99.6%, respectively, and the kit is technically easy to use and can be completed within 20 min. As a moderately complex test, it requires minimal oversight and may be used as a rapid POC test (4). Another study favored using the Binax Now RSV test as a POC test to reduce the risk of health care-associated RSV infections in pediatric wards and indicated that it can be used in the field, maintaining consistent vigilance on quality control, due to its simplicity (87). For general screening, the RSV rapid test is found to be a reasonable tool to obtain quick results. However, its lack of specificity for some patients requires confirmation by additional tests, such as culture or molecular assays, to rule out false-positive results and/or detection of other respiratory viruses (1).

It should be noted that the sensitivity and specificity of these tests are applicable to use during the epidemic season, generally the winter in temperate climates. The performance

TABLE 2 RSV rapid antigen products and their performances[a]

Technique platform	Product (company)	Compatible specimens	Length of assay (min)	Claimed sensitivity (%)[b]	Claimed specificity (%)	Validated sensitivity (%)	Validated specificity (%)	Comments	Selected reference(s)
OIA	BioStar RSV OIA (Thermo Election)	NW and NPS	15	NW, 86.8; NPS, 66.7	NW, 83.2; NPS, 96.4	87.9	99.6	FDA approved for children under age 5.	4
EIA, flow-through	Directigen RSV (BD Diagnostic Systems)	NP washes, NP aspirates, NPS, and tracheal aspirates	15	93–97	90–97	61–86	69–95	Instant, clear-cut readings. Mucous specimen hard to pass through. Two antibodies utilized to detect two RSV antigens.	24, 63, 100, 112, 115
	Pathfinder RSV EIA (BioRad Laboratories)	NW or aspirates	90	94.5	99.1	87	88	Can be read visually or with a spectrophotometer. Recalled by FDA.	73
	VIDAS RSV assay (BioMerieux Vitek)	NA	NA	NA	NA	82	94	Automated. Currently not available.	60
	TestPack RSV (Abbott Laboratories, Chicago, Ill.)	NP washes, NP aspirates, NP swabs, tracheal aspirates	15	NA	NA	67–89	87–98	Recalled by FDA and discontinued globally.	1, 15, 63, 115
CIA, lateral flow	Directigen EZ RSV (BD Diagnostic Systems)	NP washes, NP aspirates, NPS	15	89	93	59–86.5	92.3–98	Specific antibody used to avoid possible interference with the immunoglobulin therapy.	5, 100, 154
	NOW RSV(Binax)	NW or NPS	15	89–93	93–100	89–94.6	88.5–100	CLIA waived test.	5, 15, 100, 154
	SAS RSV (SA Scientific, San Antonio, Tex.)	Nasopharyngeal washes, aspirates, and swabs	15	95.6	94.1	57–97	73–100	The original equipment manufacturer for SAS, Sure-Vue, ImmunoCard, Xpect, and Clearview.	79
	Sure-Vue RSV (Fisher Scientific)	Nasopharyngeal washes, aspirates, and swabs	15	95.6	94.1	NA	NA	An 18-month shelf life.	
	ImmunoCard STAT! RSV Plus (Meridian Bioscience, Inc., Cincinnati, Ohis)	NW, nasopharyngeal aspirate, NPS	15	Wash or aspirate, 77.8–84.2; swab, 89.5–94.4	Wash or aspirate, 78; swab, 94.4–100.0	NA	NA	Analytical sensitivity from 10 to 10,000 virions/ml for both subgroups A and B.	
	Clearview RSV (Wampole Laboratories, Princeton, N.J.)	NPS or nasopharyngeal aspirates	15	93.7	97.7	NA	NA	CLIA waived test. Formerly QuickLab RSV.	
	Xpect RSV (Remel Inc., Kansas City, Kans.)	Nasopharyngeal specimens from neonatal and pediatric patients	15	95.6	94.1	67–78	96–98	Low rate of uninterpretable results. Even mildly mucoid specimens can fail to migrate.	17
	Respi-Strip RSV (Coris Bio-Concept, Belgium)	Nasopharyngeal secretions and/or culture supernatant	10	86–92	92.8–98	NA	NA	In dipstick platform. Not available in the United States.	

[a] Abbreviations: NA, not applicable or not available; OIA, optical immunoassay; NW, nasal wash; NPS, nasopharyngeal swab.
[b] Compared to cell culture unless specifically noted.

of these tests is much reduced during periods when the prevalence of virus infection is low, such as the summer months. In addition, the accuracy of these rapid tests can be influenced by the type of specimen obtained and the age of the patient. Rapid antigen detection tests for the diagnosis of RSV respiratory illness in adults are of limited value. A study performed with RSV-infected older adults showed antigen detection in nasal specimens to be very insensitive (46). Two recent studies evaluated the performances of several RSV rapid antigen detection tests including VIDAS RSV, Binax NOW, BD Directigen RSV, and BD Directigen EZ assays. The sensitivity was 0 to 25% for these rapid EIA or CIA antigen tests for adults, a population for which D-IFA remains the rapid method of choice for detecting RSV (24, 46, 100). In general, sensitivity is low when a rapid antigen test is used for the diagnosis of RSV infection in adults (24) and should be replaced by PCR (143). The extremely low sensitivity in adults is due to low and short RSV shedding in respiratory secretions and the problems associated with obtaining nasal washes from frail older adults (18, 62). Because of this relative lack of sensitivity during periods of low virus shedding, disappearance of antigen in nasal secretions during later periods of an acute illness should not be interpreted as an indication that eliminating contact isolation precautions is appropriate. Hospitalized infant patients shed infectious virus at levels that can result in nosocomial transmission far longer than the duration of positive antigen tests.

Nucleic Acid

As is the case with other viral pathogens, in vitro nucleic acid amplification (NAA) tests such as PCR have been implemented gradually in clinical laboratories for RSV detection. Different amplification and identification platforms have been described (Table 3). The NAA tests, in general, have been demonstrated to be superior to routine diagnostic methods in terms of their high sensitivity and specificity. The reported sensitivity of PCR ranges from 93.5 to 100%, and the specificity ranges from 93.9 to 100% (40, 53, 67, 126, 131). The RSV N gene has been used mainly as the target for developing NAA-based assays because (i) nucleotide sequences in the N gene are highly conserved and (ii) the N gene is located nearer the 3′ end of the genome where transcription and replication initiate, and thus this gene is preferentially transcribed. Sequence variations between RSV subgroups A and B have been used to

TABLE 3 Comparison of amplification and identification formats of published NAA tests for RSV detection and quantitation[a]

Amplification and identification format	Procedure design	Sensitivity	Carryover contamination risk	Comments	Reference(s)
RT-PCR; gel electrophoresis	One-step, seminested or nested; monoplex or multiplex	Moderate	Moderate	Earlier applications; subgroup differentiation possible when subgroup-specific primers used	1, 3, 27, 39, 41, 44, 45, 55, 59, 67, 102, 103, 114, 116, 117, 126, 140, 152
RT-PCR; RFLP	One-step; monoplex	Moderate	Moderate	First report was on isolate subgrouping; RFLP used for subgroup differentiation and further subtyping studies	49, 55, 57
RT-PCR; Southern blot hybridization	One-step; monoplex	High	Moderate to high	Subgroup differentiation possible using subgroup-specific probes	3, 50, 138
RT-PCR; colorimetric EIA	One-step; monoplex or multiplex	High	Moderate to high	Commercial ASR kit available (Hexaplex, Prodesse); subgroup differentiation possible; additional signal amplification can be incorporated	40, 47, 50, 53, 54, 58, 69, 75, 83, 108, 109, 130, 131, 143
RT-PCR; sequencing	One-step or seminested; monoplex	Moderate to high	Moderate to high	Subgroup and subtype information provided and used for molecular epidemiology studies	106, 117
RT-PCR; real-time (TM)	One-step; monoplex or multiplex	Moderate to high	Low	Subgroup differentiation and quantitation possible	18, 32, 43, 70, 80, 94, 107, 132, 137
RT-PCR; real-time (LC)	One-step; monoplex or multiplex	Moderate to high	Low to moderate	Subgroup differentiation and quantitation possible; capillary tubes are fragile	16, 101, 139, 145
NASBA; real-time (MB)	One-step; monoplex	High	Low	Basic ASR kit is commercially available (Nuclisens EasyQ, bioMerieux); target on RNA molecules	98

[a] Abbreviations: NASBA, nucleic acid sequence-based amplification; TM, TaqMan (Applied Biosystems); LC, LightCycler (Roche); MB, molecular beacons (bioMerieux).

design subgroup-specific primers and/or probes for simultaneous RSV subgrouping (1, 10, 39, 47, 49, 53, 57, 59, 69, 70, 75, 83, 101, 102, 107, 108, 126, 131, 137, 143). While cell culture remains the gold standard for RSV diagnosis, the NAA assays have been considered the "platinum" standard to be used as the reference to validate new diagnostic kits in RSV detection (1, 143, 154).

Since Paton et al. first applied RT-PCR in clinical diagnosis of RSV infections (103), numerous NAA techniques have been described in diagnostic applications. Several formats have been used to further confirm and identify the amplicons generated by PCR to further enhance the test sensitivity and specificity (Table 3). In general, diagnostic efficacy of the RT-PCR-based assays was substantially more sensitive than the combination of conventional viral culture and shell vial culture (69, 75, 83, 94, 130, 131, 143). Several new analytic-specific reagent (ASR) kits based on RT-PCR or real-time RT-PCR are commercially available from Roche, Abbott, Cepheid, and Prodesse (47, 69, 75, 83). Targeting RNA molecules, nucleic acid sequence-based amplification (NASBA)-based Nuclisens Easy Q RSV A+B Assay (bioMerieux) incorporated with real-time detection, possesses equivalent or superior sensitivity in comparison to RT-PCR procedures (98).

The mainstream use of RT-PCR in the routine diagnostic laboratories is currently hindered due to the following. First, NAA techniques have a relatively long test turnaround time, from 8 to 48 h (53, 75, 103, 131). Even with the implementation of the real-time PCR format, due to the time needed for specimen processing and nucleic acid extraction, it is still not practical to use the complicated NAA procedures as rapid, POC testing because faster and less expensive alternatives are available (18, 44, 94, 145). Second, there are currently no FDA-approved NAA kits available in the field for RSV detection. Approval requires full validation for ASR kits and user-developed procedures before they can be used for diagnostic purposes. Third, the technical complexities require extensive personnel training to perform such tests correctly, avoiding potential false-positive or false-negative results (120). Finally, the costs of reagents, instruments, and patent royalties have delayed the speed of implementation of NAA techniques in routine diagnostic services. It is predicted that NAA techniques will continue to be used to complement current conventional assays and gradually may replace culture for the detection and identification of respiratory viruses in clinical laboratories.

NAA techniques are especially useful for adult patients when viral shedding is short and low (43, 143). Their application seems efficient when multiplex formats are used for the simultaneous detection of several respiratory viruses in one clinical sample (10, 54, 58, 59, 126, 130). With implementation of the real-time format, rapid RSV viral load quantitation by RT-PCR is possible, which could be helpful to clinicians in some settings. RSV quantitation has been reported using both TaqMan and two-probe hybridization formats by RT-PCR and molecular beacons by NASBA (Table 3). Falsey and Perkins applied a quantitative real-time PCR to volunteers and patients and demonstrated that quantitative PCR has an excellent correlation with viral titers, as measured by quantitative cell culture (43, 107). Several other clinical studies with implementation of quantitative PCR discovered and/or confirmed that RSV viral loads are significantly higher in children than in adults, in acute lower respiratory tract infections than in chronic obstructive pulmonary diseases, and in subgroup A than in subgroup B infections (18, 43, 107). The exact clinical values for the application of RSV quantitative NAA assays merit further investigation.

Isolation Procedures

Isolation of the virus from respiratory secretions by cell culture remains the gold standard method for RSV diagnosis due to its excellent specificity. Appropriate clinical samples for culture include nasal washes, nasopharyngeal aspirates or swabs, tracheal aspirates, BAL fluids, and biopsy materials. Inadequate specimen collection or delays in processing often result in negative culture results. It has been shown that recovery of the virus is highly dependent on the training and interest of the personnel collecting the samples (62). Given the lability of the virus, the samples should be kept cold and should be inoculated as quickly as possible. RSV grows well in several human cell lines and may be adapted to a number of others. For primary isolation, human heteroploid cells, such as HEp-2, HeLa, and A549 cells, are usually preferred. Other cell lines that may be used but are usually less sensitive include human kidney, amnion, and diploid fibroblastic cells and monkey kidney cells (9, 33, 62). The sensitivity of all these cell lines, including the heteroploid human lines, for the growth of RSV is variable and must be monitored. Cell line cultures show characteristic syncytial CPE after 3 to 7 days of incubation (Fig. 2). However, the degree of syncytial formation depends on the type of cell culture, the heaviness of the cell monolayer, the medium, the strain of virus, the multiplicity of infection, and its laboratory adaptation. RSV does not cause syncytia in polarized epithelial cells, such as primary human respiratory tract epithelial cells or MDCK cell line cultures. While cell culture possesses the highest specificity, its sensitivity varies widely, in part due to virus viability, technical expertise required, and cell lines used in the laboratory performing the viral isolation. The reported sensitivities of the conventional tube cell culture compared with various rapid techniques have ranged from 57 to 90% (63, 76).

The major drawback for cell culture is the length of time necessary to identify viruses in culture. The shell vial assay has significantly lowered the time needed for detection of the virus to 16 h (123). The method combines centrifugation and immunofluorescence to detect expression of viral antigens on infected cells before development of CPE (110). The sensitivity and specificity of shell vial culture range from 67.4 to 92.1% and from 82.9 to 100%, respectively (73, 110, 123). Since HEp-2 cells were abandoned by many clinical laboratories, the sensitivity for routine culture performed with RhMk, MRC-5, and A549 cells can be significantly lower.

The use of Mixed Fresh Cells or R-Mix (Diagnostic Hybrids, Inc., Athens, Ohio), which is a mixture of human lung carcinoma A549 and mink lung Mv1Lu cells, has been shown to be a rapid and sensitive method for the detection and identification of respiratory viruses (8, 36, 51, 125, 142). With significantly decreased costs compared to conventional culture, R-Mix was slightly more sensitive than RMK, HEp-2, and MRC-5 cell lines used in conventional cultures, and it was several days faster (8). Screening of R-Mix cells after overnight incubation was found to be a very sensitive method for the detection of respiratory viruses, including RSV, and was more reliable than direct antigen testing (125). In addition, cell cultures have the major advantage of detection of copathogens and ideally should be used in addition to a rapid technique to diagnose RSV. R-Mix cells are useful to identify the viruses not detected with direct stains (36).

Identification

The appearance of characteristic syncytial CPE in cell culture remains adequate to establish the presence of RSV during the epidemic season. However, since other respiratory viruses such as parainfluenza virus 3 and measles virus can produce similar CPE in certain cell lines, most clinical virology laboratories confirm RSV detection by performing subgroup-specific IFA with infected cells from cell culture vials. Further identification, such as subgrouping and subtyping, can be performed by using RT-PCR followed by nucleotide sequence analysis and/or restriction fragment length polymorphism (RFLP) analysis (49, 106).

Typing Systems

Infections with RSV of subgroup A or B may result in differing clinical manifestations and outcomes; therefore, RSV subgroup information can be useful in clinical patient management (89, 141). RSV subgroup information has been widely used to facilitate epidemiological investigations (22, 50, 117). IFA procedures have been routinely used in the laboratory for subgrouping, and reagents are available from Bartels and/or Remel (Table 1). This technique is very quick but, due to its relative insensitivity, it is usually used only for confirmation and subgroup differentiation on isolates recovered by cell culture. RT-PCR followed by subgroup-specific probe hybridization or RFLP has been used to complement IFA assays by differentiating subgroups directly from the clinical specimens (22, 49, 57, 131, 138). The technique improved significantly when the real-time PCR format was implemented (70, 137).

Further analysis on genomic differences among RSV isolates beyond subgrouping has been used in epidemiologic investigations (22, 128). There was one study using heterogeneous sequences in the RSV G gene to generate subgroups, and it demonstrated that different RSV subgroups were associated with severity of clinical diseases (49). It is worth pointing out that these highly discriminatory typing techniques have been useful in monitoring possible nosocomial RSV infections and facilitating interruption of RSV outbreaks (22, 128).

Serologic Tests

The diagnosis of RSV infections by using serology has no practical application for clinical use. Diagnosis requires several weeks for induction of a serum antibody response and collection of a convalescent-phase serum specimen for identification of recent infection. A fourfold rise of titer between acute- and convalescent-phase specimens by serology is generally accepted as evidence of infection. Young infants, older individuals with repeated infections, and immunocompromised patients may not exhibit a significant rise in antibody titer following infection, depending on the assay used (35, 46). Serologic diagnosis of RSV infection has been more useful for epidemiologic and vaccine studies than for patient management because of the delay required to obtain convalescent-phase sera. Secretory class-specific antibodies to RSV in nasopharyngeal secretions may also be detected. Since these secretory antibodies may be present earlier in infection than serum antibodies, their detection has been used as an adjunct to diagnosis by antigen detection (71). Repeated infections with the same subgroup of RSV occur frequently; therefore, it is usually necessary to test acute- and convalescent-phase sera and observe an increase in titers to confirm the diagnosis. Furthermore, assays that detect immunoglobulin M antibodies to RSV show low sensitivity, particularly for infants less than 6 months of age, precluding a definitive diagnosis in a clinically relevant time frame (95).

Current means of serologic diagnosis for RSV infections include IFA, EIA, complement fixation test, and neutralization assays (62, 66, 95, 121, 144). Virus neutralization tests have been developed as plaque reduction assays or in a microneutralization format. Other test formats, including immunoprecipitation, fusion inhibition, and immunoblot assays, can be used to detect class-specific antibodies directed to specific viral proteins (56, 93). I-IFA has been used widely for RSV antibody detection (62, 144). Reagents for RSV antibody detection by I-IFA are commercially available from Bion Enterprises, Ltd. (Des Plaines, Ill.), Diagnostic Products Corporation (Los Angeles, Calif.), Globalemed, LLC (Alexandria, Va.), Hemagen Diagnostics, Inc. (Columbia, Md.), Meridian Bioscience Inc. (Cincinnati, Ohio), and PanBio Inc. (Columbia, Md.).

Antiviral Susceptibilities

Ribavirin has been used to treat severe RSV infections, although its efficacy has been questioned in recent studies. Resistance to ribavirin has not been recognized. Palivizumab prophylactic treatment of high-risk infants prevents about one-half of the hospitalizations in this group. The cause of prophylactic failure in the other patients is not clear but does not appear to be viral resistance. Several RSV escape mutants derived in the laboratory have been shown to be resistant to palivizumab prophylaxis in cell culture and cotton rats (153). An immunofluorescence binding assay that predicts palivizumab neutralization of RSV was developed, and a total of 458 RSV isolates recovered during 1998 to 2002 were tested. The results indicated that, at present, the development of palivizumab-resistant mutants does not appear to play a significant role in hospitalizations for breakthrough RSV disease occurring in infants receiving palivizumab prophylaxis. The nucleotide and amino acid sequences of the F protein epitope to which palivizumab binds were highly conserved among the clinical isolates evaluated in the present multicenter study, even in those from children receiving palivizumab prophylaxis (31).

Evaluation, Interpretation, and Reporting of Results

The availability of rapid antigen assays with a turnaround time as short as 15 min makes it possible for clinicians to receive results in a timely fashion. The potential benefits of rapid diagnosis include discontinuation or avoidance of unnecessary antibiotics, occasional indication for ribavirin as an antiviral medication, and a shortened hospital stay. Rapid detection of RSV also can be used as part of an infection control program to decrease nosocomial RSV transmission through proper isolation or cohorting of infected patients (74, 78, 84). However, due to their relatively low sensitivity, false-negative results can be expected in a small percentage of cases, and negative results should never be interpreted as excluding the possibility of RSV infection. In particular, ill children with highly characteristic illnesses in the peak period of RSV epidemics should be placed in proper isolation. In the laboratory, it is important to use culture and RT-PCR assays as backup tests, especially for those specimens that have yielded negative antigen results.

Cell culture is considered to have the highest specificity, followed by antigen tests and NAA assays. NAA assays, which have been demonstrated to be the most sensitive test for detection of RSV, are particularly useful for testing respiratory specimens collected from adults since viral shedding is

short and low (43, 137, 143). NAA assays, including RT-PCR and NASBA, will gradually be used more frequently in routine diagnostic laboratories as the techniques become more user friendly and test turnaround becomes more rapid; however, a small number of false-positive results can occur due to contamination during specimen processing or to carryover contamination. Quantitative PCR rapidly determines the viral RNA level when a real-time format is incorporated. Although the viral RNA level is correlated to the viral load measured by quantitative culture, clinical application of quantitative PCR awaits further investigation (18, 107). Before interlaboratory standardization is implemented, quantitation generated from PCR assays should be interpreted cautiously.

Immunity to RSV is short-lived, and repeated infections with RSV as well as subclinical RSV infections are common. The likelihood of identifying RSV as the etiology of a patient's infection is greatest when a specimen is obtained within the first 3 days of illness, especially when culture is used. Repeat viral cultures or viral antigen detection by several antigen assays performed serially after the initial confirmation of RSV infection usually become negative 1 week after onset of illness in about 50% of patients. However, excretion of live virus in immunocompromised infants has been documented up to 3 weeks after onset of illness (62, 77). When interpreting laboratory test results for RSV detection, it is also important to take into account the probability that RSV is associated with the patient's illness. Physicians should be aware of specific regional patterns of RSV activity when considering laboratory confirmation for infection. The positive and negative predictive values vary according to the prevalence of the disease in a particular population during certain seasons.

Effective exchange of relevant information between clinicians and the laboratory is essential to good patient care. RSV is extremely labile. Even when sensitive methods such as RT-PCR are used, false-negative results are possible, mainly due to improper specimen collection, transport, and processing. The importance of correct specimen collection and prompt specimen transport cannot be overemphasized. Prompt reporting of test results may assist clinicians in discontinuing unnecessary forms of therapy or in cohorting infected individuals, thereby preventing nosocomial spread of infection.

HUMAN METAPNEUMOVIRUS

Taxonomy

Human metapneumovirus (hMPV) is an RNA virus of the *Paramyxoviridae* family, and part of the *Pneumovirinae* subfamily along with RSV. The virus was discovered in 2001 (135). The *Paramyxoviridae* family is divided into two subfamilies, *Pneumovirinae* and *Paramyxovirinae*. The *Pneumovirinae* subfamily includes the two genera *Pneumovirus* and *Metapneumovirus*. The avian pneumoviruses (APV) are highly related to hMPV. APV and hMPV were separated by taxonomists into the separate genus *Metapneumovirus* because they can be distinguished from members of the *Pneumovirus* genus by their lack of genes encoding the nonstructural proteins NS1 and NS2 and by a different gene order in the RNA genome. APVs have been classified into four subgroups (A through D). hMPV is related most closely by sequence homology to subgroup C APV. hMPV does not infect turkeys or chickens; the only known natural host is humans.

Description of the Agent

hMPV is an enveloped respiratory virus with a single-stranded, nonsegmented, negative-sense RNA genome. Although APV and hMPV are related to the human and animal RSV viruses, they differ in that the gene order in the nonsegmented genome is slightly altered and APV and hMPV are lacking the two nonstructural proteins NS1 and NS2 that are located at the 3′ end of RSV genomes (Fig. 1). These RSV proteins are thought to counteract host alpha, beta, and lambda interferons (124). The absence of NS1 and NS2 in the metapneumoviruses may contribute to decreased pathogenicity of these viruses relative to RSV strains. Full-length sequences of a number of hMPV genomes have been reported (11, 68, 134). The genome is predicted to encode nine proteins in the order 3′-N-P-M-F-M2-SH-G-L-5′ (the M2 gene is predicted to encode two proteins, M2-1 and M2-2, using overlapping open reading frames, as in RSV) (134). The genome also contains noncoding 3′ leader, 5′ trailer, and intergenic regions, consistent with the organization of most paramyxoviruses (68). The viral promoter is contained within the 3′-terminal 57 nucleotides of the genome (12). The F, G, and SH proteins are integral membrane proteins on the surface of infected cells and virion particles. The F protein is a classic Type I integral membrane viral fusion protein containing two heptad repeats in the extracellular domain that facilitate membrane fusion. There is a predicted non-furin F1/F2 cleavage site near a hydrophobic fusion peptide, which contrasts with the furin cleavage site in RSV. The predicted attachment (G or glycosylated) protein of hMPV exhibits the basic features of a glycosylated type II mucin-like protein. The hMPV G protein differs from the RSV and APV G proteins in that it lacks a predicted cysteine noose structure. The N (nucleoprotein), P (phosphoprotein), and L (polymerase or large) proteins are replication proteins in the nucleocapsid; the M2-1 and M2-2 proteins likely are regulatory proteins. The M (matrix) protein is thought to coordinate viral assembly by interfacing viral nucleocapsids with the surface membrane proteins.

Epidemiology and Transmission

hMPV outbreaks occur in annual epidemics during late winter and early spring in temperate climates, often overlapping in part or in whole with the annual RSV epidemic. Long-term studies have shown that sporadic infection does occur year-round (147). The usual period of viral shedding has not been defined but is likely to be weeks following primary infection in infants. The incubation period is thought to be about 3 to 5 days. Humans are the only source of infection. Formal transmission studies have not been reported, but experts believe that transmission occurs by close or direct contact with contaminated secretions involving large-particle aerosols, droplets, or contaminated surfaces. Nosocomial infections have been reported, and contact isolation with excellent hand washing for health care providers is appropriate.

Clinical Significance

The virus causes upper and lower respiratory tract illnesses. A study of the association of the virus with respiratory disease was performed using prospectively collected data in a cohort of over 2,000 subjects aged 0 to 5 years, monitored during a 25-year period at Vanderbilt University Medical Center (147). This study showed that hMPV is associated with the common cold (complicated by otitis media in about one-third of cases) and with lower respiratory tract illnesses such as bronchiolitis, pneumonia, croup, and exacerbation

of reactive airways disease. The profile of signs and symptoms caused by hMPV is very similar to that caused by the related pneumovirus RSV. Approximately 12% of outpatient lower respiratory tract illness was associated with hMPV infection, which was second only to RSV in this regard. A prospective study of all infant hospitalizations for acute respiratory illness or fever without localizing symptoms in Nashville, Tenn., and Rochester, N.Y., organized by the Centers for Disease Control and Prevention found that 3.9% of 668 such child hospitalizations were associated with hMPV (99). Children with RSV or hMPV infection required supplemental oxygen and medical intensive care at similar frequencies. Many other studies at referral hospitals, most performed as retrospective studies using samples referred to a diagnostic virology laboratory, suggested that approximately 5 to 10% of such patient specimens contain the hMPV genome. hMPV has been found in association with respiratory tract disease in every continent; dozens of country-specific case series have been reported.

About one-half of the cases of lower respiratory tract illness in children occur in the first 6 months of life, suggesting that young age is a major risk factor for severe disease (147). Both young adults and the elderly can suffer hMPV infection that leads to medically attended illnesses including hospitalization, but severe disease occurs at much lower frequencies in adults than in young children (41, 42). Severe disease in older subjects is more common in immunocompromised patients (104, 136) and can be fatal in these subjects (23). A large proportion of respiratory tract secretion samples submitted to diagnostic virology laboratories are obtained from immunocompromised patients. hMPV infection is associated with wheezing more commonly in patients with underlying pulmonary disease, especially asthma (148). Although a significant number of patients with asthmatic exacerbation have hMPV infection, it is not yet clear whether there is a particular association of the virus with long-term wheezing. RSV and hMPV coinfections have been reported, which is not surprising given the overlapping seasons. Studies that identify coinfections solely on the basis of amplification of viral genome by RT-PCR, however, should be interpreted with caution for several reasons. PCR is susceptible to false-positive tests due to cross-contamination in some laboratories that perform frequent amplifications. Ideally, amplified PCR products should be confirmed by hybridization or preferably nucleotide sequence analysis to demonstrate diversity of isolates and lack of contamination by laboratory isolates. Second, viral genome can be detected by RT-PCR in respiratory secretions for several weeks or more, even after live virus shedding has ceased. It is difficult to know in this situation if the presence of a positive RT-PCR test signifies active infection or simply a recent acute infection. Coinfection with bacteria is not common, except for the local complication of otitis media, which is frequent (119, 129, 147).

Collection, Transport, and Storage of Specimens

Reports in the literature suggest that the virus can be recovered or detected by RT-PCR from nasal aspirates, nasal washes, nasal/throat swabs, and BAL specimens. It is not clear at this time what the optimal method of specimen collection from the upper respiratory tract is. Transport is generally performed using standard viral transport media, on ice. It is highly likely that optimal specimen collection and handling will be determined to be very similar to that for RSV, which is discussed in detail above. Specimens should be processed as soon as possible after collection and stored at 4°C until processing. For long-term storage of specimens or laboratory stocks, a temperature of –70°C is preferred, with specimens in O-ring-sealed cryovials.

Direct Detection

Microscopy

The virus is too small for detection by conventional microscopy but can be seen by electron microscopy (15, 135). This method, however, is not sufficiently sensitive or reproducible for clinical detection. Conventional bright-field microscopy of infected cell monolayer cultures does reveal some CPE, especially following multiple passages in culture (Fig. 3A and 3B). The characteristics of the CPE are not distinct enough that the virus could be identified on this basis, even by trained observers.

Antigen Detection

There are no direct antigen tests commercially available. Efforts have been spent in developing rapid antigen tests on nasopharyngeal aspirate samples using hMPV-specific monoclonal antibodies (37, 82, 116). Recently, a group of Italian scientists reported the development of monoclonal antibodies specific for hMPV and their use for D-IFA staining of nasopharyngeal samples or virus identification in shell vial cell cultures. A total of 40 nasopharyngeal swabs collected from infants and young children hospitalized because of acute respiratory infection during the winter-spring season

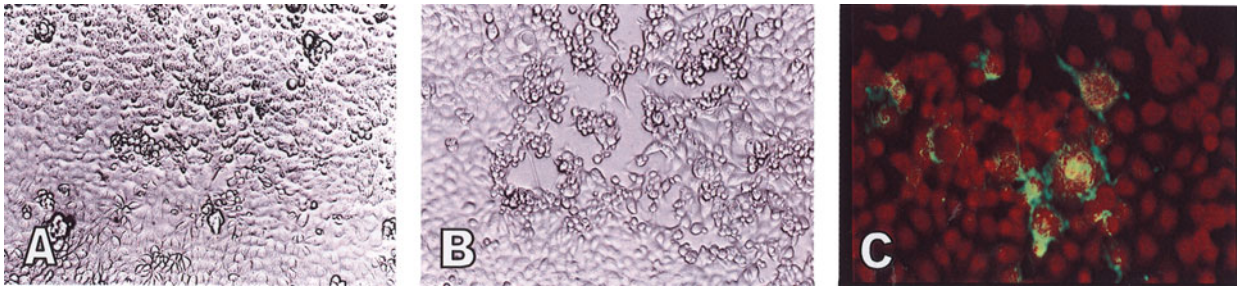


FIGURE 3 20× microscopic detection of hMPV in LLC-MK2 cell monolayer cultures. (A) Mock-infected cells form a confluent layer with only minor irregularities, as seen by light microscopy. (B) hMPV infection causes a focus of infection with rounding of cells and loss of monolayer integrity. (C) Indirect immunofluorescence of hMPV-infected LLC-MK2 centrifugation culture stained with monoclonal antibody MAb-8 (82). Images courtesy of John Williams (A and B) and David Ferguson (C).

were used to validate the D-IFA reagents. The sensitivity and specificity were 73.9 and 94.1%, respectively, in comparison to the results generated by an RT-PCR test (105).

Nucleic Acid

The most sensitive test for identification of hMPV in clinical samples to date is RT-PCR. Many of the early clinical reports used an RT-PCR test incorporating PCR primers hybridizing to the polymerase (L) gene, and the nucleotide sequence of the L gene PCR product was used to identify the virus (135, 147). Subsequently, real-time RT-PCR tests have been developed, especially targeting the N gene, which offer enhanced sensitivity and specificity, including an assay specifically designed to detect viruses from the four known genetic lineages, which are described below (29, 30, 86, 88, 118, 139, 145).

Isolation Procedures

hMPV replicates poorly in most conventional cell cultures used for respiratory virus diagnosis, such as HEK, HEp-2, and Madin-Darby canine kidney cells. Primary isolation is facilitated by the presence of a low concentration of trypsin, which is not used routinely in diagnostic virology cultures in many laboratories. The virus was first isolated in primary monkey kidney cells from a clean primate facility in The Netherlands that had been passed twice (designated tertiary monkey kidney [tMK] cells). Monkey kidney cells in the United States generally cannot be used for this purpose because many of these commercial cell cultures contain simian foamy viruses that cause CPE during the prolonged culture necessary to grow hMPV. hMPV can be isolated and grown in LLC-MK2 cell or Vero cell monolayer cultures. Growth is slow in these cell lines, and often several blind passages are required before CPE appears, especially following primary isolation. The CPE in tMK cells was reported to resemble that of RSV, although usually appearing later than with RSV. The CPE in cell lines is not particularly striking, often appearing only as focal areas of rounding of cells and minor patches of cell-cell fusion in LLC-MK2 or Vero cell cultures (Fig. 3). Virus can be isolated in about one-half of cases in which nasopharyngeal samples are positive for hMPV by RT-PCR. Shell vial centrifugation cultures may enhance recovery and shorten the time to recovery (82).

Identification

Currently, the virus is best identified when a specific RT-PCR test is found to be positive (optimally confirmed with sequence analysis of the cDNA copy of the RNA genome). The virus can be suspected when a virus isolated during the epidemic season in Vero or LLC-MK2 cells is not identified by specific immunofluorescence tests for related viruses. Recently, monoclonal antibodies specific for hMPV have been reported, and these can be used for identification of the virus by IFA, although they are not widely available currently (37, 82, 105).

Typing Systems

The nomenclature and classification of subgroups, subgroups, strains, variants, and isolates of hMPV are still being developed. Phylogenetic analyses of partial sequences of the viral genome have suggested in every case that there are at least two major genetic subgroups (designated A and B), and the phylogenetic groupings are consistent when analyses of any of five genes have been determined. Full-length sequences of hMPV genomes from viruses representing the two major subgroups have been performed by two groups using isolates from The Netherlands (strains NL/1/00 and NL/1/99) or from Canada (strains CAN97-83 and CAN98-75). These studies show that the diversity between hMPV subgroup A and B sequences is greatest for the SH and G proteins. The G glycoprotein genes exhibit nucleotide and amino acid sequence identities ranging from 52 to 58% and 31 to 35%, respectively, between the two subgroups, while the hMPV F protein is highly conserved in hMPV strains even across subgroups (with amino acid identity of 93 and 96%). hMPV phylogeny studies also suggest that the virus sequences within subgroups A and B can be further divided into two further clades per subgroup. Studies using experimental infection of animals and reciprocal cross-neutralization studies suggest, for the most part, that the viruses of varying subgroups all fall within one serotype.

Serologic Tests

Serology is not used for clinical diagnosis of acute infections, because diagnosis requires a comparison of acute- and convalescent-phase titers. Most of the serologic tests for evidence of previous infection have used relatively crude methods to date. Most reports used microtiter plate-based enzyme-linked immunosorbent assays coated with virus-infected cell lysates or fixed and permeabilized infected cell monolayers as the viral antigen, which are susceptible to high-background reading and false-positive tests. A recombinant viral protein-based assay was reported recently, which holds promise as a more reproducible assay (65). Serum neutralizing antibody assays have been used to demonstrate the induction of functional antibodies in both humans and experimentally infected animals. Most of these tests have used reduction of virus plaques or CPE as the readout; however, a more objective microneutralization test using a recombinant hMPV expressing green fluorescent protein has been reported recently (13).

Antiviral Susceptibilities

There are no licensed therapies or prophylactic treatments for hMPV infection at this time. Ribavirin and intravenous immunoglobulin, which have activity against RSV, were tested against hMPV in vitro and were found to have equivalent antiviral activity against hMPV and RSV (150). Heparin, and the sulfated sialyl lipid (NMSO3) also have been shown to have activity against hMPV (151), and a low-molecular-weight benzimidazole derivative that inhibits hMPV has been reported (6). None of these inhibitory treatments has been tested clinically.

Evaluation, Interpretation, and Reporting of Results

There are no FDA-approved tests for detection of hMPV, and diagnostic tests should be considered experimental at this time. Viral detection is available in a limited number of research and clinical laboratories, although the primers and probes for molecular testing are available in the literature and more are being developed all the time. Culture requires a very high level of technical experience, and even most research laboratories grow the virus with difficulty, especially from primary specimens. Diagnostic tests are of utility mostly in studies of epidemiology at this time. Because the average duration of shedding during infection is not defined and the role of coinfection in disease expression is not well understood, the clinical interpretation of a positive molecular test is difficult. In general, the diagnosis of hMPV infection is most likely when a positive NAA test for hMPV infection

with a replicon containing an hMPV nucleotide sequence is identified in a respiratory secretion during late winter or early spring in a patient with acute respiratory illness and negative tests for other respiratory viruses.

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Measles and Rubella Viruses

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Clinical differentiation of fever and rash illnesses caused by measles and rubella viruses has become increasingly difficult. The paucity of measles and rubella cases in the United States and other developed countries has led to a decline in clinical diagnostic acumen among physicians and health care workers (52, 86). Milder forms of measles have been reported to occur in previously vaccinated individuals (34), further obscuring diagnosis based on the clinical case definition. As global programs to control and eliminate measles expand, the medical and public health communities have become more dependent on laboratory confirmation of clinical diagnosis. Moreover, many countries where measles has been controlled by routine vaccination, coupled with mass vaccination and follow-up campaign strategies, are now implementing rubella control measures through the incorporation of rubella and measles combined vaccines in their programs. Laboratory diagnostic tests and laboratory surveillance activities for measles and rubella are performed in parallel in many clinical and public health laboratories worldwide (20). Consequently, this chapter combines the current laboratory diagnostic methods for measles and rubella for convenient review and reference.

MEASLES VIRUS

Taxonomy

Measles is the prototypic member of the genus *Morbillivirus* in the family *Paramyxoviridae*, and it is the only member of the genus that causes human disease (91). With rare exception, the members of this genus have restricted host ranges, indicative of long-term association with, and adaptation to, their respective zoonotic hosts. Other genus members include rinderpest virus, canine distemper virus, peste des petits ruminants virus, and cetacean and phocine distemper viruses.

Description of the Agent

Measles virus is an enveloped, nonsegmented, single-stranded, negative-sense RNA virus with a diameter of 120 to 250 nm. The measles virus genome is 15,894 nucleotides in length and contains six structural genes organized on the single strand of RNA in a gene order consistent with most of the paramyxoviruses, i.e., 3′-N, P, M, F, H, L-5′. The genome encodes at least eight proteins; three gene products are coded for by the phosphoprotein (P) cistron. The nucleoprotein (N) gene encodes the N protein, which encapsidates both full-length minus-strand (genome) and full-length plus-strand (antigenome) RNAs. Only encapsidated full-length RNAs (nucleocapsids) serve as templates for replication and transcription. The protein products of the P and the polymerase (L) genes interact with the full-length viral RNA and form the ribonucleoprotein complex. Viral nucleocapsid structures are surrounded by a membrane, derived from the plasma membrane, which includes the gene products from the M, F, and H genes (matrix, fusion, and hemagglutinin proteins, respectively). The F and H envelope proteins are N-linked transmembrane glycoproteins. In contrast, the nonglycosylated M protein is associated with only the innermost regions of the lipid envelope and appears to be involved in enhancing and organizing regions of the plasma membrane for nucleocapsid binding and promoting viral assembly and budding processes. The F and H proteins are responsible for fusion of virus with host cell membranes, allowing viral penetration and release of the nucleocapsid complexes into the cytoplasm, where replication occurs (44, 91).

Two receptors have been identified on the surfaces of measles virus-susceptible cells. The CD46 molecule, a regulator of complement activity present on most cell types, appears to promote the entry of vaccine and certain wild-type viruses (66). A second receptor, signaling lymphocyte activation molecule (SLAM, CDw150), has been identified on the surfaces of human lymphocytes, dendritic macrophages, and activated monocytes (98). In contrast to CD46, both attenuated and wild-type measles viruses reportedly use the SLAM receptor for entry.

Epidemiology and Transmission

Measles virus infections are transmitted via aerosols, droplets, or contaminated fomites. Measles is a highly contagious disease, and susceptible individuals who come into close contact with measles patients have a 99% probability of acquiring the disease. In the prevaccine era, greater than 90% of individuals would acquire measles infections before 10 years of age.

In unvaccinated populations, measles causes periodic epidemics, with interepidemic periods of 2 to 5 years. These periods decrease as population size and density increase and are directly related to the availability of susceptible individuals for sustained disease transmission.

In vaccinated populations, the interval between measles outbreaks increases, and sufficiently high levels of vaccination can interrupt endemic transmission. With vaccination,

the age distribution of cases is determined by which groups are likely to lack vaccine- or measles-induced immunity. In the United States, an extensive effort to achieve high levels of first-dose measles vaccination at 12 to 15 months of age and the addition of a recommended second dose of vaccine in school-age children have resulted in a decrease in reported measles cases from 400,000 to 500,000 per year in the 1960s to record lows of approximately 100 cases per year from 1998 to the present (18). Endemic transmission of measles in the United States was interrupted in 1994, and subsequent cases have occurred through periodic introduction of measles from countries where measles is endemic, occasionally followed by only limited spread (7, 89). Given widespread international travel, measles will continue to occur even in highly vaccinated populations until global eradication is achieved.

Measles remains a formidable disease of children in many areas of the world. The World Health Organization (WHO) estimates that over 30 million cases occur annually, with approximately 500,000 deaths occurring in Africa alone and a total of 777,000 associated deaths occurring worldwide (110). In marked contrast, effective use of available live attenuated measles vaccines and a variety of multidose vaccination strategies (second opportunities) have combined to eliminate measles in many large geographic regions, including the Americas, many Scandinavian countries, and the United Kingdom.

Clinical Significance

Uncomplicated Clinical Course

Approximately 1 week to 10 days following exposure, the clinical presentation begins with cough, coryza, conjunctivitis, and fever. The prodromal stage then progresses over the next 3 to 4 days, with all symptoms intensifying and the associated fever reaching as high as 105°F.

Koplik's spots, pathognomonic for measles, appear on the buccal mucosa in 50 to 90% of cases 2 to 3 days before rash onset, and may persist for 1 to 2 days following rash onset. These lesions are small, irregular red spots with a bluish white speck in the center. The erythematous rash appears approximately 2 weeks following exposure and is first evident on the forehead or behind the ears. The rash presents as red macules 1 to 2 mm in diameter, becoming maculopapules over the next 3 days. The exanthem is usually most confluent on the face and upper body and initially blanches on pressure. By the end of the second day the trunk and upper extremities are covered with rash, and by the third day the lower extremities are affected. The rash resolves in the same sequence, first disappearing from the face and neck. The lesions turn brown and persist for 7 to 10 days and then are followed by a fine desquamation. In most cases, recovery is rapid and complete.

Death resulting from respiratory and neurological causes (see below) occurs in 1 of every 1,000 measles cases, but estimates during the 1989 to 1991 outbreaks were three to four times higher (4). The risk of death is greater for infants and adults than for children and adolescents. However, rates of acute measles virus infections of infants and children in developing countries, particularly malnourished populations, can approach 10%, with rates of morbidity being much higher.

Complications

The most common complications associated with measles virus infection are otitis media (7 to 9%), pneumonia (1 to 6%), and diarrhea (6%). Pneumonia may occur as a primary viral pneumonia (Hecht pneumonia) or as a bacterial superinfection. Measles commonly involves the central nervous system (CNS), with as many as 50% of cases reported to have electroencephalogram abnormalities during the acute or convalescent phase of the illness (42).

Notable CNS complications include acute disseminated encephalomyelitis (ADEM) and subacute sclerosing panencephalitis (SSPE). ADEM occurs approximately 1 week after rash onset in 1 per 1,000 cases and is manifested clinically by seizures, lethargy, irritability, and/or coma. It results in the death of 5 to 30% of patients and causes residual deficits in about 30% of survivors (15, 60). The disease is presently considered an autoimmune disease since an immune response to myelin basic protein has been reported and measles virus has not been isolated from these patients (55).

SSPE is a progressive, inevitably fatal, late neurological complication caused by persistent measles virus infection of the CNS that occurs in approximately 1 per 100,000 measles cases (63), although recent studies estimate the rate to be 1 per 11,000 cases (8). Only wild-type measles virus nucleic acid sequences have been found in association with this disease. The average time from natural measles virus infection to manifestations of SSPE is 7 years. The disease characteristically involves personality changes, decreased motor and intellectual capabilities, involuntary movements, and muscular rigidity and ultimately leads to death of the patient. The virus is difficult to recover from brain specimens, requiring cocultivation or brain explant techniques, yet measles virus proteins and RNA can readily be detected in brain tissue (58, 94). Patients with SSPE usually have high titers of measles virus-specific antibodies in their sera and cerebrospinal fluid.

Infection during pregnancy is associated with an increased risk of miscarriage and prematurity, although there is no convincing evidence that maternal infection with measles virus is associated with congenital malformations. Clinical illness in the newborn after intrauterine exposure follows a shortened incubation period and may vary from mild to severe.

Atypical measles syndrome occurred in children who had been vaccinated with multiple doses of formalin-killed measles vaccine and subsequently exposed to wild-type measles virus. The syndrome was believed to occur as a result of immunopathological responses due to a combination of the Arthus reaction and delayed hypersensitivity (12). The syndrome has little relevance today, but the experience serves to emphasize the safety concerns that should be addressed by those wishing to develop alternative measles vaccines or to explore alternate routes of vaccine delivery.

Recently the use of combined, live attenuated measles-mumps-rubella (MMR) vaccines has been the subject of controversy. Several reports have claimed an association between vaccination with MMR and inflammatory bowel disorder, Crohn's disease, and ileal lymphonodular hyperplasia associated with regressive autism (104, 106). Thus far, reviews conducted by expert panels assembled by the Institute of Medicine, as well as several carefully controlled epidemiological and laboratory studies, have failed to confirm those reports (99). Other described complications include thrombocytopenia, stomatitis, laryngotracheobronchitis, hepatitis, appendicitis and ileocolitis, pericarditis and myocarditis, glomerulonephritis, hypocalcemia, and Stevens-Johnson syndrome.

In immunocompromised patients, syndromes such as giant-cell pneumonia and measles inclusion body encephalitis (MIBE) have been observed (10, 65). Measles-induced giant-cell pneumonia is usually unrecognized due to the absence of rash. It usually occurs in patients with deficits in cell-mediated immunity, and several cases have been diagnosed following vaccination of children with severe combined immunodeficiency syndrome (64). MIBE is generally fatal and is best

described as the unchecked replication of measles virus in the CNS in the face of an impaired or absent cell-mediated immune response. There are anecdotal reports of more severe and even fatal measles in patients with AIDS (2). However, vaccination is considered safe unless patients are severely immunocompromised.

Collection, Transport, and Storage of Specimens

In general, specimens for successful virus isolation should be collected early in the acute phase of the infection, when the virus is present in high concentrations, and transported to a laboratory under conditions that maintain the infectivity of labile viruses. Suitable samples for isolation of measles virus or for detection of viral antigen can be whole blood, serum, throat and nasopharyngeal secretions, urine, and, in special circumstances, brain and skin biopsy samples. Specimens should be processed as soon as possible after collection and are best kept at 4°C rather than frozen, since freezing causes significant loss of recoverable virus. Specimens should be frozen and shipped with dry ice only if the time between specimen collection and delivery to the laboratory is expected to exceed 48 h.

Transport medium should be used to maintain measles virus until isolation of virus from clinical specimens can be performed. Samples requiring viral transport medium and commercial sources of viral transport medium are reviewed in chapter 80. Measles virus is lymphotropic, and macrophages are a known source of infectious virus during natural infection (38). Thus, peripheral blood mononuclear cells (PBMCs) are an excellent source for the isolation of measles virus. PBMCs are obtained from heparinized blood (diluted 1:3 in saline) by sedimentation through density gradients (reviewed in chapter 80).

Serum specimens from patients with suspected measles should be assayed in an immunoglobulin M (IgM) enzyme-linked immunoassay (EIA) within 7 to 10 days of rash onset, if possible. A single serum specimen is sufficient in most cases (102). To assess seroconversion following measles vaccination, serum specimens can be tested with IgG EIAs or plaque reduction neutralization (PRN) tests (see below). Ideally, paired specimens should be obtained: the first prior to vaccination and the second approximately 3 to 4 weeks later, so that a rise in measles virus-specific IgG can be measured. Serum samples should ideally be stored at −20°C, but antibody is stable for extended periods at 4°C. Samples for IgM determinations should not be frozen and thawed more than five times. Spinal fluid samples should also be obtained if neurological complications are present or suspected.

Respiratory specimens are appropriate for testing. Nasal aspirates or bronchial lavage samples yield virus more frequently than throat swabs, because of the greater likelihood of obtaining infected cells. For immunofluorescence assays (IFAs), slides of respiratory specimens should be fixed in cold acetone for 2 min and stored at 4°C.

For isolation of virus from urine, a large volume of midstream (clean-catch), preferably morning, urine should be collected into a sterile container. Measles virus is very cell associated; thus, the urine is centrifuged at 800 × *g* for 30 min. The pelleted cells and sediment are resuspended in 1 to 2 ml of Hanks' balanced salt solution in preparation for cell culture, PCR, or other diagnostic methods (88).

Two new approaches show promise in terms of alternatives to serum specimen collection for the diagnosis of measles: the use of oral fluid and the use of blood spots on filter paper. Oral fluid samples have been used both for detection of measles virus-specific IgM and IgG and for detection of hepatitis, rubella, mumps, and other infections (46, 72, 73, 75). This technique is already being used for routine measles surveillance in the United Kingdom (13). The use of oral fluid samples has appeal because the technique is noninvasive, it can be used for rubella testing, and it does not require processing of the sample in the field, and it may be possible to detect not only measles virus-specific IgM but also the measles virus genome for molecular characterization (54). Commercial assays performed on oral fluid samples have heightened background reactivity and decreased sensitivity. These disadvantages make oral fluids less useful in settings with low measles incidence.

Like oral fluid samples, blood spots collected onto filter paper do not require processing in the field, a cold chain does not appear necessary, they can be used to test for rubella as well as for measles (24, 25, 32, 47, 78, 90, 105), and they can be used for molecular characterization of the measles virus genome (33), although virus isolation would not be possible. In addition, the eluted serum from blood spots can likely be tested in commercially available measles EIAs without the loss of sensitivity and specificity (33, 47), making this technology very attractive for widespread use. Although still considered an invasive technique, fingerstick as a method for sample collection is often more acceptable to parents than phlebotomy (107).

Direct Examination

Cytologic Examination

Characteristic cytopathic effects (CPE) of measles virus infection include giant cells and cellular inclusions (intracytoplasmic and intranuclear). Cytologic examination of various tissue specimens and secretions for these Warthin-Finkeldey giant cells can be used as a diagnostic procedure. Slides can be stained with either Wright stain or hematoxylin and eosin (H+E). Tissue samples may be fixed in 10% formalin, embedded in paraffin, sectioned, and then stained with H+E (41). Staining of tissue specimens with monoclonal antibodies to the measles virus nucleoprotein has been used for the diagnosis of giant-cell pneumonia and MIBE (112).

Immunofluorescence

Detection of measles virus can be achieved using an IFA to examine clinical specimens as well as B95a cell cultures infected with clinical material. The standard assay uses a commercially available monoclonal antibody to the nucleoprotein of measles virus and fluorescein-conjugated goat anti-mouse antiserum. Nasal secretions should be centrifuged at 800 × *g* to pellet the cells. The cell pellets are then washed several times with sterile saline before being applied to a glass slide and fixed in cold 80% acetone for 10 min at −20°C (62, 96).

Nucleic Acid Detection Techniques

Reverse transcriptase PCR (RT-PCR) has been used in research settings to detect measles virus RNA in clinical specimens and infected cells (84, 88, 93). While serologic testing is best for routine diagnosis of measles virus infections, these assays do not provide any information about the genetic characteristics (genotype) of the virus required for molecular surveillance. Therefore, RT-PCR can be considered for diagnostic use in special cases where cell culture is not a practical alternative and/or when genotyping of the virus is required. In this regard, RT-PCR from tissue specimens has been particularly useful for the diagnosis of MIBE, SSPE, and giant-cell pneumonia. Sequence data obtained from the PCR products can sometimes identify the source of the virus and differentiate between vaccine and wild-type strains of measles virus (87). RT-PCR techniques have had the greatest impact on

molecular surveillance activities. Measles virus RNA is easily extracted and amplified from infected cell cultures, and measles virus RNA has been detected in oral fluid, nasal, urinary, and, sometimes, serum samples by RT-PCR, even when virus isolation has been unsuccessful (64, 84, 88).

Isolation of Measles Virus

Measles virus can be isolated from the conjunctiva, nasopharynx, and blood during the latter part of the prodromal period and during the early stages of rash development. Although virus has been isolated from the urine as late as 4 to 7 days after rash onset, viremia generally clears 2 to 3 days after rash onset in parallel with the appearance of antibody. Thus, virus can be most readily isolated from 2 to 4 days prior to rash onset, and then about 4 days after rash onset.

Though other cell lines have traditionally been used (44), an Epstein-Barr virus-transformed B lymphoblastoid cell line derived from marmoset lymphocytes is the preferred cell line for primary isolation of measles virus (59). These cells are up to 10,000 times more sensitive for isolation of measles virus from clinical specimens than other cell lines, such as Vero cells and primary monkey kidney cells. An adherent cell population was selected from the lymphoblastoid line B95–8. The adherent cell line was renamed B95a. B95a cells are relatively easy to maintain in the laboratory, and the measles virus CPE is readily observed. However, laboratorians should note that this cell line does produce Epstein-Barr virus and should be handled as infectious material at all times.

B95a cells should be at 85% confluency when inoculated with specimens. After inoculation with clinical material, the cells should be maintained in Dulbecco's modified Eagle's medium (DMEM) plus 2% fetal bovine serum and antibiotics. The medium should be changed every 3 to 5 days, and the cells should be passaged (proceeds divided equally into two flasks) every 5 to 7 days. The medium should be replenished until the CPE become extensive (Fig. 1). When CPE is visible over at least 75% of the monolayer, viral stocks can be prepared by freezing the cells at −70°C. It is advisable to perform three or four blind passages before discontinuing efforts at viral isolation. Recently, Vero cells that constitutively express the SLAM receptor have been constructed, and they appear to have the same sensitivity for wild-type measles virus infection as B95a cells (70). Vero-SLAM cells also express the simian CD46 molecule and have the advantage that they do not produce Epstein-Barr virus.

Serologic Diagnosis

The recommended laboratory method for the confirmation of clinically diagnosed measles is a serum-based IgM EIA. Several serum-based IgM EIAs are now commercially available and are used worldwide by public health, clinical, and commercial laboratories. These assays have been used as the primary confirmatory test by the laboratory network of the Pan American Health Organization-supported measles eradication effort throughout the Americas. The WHO Global Measles Laboratory Network has also recommended use of the IgM EIA for laboratory diagnosis of measles (20). The EIAs can be done using a single serum specimen, are relatively rapid (2 to 6 h), are simple to perform, and can be used to diagnose acute measles virus infection from the time of rash onset until at least 4 weeks after rash onset. Thus, the IgM EIAs fulfill all of the basic criteria for the accurate, effective, and efficient diagnosis of measles. Both indirect and IgM capture formats have been used (36, 51). Though the IgM capture assay format is often regarded as more sensitive than the indirect format, comparative studies of some commercial indirect formatted EIA kits have demonstrated that the two formats have equivalent sensitivities and specificities (3, 81).

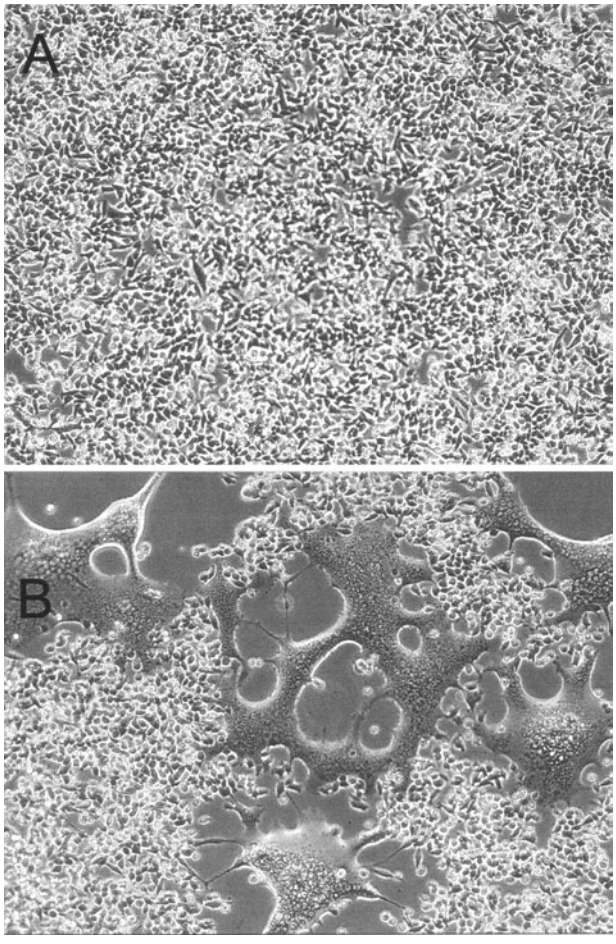


FIGURE 1 CPE of measles virus when propagated in B95a cells, a marmoset monkey lymphoblastoid cell line. (A) Uninfected monolayer of B95a cells. (B) Measles virus-infected monolayer of B95a cells with apparent syncytium formation. Reprinted from reference 7a.

Traditional antibody tests such as hemagglutination inhibition (HI), PRN, and EIA have been used extensively in the serologic diagnosis of measles. However, because of the availability of sensitive and specific commercial kits, EIA has become the most widely used test format. Commercial EIAs also have the ability to measure measles virus-specific IgM as well as IgG responses and therefore have particular importance in diagnosis as well as measles control programs. Some of the available kits were found to have sensitivities and specificities that compared favorably with PRN (30, 80). Most laboratories have trained personnel and are already equipped to run EIAs.

Standard EIAs

Many commercial assays now include measles virus antigens produced by recombinant DNA expression systems, such as baculovirus, rather than antigen produced by infected cell cultures. EIAs using baculovirus-expressed nucleoprotein in both capture and indirect formats have high sensitivity and specificity compared to those of other commercial EIAs (51). The advantages of the baculovirus expression system include extremely high yield and more consistent antigen quality.

Though the configurations of the commercial measles virus IgM kits vary, the tests are simple and straightforward to perform by following the manufacturers' protocols. Indirect EIA has been successful in the detection of IgG (51). For this indirect test, either whole virus antigen diluted in 0.05 M bicarbonate buffer (pH 9.5) or recombinant antigen diluted in phosphate-buffered saline (PBS) is distributed into polystyrene microtiter plates. Serum specimens are diluted in PBS containing 4% normal goat serum (plus 4% Sf9 cell lysate for recombinant antigen) and 0.05% EDTA and then added to the washed plates. Bound antibody can be detected with standard commercial reagents such as goat anti-human IgG conjugated to either alkaline phosphatase or biotin. Assays are developed with the appropriate substrate, and the plates can be read either by eye or with the aid of a spectrophotometer.

PRN

The PRN assay, which measures neutralizing antibodies that are directed against the surface glycoproteins of measles virus, is more sensitive and specific than HI or EIA (1). Since functional antibodies are detected, the PRN provides the best serologic correlate for the assessment of immune protection (22). However, the PRN test is not suitable for routine serologic diagnosis because it is very labor-intensive, requires paired serum samples, and takes 5 to 7 days to complete.

In the PRN assay, measles virus-specific antibody in serum combines with and neutralizes measles virus, preventing it from infecting a cell monolayer and forming a plaque under the overlay. The endpoint for the test is the highest dilution of serum which will reduce the number of plaques by 50%. Serum dilutions are made in 96-well microtiter trays, making either two- or fourfold dilutions (depending on the expected titer of the serum). Once the dilutions of serum samples are made, an equal volume (120 μl) of a dilution of virus containing 25 to 35 PFU is added to each well and incubated for 2.5 h at 36°C. After incubation, 100 μl of the serum-virus mixture is added to each of two 16-mm-diameter wells on a tissue culture plate(s) containing Vero cell monolayers. These plates are then incubated for 1 h at 36°C. Following incubation, the inoculum is removed by aspiration, and the monolayers are covered with overlay medium consisting of either 2% carboxymethyl cellulose in Leibovitz-15 medium or 1% agarose in Eagle's medium. The plates are then incubated for 4 days at 36°C, and the monolayers are stained with a solution of neutral red in cell culture medium. On day 5, the overlay is removed and the plaques are counted. The numbers of PFU in duplicate wells representing a given serum dilution are averaged.

New EIAs

A new and potentially promising development has been the use of EIAs to measure the avidity of IgG antibodies to measles virus (31, 103). As the immune response matures, low-avidity antibodies are replaced with high-avidity antibodies. These avidity differences can be detected by using protein denaturants, 6 to 8 M urea, in the washing step of the indirect EIA for measles virus IgG. An avidity index is then calculated by comparing the optical densities obtained with and without urea in the wash buffer. These tests have been able to successfully differentiate between primary and secondary responses to vaccination and to natural infection (74).

Evaluation, Interpretation, and Reporting of Results

EIA Interpretation

Although the EIAs are very good, they are nonetheless imperfect. These imperfections are manifested in both the positive and negative predictive values of the tests, and they are particularly apparent as the prevalence of measles disease decreases in the population. The vast majority of false-negative results stem from testing serum specimens taken too early with respect to rash onset. For example, the CDC capture IgM assay, which has a sensitivity and specificity equivalent to those of commercial EIAs, has been shown to detect IgM in only 77% of true measles cases within the first day of rash onset (45). In the case of indirect IgM assays, false-negative values can be the result of insufficient removal of high levels of measles virus-specific IgG from a test specimen. The residual IgG competes with IgM for viral antigen placed on the solid-phase support, thereby blocking IgM binding to the antigen and interfering with IgM detection. Current-day immune absorbent reagents are much improved over earlier reagents; thus, fewer problems of this nature occur.

False-positive tests due to the presence of rheumatoid factor (RF) appear to be more frequently encountered when using the indirect IgM assay. RF is an IgM class immunoglobulin that reacts with IgG and is produced as a result of some viral diseases and autoimmune diseases. Immune complexes may form that contain test antigen-specific IgG and RF IgM. By virtue of the IgG binding to the viral antigen, the IgM component of the RF immune complex is recognized by the detector system, thus rendering a falsely positive result. Similar false-positive results can occur in capture assays but appear to be enhanced by the presence of high levels of both antigen-specific antibody and RF (36).

The inherent sensitivities and specificities of the EIAs in times of low disease prevalence become a factor in interpretation. For instance, in a setting where measles has a prevalence of 50%, if the sensitivity of the EIA is 90% and the specificity is 99%, the positive predictive value is extremely high (99%). However, if the prevalence of measles drops to 1%, the positive predictive value falls to 48%. Moreover, a relatively small reduction in the specificity of the assay can have major effects on the positive predictive value of the assay. A reduction of specificity from 99 to 95%, when measles prevalence is 1%, decreases the positive predictive value from 48 to 15%. In other words, 85% of the positive test results would be "false-positive" results, even in the context of a highly specific assay. This situation occurs in countries that have eliminated endemic measles but remain vigilant in performing case-based surveillance of rash illnesses. In these geographic regions, both measles IgM and IgG EIAs are performed on serum specimens from suspected cases due to high vaccine-induced seroprevalence. In this setting, if the IgG test is positive and the IgM test is negative, the case is discarded (depending upon the timing of serum collection).

Unfortunately, EIA specificity is not 100%. Serum specimens from patients with parvovirus B19 and rubella have inherent rates of false-positive reactions (overall rate of about 4%) when tested in measles IgM EIAs and vice versa (53, 100). The reason(s) for these false-positive reactions is not understood, and attempts to remove immune complexes using anti-RF have proven unsuccessful.

There is also evidence that case contacts that have a resident IgG response, due either to a history of natural infection or to vaccination, may develop a secondary IgG or an IgM response to currently circulating virus (37, 48). The IgM is generally fleeting and weakly positive and, except for rare instances, should not pose as a source of confusion to the diagnostic laboratory.

Table 1 summarizes the possible interpretations of EIA results. Despite the vagaries associated with the EIAs, they are the best assays available for laboratory confirmation of

TABLE 1 Interpretation of measles EIA results[a]

IgM result	IgG result	Infection history	Current infection	Comment(s)
+	+ or −	Not previously vaccinated, no history of measles	Recent first MCV[b]	Seroconversion,[c] postvaccination
+	+ or −	Not vaccinated, no history of measles	Wild-type measles virus	Seroconversion,[c] classic measles
+	+ or −	Previously vaccinated, primary vaccine failure	Recent second MCV vaccination	Seroconversion,[c] postvaccination
−	+	Previously vaccinated, IgG positive	Recent second MCV vaccination	IgG level may stay the same or increase
+	+	Previously vaccinated, IgG positive	Wild-type measles virus	May have few or no symptoms[d]
+	+	Recently vaccinated	Exposed to wild-type measles virus	Cannot distinguish if vaccine or wild-type virus infection; evaluate on epidemiological grounds[e]
+ or −	+	Distant history of measles	Wild-type measles virus	May have few or no symptoms[d]; if clinically compatible may have been misdiagnosed initially

[a] Reprinted from reference 7a.
[b] MCV, measles virus-containing vaccine.
[c] IgG level depends on timing of specimen collection.
[d] Rare occurrence; do not consider contagious unless clinical presentation is consistent with measles.
[e] If result is IgM negative, it is helpful to rule out wild-type measles virus infection.

clinically diagnosed measles. It should be emphasized that the vast majority of serum specimens submitted for serology will yield a test result that will be easily interpretable. However, laboratorians should be provided with as much information as possible about the clinical disease and epidemiological investigation to aid in the final interpretation of the test result.

RUBELLA VIRUS

Taxonomy

A number of small, enveloped viruses having the same overall genetic organization and replication strategy as rubella virus exist, and they are grouped into the *Togaviridae* family. The *Togaviridae* family consists of the *Rubivirus* genus, containing only rubella virus, and the *Alphavirus* genus, containing about 25 other viruses, all of which are transmitted by arthropods (e.g., western equine encephalitis virus). Rubella virus has a restricted host range and appears to infect only humans.

Description of the Agent

Rubella virus virions are particles about 70 nm in diameter that are comprised of a core surrounded by a lipid envelope. The core consists of the positive-strand genome (~10,000 nucleotides) and the virus protein C. The viral envelope contains two viral glycoproteins, E1 and E2. The viral RNA replicates in the cytoplasm of infected cells, with nonstructural proteins being translated from the 5′ two-thirds of the genomic RNA and the structural proteins being translated from a subgenomic RNA that is a copy of the 3′ one-third of the genomic RNA. New virions are produced when genomic RNA, the E1 glycoprotein, the E2 glycoprotein, and the C protein assemble at cellular membranes (21, 85).

Compared to many RNA viruses, the rubella viral RNA is highly conserved. Nevertheless, rubella viruses currently circulating in the world contain RNAs that differ sufficiently that two clades of rubella viruses, differing by about 10% in the nucleotide sequence, have so far been identified (40, 56). Groups of related viruses within the clades have been classified as genotypes. At present, seven genotypes and three provisional genotypes of rubella viruses have been recognized (111). Only minor immunologic differences exist among circulating viruses. Immunity to one rubella virus is sufficient to protect against other known rubella viruses.

Clinical Significance

Rubella (German measles or 3-day measles) was first described in the 18th century and was accepted as a disease independent of measles and scarlet fever in 1881 (26). Postnatal rubella is characterized by an acute onset and generalized maculopapular rash with mild fever (greater than 99°F) and may include arthritis or arthralgia (mostly in postpubertal females), lymphadenopathy (specifically postauricular and suboccipital lymphadenopathy), and conjunctivitis. Because disease caused by rubella virus is mild, about 50% of postnatal rubella cases are not diagnosed. Although rubella had been largely ignored for 60 years, N. McAlister Gregg first recognized that cataracts in children followed maternal rubella during gestation (43). The association between congenital rubella and a spectrum of significant birth defects including sensorineural hearing loss, cardiovascular abnormalities, cataracts, congenital glaucoma, and meningoencephalitis is now accepted. Rubella virus is now recognized as the most potent infectious teratogenic agent yet identified (61, 92, 108).

When rubella occurs in a pregnant woman in the first 11 weeks of gestation, there is a high likelihood of defects in the infant (about 90%). After 18 weeks, the likelihood of birth defect is much lower, although the infant may still be infected with rubella. Congenital rubella infection (CRI) refers to infants born with rubella virus infection with or without birth defects. The pathogenesis of CRI leading to congenital rubella syndrome (CRS) is not well understood, but rubella virus infection early in gestation results in an altered immune response to the virus and altered organogenesis. The significant effect on the fetus of rubella virus infection during the first trimester is likely related to the facts that the fetus cannot synthesize IgM until about 20 weeks of gestation and cell-mediated immunity does not develop until about 27 weeks. Congenitally infected infants shed virus for long periods, have a slowly developing immune response to rubella virus, and

respond to particular rubella virus proteins differently than individuals with postnatal rubella. These three characteristics of congenital infection are all consistent with selective immune tolerance to rubella virus proteins in such infants (21). Infection during the first 11 weeks of gestation often results in multiple organ involvement and necrosis in many tissues (61). Organogenesis is affected by rubella virus infection, since specific organs are involved and small but normal organs in CRS infants have a reduced numbers of cells. Reinfection with rubella virus can occur, but viremia is rare, resulting in very low risk to the fetus in a reinfected pregnant woman (5).

CRI results in both shedding of virus and IgM antibodies in the neonate. Diagnosis is based on detection of rubella virus or rubella virus-specific IgM in such patients. If congenital defects characteristic of CRS are not present, the infant is diagnosed as having CRI only. The clinical definition of CRS is standardized, but laboratory confirmation of rubella virus infection in the newborn by either serologic or culture techniques is critical, especially when only a single defect presents, since the defects characteristic of CRS can occur for other reasons (16).

Epidemiology and Transmission

Rubella virus was not isolated until 1962, largely because infected cells are difficult to identify in tissue culture (71, 109). Introduction of rubella vaccine (licensed in 1969) in the United States, mostly through childhood immunization, immediately broke the 6- to 9-year epidemic cycle of rubella. The last major U.S. rubella epidemic was in 1964 to 1965, when 20,000 CRS cases occurred (50). The combined MMR vaccine was recommended for the United States in 1972. The current incidence of CRS in the United States is ~10 CRS cases per year. Most mothers of affected infants were born in countries without rubella immunization programs or with recently organized programs (83). Occasional outbreaks of rubella in some U.S. populations (e.g., foreign-born Hispanics) have not spread to the undervaccinated populations (e.g., those in inner cities), suggesting that herd immunity may be giving a protective effect (82). Indigenous rubella and CRS have been eliminated from the United States, and these diseases are targeted for elimination from the Americas and Europe by 2010 (14, 19) (http://www.euro.who.int/Document/E87772.pdf). Despite the possibility of worldwide rubella eradication, rubella remains endemic in many countries and about 100,000 to 200,000 CRS cases occur annually in the world (6, 28, 77). Postnatal transmission of rubella virus is usually by close contact with an infected individual, such as occurs in correctional institutions or day care centers.

The safety of live rubella virus vaccine strains is well documented (76). None of 293 infants born to susceptible mothers who were inadvertently vaccinated 1 to 2 weeks before to 4 to 6 weeks after conception were born with CRS. However, a small theoretical risk remains. Thus, the Advisory Committee on Immunization Practices recommends avoiding pregnancy after receipt of rubella-containing vaccine for 28 days (17).

Collection, Transport, and Storage of Specimens

Clinical specimens for culture of rubella virus are usually throat swabs or nasopharyngeal secretions diluted into transport medium (e.g., Culturette collection and transport devices). The virus can also be isolated from a number of other specimens, including cataract tissue and urine (provided that pH is controlled) (5). Urine is often a source of infectious virus from CRS patients. Specimens should be stored at 4°C for short periods (days) or at −70°C for longer periods (weeks); virions lose infectivity at higher temperatures (e.g., 37°C). Virions are rapidly inactivated by mild heat (56°C), detergents, or lipid solvents. Rubella virus-specific IgG can be detected in urine (97). Specimens for serology or culture can be transported by standard methods (e.g., overnight carrier) at 4°C since virions are relatively stable at 4°C. Oral fluid/saliva and blood spots dried onto filter paper may be used as alternatives to serum for rubella virus-specific antibody testing as discussed for measles (47, 54, 69, 79).

The timing of specimen collection is important. Rubella virus-specific IgM is the laboratory diagnostic criterion typically used for rubella, but about 50% of rubella cases are IgM negative on the day of rash (see Table 2 and related text below). Since postnatal rubella is a mild disease of short duration, special effort may be required to obtain a serum sample 5 to 7 days after rash, when most rubella patients are strongly IgM positive. Patients with CRS are IgM and virus positive for months; therefore, timing is less critical for these patients.

Direct Examination

Nucleic Acid Detection Techniques

Amplification of rubella virus RNA directly from a clinical specimen using RT-PCR can be used to determine if a patient

TABLE 2 Timing of biological markers of rubella virus infection[a]

Diagnostic criterion	Convenient time when many cases are positive (%)	An example of a time when >90% of cases are positive	Approximate time for 50% decline[b]
Postnatal rubella			
Virus in throat by culture	Day of rash (90%)	2 days **before** rash	4 days after rash
IgM in serum by ELISA	Day of rash (50%)	5 days after rash	6 wk after rash
IgG in serum by ELISA	3 days after rash (50%)	8 days after rash	Lifetime
Virus in blood by culture[c]	Day of rash (50%)	5 days **before** rash	1 day after rash
CRS[d]			
Virus in throat by culture	At birth (almost all)	2 wk after birth	3 mo of age
IgM in serum by ELISA	At birth (80%)	1 mo of age	6 mo of age
IgG in serum by ELISA[e]			

[a] Times and percentages given are approximate and are meant to guide typical specimen collection. Percentages will vary depending on the sensitivity of the assay used. Note that the times listed in the third column were chosen to help guide specimen collection and may not be the earliest time when >90% of cases are positive.
[b] After maximum number of cases are positive for a given criterion, the approximate time for 50% of cases to become negative.
[c] Data taken from reference 29.
[d] Information given is for fetal infection in the first trimester.
[e] Declining maternal IgG and developing IgG response in a CRS patient lead to high (steady) or increasing IgG levels in the CRS patient through the first year of life.

is infected with rubella virus. Not all RT-PCR protocols are sufficiently sensitive to be used directly with clinical specimens. Assays that can reliably detect 3 to 10 copies of rubella virus RNA are sufficiently sensitive. Nested RT-PCR protocols, although difficult to maintain, usually meet this criterion (11, 27). Direct detection of rubella virus RNA by RT-PCR requires the laboratory to evaluate the significance of results from such tests. Multiple negative controls and amplified product in more than one specimen from a given patient will increase confidence of a positive diagnosis based on direct RT-PCR. The significance of negative results is usually difficult to determine since false-negativity rates are usually not available. Nevertheless, when serum from a patient cannot be obtained, or when confirmation of serologic results is desired, direct detection of rubella virus RNA by RT-PCR may be necessary since it is more rapid than viral culture.

Isolation and Identification of the Virus

Growth of rubella virus from clinical specimens can be used to diagnose postnatal rubella, CRS, and CRI (Table 2). Throat swabs taken on the day of rash, typically a convenient time for sample collection, are usually positive for rubella virus, even though a slightly higher percentage of cases are positive 2 days before rash onset. Virus shedding in the throat declines rapidly, and by 4 days after rash onset, only about 50% of cases are positive. In addition, viral culture is used to monitor virus in CRS and CRI patients for the purpose of determining when isolation of these patients from susceptible contacts can be stopped. The virus will grow in a variety of cell types, including Vero, BHK21, AGMK, and RK-13 cells. The primary problem encountered with tissue culture is the lack of a cell type that produces CPE in a single passage of wild-type viruses. Historically, this problem was overcome by clever assays exploiting the fact that rubella virus growth interferes with the replication of lytic enteroviruses such as coxsackievirus A9. However, interference assays are quite difficult to maintain (23). Virus growth can now be identified in the absence of CPE using methods such as RT-PCR and IFA to detect viral RNA and proteins, respectively.

Bacterial and fungal contamination of tissue culture cells is prevented by treating specimens with high concentrations of antibiotics or by filtration through low-retention (pore size, 0.2 μm) filters prior to inoculation. Tissue culture cells (usually Vero) are inoculated with approximately 0.5 ml of the specimen. Virus is allowed to attach to cells for 1 h at 35°C, and then DMEM with about 5% fetal calf serum (FCS) is added. Virus is then allowed to grow for 1 week at 35°C. After 1 week, a portion of the medium from this initial culture is either stored or passaged immediately to another confluent monolayer of cells. The cells from the initial culture are processed for RT-PCR, IFA, or both. Two passages should be performed before a specimen is considered negative.

The sensitivity of the RT-PCR system used to detect viral RNA from infected tissue culture cells is not critical, since the amount of rubella viral RNA has been amplified by passage in tissue culture (about 10^6-fold) (40). Cell-associated RNA is extracted by standard means, cDNA is prepared by standard reverse transcription reaction [typically using oligo(dT) primers], and virus-specific primers are then used to amplify cDNA. Ethidium bromide staining of amplicons after agarose gel electrophoresis can be used for postamplification detection. Sequencing of the resulting amplified nucleic acid can provide useful information (e.g., vaccine or wild-type virus) (39). Detection of rubella virus-infected cell monolayers can also be accomplished by IFA. It is crucial that IFA reagents have low background, since rubella virus culture does not produce high levels of progeny virus (about 10^7 PFU/ml for laboratory strains such as f-Therien). Infected cells are easily identified when stained with high-quality reagents (Fig. 2). Dilution of specimens may be desirable since it is useful to have both infected and uninfected cells in the same field. Monoclonal antibodies to the E1, E2, and C proteins, which react with both reduced and nonreduced antigens on Western blots, often work well in the IFA.

Serologic Tests

ELISA

Detection of rubella virus-specific IgM by either IgM capture enzyme-linked immunosorbent assay (ELISA) or indirect IgM ELISA is the fastest and most cost-effective diagnostic test for recent postnatal infection. Unfortunately, only about 50% of postnatal rubella cases are IgM positive on the day of symptom onset (Table 2). Most postnatal rubella cases have virus-specific IgM detectable by capture ELISA from 5 until 20 days after symptom onset and IgG by indirect ELISA ≥8 days after symptom onset (Fig. 3) (35, 101). A negative IgM serologic result 4 to 5 days after onset should be followed with testing of a serum sample taken as soon as possible thereafter to avoid false-negative results (16). If acute- and convalescent-phase sera are available, a fourfold rise in rubella virus-specific IgG is diagnostic for postnatal rubella infection; such sera should be taken as early as possible after disease onset and about 2 to 3 weeks after disease onset. When IgG titers are used for diagnostic purposes, a dilution series of each serum sample should be made and ELISA results for each dilution series compared, since optical density values at a single dilution are unreliable.

The same ELISAs may be used to confirm CRS. Most congenitally infected infants have IgM detectable from birth to 1 month of age (Table 2). The percentage of infants who are IgM positive declines over the first year of life. At 1 year, most infants are negative. In CRS patients, the IgG response increases gradually over the first 9 months, while maternal IgG declines. Thus, high or increasing IgG levels in the first year of life are consistent with CRS. Precise recommendations for the best times for collection of samples from postnatal rubella and CRS cases have been published (16).

Latex Agglutination

Latex spheres coated with rubella virus antigen can be aggregated by either rubella virus-specific IgG or IgM, and they are the basis for commercially available rapid latex particle agglutination assays. These assays are often used when immunity determination is required (e.g., prenatal screening), since they are fast and easy to perform.

HI

The HI test was once the standard test for antibodies to rubella virus, and many current tests are calibrated using HI assays. However, indicator erythrocytes are not readily available and the assay is difficult to perform since removal of nonspecific inhibitors and internal standardization are required. If HI is used, it is usually desirable to verify results with another assay.

IFA

IFA is an option for detection of IgG and IgM antibodies to rubella virus. Commercial IFAs are available. Typically cells expressing rubella virus proteins and control cells are reacted with test serum and any rubella virus-specific antibodies are then detected with fluorescent dye-labeled goat anti-human IgG (or IgM) and fluorescence microscopy. Negative human

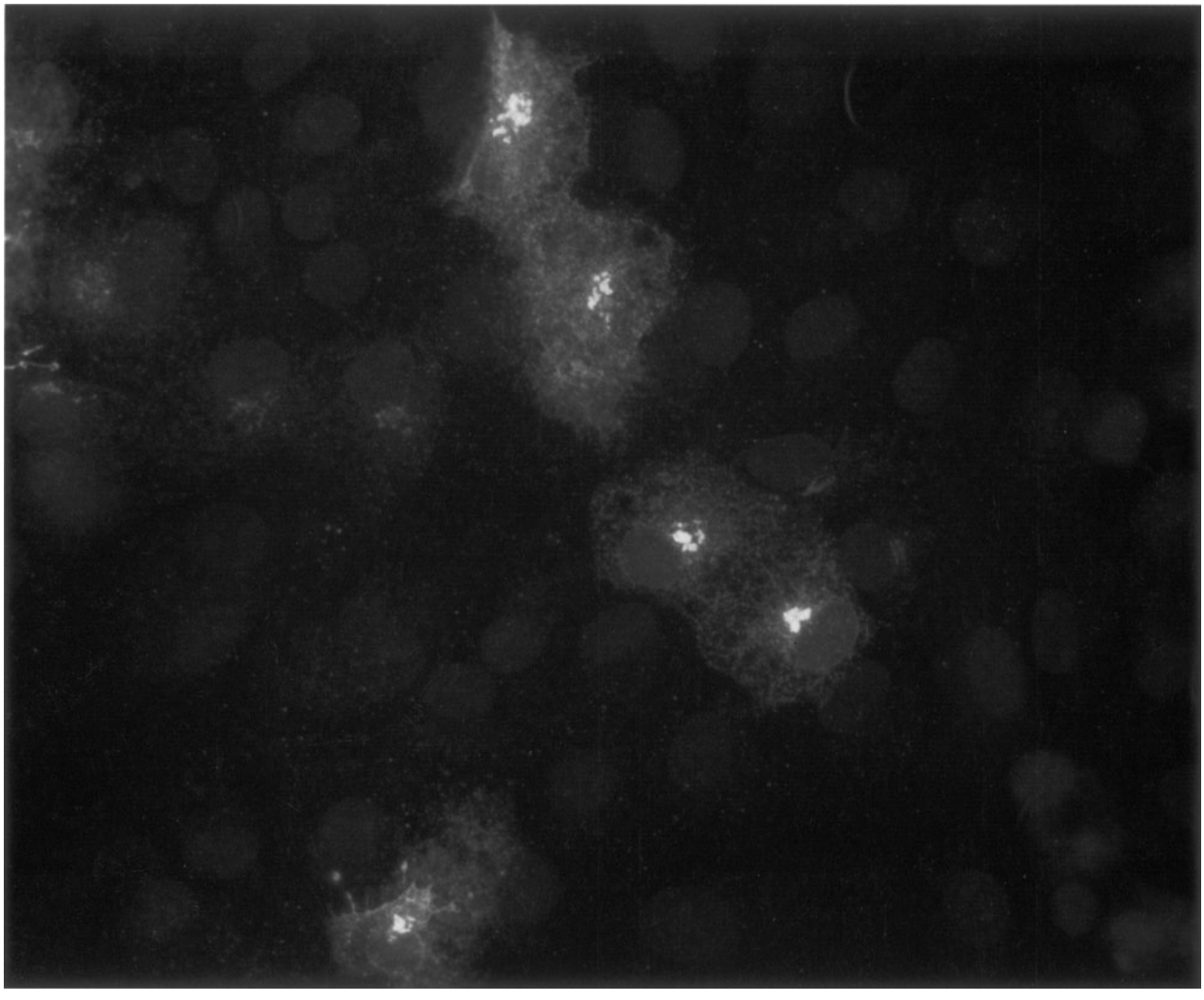


FIGURE 2 IFA for rubella virus-infected cells using monoclonal antibody to the E2 protein. Vero cells were infected with wild-type rubella virus and 3 days after infection were fixed with cold paraformaldehyde followed by permeabilization with –20°C methanol. Reaction of infected cells with monoclonal antibody to the E2 protein (monoclonal antibody 24–26; Viral Antigens, Memphis, Tenn.) was followed by detection of bound monoclonal antibody with Alexa Fluor 488-conjugated, highly cross-absorbed goat anti-mouse IgG (Molecular Probes Inc., Eugene, Oreg.) (green fluorescence in the original photograph). Cell nuclei were visualized by staining with propidium iodide (red fluorescence in the original photograph). About 25% of the cells which stained with propidium iodide were positive for E2 protein.

sera are useful for monitoring nonspecific signal. Fluorescence should be cell associated. Staining restricted to the periphery of the cell monolayer is not indicative of a true-positive result.

PRN

When a quantitative result on the neutralizing capacity of an antiserum is necessary, a PRN assay should be done. A laboratory strain (e.g., f-Therien) should be used since viruses from clinical specimens do not exhibit CPE. The assay follows a format common to many viruses. The initial step is incubation of 2- or 10-fold dilutions of antiserum and a standard amount of rubella virus (usually about 100 to 200 PFU) in medium or buffer containing protein to inhibit losses on surfaces (e.g., 0.1% bovine serum albumin) for 1 h at 35 to 37 °C followed by overnight in a refrigerator. A virus control must be carried through all steps, since some reduction in the number of plaques is observed at 35°C. Virus-antiserum specimens are then allowed to attach to confluent Vero cell monolayers for 1 h at 35°C and then overlaid with DEAE dextran (100 μg/ml)-containing medium with 0.5% agar (e.g., Oxoid). Medium in the agar overlay is typically DMEM; 1% FCS may also be included to maintain the monolayer. After 6 days, agar is removed and plates are stained with neutral red. Crystal violet can also be used; however, a wash step to remove dead cells should be used prior to crystal violet staining. The neutralizing capacity of the antiserum is the inverse of the antiserum dilution giving a standard reduction in plaques, typically 90% reduction.

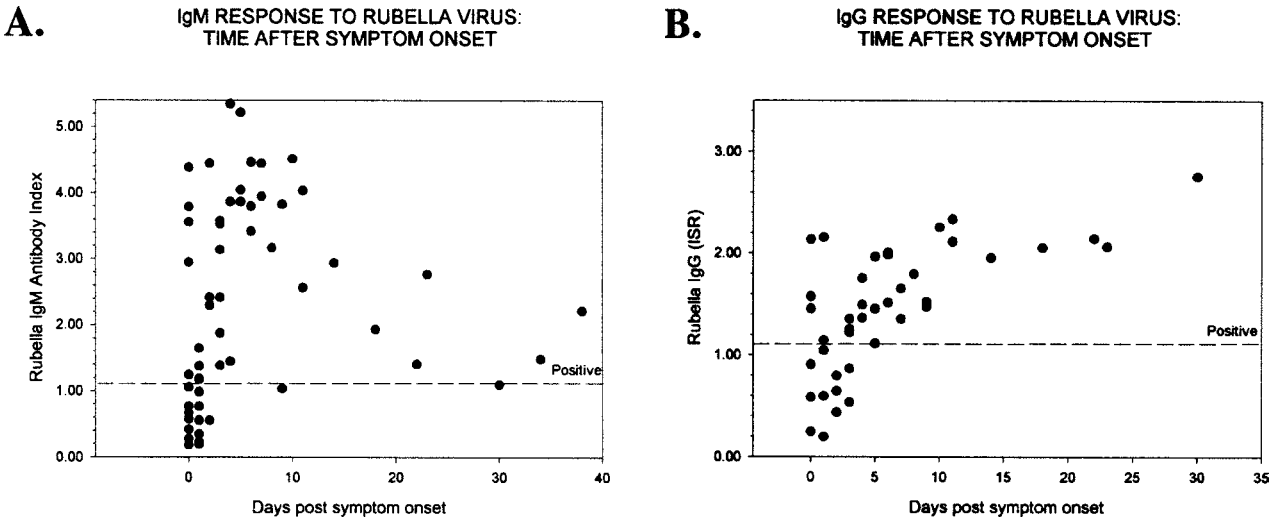


FIGURE 3 Time course of rubella virus-specific IgM and IgG detection by ELISA in sera of rubella patients. Commercial IgM capture ELISA (A) and IgG indirect ELISA (B) were used to detect rubella virus-specific antibodies at the indicated number of days after onset of symptoms (usually rash); antibody index and ISR are the commercial test designations for the ratio of the optical density obtained for the test serum to the optical density obtained for a standard (cutoff) serum. The minimum signal considered positive in each test is indicated by a dashed line. Only results from patients who tested positive for IgM to rubella virus at some time after the onset of symptoms are shown.

Other Serologic Tests

In cases where an IgM serologic diagnosis of recent infection is not reliable (e.g., first serum sample was collected months after clinical symptoms), low avidity of IgG to rubella virus proteins has been used to indicate recent infection (49, 67, 100). This test compares the ability of detergents or chaotropic agents to dissociate case IgG and mature IgG from rubella virus proteins.

Since the clinical symptoms of postnatal rubella and CRS are dramatically different, it is not surprising that there are significant differences in the immune responses of patients with these diseases. In Western blots, samples from CRS patients often have an altered distribution of antibodies to the viral glycoproteins compared to the distribution in samples from postnatal rubella patients (57).

Prenatal Screening

The present description of laboratory testing for rubella emphasizes identification of postnatal and congenital rubella cases. However, in the United States much of the testing is for immunity to rubella, since health care providers should test for immunity by a serum IgG test in all pregnant women at the earliest prenatal visit. There are slightly different criteria for rubella immunity that are recommended by various groups (range is about 10 to 15 IU/ml) (76). Commonly used tests, e.g., ELISA, are standardized to give positive results for 10 IU/ml, the breakpoint defining immunity to rubella in the United States (95). Much of the screening for immunity to rubella virus in the United States is done by automated random-access systems using microparticle immunoassays.

Evaluation, Interpretation, and Reporting of Results

Noncommercial tests must be standardized by the laboratory. Standard operating procedures should be written and followed. All necessary controls should be performed. Laboratories utilizing commercial assays are advised to adopt a quality assurance program that includes both testing with standard sera and data analysis to detect potential changes in assay performance due to defective reagents.

IgM and IgG testing should be done routinely with sera from individual patients, since results from both immunoglobulin classes often provide confirmatory data. For example, a serum sample taken on the day of rash may be negative for both rubella virus-specific IgM and IgG, while one taken 8 days postrash should be positive for both.

There is often a considerable burden on the laboratory in the diagnosis of rubella. For example, when primary rubella virus infection is suspected in a pregnant woman, false positives and false negatives may lead to incorrect clinical decisions (9). However, the laboratorian may be asked to go beyond just communicating false-positivity and false-negativity rates. Testing for recent infection with other viruses that cause clinically similar diseases (e.g., human parvovirus B19) is often prudent. Positive rubella results may be more believable if no other infection is found. Retesting of specimens and testing of multiple specimen types (e.g., serology and viral culture) from the same patient taken at the proper times (Table 2) often give a consistent set of results and reduce the likelihood of false-positive results. False positives can occur even with modern tests such as IgM capture ELISA. For example, in one report, 1 of 87 sera testing positive for rubella virus-specific IgM by IgM capture ELISA was from a patient whose final diagnosis was primary human parvovirus B19 (100). False negatives often can be identified by retesting of the same specimen or testing multiple specimens from a patient (e.g., sera taken 1 week apart). If only a single specimen is available, it may be tested by multiple assays. For example, IgG avidity may resolve the diagnosis from a single serum sample that is IgM positive for both rubella virus and human parvovirus B19 (100).

Postnatal rubella can be clinically similar to other diseases, or it can be asymptomatic. Additionally, birth defects characteristic of CRS occur for other reasons. Thus, correct

classification of suspected postnatal rubella and CRS is based on laboratory results rather than clinical presentation. Classification of a postnatal rubella case results in its categorization as suspected, probable, confirmed, or asymptomatic confirmed; for a case of congenital rubella, categories are suspected CRS, probable CRS, confirmed CRS, or infection only. Positive laboratory results are required to correctly classify asymptomatic confirmed or confirmed cases of postnatal rubella and CRS. Clinical, laboratory, and epidemiological information (e.g., international travel) all may enter into the final clinical decision(s). A full description of classification criteria and recommendations should be consulted (16). One specific diagnostic situation should be noted. A series of tests including a rubella virus IgM test should not be used to determine immunity in a pregnant woman because of the possibility of a falsely IgM-positive test; immunity should be determined by IgG testing alone. Since the TORCH (toxoplasmosis, other, rubella, cytomegalovirus, and herpes simplex virus) panel includes testing for rubella virus IgM, it should not be used to determine immunity.

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Enteroviruses and Parechoviruses*

JOSÉ R. ROMERO

89

TAXONOMY

Members of the genus *Enterovirus* currently officially comprise 64 distinct serotypes (49) within the family *Picornaviridae* (with "pico" meaning small, "rna" indicating an RNA genome, and "viridae" signifying viruses). The traditional taxonomic criteria for the identification of enteroviruses (EVs) to the species level were based on the patterns of replication of the individual serotypes in cell cultures and on disease manifestations in animal systems (25, 26).

These original distinctions have been blurred significantly by the identification of new strains and the development of new cell lines, resulting in crossover EV growth patterns. Additionally, molecular methods for EV typing have replaced traditional serologic methods of serotype assignation (92, 96). EV taxonomy was effectively defined through phylogenetic analysis using the complete coding sequence of VP1, the major enteroviral capsid protein (48, 91, 93). This approach demonstrated that several EVs originally accepted as individual serotypes are likely strains of one another (93). Additionally, echoviruses 22 and 23 have been reclassified into a distinct genus, *Parechovirus* (49), since sequence analysis demonstrated that they are genetically distinct from other members of the genus (47), and they are now designated *Human parechovirus 1* (HPeV 1) and HPeV 2, respectively. The International Committee on Taxonomy of Viruses has issued revised species demarcation criteria for the genus *Enterovirus* (49). Based on these criteria, EVs are now classified into five species (poliovirus and human enteroviruses A through D) based on their molecular and biologic characteristics (Table 1).

The genus *Parechovirus* is comprised of three HPeV serotypes (8, 50, 124). HPeV 1 and 2 were originally identified in 1956. It is likely that, as with the EVs, molecular diagnostics will lead to the identification of additional members of the genus.

DESCRIPTION OF THE AGENTS

Similar to other members of the family *Picornaviridae,* the EVs are small (30-nm diameter in the hydrated state), nonenveloped viruses that possess a single-stranded positive (message)-sense RNA genome. Their buoyant density in cesium chloride is 1.30 to 1.34 g/cm^3 (49, 104). The EVs are stable in acid, ether, and chloroform and are insensitive to nonionic detergents. They are inactivated by heat (>56°C), UV light, chlorination, and formaldehyde. The EVs replicate optimally at 36 to 37°C.

The EVs are quite stable in liquid environments and can survive for many weeks in water, body fluids, and sewage. This is due to a number of virion properties, including thermostability in the presence of divalent cations, acid stability, and the absence of a lipid envelope.

The EV RNA genome is ~7.4 kb long (Fig. 1) and serves as a template for both viral protein translation and RNA replication (104). The genome is organized into a long (~740 nucleotides) noncoding region, designated the 5′ nontranslated region (5′NTR), which immediately precedes a single open reading frame (ORF). The ORF begins at approximately nucleotide 740 from the 5′ end and terminates at approximately nucleotide 7370. The ORF is subdivided into three regions, P1 to P3. The P1 region encodes the four structural proteins (VP1 through VP4) that comprise the viral capsid. These are organized 5′ to 3′ as VP4 (1A), VP2 (1B), VP3 (1C), and VP1 (1D). The P2 and P3 regions encode seven nonstructural proteins (organized 5′ to 3′ as 2A, 2B, 2C, 3A, 3B, 3C, and 3D) that are essential for the viral life cycle. The ORF is translated into a single polyprotein that is posttranslationally cleaved into several functional intermediates and the final 11 proteins by virus-encoded proteases. Immediately downstream of the ORF is a short noncoding region of approximately 70 to 100 bases (3′NTR) and a terminal polyadenylated tail.

The EV 5′and 3′ NTRs play critical roles in the EV life cycle (104). The 5′NTR contains multiple regions of predicted higher-order structure (i.e., stem-loops or domains). A domain located at the extreme 5′ terminus of the 5′NTR is essential for replication of viral RNA. Cap-independent translation of the EV genome is regulated by the internal ribosome entry site, which spans a discontinuous region within the 5′NTR. For polioviruses and several nonpolio enteroviruses (NPEVs), the 5′NTR has been documented to be a determinant of virulence phenotype and cell type specificity (9, 31, 77). Given the crucial nature of the functions controlled by the 5′NTR, it is not surprising that nucleotide sequences with absolute (or near absolute) conservation among the EVs exist within this region (110, 113). These regions of high nucleotide identity have been exploited for the design of

* This chapter contains information presented in chapter 93 by José R. Romero and Harley A. Rotbart in the eighth edition of this Manual.

TABLE 1 Human EVs and HPeVs

Human EVs[a]
Poliovirus (3 serotypes)
Human polioviruses 1–3
Human EV A (10 serotypes)
Human coxsackieviruses A2, A3, A5, A7, A8, A10, A12, A14, and A16
Human EV 71
Human EV B (36 serotypes)
Human coxsackieviruses A9 and B1 to B6
Human echoviruses 1–7, 9, 11–21, 24–27, and 29–33
Human EV 69
Human EV C (11 serotypes)
Human coxsackieviruses A1, A11, A13, A15, A17 to A22, and A24
Human EV D (2 serotypes)
Human EV 68 and EV 70
Serotypes not yet assigned to a species (2 serotypes)
Human coxsackieviruses A4 and A6
HPeVs
Human parechoviruses 1–3

[a] Coxsackievirus A23, echoviruses 8, 10, 22, 23, and 28, and EV 72 have been reclassified. New rules for species demarcation issued by the International Committee on Virus Taxonomy require that EV species members possess a genome base composition of guanine and cytosine that varies by no more than 1% and share the following characteristics: (i) >70% amino acid identity in the P1 coding region and 2C plus 3CD proteins, (ii) a limited range of natural hosts and host receptors, and (iii) proteolytic processing, replication, encapsidation, and genetic recombination schemes (83).

primers and probes used for the detection of EVs through nucleic acid amplification techniques (reverse transcription-PCR [RT-PCR] and nucleic acid sequence-based amplification [NASBA]). The 3′NTR has also been demonstrated to play a role in viral RNA replication.

The EV capsid is arranged in 60 repeating protomeric units that confer an icosahedral shape to the virion (104). VP1, VP2, and VP3 comprise the surface of the virion and possess an eight-stranded "beta-barrel" core structure. The external loops that connect the beta strands are responsible for the differences in surface topography and antigenic diversity among the EVs. Neutralization sites, typically three or four per protomer, are most densely clustered on VP1. VP4 is located internally within the capsid and has no surface-exposed regions.

The near-atomic structures of multiple human picornaviruses (104) have been resolved and reveal that the EVs (and rhinoviruses) share a number of conserved structural motifs. Surrounding a conserved protrusion at the fivefold axis of each pentameric unit (i.e., five protomers) is a narrow, deep cleft (25 Å) or canyon. It is into this site that the specific receptors for the EVs bind when the virus encounters a susceptible host cell.

The genomic organization of HPeVs is similar to that of EVs. However, unlike the EVs, HPeVs possess only three capsid proteins, VP0, VP3, and VP1 (124). No crystallographic structural information exists for the HPeVs. Sequence similarities between the HPeVs and other picornaviruses suggest that the major capsid proteins share the typical beta-barrel core structure. Relative to EVs, HPeVs are predicted to have a flatter surface topography (124), with antigenic variance determined by the external loops, as with EVs.

EPIDEMIOLOGY AND TRANSMISSION

The EVs and HPeVs are ubiquitous agents that are found worldwide (21, 124). In regions with temperate climates, the majority of EV infections occur in the summer and fall (21, 125), but it is important to note that EV infections continue to occur during the winter (13, 19). In tropical climes, EV infections occur year-round or with increased incidence during the rainy season (72). HPeV infections also occur in late summer to autumn as well as in winter to early spring (8). A recent report suggested that seasonal differences in incidence may occur among the serotypes (8).

The majority of the EVs were identified between 1948 and 1963 during the course of investigations of cases of paralytic poliomyelitis and other central nervous system (CNS) syndromes (25, 26, 99). Since the introduction of vaccines in the late 1950s and early 1960s, much of the world is now free or virtually free of poliovirus infections (56). The control of poliovirus infections in much of the world has focused attention on the NPEVs (Table 1).

It is estimated that the EVs infect a billion or more individuals worldwide each year (91). In the United States, NPEVs are estimated to cause 30 to 50 million infections each year (91, 93), which result in 10 to 15 million symptomatic NPEV infections annually (17). While several modes of transmission exist for the EVs and HPeVs (fecal-oral, respiratory, transplacental, perinatal, and self-inoculation routes), the major one is the fecal-oral route. For some EV serotypes (i.e., coxsackievirus A21 and EVs 68, 70, and 71), transmission via the respiratory and self-inoculation routes is important. The respiratory route may be an important mode of transmission for the HPeVs (124).

The highest incidence of EV infection is observed in young infants and in children of 5 to 10 years of age (117). Secondary infections are observed in >50% of susceptible household members (79). The incidence of HPeV infection is highest in children of <3 years of age, with the majority of infections occurring in infants of <12 months of age (8, 124).

The rank order of the dominant circulating EV serotypes varies annually (17, 18). The predominant NPEV isolates

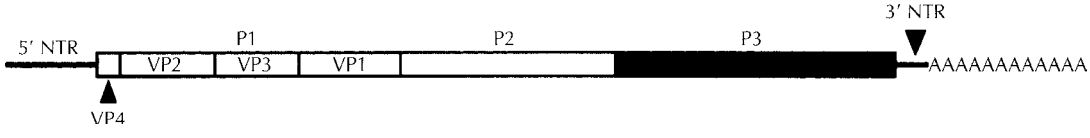


FIGURE 1 Organization of the EV RNA genome. (Reprinted with permission from reference 110.)

identified in the United States from 1997–2001 were echoviruses 30, 18, 13, 11, and 6, coxsackievirus B5, echovirus 9, coxsackievirus B2, coxsackievirus A9, and coxsackieviruses B3, 4, and 1 (listed in descending order). These 12 serotypes represent approximately 83% of all EVs identified during that period. Uncommon serotypes can cause periodic epidemics, such as the emergence of echovirus 13 as a major cause of EV disease (including meningitis) in the United States during the 2001 enteroviral season (83).

CLINICAL SIGNIFICANCE

The EVs are responsible for a wide array of clinical diseases affecting many organ systems. Despite the name "enterovirus," enteric disease is not a prominent manifestation, although diarrhea and vomiting may be significant manifestations of certain outbreaks of "summer flu" due to EVs. Mild hepatitis, almost always in association with other, more significant findings, is common during infection with many EV serotypes. In addition to the actual diseases that they cause, the EVs are of great concern because they mimic other pathogens. Distinguishing EV infections from those due to common bacteria and other viruses on clinical grounds alone is often difficult (36). Hence, unnecessary treatment for other infections is frequently instituted during EV infections.

No disease is uniquely associated with any specific EV serotype, and no serotype is uniquely associated with any one disease (21). This is true even of paralytic poliomyelitis, which has been associated with numerous NPEV serotypes. In most circumstances, it is therefore unnecessary to identify or diagnose the specific disease-producing EV serotype.

The majority of EV infections are asymptomatic (21). The most common symptomatic manifestation of EV infection is an acute nonspecific febrile illness, with or without an exanthem. This so-called viral syndrome is one of the most numerically important causes of fever among children. During the summer and fall, the EVs are responsible for 50 to 60% of hospital admissions for the evaluation of acute, nonfocal febrile illnesses in infants and children (8, 79). When accompanied by upper respiratory symptoms, the "summer cold" is indistinguishable from the same illness caused by rhinoviruses in the fall months.

The paralytic potential of the polioviruses, the prototypic EVs, is well recognized. In the Americas and regions of the world where the polioviruses have been eradicated, the NPEVs and circulating vaccine-derived polioviruses (VDPVs) are now the principal causes of EV-associated acute flaccid paralysis (AFP). Multiple NPEV serotypes are known to cause AFP (21). Recently, cases of AFP associated with VDPVs have been reported from Haiti, Dominican Republic, Madagascar, and China (56). These viruses are circulating strains derived from the vaccine (Sabin) strains of poliovirus.

By far the most vexing clinical EV syndrome that the physician encounters is aseptic meningitis. The EVs are the most common cause of meningitis in the United States and account for over 80% of all viral meningitides (125). The echoviruses and group B coxsackieviruses, particularly echoviruses 4, 6, 9, 11, 16, and 30 and coxsackieviruses B2 and B5, constitute the principal EVs associated with this syndrome (79). An estimated 30,000 to 50,000 hospitalizations for NPEV meningitis occur each year.

Approximately 10 to 20% of viral encephalitides are due to EVs (78). The group A coxsackieviruses are the predominant EV subgroup associated with this syndrome (79). A severe form of brain stem encephalitis, rhombencephalitis, has been associated with EV 71 infections (43). For patients with B-cell immunodeficiencies, a syndrome known as chronic EV meningoencephalitis in agammaglobulinemia has been described (70). The echoviruses, particularly echovirus 11, are the group of EVs most frequently associated with this syndrome.

The group B coxsackieviruses are responsible for one-third to one-half of all cases of acute myocarditis and pericarditis. Group B coxsackieviruses 2 to 5 are the predominant serotypes identified in clinical studies (21). The echoviruses and polioviruses have also been associated with myopericarditis, but significantly less so than group B coxsackieviruses.

Acquisition of EV infection in the perinatal period or within the first 2 weeks of life may result in a severe sepsis-like syndrome with multiorgan involvement in the neonate (1). The morbidity and mortality associated with this syndrome are significant. The echoviruses, particularly serotype 11, and the group B coxsackieviruses are frequently associated with this syndrome.

Although originally described for agammaglobulinemic patients (70), persistent, life-threatening EV infections can also occur in patients with combined variable immunodeficiency, severe combined immunodeficiency, hypogammaglobulinemia, or hyper-immunoglobulin M (hyper-IgM) syndrome and those undergoing bone marrow and solid organ transplantation. Manifestations almost always include meningoencephalitis.

Other acute clinical EV syndromes of significance include hemorrhagic conjunctivitis (predominantly caused by EV 70 and a variant of coxsackievirus A24), hand-foot-and-mouth syndrome (predominantly caused by coxsackievirus A16), herpangina, Bornholm disease, and pleurodynia (the major causes are coxsackieviruses B3 and B5) (21).

Symptomatic infections due to HPeVs are primarily respiratory (i.e., colds or pneumonia) and gastrointestinal (gastroenteritis) in nature (8, 124). However, as with the EVs, cases of neonatal sepsis, AFP, aseptic meningitis, encephalitis, and myocarditis have been reported as a result of HPeV infection.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

The most commonly used and most clinically significant specimens for nucleic acid amplification-based diagnosis of EV infections are cerebrospinal fluid (CSF) and serum (Table 2). If obtained sterilely, CSF and serum require no additional processing prior to extraction of the EV genome. Residual samples found in clinical laboratory refrigerators may be suitable for nucleic acid amplification-based detection of EVs due to the inherent environmental stability of EVs. Specimens that have been handled on automated chemistry or hematology analyzers should be considered compromised due to the potential for cross-contamination with other specimens.

Specimen processing procedures for nucleic acid amplification methods should preserve viral capsid integrity so that the EV RNA genome is protected from ubiquitous nucleases in body fluids and the environment. Biopsy specimens should be snap frozen or transported to the laboratory in a small amount of sterile isotonic saline solution for immediate processing. A recently developed alternative to immediate freezing for transport of tissue samples may be the use of a high-salt RNA protection buffer (RNALater; QIAGEN, Valencia, Calif.) (127).

Specimen storage at room temperature has been documented to negatively impact RT-PCR detection of EVs (129). No appreciable loss in PCR signal may be seen in unextracted

TABLE 2 Clinical samples suitable for detection of EVs and parechoviruses by nucleic acid detection or viral isolation

Clinical sample	EV detection			Parechovirus detection		
	NAAT[a] only	Isolation only	NAAT and isolation	NAAT only	Isolation only	NAAT and isolation
CSF			X			X
Serum	X			X		
Respiratory tract secretions			X			X
Feces			X			X
Urine		X			X	
Myocardium	X			X		
Pericardial fluid	X			X		
Throat swab			X			X
Rectal swab			X			X

[a] NAAT, nucleic acid amplification test.

specimens kept at 4°C to −20°C for up to a month. However, longer storage at these temperatures can result in a gradual decline of viral nucleic acid copy number that could, in turn, potentially affect detection by RT-PCR or NASBA. Thus, specimens are best stored at −70°C. Freezing and thawing disrupt the viral capsid and should be avoided. Large-volume samples should be divided into aliquots prior to being frozen. Storage of samples in frost-free refrigerators should also be avoided. At no time should a specimen (i.e., CSF) be subjected to boiling.

Certain clinical specimens (Table 2) require processing prior to inoculation of cell cultures (71). Feces (1 ml if liquid and approximately pea-sized samples if solid) are added to viral transport medium (a 4-ml volume is sufficient), vortexed well, and centrifuged (1,000 × *g* 30 min, 4°C). One milliliter of supernatant is removed, added to 2 ml of viral transport medium, and vortexed. Monolayers are inoculated with 0.2 to 0.3 ml of diluted supernatant. Specimens (rectal, nasal, or throat) collected on swabs should be transported in viral transport medium. In the laboratory, the specimen is vortexed and the swab is removed. The specimen is then centrifuged, and cell cultures are inoculated as described for fecal samples. Nasopharyngeal aspirate and wash specimens are centrifuged (2,500 rpm, 15 min, 4°C), and cell cultures are inoculated with 0.2 to 0.3 ml of supernatant. Serum, CSF, and urine need no pretreatment and may be inoculated directly onto cell cultures. Serum and urine can be toxic to monolayers. Cell death can often be prevented by replacing the culture medium within 24 h of inoculation. Acid dissociation of antibody bound to virus may improve detection when attempting to isolate EVs from serum and CSF (71, 73).

NUCLEIC ACID DETECTION

Numerous compelling reasons exist for seeking rapid diagnostic assays, such as nucleic acid detection tests, for the EVs. The time required for isolation and identification of EVs by traditional cell culture is too prolonged to be of clinical utility. Some serotypes, particularly within the coxsackievirus A group (i.e., A1, A19, and A22), fail to grow in cell culture (42, 71, 123, 136), and 25 to 35% of specimens from patients with characteristic clinical findings of EV infections are negative by cell culture (22).

Detection of EV RNAs is best performed with nucleic acid amplification methods. The concentration of EVs in clinical specimens is too low (1 to 10 titratable virions per ml of CSF) for detection through direct hybridization (137). Options for nucleic acid amplification include different permutations of RT-PCR and NASBA. The limited available data suggest that the sensitivities of RT-PCR and NASBA assays may be comparable (14, 64). A growing number of laboratories have adopted the most rapid, least labor-intensive approaches, including one-step real-time RT-PCR using a DNA polymerase that performs RT and PCR.

Specimens must be extracted prior to being tested to dissociate the EV RNA genome from the protein capsid, to eliminate ubiquitous RNases, and to remove potential inhibitors of amplification reactions (38, 86). The most common method reported in the literature for RNA extraction from CSF prior to RT-PCR is the use of a nucleic acid-binding, silica gel-based membrane contained within a spin column (QIAamp Viral RNA mini kit; QIAGEN). The extracted RNA is subsequently eluted from the membrane and used directly for RT. Silica-based extraction has also been used for NASBA (20, 56, 99). Extraction reagents are provided with NASBA kits (NucliSens basic kit; Biomerieux, Durham, N.C.). Many laboratories are now replacing manual nucleic acid extraction with automated methods. EV RNA detection protocols that include robotic extraction (BioRobot M48 [QIAGEN] and MagNA Pure LC [Roche Diagnostic Systems, Indianapolis, Ind.]) of CSF, respiratory, and enteric specimens have been published (66, 103).

The design of nucleic acid amplification assays for EV detection in clinical specimens should optimally include internal controls to detect amplification inhibitors. Ideally, these controls are added to specimens prior to extraction to assess whether extraction has removed endogenous inhibitors. Internal control strategies have been reported for RT-PCR (5, 81) (Armored RNA; Asuragen Diagnostics, Austin, Tex.) and NASBA (14, 16, 33, 65, 66) methods.

EV RNA assay verification experiments and quality assurance protocols demand the use of standards. Unfortunately, no universal or nominal EV standard preparations exist. In place of such standards, most laboratories utilize propagated EV strains that have been quantified by plaque assay.

Conventional RT-PCR

The most significant development in rapid direct detection of the EVs and HPeVs has been the application of PCR (8, 27, 50, 52, 53, 67, 110, 113). The following three general

strategies have been employed: universal detection of all EV serotypes, species-specific detection of a limited number of serotypes, and serotype-specific or strain-specific detection within a single serotype. Universal and species-specific detection methods are clinically useful. Strain- or serotype-specific PCR is of use mainly for molecular epidemiology, phylogeny, and molecular virulence studies (10).

The development of PCR technology and its subsequent adaptation for detection of the RNA genomes of EVs via RT-PCR (20, 46, 114) have provided a sensitive, specific, rapid, versatile, and clinically useful (105, 109, 110) method for the detection of EVs. The wide array of reported conditions for EV PCR extraction, RT, amplification, and detection have been reviewed (110, 113). The assay requires only a small sample size, generally 100 to 200 μl of fluid or 1 mg of tissue.

RT-PCR for EV detection can detect as few as 1 to 100 molecules of EV genome, 0.001 to 1 PFU, or 0.003 to 0.1 50% tissue culture infective dose (110, 113). The robustness of RT-PCR assays in the clinical setting may be significantly lower. For CSF, the sensitivity and specificity of RT-PCR compared to those of the traditional "gold standard," cell culture, range from 86 to 100% and 92 to 100%, respectively (97, 110, 114, 122). Other studies have found RT-PCR with CSF to be more sensitive than cell culture (15, 36, 38, 63, 101, 110, 115, 118, 122, 132). For agammaglobulinemic patients with meningoencephalitis or encephalopathy, EVs have been detected in CSF by RT-PCR but not by cell culture (119, 135). A limited number of studies have evaluated the sensitivity and specificity of RT-PCR with other sample types. For serum, the sensitivity and specificity of RT-PCR range from 81 to 92% and 98 to 100%, respectively (2, 118). Urine appears to be the poorest sample source for RT-PCR (2, 87, 119). This may be due to the presence of an unidentified inhibitor(s) (86).

No cross-amplification has been found with 41 different nonrelated viruses, bacteria, fungi, and yeast species tested (110). However, certain primer pairings can result in the amplification of a limited number of rhinoviruses (14, 55, 63) due to shared 5′NTR sequences. Cross-amplification should be of minor importance in specimens where rhinoviruses should not be found, such as CSF, serum, or non-respiratory-tract tissues.

Reagents for EV RNA amplification by conventional PCR are available commercially (Enterovirus Consensus kit; Argene Biosoft, Varilhes, France) but are not approved by the U.S. Food and Drug Administration (FDA). Most laboratories develop and verify assays in-house, using published primers and probes. Conserved sequences within the 5′NTR and VP2 have been exploited to design primers and probes capable of nearly universal amplification of the EVs (20, 35, 46, 97, 115) (Fig. 2). Compared to homologous regions of the sequenced EVs, these primers and probes exhibit homologies to their target regions that ranged from 81 to 100% (110, 113). The two 5′NTR targets most frequently used are those reported by Rotbart et al. and Chapman et al. (20, 118, 120). Primers and probes targeting the highly conserved nucleotide regions 444 to 468, 542 to 560, and 577 to 596 of the EV 5′NTR (numeration relative to the poliovirus type 1 genome) (118, 120) are pan-reactive with the EVs (39, 55, 110, 141). While these primers do not detect the parechoviruses (discussed below), it is important that they do amplify the genomes of several rhinoviruses (14, 52, 63). The latter may be of importance only in the testing of throat and nasopharyngeal specimens because the rhinoviruses are not causes of CNS or systemic disease.

RT is accomplished by using oligonucleotide primers (either random hexamers or sequence-specific oligonucleotides) and one of several reverse transcriptases, such as that of avian myeloblastosis virus or Moloney murine leukemia virus (121). The former enzyme is available in its native form or as a genetically engineered version that exhibits greater thermostability (62). Theoretical and practical advantages exist in favor of the use of each reverse transcriptase (113). The cDNA is amplified by using a set of sequence-specific oligonucleotide primers and a thermostable DNA polymerase. The most commonly used thermostable DNA polymerase for PCR of the EVs is that of *Thermus aquaticus, Taq* polymerase. Other thermostable DNA polymerases, such as Vent polymerase (from *Thermococcus litoralis*) and *Tfl* polymerase (from *Thermus flavus*), have also been used (110).

RT and amplification can be performed with a single thermostable DNA polymerase, *Tth* polymerase (from *Thermus thermophilus*), with inherent reverse transcriptase activity that is enhanced in the presence of manganese (84, 120). The thermal stability of *Tth* polymerase permits the RT reaction to be carried out at higher temperatures. This single-tube RT-PCR approach offers a number of potential advantages, including decreased risk of cross-contamination, increased efficiency of RT due to greater destabilization of 5′NTR secondary structures at high temperatures (12, 62), and improved specificity of the primer-template interaction at high temperatures.

Amplicons can be detected by a number of methods (110). While agarose gel electrophoresis detection can be used, it does not verify amplicon authenticity. The use of seminested or nested primers to verify the authenticity of the amplicon has been reported. The internal PCR product can be detected by using agarose gel electrophoresis. Nucleic acid hybridization of the amplicon to a biotin-, digoxigenin-, or radioactively labeled probe targeted to conserved sequences internal to those of the amplification primers is a better approach since it is more sensitive and less prone to contamination than nested

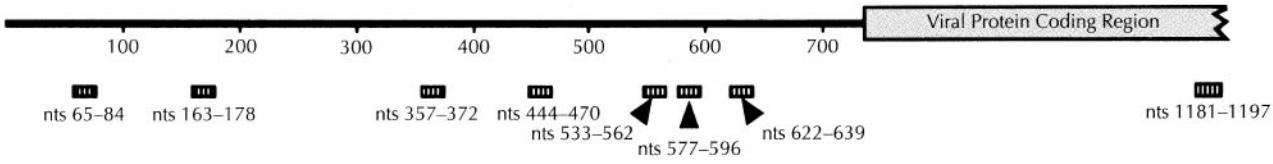


FIGURE 2 Relative locations of originally described primers and probes for detection of EVs. The diagram is a linear representation of the enterovirus 5′NTR and a portion of the viral protein coding region. Primers and probes are designated as hatched boxes below the genome. Numbering corresponds to the nucleotides in the poliovirus type 1 genome. (Reprinted with permission from reference 110.)

methods. Hybridization may be accomplished through slot- or dot-blot, Southern blot, or liquid-phase techniques. Colorimetric detection using biotinylated probes has been described previously (71). Two commercial tests have used this approach; one is no longer on the market (Amplicor EV; Roche Diagnostics, Branchburg, N.J.), but the other is still available (Enterovirus Consensus kit; Argene Biosoft) (64).

Multicenter proficiency testing programs for EV RT-PCR assays developed in-house indicated that properly designed tests can be equally effective in the detection of EV, regardless of the format used (68, 82). However, other studies have reported marked center-to-center variation in testing proficiencies. In one survey, one-third of participating laboratories were nonproficient in the detection of EV by RT-PCR (129). In another report, 6.8% of participating laboratories recorded false-positive results, pointing to the need for fastidious attention to methods for the prevention of cross-contamination (82). Overall, these findings underscore the importance of rigorous internal quality control standards and periodic proficiency reassessment to ensure uniformly high-quality testing.

Although only a small number of reports detailing the detection of HPeVs by RT-PCR have been published (8, 50, 52, 53, 67), these assays parallel EV RT-PCR assays in terms of sensitivity (52, 67). All reported assays have used primer pairs targeted to the HPeV 5′NTR in combination with amplification and detection methodologies similar to those for EV detection systems. In one report, a multiple RT-PCR assay was developed to detect EVs, HPeVs, and rhinoviruses from clinical specimens (52).

Real-Time RT-PCR

Sufficient information has accumulated to begin to assess the utility and pitfalls of real-time RT-PCR for EVs (3, 27, 52, 63, 80, 81, 87, 103, 130, 131, 134) and HPeVs (27). As with conventional PCR, careful optimization, including primer/probe design and amplification conditions, is required prior to assay implementation. However, well-designed assays appear to be at least as sensitive as traditional in-house assays (3, 27, 54, 63, 80, 87).

The major advantages of real-time RT-PCR compared to conventional PCR are decreased labor and a decreased time to result. These savings are due in part to simultaneous target amplification and amplicon detection. Additionally, the thermal conduction characteristics of real-time PCR vessels allow for rapid temperature transmission and permit ultrashort cycling times, further reducing the time to detection. Almost all reports of the clinical application of real-time RT-PCR for EV detection indicate that the assay can be completed in 4 h or less, including the time required for viral RNA extraction (3, 27, 54, 63, 80, 81, 87, 103, 130, 134).

Single-tube protocols, in which RT and subsequent cDNA amplification are carried out in a single reaction vessel, may be less sensitive and more variable (27, 54, 63, 134) than protocols where RT is initially performed in a separate tube prior to amplification. Several methods to increase the sensitivity of *rTth* polymerase- or Moloney murine leukemia virus polymerase-based assays have been employed, including incorporation of the T4 phage gene 32 protein (3, 27, 81). This phage-derived helicase may help to destabilize RNA secondary structures that are prevalent within the EV 5′NTR. Other approaches have included increasing the concentrations of the *rTth* polymerase and manganese (80) and using a second real-time amplification reaction to detect the product (103, 134) in a seminested manner.

Amplicons have been detected using either nonspecific DNA-binding fluorophores, such as SYBR green, or specific fluorophore-labeled oligonucleotide probes that exploit fluorescent resonance energy transfer (FRET) technology (69). SYBR green binds to the minor groove of DNA, and this association increases its fluorescence intensity. The intensity of the fluorescent signal increases further as amplicons are generated. Assays that employ melting-curve analysis of PCR products to verify amplicon authenticity (3) are preferable to tests that employ fluorescence intensity measurement alone (54).

FRET is the most commonly used detection method in real-time RT-PCR assays (3, 27, 54, 63, 80, 81, 87, 103, 130, 134). The biochemistry of FRET probes is reviewed in chapter 16. One major advantage of these dually labeled probes is the capability to simultaneously detect amplicons and confirm their identity. This approach has been used successfully to discriminate between amplicons generated from EVs and rhinoviruses (54) when using primers that target sequences common to both picornavirus genera (55) or from EVs and HPeVs in a duplex assay (27).

There are currently no FDA-approved real-time RT-PCR assays for EV detection. Components can be obtained commercially as analyte-specific reagents (Cepheid, Sunnyvale, Calif.; Chemicon Light Diagnostics, Temecula, Calif.; QIAGEN). Detection kits are also available in research-use-only formats (Nanogen, San Diego, Calif.).

NASBA

The biochemistry of NASBA assays is reviewed in chapter 16. NASBA assays are reported to detect 10 to 100 RNA copies/reaction (14, 32, 33, 40). Like RT-PCR, NASBA assays are significantly more sensitive than cell culture for the detection of EVs in clinical specimens (33, 65). A real-time NASBA assay utilizing molecular beacons (reviewed in chapter 16) as detection probes has been reported to have a similar clinical sensitivity to that of conventional NASBA (66). The advantages of this method are a decreased time to result and ease of use. Conventional and real-time NASBA reagents are commercially available as analyte-specific reagents (NucliSens Basic reagents and EasyQ Enterovirus reagents, respectively; Biomerieux), but primers and probes are supplied by the user. One advantage of commercial NASBA assays is the provision of internal controls. However, one problem with these assays has been the frequency of invalid results as a result of reaction inhibition (14, 33, 65). This appears to occur more frequently with the use of stool, rectal, throat, and nasopharyngeal samples than with CSF (14, 64, 65). In one report, CSF specimens that yielded indeterminate NASBA results were all significantly contaminated with blood (33).

ISOLATION PROCEDURES

Reported mean isolation times for EVs from CSF using traditional cell culture techniques range from 3.7 to 8.2 days (116); EVs from other sites, where viral titers are higher, often grow more rapidly (1 to 3 days) (41, 76, 137). Infections of susceptible cell monolayers with EVs and HPeVs result in a characteristic cytopathic effect (CPE) consisting of shrinkage and rounding of individual cells within the monolayer. The nuclei of infected cells exhibit pyknosis. As infection progresses, the cells degenerate and separate from the surface of the plate.

The general susceptibilities of commonly used cell lines for the EVs are summarized in Table 3. No single cell line is optimal for the replication of all EV serotypes. Monkey kidney cell lines have good sensitivities for the polioviruses, group B coxsackieviruses, and echoviruses, whereas human diploid fibroblasts, such as MRC-5 cells, WI-38 cells, and human embryonic lung fibroblasts (HELF), give higher yields

TABLE 3 Susceptibilities of cell lines commonly used for isolation of EVs[a]

Cell line	Susceptibility to virus			
	Polioviruses	Coxsackieviruses		Echoviruses
		Type A[b]	Type B	
Monkey kidney				
Rhesus	+++	+	+++	+++
Cynomolgus	++++	+	+++	+++
Buffalo green (BGM)	+++	+	++++	++
Human				
HeLa	+++	+	+++	+
Kidney (HK)	+++	+	++	+++
WI-38	++	++	+	+++
Embryonic lung (HELF)	+++	++	+	+++
MRC-5	+++	+	+	+++
Rhabdomyosarcoma (RD)	+++	+++	+	+++
HEp-2	+++	+	+++	+
A549	+++	+	+++	+++
NCI-H292	+++	+++	+++	+++

[a] Relative susceptibilities: +, minimally susceptible; ++++, maximally susceptible. Some EVs are difficult to isolate even on minimally susceptible cell lines.
[b] Some type A coxsackieviruses are not readily isolated (A1, A19, and A20).

for type A coxsackieviruses. Most laboratories that still perform culture for EV detection therefore use a combination of a primary monkey kidney cell line, such as cynomolgus monkey kidney cells, and a human diploid fibroblast line. The yield and speed of EV recovery can be improved with the addition of Buffalo green monkey kidney (BGMK) and rhabdomyosarcoma-derived RD cells (22, 23, 28). Recently, it was shown that in addition to BGMK cells, the cell line A549 is suitable for detection of the group B coxsackieviruses (98). RD cells are the most sensitive for detection of group A coxsackieviruses but fail with most type B coxsackieviruses, and successful isolation frequently requires blind passaging. Some type A coxsackieviruses (A1, A19, and A20) cannot be isolated in cell cultures. If enough cell lines are used, the pattern of growth in each by a single EV isolate may accurately predict the subclassification of the isolate (42, 51), although the clinical value of this information is limited.

A variety of nontraditional cell lines are also useful for EV isolation. Human colon adenocarcinoma-derived CaCo-2 cells can be used to isolate a range of EVs (106). BGMK cells genetically engineered to express the human group B coxsackievirus receptor (the complement regulatory protein decay accelerating factor) exhibit an expanded range of EV permissiveness (11, 45). The genetically engineered mouse cell line L20B, which carries the human poliovirus receptor, selectively supports the replication of polioviruses over that of NPEVs (85, 138).

Shell vial culture (SVC) combined with the use of monoclonal antibodies for virus detection in the absence of CPE reportedly reduces the time to detection to 2 to 3 days but appears to be less sensitive than traditional cell culture (60, 128). SVC using a mixture of cell lines in a single vial has shown promise. A mix of decay accelerating factor-expressing BGMK cells and A549 cells (Super E-Mix, Diagnostic Hybrids Incorporated, Athens, Ohio) was more sensitive than conventional culture but less sensitive than RT-PCR (11). The use of two shell vials containing mixtures of NCI-H292, BGMK, A549, and RD cells may achieve optimum sensitivity for EV detection (44). The greatest limitation of SVC may be the shortcomings of the monoclonal antibodies used in virus detection steps (discussed below).

Although no comparison studies have been carried out to determine the optimum cell line for isolation of HPeVs, they have been reported to grow on tertiary monkey kidney cells, embryonic lung cells, African green monkey kidney (Vero) cells, and A549 cells (9, 124).

SPECIFIC IDENTIFICATION OF EV ISOLATES

Determination of the specific serotypes of infecting EVs is often unnecessary because the diseases caused by the EVs are not serotype specific. In most circumstances, therefore, it is adequate and useful for the diagnostic laboratory to report the presence of an EV without further detail. Until recently (24), the most common exception to this principle was in pediatrics, where distinguishing between vaccine strain polioviruses and NPEVs is critical to the interpretation of viral culture results. Until 2000, children in the United States were repeatedly immunized with the trivalent oral polio vaccine (Sabin strains) during the first 2 years of life. This practice continues in many parts of the world. Like all EVs, the Sabin poliovirus strains may be shed from the throat for 1 to 2 weeks and in the feces for several weeks to months. Hence, isolates from those two sites must be identified as either nonpolio or polio serotypes. Vaccine poliovirus has only rarely been recovered from CSF or blood (37, 73); further characterization of EV isolates from these sites is thus less important.

Distinguishing between polioviruses and NPEVs can be accomplished by using traditional or molecular methodologies. The traditional method employs neutralization of the isolate with a pool of antisera directed against the three poliovirus serotypes (42). Successful inhibition of growth by the antisera confirms the poliovirus identity of the isolate, whereas failure to neutralize an isolate with typical CPE implies the presence of an NPEV.

Various molecular methods have been described for specific identification of the polioviruses, including PCR-restriction fragment length polymorphism analysis (4), probe hybridization

(29), and diagnostic PCR assays (57, 58, 59). Species-specific identification of the three poliovirus serotypes can be accomplished by testing a culture-passaged isolate with a set of degenerate pan-poliovirus oligonucleotide primers targeted to the VP1 coding region (59). These primers identify all poliovirus strains (wild-type and vaccine strains, i.e., Sabin strains) and accurately discriminate polioviruses from NPEVs. Serotype-specific identification of poliovirus isolates is possible by using degenerate oligonucleotide primers targeted to VP1 sequences that are conserved within but not across poliovirus serotypes (58). Most recently, the need to differentiate recombinant VDPV isolates from wild-type and vaccine poliovirus strains has been met by the use of a multiplex PCR assay that uses six Sabin strain-specific primer pairs targeted to the 2C and 3D viral coding regions (57).

Further identification of an NPEV's specific serotype is useful under certain circumstances. Epidemiologic studies of patterns of EV infections require knowledge of specific serotypes, as do descriptions of unusual or novel clinical manifestations, such as flaccid paralysis due to NPEVs (15, 16, 62) or, recently, echovirus 13 (83). While the criterion standard for EV serotype determination has traditionally been the use of the Lim and Benyesh-Melnick (LBM) pools of antisera (74, 75, 76), several limitations exist with this methodology. The pools identify only 40 of the 64 originally described EV serotypes and fail to identify newly identified EVs (88, 94, 95). Because the LBM pools were developed over 30 years ago, genetic drift of the EVs over time has given rise to antigenic variants of the prototypic strains, which may be difficult to conclusively serotype. The procedure is labor-intensive and time-consuming, and the supply of LBM pools available from the World Health Organization is limited (distribution is restricted to reference laboratories).

The confluence of nucleic acid amplification technology and bioinformatics has resulted in a novel and powerful method for "molecular serotyping" (93) of the NPEVs (96, 99) and HPeVs (8, 124). This approach is based on the use of sets of degenerate primers targeted to sequences within the VP3, VP1, and 2A coding regions of the NPEVs. The primers permit the amplification of approximately one-half of the upstream portion of the VP1 coding region. The amplicons generated are rapidly sequenced by using PCR and computationally compared to a VP1 sequence database for known EVs (93). The method greatly reduces the time required for identification of the EV from weeks to days (92). Furthermore, it has been extremely useful in typing isolates previously classified as "nontypeable" EVs because they failed to be neutralized by the traditional LBM pools (91). Lastly, this approach has led to the identification of multiple new EV serotypes (88, 89, 94, 95, 96) and a novel HPeV (50). Serotype-specific primers have been developed for other NPEVs (10).

Broadly reactive and serotype-specific EV monoclonal antibodies have been developed (71) and applied to cell culture confirmation by immunofluorescence. While preliminary studies suggested that these reagents, used singly and in blends (DAKO-Enterovirus 5-D8/1 [DAKO, Glostrup, Denmark] and Pan-Enterovirus blend [Chemicon International, Inc., Temecula, Calif.]), might find an important role in serotype identification, further studies have identified several limitations (60, 108, 128). Most notable is the lack of sensitivity and reported cross-reactivity with the rhinoviruses (60). The concordance of results for identification of clinical EV isolates to the species level by using monoclonal blends versus the neutralization assay has demonstrated the latter to be significantly superior for identification of EVs (108). Multiple studies have documented the lack of reactivity of monoclonal blends in detecting EV 71 (128). These studies appear to indicate that monoclonal antibodies for EV identification should best be used as a preliminary screen for species or serotype identification.

SEROLOGIC DIAGNOSIS

Homotypic enzyme-linked immunosorbent assays for the detection of EV serotype-specific immunoglobulin M (IgM) antibodies (i.e., coxsackievirus B-specific IgM and EV 71-specific IgM) as well as heterotypic assays for the detection of IgA, IgM, and IgG antibodies against EVs in general have been reported (6, 7, 28, 32, 101, 125, 132). Homotypic assays are generally impractical unless there is suspicion of a specific EV serotype. The IgM response to EVs may be nonspecific and may lead to a false-positive result and lack of sensitivity (102, 106, 133). Cross-reactivity with non-EV pathogens has not been studied thoroughly. In one report, sera from patients infected with acute hepatitis A virus (a member of the *Picornavirus* family) produced a significant number of false-positive results in an enzyme-linked immunosorbent assay for detection of enterovirus IgM antibodies (7). Because of the inherent difficulties associated with assays for the detection of EV serotype-specific IgM antibodies, these tend to remain in the realm of epidemiologic investigation.

ANTIVIRAL SUSCEPTIBILITY TESTING

There is no FDA-approved antiviral agent available for the treatment of EV or HPeV infections. A promising investigational drug, pleconaril, has undergone extensive in vitro susceptibility testing against EVs but is unavailable in the United States (111). A well-characterized 96-well cell culture protection assay to test the effectiveness of potential antiviral compounds has been reported (100). Briefly, cells in 96-well plates are infected with the EVs of interest, and a serially diluted test compound mixed with medium is added to each well and incubated for 3 days. The monolayers are then examined optically or manually. The 50% inhibitory concentration of the compound being studied is defined as the concentration that protects 50% of the cell monolayer from virus-induced CPE.

EVALUATION AND INTERPRETATION OF RESULTS

An understanding of the sites where asymptomatic shedding and disease-inducing replication occur is critical to the interpretation of EV assays. Detection of EV in the CNS, bloodstream, lower respiratory tract, and genitourinary tract implies a true invasive infection and a high likelihood of association with current illness. In contrast, EVs can be shed asymptomatically from the nasopharynx and the gastrointestinal tract for weeks to months. Detection of EVs by virus isolation or RT-PCR at these sites must be interpreted cautiously, because their presence alone does not establish a diagnosis. EV in the feces or nasopharynx of a patient with meningitis may represent residual shedding from an infection from weeks before and may have nothing to do with the current illness.

An additional factor potentially complicating the evaluation of results from these body sites in young children is the administration of live attenuated oral poliovirus in the first years of life. While this vaccine is no longer used in the United States (24), the majority of countries worldwide, including some in Europe, still employ oral poliovirus as part of their vaccination regimens. Reporting an EV isolate in a setting where oral poliovirus is used without specifying if it is

a poliovirus or NPEV can lead the physician to wrongly discontinue antibiotics or antiherpes therapy in the belief that an EV etiology has been established. The distinction between polioviruses and NPEVs from the CNS, bloodstream, lower respiratory tract, and genitourinary tract is less important, since vaccine strains of poliovirus have rarely been isolated from such specimens and, in those rare instances, may actually be causing the illness in question (37, 73).

A further cautionary note is warranted with regard to infections with dual agents. Rare reports of coinfections of the CSF by bacteria and EVs have appeared (139). In such cases, the bacterium-associated clinical sequelae dominated, i.e., the patients were clinically suspected of having bacterial meningitis and the virus was isolated incidentally. The patients were sick enough that identification of a virus before identification of the bacterium would have been unlikely to dissuade the clinician from continued use of antibiotics. In the much more common situation where the clinical presentation is typical of viral meningitis, coinfection with a clinically "silent" bacterium would be extraordinarily unlikely. Hence, identification of an EV from a nonpermissive site in a patient with a clinically compatible illness is sufficient evidence for establishing EV causality.

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Rhinoviruses

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90

TAXONOMY

Human rhinoviruses (HRV) constitute a genus of the *Picornaviridae* family, which includes three additional genera that are pathogenic for humans: *Enterovirus, Parechovirus,* and *Hepatovirus*. The rhinovirus genus derives its name from the predominant site of its replication and symptomatology, the nose.

At present, more than 100 rhinovirus serotypes have been characterized on the basis of neutralization tests. While some cross-neutralization occurs, there is no group antigen (46, 49). Based on receptor binding, rhinoviruses can be divided into two groups (76, 78). Intercellular adhesion molecule 1 (ICAM-1) is the cell receptor for the major group, comprising approximately 90% of known HRV serotypes. ICAM-1 is a member of the immunoglobulin supergene family and may be expressed on the surface of many different cells, including those that are active in respiratory immune responses (64, 82). The natural ligand for ICAM-1 is lymphocyte function-associated antigen (LFA-1), and since the binding sites for LFA-1 and rhinovirus overlap, rhinovirus may block ICAM-1/ LFA-1 binding on leukocytes and disrupt local airway immune responses. It has been postulated that this may be a factor in the pathogenesis of secondary viral or bacterial respiratory tract infections (24). The minor group of serotypes binds to members of the low-density lipoprotein receptor family (36). Originally comprised of 10 serotypes, the minor group now includes two additional serotypes previously classified in the major group (78). Rhinovirus type 87 (HRV 87) does not fit either receptor group and is now considered to be an acid-sensitive strain of enterovirus type 68 (8, 76).

Based on antiviral susceptibility, rhinoviruses have been divided into two groups, A and B, which do not coincide with receptor groups. Serotypes within each group share sequence homologies for VP1 and antiviral binding site (2). Phylogenetic analysis, based on sequencing of capsid protein genes VP4/VP2 and VP1, also supports dividing the genus into two major species, A and B (49). Again, HRV 87 does not fit this subdivision but belongs instead to human enterovirus species D (8). As more is learned about the molecular genetics of HRV, further revisions in classification and taxonomy are anticipated in the future (65).

DESCRIPTION OF THE AGENT

The rhinovirus virion consists of a nonenveloped icosahedral nucleocapsid, 20 to 27 nm in diameter, with 60 protomeric units consisting of four protein subunits: VP1, VP2, VP3, and VP4. These structural polypeptides are obtained by posttranslational cleavage of a large polypeptide precursor (14). The 3C protease that participates in cleavage of this polypeptide is a target for antiviral drugs (52). VP1, VP2, and VP3 reside on the exterior of the virus and make up its protein coat. Located within VP1 is a hydrophobic pocket into which antiviral compounds such as pleconaril bind (85). VP4 resides inside the protein shell. X-ray crystallography and cryoelectron microscopy studies have identified large depressions or "canyons" on each of the 60 protomeric units, which appear to be sites for cell receptor binding and also play a critical role in conformation changes that follow attachment (84). Conformational changes in the canyon floor can also be induced by certain antiviral agents, thus inhibiting virus attachment to cells or virus uncoating.

The HRV genome is single-stranded, plus-strand RNA, approximately 7,200 nucleotides in length, with a single open reading frame, and coupled at the 5′-noncoding region (5′-NCR) with a short peptide (VPg). Rhinovirus replicates in the cytoplasm of infected cells, producing infectious virions that sediment at a buoyant density of 1.40 g/l in cesium chloride. Empty capsids and particles lacking one or more structural polypeptides are also produced.

As they have no lipid envelope, rhinoviruses are fairly resistant to inactivation by organic solvents such as ether, chloroform, ethanol, and 5% phenol. Although rhinoviruses are relatively thermostable, heating at 50 to 56°C progressively decreases infectivity. Rhinoviruses are traditionally differentiated from enteroviruses by their loss of infectivity upon exposure to pH 3 for 3 h at room temperature, although this may not be the case with all serotypes and strains (65).

EPIDEMIOLOGY AND TRANSMISSION

In temperate zones, rhinovirus infections occur year-round, with a peak in September and a second peak in late spring. Seasonal fluctuations relate in part to improved survival of rhinoviruses under conditions of high relative indoor

humidity and herding of children together when school opens in the fall (53). Prevalent serotypes can change from year to year, with multiple types circulating in a given area at any time, and epidemic strains can also persist over consecutive seasons. Although the molecular epidemiology of HRV strains is still in its infancy, it appears that genetic variability is common, due to error-prone replication (65).

Studies with volunteers have shown that inoculation of virus into the nose or conjunctivae is the most efficient way to initiate infection (19). Rhinovirus is present in highest titers in the nose of infected persons and commonly contaminates their hands. Consequently, investigators have found that rhinovirus can be transmitted via hand-to-hand contact (27) or by contaminated fomites, followed by self-inoculation of virus into the nose or conjunctivae (3, 28). Furthermore, transmission can be interrupted by using tissues impregnated with virucidal agents for nose blowing, by treating surfaces with disinfectant, or by applying iodine to the fingers (35). Studies with volunteers have also shown that, with very prolonged exposure times, rhinovirus transmission can occur by the aerosol route (18).

Rhinoviruses replicate primarily in the ciliated epithelial cells in the nose, and the incubation period after inoculation is 2 to 3 days. The peak of virus shedding coincides with acute rhinitis. Virus may become undetectable by culture at 4 or 5 days or may be present in low titers for up to 2 to 3 weeks. Symptoms persist for 7 days on average and include profuse watery discharge, nasal congestion, sneezing, headache, mild sore throat, cough, and little or no fever. Rhinovirus causes minimal pathology in the nasal epithelium, and the symptoms of rhinovirus infection parallel the rise and fall of chemical mediators of inflammation (28, 34, 43, 57). Psychological stress appears to increase susceptibility and the development of clinical symptoms (13), whereas exposure to a cold environment does not (21). Immunity is type specific and correlates best with local production of immunoglobulin A (IgA) (5, 10).

CLINICAL SIGNIFICANCE

The rhinoviruses cause approximately two-thirds of the cases of the common cold and thus are responsible for more episodes of human illness than any other infectious agent (34). Although a trivial illness, the common cold is acutely disabling, and its cost in days lost from work, cold remedies, and analgesics is in the billions of dollars. In recent years, HRV has been increasingly implicated as the major viral cause of asthma exacerbations and decompensation of chronic lung disease (25, 81). Severe asthma exacerbations have been linked to prolonged shedding of rhinovirus RNA (42). Consequently, the pathogenesis of rhinovirus infection is currently the subject of intense study (38, 43, 57, 61, 69, 80, 86). In addition, rhinovirus infection appears to have a more important role in sinusitis and otitis media than has been previously thought (56, 62, 73).

Since rhinovirus has long been considered an upper respiratory pathogen of minor clinical consequence, most diagnostic laboratories do not use optimal conditions for its recovery. With use of more-sensitive molecular diagnostic methods, rhinovirus has been increasingly recognized as an important etiology of lower respiratory tract infections (22, 33, 59, 60), especially in the very young (40, 44, 57), school-age children (40, 72), the elderly (51, 55, 71), and in those with chronic illnesses (22), cancer, immunosuppressive illnesses, or transplants (11, 45, 63, 77), or underlying pulmonary disease (47, 81). In fact, the clinical manifestations of rhinovirus infection in hospitalized infants are similar to those caused by respiratory syncytial virus (RSV). However, the mean age of infants affected by HRV tends to be slightly older (44). Although rhinovirus viremia has rarely been found by culture, rhinovirus RNA was recently detected, using sensitive molecular methods, in the blood of 11.4% of HRV-infected young children, including 25% of those with HRV-associated asthma exacerbations (83).

While many antivirals show activity against rhinoviruses in the laboratory, inadequate drug delivery to the site of infection has reduced clinical benefit for patients and treatment remains experimental. Studies have included intranasal administration of soluble ICAM-1 to rhinovirus-infected volunteers, use of intranasal ipratropium bromide, an anticholinergic agent, intranasal imiquimod, or administration of antivirals and anti-mediators in combination (12, 28, 30, 74, 75, 85). A picornavirus capsid binding agent, pleconaril, has shown some benefit against natural colds in clinical trials (31). Recently, inhibitors of rhinovirus 3C protease have shown potent antiviral activity and are undergoing clinical trials (32, 52). The usefulness of these approaches requires further study and validation. Due to numerous HRV types, prospects for a vaccine in the near future are negligible.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

In natural infections, rhinovirus can generally be isolated from 1 day before to 6 days after onset of cold symptoms, but it is shed in highest concentration on day 1 or 2 of illness. Of note, with sensitive molecular methods, rhinovirus can be detected for several weeks after infection. Since rhinovirus is excreted in highest titers from the nose, nasal rather than throat specimens should be obtained for virus isolation. Likewise, sputum is a low-yield specimen for the isolation of rhinovirus. Comparisons of nasal wash, nose swab, throat gargle, and throat swab specimens for the isolation of rhinovirus from clinical specimens revealed nasal wash to be the best (1, 4, 10). Nasal wash specimens are obtained as follows. Tilt the patient's head backwards and instill 1 ml of sterile phosphate-buffered saline into one of the nostrils. Then, ask the patient to lean forward and allow the washing to drip into a sterile petri dish or other collection container. Repeat with the other nostril until each nostril has been washed with 5 ml of phosphate-buffered saline. The washings are then transferred into a sterile container with an equal volume of viral transport media (VTM) containing antibiotics. The inclusion of phenol red in the VTM allows the detection of acid pH, which would adversely affect the isolation of rhinovirus.

If a nasal wash cannot be performed, a nasopharyngeal aspirate is preferred, or a deep nasal or nasopharyngeal swab and a throat swab should be collected and combined (10, 34). Of note, a nasal swab or throat swab yields only half the number of positives detected by a nasal wash or aspirate method (10). The swabs are then immersed in a vial containing 2 ml of VTM. An endotracheal aspirate, bronchoalveolar lavage, or bronchial wash specimen should be collected to diagnose lower respiratory tract disease.

Specimens should be transported promptly to the laboratory. Best results are obtained with rapid inoculation of cell cultures; however, specimens can be held up to 24 h at 4°C in VTM with neutral pH. If longer delays are necessary, specimens should be frozen at −70°C and thawed just prior to inoculation. Freezing does not appear to be detrimental to virus recovery (4). In general, a similar

sample handling is acceptable for molecular analysis; however, the compatibility of various VTM with different extraction reagents must be confirmed in each laboratory.

DIRECT DETECTION

Antigen

Due to the large number of serotypes and the lack of a common group antigen, it has been difficult to develop direct antigen tests for rhinovirus in clinical specimens. The enzyme immunoassays that have been developed have suffered from narrow reactivity and low sensitivity and are no longer used (16, 48).

Nucleic Acid

Fortunately, picornavirus genomes have a high degree of nucleic acid sequence homology in the 5′-NCR. By using primers or probes based on sequences in the 5′-NCR, all rhinovirus serotypes can theoretically be detected in a single assay. However, enteroviruses as well as rhinoviruses can be detected. Reverse transcriptase PCR (RT-PCR) and nucleic acid sequence-based amplification (NASBA) have been much more sensitive than culture methods, doubling or even tripling the number of HRV infections detected (7, 29, 37, 39, 50, 67, 77, 79). Nevertheless, culture can occasionally detect some rhinoviruses missed by RT-PCR because of variability in techniques and primer or probe mismatches (7, 37, 79). Although in these comparative studies suboptimal culture conditions are often used (34), amplification methods are clearly superior and continue to improve in sensitivity and in time to result.

At present there is no standardization of molecular methods, and blinded comparative studies have not been reported. The published techniques have differed in types of samples tested, RNA extraction methods, primer selection, and amplification and detection protocols. Since primer pairs from the 5′-NCR region will commonly detect both enteroviruses and rhinoviruses, a universal problem is differentiating these two picornavirus groups from each other. Unfortunately, primers that are best able to distinguish rhinovirus from enterovirus may not be the most sensitive for detection of rhinovirus in clinical specimens (39). Thus, more broadly reactive primers are often used. Sequencing of amplicons to differentiate rhinovirus from enterovirus in all positive clinical samples may be feasible in the near future; however, the necessary equipment and expertise are not widely available at present in the routine clinical lab (17). Alternative strategies to distinguish rhinovirus from enterovirus have included primer pair design to generate amplicons of different sizes for rhinovirus and enterovirus (29), restriction enzyme digestion of a common-sized amplicon (58), differential internal probe hybridization (7, 17, 29), and nested PCR with annealing and extension times optimized for rhinovirus detection (67). In general, methods relying on simple gel electrophoresis for detection are cost- and time-saving but tend to be 10-fold less sensitive than either nested PCR or detection by hybridization. Unfortunately, Southern blot hybridization can extend time to result by a day, and nested PCR increases the risk of cross-contamination. Furthermore, most methods reported have failed to detect or accurately differentiate a few of the viruses tested, and to date no study has evaluated all rhinovirus and enterovirus serotypes.

Important recent advances have been the use of real-time amplification using SYBR green, TaqMan hybridization probes, or molecular beacons (15, 17, 41, 50, 77), and multiplex PCR to detect multiple respiratory viruses (6, 26, 66). Real-time amplification methods have the advantages of simplicity, reduced risk of contamination by use of closed tubes, the potential for quantitation, and a shortened time to result since detection occurs concurrently with amplification.

Since some antivirals, such as pleconaril, may be effective against both enterovirus and rhinovirus infections (31), a single assay that detects both virus groups could ultimately turn out to be an advantage.

ISOLATION PROCEDURES

Cell Culture

Rhinoviruses grow only in cells of human or monkey origin. Although the original isolation of rhinoviruses was in primary monkey kidney cells, these cells have not been consistent in yielding a broad range of isolates. The use of sensitive human embryonic lung fibroblast (HELF) strains has provided superior results. Rhinoviruses were originally separated into M and H strains based on their ability to replicate in monkey and human cells (M strains replicate in both monkey and human cells, whereas H strains replicate only in human cells). However, this distinction is no longer used since H strains can be adapted to grow in monkey cells.

The most commonly used cells in clinical laboratories are the HELF strains WI-38 and MRC-5. WI-38 cells are significantly more sensitive than MRC-5 (4), but MRC-5 cells are more commonly available in clinical laboratories. Human embryonic kidney (HEK) can also support rhinovirus replication. Unfortunately, different lots of normally sensitive cell lines have been found to vary over 100-fold in sensitivity to rhinovirus (9); the reasons for this variation are not known. Therefore, for optimal results, simultaneous use of at least two sensitive systems is recommended.

In the research setting, several HeLa cell clones, such as HeLa M or Ohio HeLa cells, HeLa H, HeLa R-19, and HeLa I cells have been shown to support the replication of rhinoviruses to high titers (1, 4). A human fetal tonsil cell line was initially reported to be more sensitive than MRC-5 and HeLa cells for rhinovirus isolation (23). However, HeLa I cells were recently found to be more sensitive than WI38, MRC-5, fetal tonsil, or HeLa H or M cells for recovery of rhinovirus from clinical specimens (4). These specialized cell lines can be obtained only from research laboratories.

After inoculation, cultures are incubated in standard cell culture medium such as Eagle's minimum essential medium with 2% fetal calf serum and antibiotics at a neutral pH. It is important that culture conditions mimic those of the nose; cultures should be incubated at 33 to 35°C with continuous rotation in a roller drum to provide aeration of the monolayer (68). Of note, these incubation conditions also promote the replication of other respiratory viruses that may be present in clinical specimens. Rhinovirus cytopathic effects (CPE) can be observed as early as 24 to 48 h after inoculation and are often detected by day 4. Passage of infected HeLa cell cultures may be necessary for some isolates before CPE are apparent. In fibroblasts, cellular changes are easier to read and are often detected earlier than in epithelial cell lines. Both large and small rounded, refractile cells with pyknotic nuclei are observed in foci that also contain cellular debris (Fig. 1). Rhinovirus CPE are similar to enterovirus CPE but may sometimes be confused with nonspecific changes. The CPE progress over a 2- to 3-day period with the degree of cellular

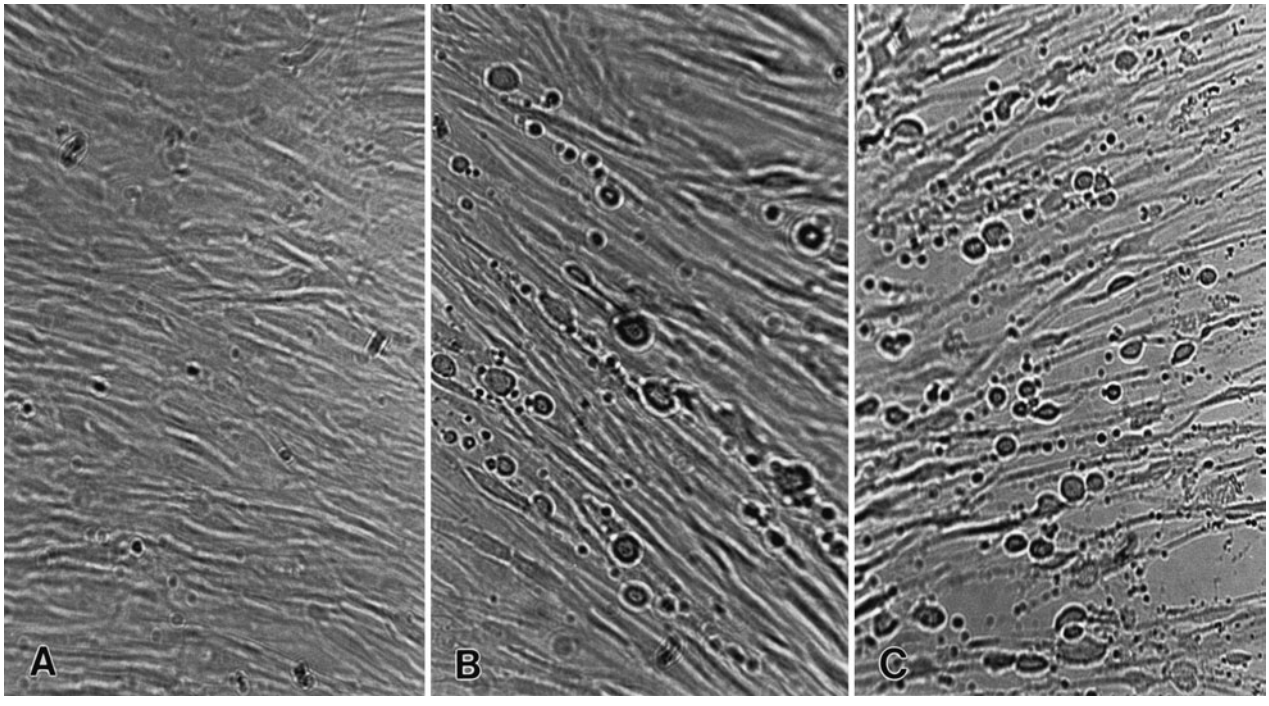


FIGURE 1 Rhinovirus cytopathic effects in human embryonic lung fibroblasts. (A) Uninfected cells. (B) Early focus of rhinovirus CPE. (C) More advanced rhinovirus CPE. Magnification, ×100.

change depending on the serotype and the inoculum dose. It should be noted that rhinovirus CPE can regress or virus may inactivate if left too long. Therefore, cultures should be promptly passaged. Passage is also necessary to increase viral titers prior to the performance of identification tests.

HELF cell cultures should be observed for 14 days, with refeeding at 7 days to increase recovery of virus. HeLa cell cultures can be observed for only up to 7 to 8 days, when passage becomes necessary due to nonspecific cell degeneration and rounding.

Organ Culture

Organ cultures of human fetal nasal epithelium or trachea were used in the past to isolate rhinoviruses not grown in standard cell cultures but are no longer used for this purpose. Details of the procedure have been reviewed elsewhere (1). Recently, however, cultures of nasal and tracheal mucosa obtained from biopsy material have been used for research studies of rhinovirus pathogenesis (38).

IDENTIFICATION OF VIRUS

A presumptive diagnosis of a rhinovirus isolate is made by the appearance and progression of characteristic CPE in the appropriate clinical setting. Further identification of a presumed rhinovirus isolate is usually limited to differentiating it from enterovirus by determining sensitivity to acid pH (Fig. 2). First, passage of the isolate is necessary to obtain a minimum titer of 10^3 50% tissue culture infective doses ($TCID_{50}$)/ml. Testing for other properties of rhinovirus, such as sensitivity to temperature and resistance to lipid solvents, is rarely done in a routine viral diagnostic laboratory. With over 100 rhinovirus serotypes, neutralization testing is time-consuming, costly, and not routinely available. Further identification of presumptive rhinovirus isolates can be obtained by genotypic analysis in research laboratories.

Acid pH Stability

Rhinoviruses are sensitive to low pH, whereas enteroviruses are stable. Thus, a reduction in virus titer by 2 to 3 $\log_{10}$ $TCID_{50}$ can be expected upon exposure of rhinovirus to low pH. First, prepare two solutions of a buffer, such as HEPES, one at pH 3.0 and one at pH 7.0. Add 0.2 ml of unknown virus suspension to 1.8 ml of pH 3.0 HEPES and 0.2 ml of virus to 1.8 ml of pH 7.0 HEPES. Keep the mixtures at room temperature for 3 h, adjust the pH to 7.0, make serial dilutions of the mixtures, and inoculate them into the cell culture. If the unknown virus is a rhinovirus, a minimum 2 $\log_{10}$ reduction in viral titer should be evident in the acid-treated sample. A known rhinovirus and a known enterovirus should be treated in a similar fashion as controls.

Resistance to Lipid Solvents

Since rhinoviruses lack a lipid envelope, they are resistant to treatment with lipid solvents such as chloroform or ether, and infectivity titers should not be affected. Add 0.1 ml of chloroform to 1 ml of isolate, shake vigorously for 10 min, and then centrifuge at 1,000 × *g* for 5 to 10 min. Remove the top or aqueous phase, prepare serial dilutions, and inoculate them into the cell culture. Alternatively, add 0.2 ml of diethyl ether to 0.8 ml of isolate in a stoppered tube and shake vigorously. Allow the mixture to react at room temperature for 1 to 2 h; shake the tube intermittently. Then, transfer the mixture into an open glass petri dish in a fume hood and allow the ether to evaporate. Prepare serial dilutions and inoculate them into the cell cultures.

An equal volume of unknown virus should be handled in a similar manner but not treated with chloroform or ether. A known picornavirus and a chloroform-sensitive virus, such as herpes simplex virus (HSV), should also be included as controls. The picornavirus should be unaffected, and the HSV should be completely inactivated.

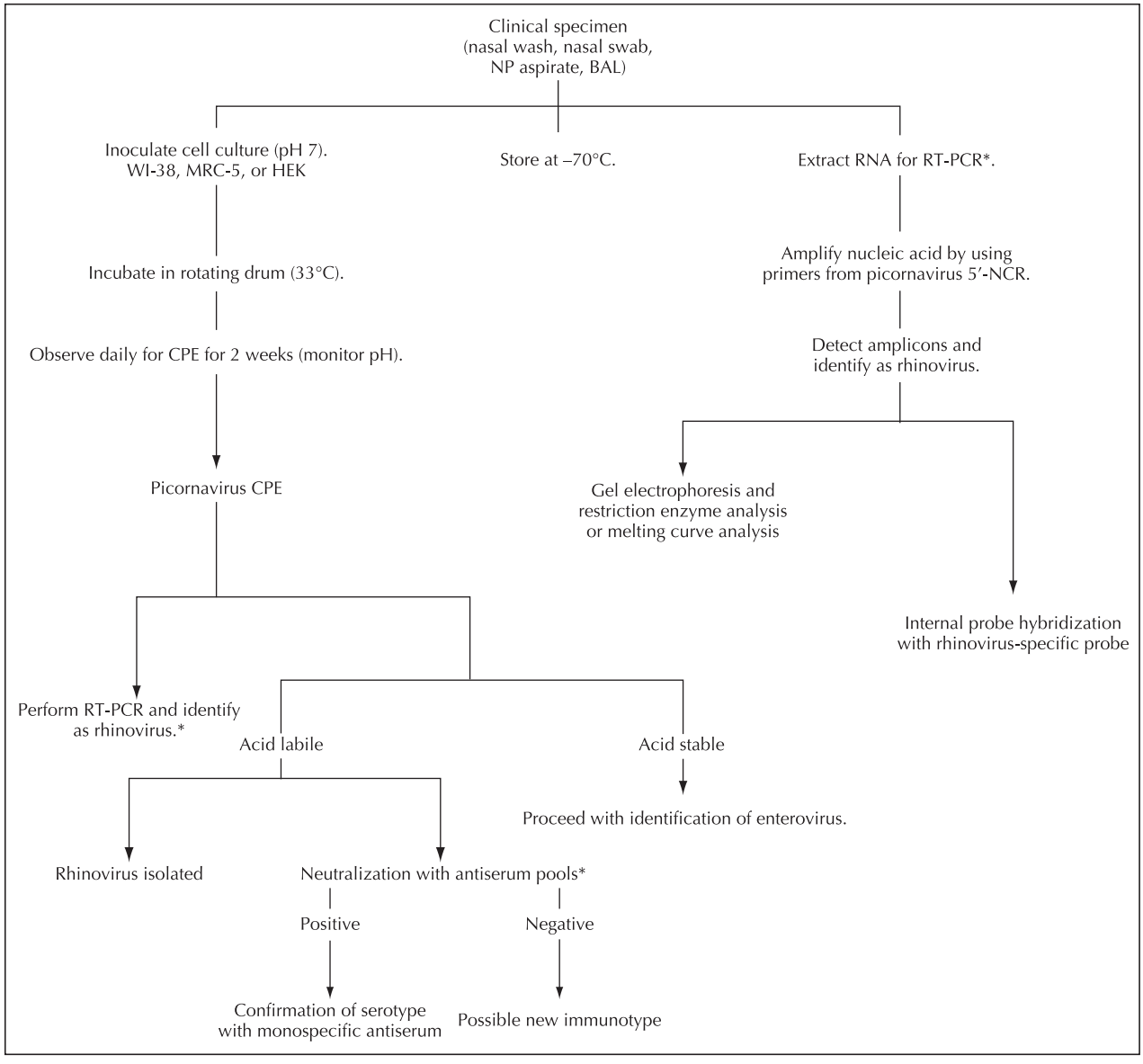


FIGURE 2 Flow scheme for the isolation or detection and identification of rhinovirus. Techniques usually reserved for the research laboratory are marked by an asterisk. HEK, human embryonic kidney cells; BAL, bronchoalveolar lavage.

Temperature Sensitivity

Since rhinoviruses often grow best at 33°C, they may be distinguished from enteroviruses by inoculation of serial dilutions of the unknown virus and incubation of one set of cultures at 33°C and a replicate set of cultures at 37°C. The onset of CPE should be more rapid and the titer of virus obtained should be higher at the lower temperature. It is important to note that some rhinovirus isolates may not show this temperature sensitivity (59).

Virus Identification by RT-PCR

Nucleic acid amplification techniques can also be used for identification of rhinovirus isolates in cell culture. However, cross-reactions with enteroviruses can occur more often with high-titered culture isolates than with clinical samples, especially when 5′-NCR regions are targeted (70).

TYPING SYSTEMS

Neutralization Tests

Specific identification of rhinoviruses by serotyping is an expensive and labor-intensive procedure that is rarely performed. Hyperimmune antisera for types 1A to 100 are available through the American Type Culture Collection. Intersecting serum pools similar to those used for enterovirus identification have been prepared, and isolates are usually tested in a microneutralization procedure to conserve reagents. Confirmation of serotype is then performed using monospecific antiserum. Neutralization generally involves the neutralization of 30 to 100 $TCID_{50}$ of virus-induced CPE by 20 U of antiserum. Nontypeable isolates are occasionally found. Detailed procedures are described elsewhere (1).

Virus Typing by RT-PCR

PCR of variable regions of 5′-NCR has also been used successfully to type a limited number of serotypes (54). In addition, HRV serotypes can be differentiated by sequencing of capsid protein genes (46, 49).

SEROLOGIC TESTS

The number of HRV serotypes makes blind serologic testing impractical in the clinical setting. In research studies, the standard serologic assay to measure antibody in serum or nasal wash specimens is the neutralization test (20). For maximum sensitivity, a low challenge dose (3 to 30 $TCID_{50}$) should be used. Plaque reduction neutralization has also been described. Hemagglutination inhibition has also been used but is not as sensitive as neutralization, and not all rhinoviruses react with red blood cells. EIAs using HRV-EL and HRV-2 antigens have been used to detect serum and nasal IgG and IgA in volunteers inoculated with these two rhinovirus types (5); EIA was 100 to 10,000 times more sensitive than neutralization. The problem of detecting antibodies to multiple serotypes in natural infection has not been addressed.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

A comparison of HRV diagnostic methods is shown in Table 1. The diagnosis of rhinovirus infection in the clinical laboratory is currently based upon isolation of virus with characteristic CPE in commonly available cell systems, such as WI-38, MRC-5, or HEK. Incubation at 33 to 35°C and rotation of cultures provide optimal conditions for virus replication. Differentiation of rhinoviruses from enteroviruses, with which their CPE can be confused, is based primarily on acid stability testing of isolates. Rhinovirus infectivity is reduced 2 to 3 $\log_{10}$ after incubation at pH 3 to 5, whereas enterovirus infectivity is unaffected. Of note, exceptions to both acid sensitivity and low incubation temperatures have been recognized (65). Specific identification of rhinovirus serotypes by neutralization test or molecular methods is reserved for epidemiologic research studies. Serologic testing is impractical and not commercially available.

Nucleic acid amplification assays have revolutionized rhinovirus detection. Yet application of this methodology to rhinovirus diagnosis has not yet made the transition into the routine clinical laboratory, due to the expense and expertise required for in-house test development and the

TABLE 1 Comparison of diagnostic methods for rhinoviruses

Method	Advantages	Disadvantages and limitations	Clinical applicability	References
Virus isolation	Rhinoviruses replicate in cell cultures commonly used in clinical laboratories (e.g., WI-38, MRC-5, HEK) These cultures may detect other viruses as well as rhinovirus (e.g., CMV, VZV, HSV, adenovirus, enterovirus, RSV)[a]	Optimal recovery of multiple rhinovirus serotypes requires use of additional sensitive cell cultures not routinely used in diagnostic laboratories (e.g., Ohio HeLa cells, HeLa I cells, or fetal tonsil) Normally sensitive cells can vary over 100-fold in sensitivity Differentiation of rhinovirus from enterovirus isolates is time-consuming (e.g., quantitative acid sensitivity test)	Most common test in clinical laboratories	1, 4, 9, 23, 68
RT-PCR or NASBA	More sensitive and more rapid than culture methods Broadly reactive primers that are most sensitive for the detection of rhinoviruses in clinical specimens usually also detect enteroviruses Amplicons can be detected in real-time assays by rhinovirus-specific probes Multiplex assays can detect multiple respiratory viruses	Molecular assays must be established and validated in-house, which is time-consuming and expensive Differentiation of rhinovirus from enterovirus can be difficult Rhinovirus-specific diagnosis is rarely requested Since the same clinical syndrome may be caused by other viruses, samples must be tested for additional viruses	Expected to increase with advent of real-time assays	6, 7, 15, 17, 26, 29, 37, 39, 41, 50, 58, 66, 67, 77, 79
Organ culture	May detect some infections not detected by virus isolation in cell culture Useful in studies of pathogenesis Biopsy specimens or surgically removed tissues can be used	Fetal nasal or tracheal tissues are not readily available Largely supplanted by molecular assays for detection of rhinovirus in clinical specimens	Occasionally used in pathogenesis research	1, 38
Serology	May detect some infections not detected by virus isolation Research tool used after infection of volunteers with a known serotype or in epidemiologic studies	The number of serotypes in natural infections makes blind serologic testing impractical Serologic tests are not commercially available	Used only in research settings	5, 18

[a] CMV, cytomegalovirus; VZV, varicella-zoster virus.

lack of clinical demand. At present in the clinical laboratory, rhinoviruses are rarely if ever specifically requested. Rather, rhinoviruses are isolated when the suspected virus is influenza or RSV. Although molecular techniques applied in the research setting will continue to elucidate the important role of rhinoviruses as lower respiratory tract pathogens and as significant causes of asthma and chronic obstructive pulmonary disease exacerbations, the standard diagnostic approach may not change substantially until either effective therapy becomes available or the impact of rapid and sensitive rhinovirus diagnosis on the management of hospitalized patients can be demonstrated.

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Coronaviruses

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TAXONOMY

All coronaviruses (CoV) belong to the genus *Coronavirus* within the family *Coronaviridae* and are enveloped viruses possessing a positive-strand RNA genome of 27 to 32 kb. CoV contain the largest known genome among all RNA viruses. There are five CoV known to infect humans, including strains OC43 and 229E, severe acute respiratory syndrome-associated CoV (SARS-CoV), and strains NL63 and HKU1. Based on genotypic and serologic characterization, CoV have been divided into three distinct groups: 229E and NL63 are group 1 CoV, and OC43, SARS-CoV, and HKU1 are group 2 CoV. No human pathogens have been reported for group 3 CoV, which include avian infectious bronchitis virus. The genomic RNA is capped and polyadenylated and comprises several genes encoding both structural and nonstructural proteins. The genome organization of CoV has the characteristic order 5′-replicase gene, spike (S) gene, envelope (E) gene, membrane (M) gene, and nucleocapsid (N) gene-3′ (22). The G+C content ranges from a low of 32% for CoV HKU1 to a high of 41% for SARS-CoV. In addition to the S, E, M, and N structural proteins, the genome also encodes a chymotrypsin-like protease, replicase (polymerase), helicase, and hemagglutinin-esterase. In the replicase gene, a frameshift interrupts the protein coding regions and separates open reading frames 1a and 1b. Both the 5′ and 3′ ends contain short untranslated regions. The RNA genome is translated as a polyprotein which is subsequently cleaved by the chymotrypsin-like protease activity. The S gene encodes the S glycoprotein which binds to the host cell receptor (ACE-2 for SARS-CoV and aminopeptidase N [CD13] for CoV 229E) and leads to membrane fusion and viral entry.

DESCRIPTION OF THE AGENTS

CoV have been associated with a very high mutation rate that has led to altered properties, host ranges, and disease manifestations in animals. Recombination rates are very high, which is unusual for a nonsegmented RNA virus, due to discontinuous transcription and polymerase jumping. Group 2 CoV have apparently acquired the hemagglutinin/esterase (HE) gene from influenza C virus in this manner. CoV have been identified in a wide range of animals including mice, rats, chickens, turkeys, swine, dogs, cats, bats, rabbits, horses, cattle, and humans and cause a variety of severe diseases including gastroenteritis and respiratory tract diseases. CoV-like particles have been identified in human feces since the early 1980s, but the link with gastroenteritis has not been firmly established. Prior to 2002, there were only two CoV known to infect humans, namely, CoV OC43 and CoV 229E. Since 2002, three additional human CoV have been discovered, bringing the total number of CoV infecting humans to five. Severe acute respiratory syndrome (SARS) emerged from China in the fall of 2002, and SARS-CoV was identified as the causative agent (34). On the basis of genome analysis, SARS-CoV was originally assigned to a fourth CoV group but more recently has been recognized as a distant relative to the group 2 CoV and is now considered a group 2 subgroup (12, 22). In 2004, a novel group 1 CoV associated with respiratory tract infections, CoV NL63, was discovered in The Netherlands and its genome was sequenced (45). In 2005, a fifth human CoV, a group 2 CoV, was discovered in patients with pneumonia in Hong Kong (47). All CoV are pleomorphic, are 80 to 160 nm in size, and contain a corona consisting of club-shaped peplomers (Fig. 1). CoV replicate in the cytoplasm and bud off the cytoplasmic membrane. Virion release occurs via membrane fusion and exocytosis, with eventual cell death.

EPIDEMIOLOGY AND TRANSMISSION

Many CoV have been identified in a variety of different animals, and for the most part, these viruses do not cross over into other species. SARS-CoV is believed to be zoonotic in origin, having crossed into humans most likely from animal hosts in the wild-game markets in Guangdong, China. Many of the first infected individuals in November and December 2002 had contact with the live-game trade. The disease was first called "infectious atypical pneumonia" because it caused clusters of disease in families and health care workers. The etiologic agent of SARS was identified as a new CoV by Peiris and coworkers at the University of Hong Kong (34), and the viral genome was fully sequenced in a record time of 3 weeks, first by workers at the University of British Columbia, Vancouver, Canada (32). Genomic analysis revealed that SARS-CoV was a new CoV not previously found in humans. The absence of antibody in healthy humans suggested that SARS-CoV had recently emerged in the human population and that animal-to-human interspecies transmission was the most probable explanation for its emergence. Specimens collected from apparently healthy animals in the wild-game animal markets in China indicated that a number of animals

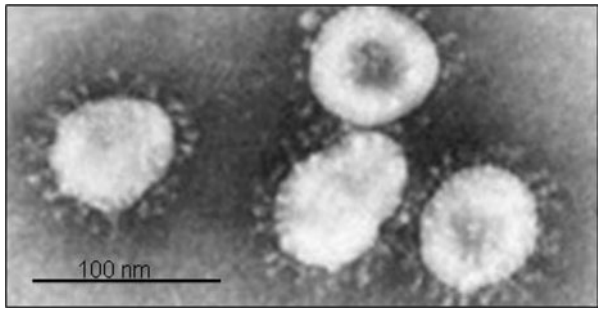


FIGURE 1 Electron micrograph of CoV OC43 showing pleomorphic shape and characteristic coronas made up of surface peplomers.

including the Himalayan palm civet cat and raccoon dogs yielded a SARS-CoV-like virus with more than 99% nucleotide homology to human SARS-CoV (15). Many animal handlers with no previous history of a SARS-like illness had serum antibodies to SARS-CoV. Taken together with the fact that a number of SARS cases had an epidemiologic link to wild-game animals, it is very likely that these markets in Guangdong provided the interface for transmission to humans. The early transmissions to humans were probably inefficient, causing little disease or human-to-human transmission. The animal SARS-like virus probably adapted in humans for efficient human-to-human transmission, and SARS-CoV emerged. There is evidence that SARS-CoV evolved toward greater fitness during the SARS outbreak as human viruses isolated late in the outbreak contain a 29-nucleotide deletion in open reading frame 8 that is absent both from human viruses isolated early in the outbreak and from animal viruses (15). Similarly, the gene encoding the S protein that mediates viral entry shows high rates of nonsynonymous mutation in early isolates that are absent in later isolates, probably reflecting the ongoing adaptations to the new host. Recent reports on the detection of SARS-like CoV from the blood and feces of Chinese horseshoe bats in the wild and Chinese markets raise the possibility of a bat origin of SARS-CoV (25).

The major routes of CoV transmission in humans are droplet infection, aerosolization, and fomites. Deposition of droplets onto the respiratory epithelium probably initiates infection. Whether infection can occur through the oral or conjunctival epithelium remains unknown, but SARS-CoV has been detected in tears. As mentioned above, SARS first appeared as a potentially fatal cause of pneumonia in the Guangdong province of China in 2002. The first human case was identified on 16 November, and within 6 months SARS had spread to 29 countries, infecting 8,098 and killing 774 individuals (50). The last known case in this outbreak occurred 15 July 2003 in Taiwan. This outbreak had a profound effect on public health and economies worldwide, with an estimated $100 billion lost from global economies. SARS has arguably been the most significant event in medical virology since the emergence of human immunodeficiency virus and AIDS in the early 1980s.

At the beginning of the SARS outbreak, nosocomial infections played an important role in the outbreak. The first major outbreak in Hong Kong occurred in the Prince of Wales Hospital around 10 March 2003, resulting in 138 SARS cases, 69% of which were in hospital workers. By the end of the outbreak, there were 1,755 SARS cases in Hong Kong, 339 of which were in workers in 16 hospitals. In Canada, the first large outbreak also occurred in a community hospital, affecting 128 patients, of which 37% were members of the hospital staff (37). Asymptomatic infection of humans appears to be a rare event. Despite the rapid spread of SARS worldwide, the average number of secondary infections caused by any one case is low (2.2 to 3.7) compared with that in influenza (5 to 25) and household transmission of SARS is relatively inefficient (13). Superspreading events in which a few infected individuals disproportionately contribute to transmission (e.g., a difficult intubation in the intensive care unit's resulting in infection of a number of house staff) were characteristic of the outbreak. Factors associated with superspreading events are not well understood but may include coinfection with other viruses, host factors such as immunosuppression, and/or environmental factors. The number of infected staff members in hospitals was strongly correlated with the number of admitted SARS patients, the length and type of exposure, and the use of personal protective equipment (PPE) such as gloves and masks. Late in the outbreak, it was shown that PPE significantly decreased the occurrence of nosocomial infections and played a significant role in outbreak management (29). These and other studies clearly indicate that exposure to infected individuals is the driving force behind the horizontal transmission of SARS.

CLINICAL SIGNIFICANCE

Human CoV OC43 and 229E were among the first human CoV to be discovered in the mid-1960s. These viruses were isolated from adults with respiratory illness and grew only in embryonic tracheal organ cultures. They usually cause a mild, self-limited upper respiratory tract infection with common cold-like symptoms in adults. Overall, OC43 and 229E account for between 5 and 30% of respiratory tract infections, with outbreaks occurring at 3- to 4-year intervals (44). Like those with other respiratory viruses, infections with human CoV can present with a wide range of signs and symptoms, from a common cold with a cough, runny nose, and fever to a lower respiratory tract infection with bronchiolitis or pneumonia. Human CoV OC43 and 229E have been associated with respiratory tract infections in a variety of settings and have been linked to nosocomial infections in high-risk immunocompromised children, in hospitalized elderly patients with noninfluenza severe respiratory tract infection and pneumonia, and in newborns, children, and hospital staff (10, 35, 43). In one recent prospective study of 501 patients in France, OC43 was found in 6% of patients compared with 6.1% for respiratory syncytical virus, 7.8% for influenza virus, 6.4% for rhinovirus, 1% for parainfluenza 2 virus, 2% for adenovirus, and 1% for enterovirus (43). Symptoms included fever (59%), rhinitis (37%), pharyngitis (30%), laryngitis (3%), otitis (13%), bronchitis (17%), bronchiolitis (10%), and pneumonia (7%). Like other respiratory viruses, human CoV OC43 and 229E are spread by large-droplet infection. Of concern to infection control practitioners is a recent finding that 229E can survive for up to 3 h when dried on solid surfaces and for up to 6 days in saline solution at room temperature.

The first reported SARS death was that of a restaurant chef who first presented with pneumonia. The natural history of SARS-CoV has now been documented in several studies. The initial symptoms are unremarkable and typical of an upper respiratory tract viral infection. After a few days, coughing and low-grade fever progress rapidly into full-blown pneumonia requiring hospitalization and often mechanical ventilation. Fever, malaise, lymphopenia, and

elevated liver enzymes, together with infiltrates and consolidation visible upon chest X ray, are usually present. Quantitative PCR studies have shown that the viral load is high in the lower respiratory tract but low in the upper respiratory tract. Viral loads in the upper respiratory tract and feces are low during the first 4 days and peak at around day 10 of illness (37). This is in marked contrast to viral loads in other respiratory viral infections such as influenza, in which the loads peak soon after the onset of symptoms. This unusual feature of SARS-CoV infection explains its low transmissibility early in the illness and perhaps explains why outbreaks in some countries were limited to only a few cases. More importantly, it explains the poor sensitivity of early reverse transcriptase (RT)-PCR tests on nasopharyngeal (NP) specimens collected early in the illness.

Although the main signs and symptoms of SARS are associated with the respiratory tract, the virus also infects other organs. About a quarter of SARS patients had watery diarrhea, and virus can be cultured from the feces and urine as well as the respiratory tract. Virus also can be detected by RT-PCR in serum, plasma, and peripheral blood leukocytes; however, the viremia may be short lived. Patients have pronounced peripheral T-cell lymphocytopenia with reduced CD4 and CD8 cell counts, with a third of individuals having a CD4 count of less than 200 cells/mm^3. Infected individuals with high serum viral loads have a poor prognosis. Between days 10 and 15 of illness, high viral loads in NP aspirates (NPA), feces, and serum are independent predictors of adverse clinical outcome. SARS-CoV is invariably found in the lungs of individuals dying of SARS, but viral loads are usually higher in those dying earlier in the course of illness (<21 days after the onset of symptoms). About one-quarter of patients with SARS require management in intensive care units, and the overall case fatality rate was 10%. Disease severity and mortality are correlated with age, with the highest mortality rates (52%) occurring in those >65 years of age and the lowest rate in the 0- to 24-year-old group. Children acquiring SARS seldom require intensive care or mechanical ventilation (18). Although SARS-CoV can be found for months in the feces of an infected individual, there is no evidence that the virus persists following resolution of the illness.

The fourth human CoV, NL63, was first discovered in The Netherlands in a 7-month-old boy who presented with coryza, conjunctivitis, and fever and who had chest X-ray findings consistent with bronchiolitis (45). The virus grew in tertiary monkey kidney cells, which distinguished it from human CoV OC43 and 229E. Sequencing of the genome indicated that it was not a recombinant virus and was genetically distinct from all other human CoV. Following the first case of infection, NL63 was detected in five additional children and three adults, two of which were immunocompromised. NL63 has also been detected in 2% of prospectively studied patients with respiratory tract infections in Brisbane, Australia. Fouchier et al. independently reported a novel group 1 CoV with a 27,555-nucleotide genome with a 34% G+C content (11). This virus was first isolated in 1988, from an 8-month-old with pneumonia by using monkey kidney cells and is most likely the same virus as NL63. Use of an RT-PCR assay and primers based on the first isolate permitted detection of this virus in 4 of 139 specimens collected from pediatric patients with upper respiratory tract infections between 2000 and 2002, indicating that this virus has been circulating in The Netherlands for a number of years.

The fifth human CoV, HKU1, was discovered in January 2004 in Hong Kong in a 71-year-old man returning from Shenzhen, China. He presented with fever and a productive cough with purulent sputum and had a chest radiograph showing patchy infiltrates. All attempts to grow a virus from NP specimens failed, but CoV RNA was detected in the NPA by RT-PCR using *pol* gene consensus primers (47). Quantitative PCR indicated high titers of virus (10^6 genome equivalents) present in the NPA during the first week of illness, with decreasing titers in the second week and undetectable levels of virus in the third and fourth weeks. The virus was absent from urine or stool specimens. A recombinant-N-protein-based enzyme-linked immunosorbent assay (ELISA) indicated seroconversion in the first week of infection, with both immunoglobulin M (IgM) and IgG antibody detectable. A second case was subsequently identified, that in a 35-year-old woman with pneumonia with unknown etiology. The recent discovery over the past 2 years of two new human CoV isolated from the upper respiratory tracts of symptomatic patients suggests that additional, as yet unidentified human CoV may await discovery.

COLLECTION, TRANSPORT, AND HANDLING OF SPECIMENS

Specimens for the detection of the non-SARS-CoV may include either upper or lower respiratory tract specimens. NP, oropharyngeal, endotrachial, bronchoalveolar lavage (BAL), and postmortem lung specimens are all useful for detecting CoV. With respect to SARS-CoV, NP specimens, which are often negative during the first week of infection, have the highest positivity rates in the second week of illness, with rates peaking at about day 10. Quantitative PCR revealed that viral loads were highest in lower tract specimens (BAL and sputum specimens and endotracheal aspirates), and BAL specimens have been positive more frequently than NP specimens (4, 8, 40). The collection of BAL specimens, however, has been associated with an increased risk of nosocomial transmission and risk to health care workers and was halted in many institutions. The low virus detection rate for NP specimens early in the course of infection underscores the importance of optimal timing and specimen type for diagnosis. The Centers for Disease Control and Prevention, Atlanta, Ga., has recommended that multiple specimen sources be tested, namely, NP plus oropharyngeal and serum or plasma specimens during the first week of illness and NP plus oropharyngeal and stool specimens after the first week of illness. For the interpretation of a positive diagnosis based on PCR testing, it is generally recommended that a patient have either two positive clinical specimens from different anatomical sites or positive specimens from the same site collected on two separate occasions (http://www.cdc.gov/ncidod/sars/guidance/f/pdf/app4.pdf).

Natural history studies of SARS patients indicate that SARS-CoV is also present in nonrespiratory specimens including blood, cerebrospinal fluid, urine, tears, and feces. Serum, plasma, and buffy coat specimens are prepared from coagulated or noncoagulated blood specimens, the latter including EDTA, heparin, or mononuclear cell tubes (Becton Dickinson), for use in SARS-CoV detection. Rectal swabs or fecal specimens are good diagnostic specimens, especially late in the course of infection. Replication of SARS-CoV in the gastrointestinal tract has been confirmed by electron microscopy, in situ hybridization, and RT-PCR testing of biopsy and postmortem specimens (42). The discovery of SARS-CoV in feces indicates that the virus rapidly disseminates from the NP to other tissues and suggests that the virus could be transmitted by the fecal route. In the case of stool specimens, SARS-CoV RNA can be detected at high levels for several weeks or even months whereas virus culture

is positive only during the first 2 to 3 weeks of illness (33). Fecal specimens have a high viral load at the end of the first week of illness and are the specimen of choice for diagnosis during the second week of disease. It is possible that virus replication continues for weeks in the gastrointestinal tract but that virus is complexed with antibody and is no longer infective or transmissible. This possibility is consistent with epidemiologic findings. Of the nonrespiratory specimens, stool specimens have shown the highest positivity rates and highest viral loads. Testing of serum or plasma specimens for viral RNA was disappointing at first, but a subsequent study has shown 50 and 78% positivity rates for serum and plasma samples during the first week of illness (46). A second study confirmed these results for plasma with a 79% positivity rate during the first 3 days of illness (14).

Given the severity of SARS and recent reports of laboratory-acquired infections (27), appropriate precautions should be followed for handling specimens submitted for SARS investigation. Testing of respiratory or stool specimens must be done using biosafety level 2 precautions in a class II biological safety cabinet with laboratory workers wearing PPE including long-sleeved gowns, gloves, eye protection, and N95 masks. Virus isolation should not be attempted by routine clinical laboratories since SARS-CoV is a level III agent and propagation of virus requires a biosafety level 3 facility.

DIRECT DETECTION

Microscopy

Histopathologic changes due to CoV infections vary greatly depending on the virus, the host species, and the cell type infected, and ergo, there are no tissue changes indicative of a CoV infection. CoV-infected cells can be detected by indirect immunofluorescence (IF) staining using antibody specific to the desired CoV and a fluorescein isothiocyanate-conjugated anti-species antibody. IF staining can be used in combination with the shell vial technique employing centrifugation-assisted inoculation and staining at 24 h postinfection to detect early antigens. Electron microsocopy can be used to identify CoV directly in clinical specimens or in infected cell cultures. Negative staining of tissue culture media or aqueous specimens with phosphotungstic acid reveals the pleomorphic virions with diameters of 80 to 160 nm and characteristic coronas consisting of club-shaped peplomers (Fig. 1).

Antigen Detection

Human CoV antigens can be detected in cells from NP or throat wash specimens by standard methods and stained with CoV-specific monoclonal antibodies and a fluorescence-labeled anti-mouse IgG conjugate (28, 38). Specific CoV antigens can also be detected by ELISA using immobilized antibody to capture antigens from clinical specimens and a second species of CoV antibody and anti-species IgG conjugate. This approach has recently been used for detection of SARS-CoV N antigen in serum or NP specimens (7, 17, 26, 28). In one study involving serially collected specimens, detection of N protein in serum was more sensitive than IgG ELISA or RNA PCR tests for diagnosing SARS in the first 10 days after onset (26). To date, no commercial assays are available for CoV antigen detection.

Nucleic Acid Analysis

A variety of nucleic acid amplification tests (NAAT) including RT-PCR, nucleic acid sequence-based amplification (NASBA), and loop-mediated isothermal amplification (LAMP) have been used to detect CoV RNA. The first RT-PCR assays described for SARS-CoV RNA targeted the polymerase *(pol)* 1B region of the 5′-replicase gene. The *pol* 1B primers have been used for the development of both nested and nonnested, 1- and 2-step (involving combined and separate RT reactions, respectively) conventional heat block RT-PCRs as well as real-time PCR assays (Table 1). Drosten et al. (2003) used two sets of primers in a nested PCR format to amplify a 109-bp fragment and the same primers in a real-time assay and showed that the real-time assay had sensitivity equivalent to that of the nested assay for detecting SARS-CoV RNA in 18 clinical specimens (9). Ksiazek et al. (2003) used different *pol* 1B primers and described a 2-step assay with a sensitivity of 100 genome equivalents (GE) that was more sensitive than culture (21). Poutanen et al. (2003) also described a 2-step *pol* 1B assay for detecting SARS-CoV RNA in clinical specimens obtained from 10 patients in the Toronto, Canada, SARS outbreak (37). These first three assays were used to test only a small number of clinical specimens and were not compared to any other NAAT, precluding an assessment of their performance.

Lau et al. (2003) described an enhanced RT-PCR involving target preamplification by using two separate amplifications within the *pol* 1B gene (23). The first PCR was a conventional heat block assay followed by real-time amplification using the product from the first reaction. The product of the enhanced RT-PCR was a 68-bp amplicon. The assay was used to test 120 specimens from 80 patients in Hong Kong and China and detected 28 of 120 positive specimens versus 21 of 120 positive specimens detected by the real-time assay (23). Additional testing of the discordant specimens was not performed to allow a comparative assessment of assay performances.

Following the introduction of these original assays, additional tests were developed that amplify alternative gene targets. Mahony et al. (2004) introduced 1- and 2-step real-time assays that amplify a 149-bp fragment of the N *(nuc)* gene (31). The 1-step real-time assay was evaluated by testing 68 specimens and has an analytical sensitivity similar to that of the *pol* 1B assay (10 GE). Bressler and Nolte (2004) described a 2-step real-time Taqman assay amplifying a 66-bp fragment of the *nuc* gene with an analytical sensitivity of 90 GE (2). They showed that the *nuc* gene assay has a sensitivity similar to that of their *pol* 1B assay using armored-RNA-spiked specimens. Drosten et al. (2004) also showed that a 1-step real-time assay targeting the *nuc* gene detected numbers of positive specimens similar to those detected by two other *pol* 1B assays when 66 specimens were evaluated (8).

Real-time assays offer faster turnaround time and the ability to quantitate viral load. Wang et al. (2004) tested 116 SARS specimens by using a 2-step assay amplifying a 123-bp fragment of the *pol* 1B gene (46). Houng et al. (2004) introduced a 2-step real-time assay that amplifies a region of the 3′ noncoding region of the genome (19). This assay has an excellent analytical sensitivity of 6 to 8 GE but has not been evaluated with SARS specimens. Poon et al. (2004) introduced a real-time multiplex assay that targets both the *pol* 1B gene and 18S rRNA as an internal control (36). This 1-step Taqman assay has an analytical sensitivity of 10 GE and was evaluated by using 86 NP specimens from SARS cases. An internal control was used to identify specimens that contained amplification inhibitors or that lacked specimen RNA because of a failed extraction. In their study, 0 of 86 NP specimens failed to amplify the internal control, indicating successful RNA isolation and the absence of amplification inhibitors.

TABLE 1 Description of 14 nucleic acid amplification assays for detection of human CoV RNA[a]

Target	Primer name	Primer sequence	Amplicon size (bp)	Format[b]	Analytical sensitivity[c]	No. of clinical specimens	Comments	Reference
pol 1B	BNIoutS2 BNIoutAS BNIinS BNIAs	5′-ATGAATTACCAAGTCAATGGT TAC-3′ 5′-CATAACCAGTCGGTACAGCTAC-3′ 5′-GAAGCTATTCGTCACGTTCG-3′ 5′-CTGTAGAAAATCCTAGCTGGAG-3′	190 109	2 step nested, 1 step Taqman, real time	10 GE	49	Sensitivities of nested and real-time assays were equivalent for detecting SARS-CoV in 18 specimens.	8
pol 1B	Cor-p-F2 Cor-p-R1	5′-CTAACATGCTTAGGATAATGG-3′ 5′-CAGGTAAGCGTAAAACTCATC-3′	368	2 step	100 GE	19	PCR more sensitive than culture for detecting SARS-CoV	21
pol 1B		5′-TGATGGGATGGGACTATCCTAAGTGTGA-3′ 5′-TTGCATCACCACTAGTTGTGCCACCAGGTT-3′	ND	2 step	ND	10	5 of 6 patients were PCR positive for SARS-CoV.	37
nuc	APNF APNR	5′-TGAATACACCCAAAGACCAC-3′ 5′-TGATGAGGAGCGAGAAGAG-3′	149	1 step, real time	10 GE	68	Comparison of 5 in-house nested, nonnested, and real-time assays and 1 commercial assay for detecting SARS-CoV	31
pol 1B	Sense Antisense	5′-TTATCACCCGCGAAGAAGCT-3′ 5′-CTGTAGAAATCCTAGCTGGAG-3′ FAM-TCGTGCGTGGATTGGCTTTGATGT-TAMRA	123	2 step, real time	ND	116	SARS-CoV detected	46
pol 1B	Coro1 Consensus Coro2	5′-TGATGGGTTGGGACTCTAAATGTGA-3′ 5′-GTAGTTGCATCACCGGAAGTTGTGCCACC-3′	220	1 step	1–10 GE	44	Uses consensus primers, detects other CoV as well as SARS-CoV	1
pol 1B		5′-GGTTGGGACTATCCTAAGTGTGA-3′ 5′-CCATCATCAGATAGAATCATCATA-3′	440	2 step	ND	0	Uses consensus primers, detects other CoV as well as SARS-CoV	47
3′ NCR	Sense Antisense	5′-GGACCTGAAAGAGCCACCACA-3′ 5′-CATTATTCACTGTACCCTCGATCG-3′ FAM-TTTCATCGAGGCCACGCGGAG-TAMRA	ND	2 step, real time	6–8 GE	16	SARS-CoV detected	19
ND	Cor-1 Sense Cor-2 Antisense	5′-CACCGTTTCTACAGGTTAGCTAACGA-3′ 5′-AAATGTTTACGAAGGTAAGCGTAAAA-3′	286	2 step	ND	0	RT-PCR more sensitive than culture for detecting SARS-CoV	4
nuc	SANS1 SANPAs2	5′-TGGACCCACAGATTCAACTGA-3′ 5′-GCTGTGAACCAAGACGCAGTAT-3′ FAM-TAACCAGAATGGAGGACGCAATGG-TAMRA	ND	1 step, real time	ND	66	Compared 2 commercial assays to in-house assays for detecting SARS-CoV	8

nuc	Forward Reverse	5′-GGAGCCTTGAATACACCCAAAG-3′ 5′-GCACGGTGGCAGCATTG-3′ FAM-CCACATTGGCACCCGCAATCCTAATA-TAMRA	66	2 step, real time	90 GE	0	*pol* 1B and *nuc* RT-PCRs had similar sensitivities for detecting SARS-CoV	2
pol 1B		5′-CAGAACGCTGTAGCTTCAAAATCT-3′ 5′-TCAGAACCCTGTGATGAATCAACAG-3′ FAM-TCTGCGTAGGCAATCC-TAMRA	ND	1 step, real time	10 GE	86	Used 18S rRNA as internal control for detecting SARS-CoV	36
pol 1B	Antisense Sense	5′-AGTTGCATGACAGCCCTCTACA-3′ 5′-CCCGCGAAGAAGCTATTCG-3′ FAM-CGTTCGTGCGTGGATTGGCTTTG-TAMRA	68	2 step, 2 PCR, real time	ND	120	First PCR followed by second Taqman PCR assay for detecting SARS-CoV	23
LAMP *pol* 1B		6 primers used included 2 outer, 2 inner, and 2 loop primers that recognize 8 distinct regions on the target zone.	196	1 step	0.01 PFU	59	LAMP detected 7 additional SARS-CoV positives.	41

[a] ND, no data available; 3′NCR, 3′ noncoding region.
[b] Format refers to whether the RT step is combined with (1 step) or separate from (2 step) the PCR.
[c] Sensitivity refers to the number of SARS-CoV RNA GE per reaction.

Two recent reports have described consensus *pol* 1B gene primers for the detection of CoV RNA including that of SARS-CoV. Adachi et al. (2004) used consensus primers to amplify a 220-bp fragment of *pol* 1B in a 1-step assay with an analytical sensitivity of 10 GE (1). They tested 44 SARS specimens as well as cultured CoV 229E and OC43 and turkey infectious bronchitis virus and showed that these consensus primers could detect all four CoV. Woo et al. (2005) described a 2-step conventional heat block (non-real-time) assay that uses consensus *pol* 1B primers to amplify a 440-bp fragment of the *pol* 1B gene (47). This assay was used to detect a novel human respiratory CoV, HKU1, in a 71-year-old with non-SARS pneumonia during the SARS outbreak in Hong Kong. This assay, which uses consensus primers, should be able to detect SARS-CoV, although the authors did not show that the assay was able to do so.

Isothermal amplification formats including NASBA and LAMP have also been used to detect SARS-CoV RNA. Thai et al. (2004) developed a LAMP assay using six primers, including two outer, two inner, and two loop primers, that recognize eight distinct regions of the *pol* 1B gene (41). The 1-step LAMP assay amplifies a 196-bp fragment and has an analytical sensitivity of 0.01 PFU. They evaluated the assay by testing 59 specimens from Vietnamese patients by both LAMP and conventional RT-PCR. LAMP detected 13 positive specimens compared to 6 detected by RT-PCR. Analysis of the discordant results was not performed to determine whether the additional positive specimens were true positives or false positives. A NASBA test for SARS-CoV RNA has been described, but this test has not yet been evaluated with clinical specimens (5). Juang et al. recently described a multiplex RT-PCR that amplifies two SARS-CoV genes and uses a gene chip detection platform (20).

Commercially available tests have recently appeared and include the RealArt HPA CoV RT-PCR assay from Artus GmbH (Hamburg, Germany), the GeneCode SARS-CoV NP and POL MultiCode-RTx kits from EraGen Biosciences (Madison, Wis.), and the LightCycler SARS-CoV kit from Roche Diagnostics (Branchburg, N.J.). Limited evaluations of these assays have been performed, and their true performance has yet to be determined. These first-generation assays appear to have suboptimal performance with low clinical sensitivities (36.4 to 94.4%) and low specificities (87 to 100%) when a combined reference standard of positivity is used (30). The introduction of second-generation tests with improved sensitivities and specificities is eagerly anticipated. Diagnostic companies are presently working on chip- and bead-based assays for the detection of human CoV and other respiratory viruses.

ISOLATION PROCEDURES

Cell culture has proven to be unreliable for primary isolation of CoV, and some strains grow in cell culture only after adaptation. Human CoV OC43 and 229E can be isolated in human embryonic lung cells (L132) or MRC-5 cells but require incubation at 32 to 34°C (6). Human CoV NL63 and HKU1 can be isolated in tertiary monkey kidney (cynomolgus monkey) or LLC-MK2 cells. Cytopathic effects may take 7 to 10 days to appear and vary from a ground-glass refractive appearance to cell rounding and detachment depending on the host cell (Fig. 2). SARS-CoV can be isolated in Vero-E6 cells, although this must be attempted only in a biosafety level 3 laboratory.

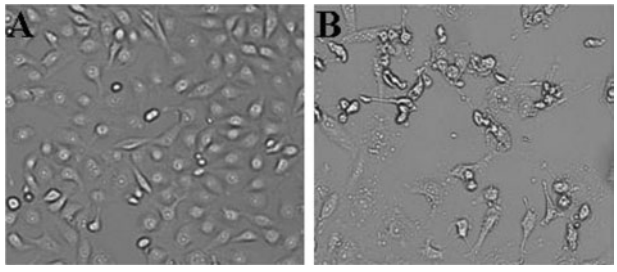


FIGURE 2 Cytopathic effect at 5 days postinfection for NL63-infected LLC-MK2 cells (B). Panel A shows uninfected LLC-MK2 cells.

TYPING SYSTEMS

Although human CoV can be isolated by using cell culture, there are no type-specific antibodies for typing of isolates. Nucleic acid sequence analysis must therefore be used for viral typing. CoV typing can be performed by PCR using consensus primers followed by amplicon sequencing or PCR using type-specific primers.

SEROLOGIC TESTS

Due to the benign nature of the non-SARS-CoV infections, CoV antibody tests have not been widely used for diagnosis but have been used primarily by research laboratories for seroepidemiologic studies. Early in the SARS outbreak, detection of SARS-CoV antibody was considered the "gold standard" for diagnosis. The first antibody tests were IF assays using virus-infected cells spotted onto microscope slides or ELISAs using crude virus-infected cell lysates to coat microtiter plates. Both methods proved useful in diagnosing SARS, but it soon became clear that serology had a major limitation for diagnosis. Seroconversion following infection with SARS-CoV usually occurred in weeks 2 and 3 of illness, and some patients seroconverted as late as 28 days postinfection. Given the delayed seroconversion, serology has limited utility for the diagnosis of infection during the acute phase of illness. SARS-CoV IgM and IgG class antibodies appear at the same time following infection, and therefore IgM testing does not assist in the serodiagnosis. IgA antibody appears about the same time as IgG and persists in serum for 1 to 2 months. IgM antibody persists for about 11 weeks and is then undetectable, whereas IgG persists for months and probably years. The SARS whole-cell immunofluorescence (IF) test detects mostly antibody to the N protein, and about 4% of sera from healthy individuals give a positive result, due to the presence of antibodies cross-reacting with N proteins of other CoV. ELISAs using crude antigens showed sensitivities and specificities similar to those of the IFA test. Western blot (WB) assays using whole virus cell lysates show the presence of a predominant N protein band (often a doublet or triplet of around 36 to 48 kDa), an S antibody band at 150 kDa, and less predominant antibody bands at 80, 60, 32, and 24 kDa (24). Recombinant S and N proteins have quickly replaced viral lysates in ELISAs and WB and immunodot assays and provide a safe, low-cost alternative for serodiagnosis. The N protein has been shown to be the immunodominant protein of SARS-CoV and an excellent antigen for use in the ELISA. The recombinant-N-protein-ELISA has improved sensitivity and specificity compared with the whole-lysate assay and can be used to measure IgG, IgA, and IgM class antibody by using specific conjugates. Several laboratories have compared the use of the recombinant N protein in a WB assay and an immunodot assay or ELISA and found sensitivities and specificities in the range of 94 to 96 and 96 to 98%, respectively (48). Although the S protein is an important viral protein and elicits neutralizing antibody, the recombinant-S ELISA is less sensitive than a recombinant-N ELISA (49) and it has not been widely used in serologic tests. The development of commercial tests for the measurement of SARS-CoV antibody has been slow; the Euroimmun IFA test from Germany has been the only test to be introduced into North America. Further evaluations using large numbers of sera will be required to determine the true performances of individual antibody tests compared with that of the neutralization test (NT).

The gold standard test for detecting SARS-CoV antibody is the NT. The performances of all new serologic tests should be evaluated by comparing them with that of the NT. Because this test measures neutralizing antibody by reacting serum with live virus, the test can be performed only in a biosafety level 3 facility and is limited to only a few laboratories. For this reason, the kinetics for the appearance of NT antibody and the role of IgM and IgA class antibody in virus neutralization are poorly understood. The recent development of SARS-CoV pseudotype virions and their use in neutralization and virus entry assays provide a novel method for assessing neutralizing antibody in SARS patients' sera that is safe (does not require biosafety level 3 containment) and cost-effective (16). This assay can easily be automated for high-throughput capacity for the large-scale screening of neutralizing antibody for vaccine trials. Recent comparisons of different SARS antibody tests indicate that the NT has the highest specificity and that the ELISA using recombinant N or S proteins has higher sensitivity (49; M. Smieja, J. Mahony, M. Petric, T. Mazzulli, M. Fearon, G. Hawes, D. Dick, T. Booth, L. deKoning, R. Spafford, L. Haynes, and S. Richardson, *20th Annu. Clin. Virol. Symp.,* abstr. B40, 2004). A WB assay can be used to confirm N protein ELISA positive results (48).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

In the event that SARS returns, laboratories will be faced with significant challenges to diagnose SARS when other common respiratory viral infections and possibly new, unconventional agents such as avian influenza virus are cocirculating in the population. Of the nearly 20 different NAAT that have been described for the detection of SARS-CoV RNA, most of the assays have been evaluated only with small numbers of clinical specimens from infected patients, and their true performance characteristics are poorly defined. This is particularly the case for the first generation of commercially available assays. The unique natural history of SARS-CoV infection, together with its clinical importance, dictates the testing of both respiratory and nonrespiratory specimens, the testing of multiple specimens from the same patient, and confirmatory testing performed by a reference laboratory before a positive diagnosis is reported.

Laboratory policy for reporting positive test results should be based on recommendations from the Centers for Disease Control and Prevention and the World Health Organization as follows: for a positive RT-PCR result, (i) repeat the test using a second aliquot of the original specimen and, if positive, confirm the result by sending the specimen to a reference laboratory, or (ii) test the positive specimen by using

a second RT-PCR test targeting a different part of the genome and, if positive, confirm by sending the specimen to a reference laboratory (http://www.cdc.gov/ncidod/sars/guidance/f/pdf/app2.pdf). A negative RT-PCR test result for an NP specimen from a suspected SARS patient does not rule out SARS since SARS-CoV is present in low levels in the upper respiratory tract in the first week of infection (3, 40). A negative result from an infected patient may be due to the presence of PCR inhibitors that copurify with RNA, a poor-quality specimen, or a specimen lacking virus. Specimens containing a small amount of virus may test negative if an insufficient volume of the specimen is used for nucleic acid extraction or if a low number of target molecules are partitioned into a large aqueous volume and only a portion of the sample is tested (39). Since the levels of viral RNA in NP and stool specimens rise in the second week of illness, a follow-up NP and/or fecal specimen should be collected and tested by PCR. Detection of SARS-CoV in plasma or peripheral leukocytes should be considered since this approach provides sensitivities of around 80% during the first week of illness. The use of multiple and serial respiratory tract and fecal specimens will improve the sensitivity of PCR diagnostic testing especially when a large volume of the sample is extracted and a large proportion of the sample RNA is used for amplification (4, 36). Recognizing that the first human infections were caused by a zoonotic virus that adapted to its new human host and that humankind faces the continuing possibility of the introduction of new variants of animal SARS-associated viruses, greater vigilance will be required to make a diagnosis of infection if new animal CoV are introduced into the human population. The use of RT-PCR assays employing consensus primers and sequencing that can detect all known CoV would be recommended in this scenario.

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Hepatitis A and E Viruses*

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Viral hepatitis is the general term for inflammatory disease of the liver caused by at least five different viruses, with hepatitis A, B, C, D, and E viruses having a definite association with acute viral hepatitis. Of these, only hepatitis A virus (HAV) and hepatitis E virus (HEV) are enterically transmitted, and both cause acute and generally self-limiting infections without any long-term carrier status. While there are no serious long-term sequelae in patients who recover from hepatitis A or hepatitis E, both viruses are associated with significant risks of acute, fulminant hepatitis and liver failure. These severe outcomes are most commonly seen in patients with acute hepatitis A on a background of chronic hepatitis C or hepatitis B virus infection, and for patients with hepatitis E during pregnancy, especially during the third trimester. In the absence of these cofactors, the diseases show a general trend toward greater severity with increasing age, with the majority of infections being subclinical in children; however, severe and fulminant HAV and HEV infections can occur at any age.

TAXONOMY

HAV

HAV is the type species of the *Hepatovirus* genus within the family *Picornaviridae*. No other members of this genus have been identified. HAV is distinguished from other picornaviruses (such as poliovirus) by its tropism for the liver, its high thermal stability, the small size of the VP4 protein, and the assembly of a unique capsid intermediate (PX) during virus assembly (Fig. 1). Although there is significant genetic diversity among HAV strains, with two major genotype groups found in humans and a third genotype considered to be a true simian strain (AGM-27) (34, 100), all strains of HAV represent a single serotype, and this diversity does not affect the performance of serological assays. Genotyping of HAV is based on sequence analysis around the VP1-2A region and is useful as a research tool for outbreak investigations (81, 82).

HEV

HEV is the type species of the *Hepevirus* genus within the family *Hepeviridae* (38). Recent descriptions of an HEV-related virus from chickens which causes big liver and spleen disease (77) and hepatitis-splenomegaly (45, 50) demonstrate that this viral family is more extensive than previously suspected. The avian HEV-like virus has been fully sequenced (50), but due to its rather low sequence homology with human and swine strains (around 50% at the nucleotide level, compared to a minimum of 75% between mammalian strains), it is uncertain whether this should be considered a strain of HEV (avian HEV) or, rather, the type species of a second genus within the *Hepeviridae* family. This would be analogous to the taxonomy for mammalian and avian hepatitis B viruses (*Orthohepadnavirus* genus and *Avihepadnavirus* genus, respectively, within the family *Hepadnaviridae*).

Four genotypes of HEV have been described to occur in humans, at least two of which appear to represent zoonotic infections originating from viruses endemic in swine and other animals (see below) (36, 49, 86, 87, 109).

DESCRIPTION OF THE AGENTS

HAV

HAV is a small virus with a nonenveloped, icosahedral capsid approximately 27 nm in diameter enclosing a single-stranded, positive-strand RNA genome of around 7,500 nucleotides (nt) (Fig. 1A). Viral particles have a sedimentation coefficient of around 156S and a buoyant density of 1.325 g/cm^3 in cesium chloride (60). HAV is resistant to low pH (3.0) and shows remarkable thermal stability, especially in the presence of magnesium salts (3). Thorough cooking is necessary to inactivate HAV in contaminated foods.

An outline of the viral replication mechanism is shown in Fig. 1A. The positive-strand RNA genome contains a single open reading frame (ORF) which is translated to yield a giant polyprotein, and the virus-encoded 3C protease cleaves the polyprotein at a number of sites, liberating eight proteins in the first instance, including the RNA-dependent RNA polymerase (RDRP or 3D^{pol}) and protease, 3C^{pro}. The small VPg protein (part of 3AB) then serves as a primer for the synthesis by RDRP of a complementary, negative-strand RNA, and subsequently for further copies of positive-strand RNA. These identical "daughter" positive-strand RNAs serve as templates for further transcription to amplify the RNA pool, as mRNA for synthesis of viral proteins, and finally as new genomes for encapsidation within the viral particle (see reference 83 for a

* This chapter contains information presented in chapter 95 by Edwin A. Brown and Jack T. Stapleton and chapter 98 by George G. Schlauder and George J. Dawson in the eighth edition of this Manual.

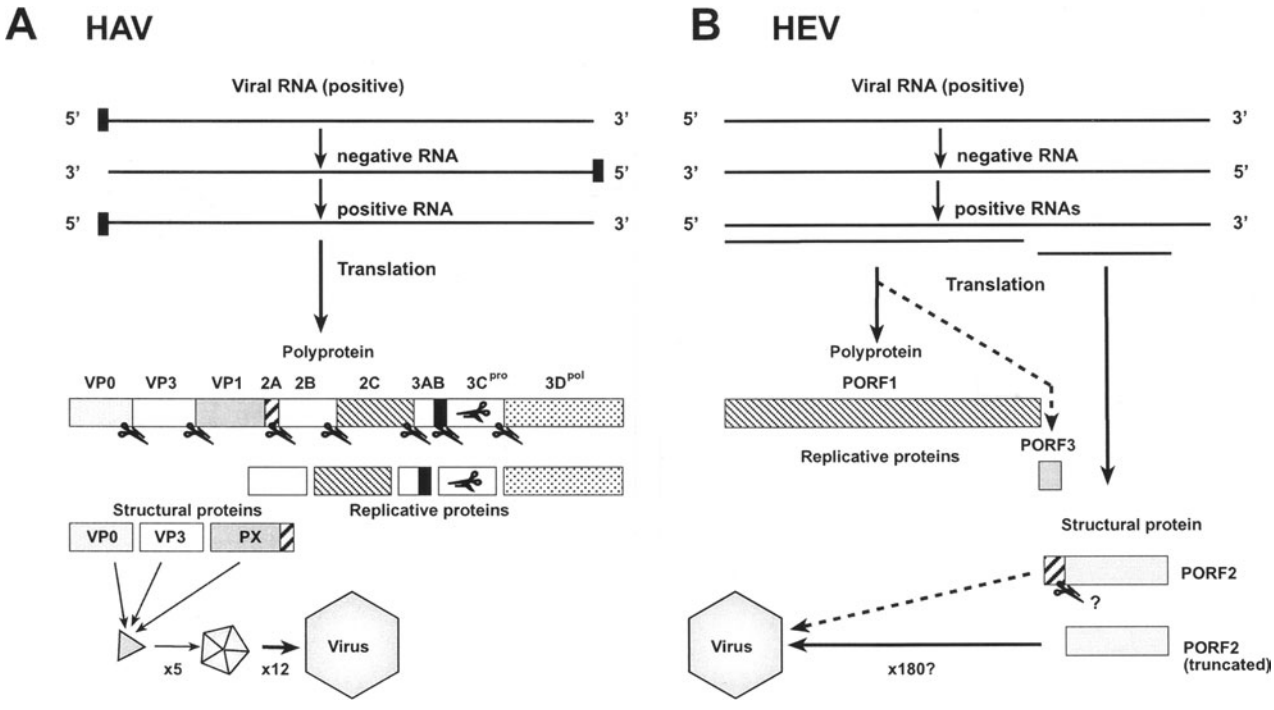


FIGURE 1 Genome replication and proteins of HAV and HEV. Both viruses have positive-strand RNA genomes of around 7,200 to 7,500 nt. (A) HAV replication proceeds via transcription from the genome to give full-length negative-strand and then positive-strand RNA, which can be either assembled into the virus particle or used to translate further copies of a single, giant polyprotein which is processed by viral protease to yield the replicative proteins and capsid proteins. Assembly of five copies of each of the three capsid proteins (VP0, VP3, and PX) into pentamers and then 12 pentamers into capsids is required to form the antigenic sites of the virus. (B) Details of HEV replication are unknown, but it most likely produces a full-length negative-strand RNA and then full-length positive-strand RNA (new viral genomes) as well as subgenomic mRNAs which are used to translate the ORF2 (capsid) and ORF3 proteins, as well as the ORF1 polyprotein, which is cleaved at unknown sites to yield the replicative proteins. Cleavage of full-length PORF2 results in assembly of virus-like particles from the truncated product, but the size of the authentic viral capsid protein is unknown. Modified from reference 4.

comprehensive review of picornaviral replication). The HAV particle is initially composed of three proteins, VP0, VP1–2A (also known as PX), and VP3 (6), that are liberated from the polyprotein by 3C protease (Fig. 1A). Five copies of each of the three proteins associate to form pentamers, and 12 copies of the pentamer form virions (with viral RNA) or empty capsids with the same antigenicity. Following assembly, 2A is removed from VP1 by cellular enzymes and/or 3C protease, and VP0 is cleaved to yield VP2 and VP4. The mechanism by which virus is released from the liver is unknown; liver damage is most likely a result of cellular immune responses to the virus rather than direct lysis of hepatocytes by the virus.

Diagnosis and immunization for hepatitis A are not complicated by strain differences, despite significant levels of amino acid variation in the capsid proteins, because HAV exists as a single serotype worldwide. This is especially notable in the case of the highly divergent AGM-27, which is considered a true simian strain of HAV and causes disease in African green monkeys and other lower primates (34, 100). The antigenic sites of HAV are formed through the complex interactions of the proteins within and between pentamers (95), which has hampered the production of HAV antigenic material through recombinant DNA techniques. As a result, cell culture remains the only reliable source of HAV antigens for serological diagnostic tests and for vaccines. The replication of HAV in cell culture is rather inefficient compared to that of most picornaviruses, which necessitates very-large-scale cell culture production, but the virus is highly immunogenic. A number of highly effective licensed vaccines are available against HAV: Havrix (GlaxoSmithKline) and Vaqta (Merck) within the United States, together with Avaxim (Sanofi Pasteur) and Epaxal (Berna Biotech) worldwide. A combination hepatitis A and hepatitis B vaccine, Twinrix (GlaxoSmithKline), is also widely available.

HEV

Virions of HEV are nonenveloped, icosahedral particles approximately 32 to 34 nm in diameter, most likely comprising a single viral protein which encapsidates the single-stranded, positive-sense RNA genome of approximately 7,200 nt. Virions of HEV are much less stable than those of HAV, being sensitive to conditions such as CsCl and freeze-thawing that have no effect on HAV, and showing much greater sensitivity to heat than does HAV (32). This lower stability is likely to be a major factor in the low secondary attack rate for HEV. Virions have a sedimentation coefficient of 183S and a buoyant density of 1.29 g/cm^3 (measured in potassium tartrate-glycerol) (17). The single capsid protein is

encoded by ORF2, expected to yield a protein of 660 amino acids; however, the capsid protein of authentic viral particles has not been characterized.

Extensive progress has been made in the development of HEV diagnostics and experimental vaccines based on heterologous expression systems for viral proteins, but HEV has proven refractory to propagation in conventional cell cultures. Studies using infectious cDNA clones of HEV (35) are now revealing many aspects of HEV replication.

A schematic of the HEV genome and encoded proteins is shown in Fig. 1B. The 7,204-nt infectious cDNA clone of the SAR-55 strain of HEV described by Emerson and colleagues (35) can be assumed to be full length. Most isolates of HEV are close to this length, with the larger swine (69), US-2 (85), and T1 strains (110) having insertions of around 54 nt within ORF1, while the recently described avian HEV is substantially smaller, at 6,654 nt, due to deletions in both ORF1 and ORF2 (50).

The 5′ end of the genome has a 7-methylguanosine cap, while both the 5′ and 3′ ends of the viral RNA have short, highly conserved untranslated regions which are likely to function in viral replication. Three ORFs, organized as 5′-ORF1-ORF3-ORF2–3′, with ORF3 and ORF2 largely overlapping, encode the viral proteins (described here as PORFs) PORF1 (replicative polyprotein), PORF3 (unknown function), and PORF2 (capsid protein), respectively (Fig. 1B).

HEV shows a much greater degree of genetic diversity than HAV (see references 50 and 87 for detailed reviews of HEV genotypes). HEV can be classified into four putative "genotypes" (as defined by Wang and colleagues) (109); the Burmese and related strains (including most Chinese strains) are classified as genotype 1, the Mexican strain is classified as genotype 2, the swine HEV strain discovered in the United States (67) and closely related strains isolated from patients infected in the United States (85) are classified as genotype 3, and distinct isolates from patients in China (T1 strain) (109) and both patients and swine in Taiwan (48, 49) are classified as genotype 4. Until recently, the original Mexican strain was the only known representative of genotype 2; however, strains isolated in Nigeria also appear to be genotype 2 (18), whereas other African strains have been genotype 1 (105).

In contrast to HAV, the genotypic differences between HEV strains are associated with significant antigenic differences, with corresponding effects on the sensitivities of many serological assays (see below). However, these antigenic differences appear to lie outside the principal neutralizing antibody domains, since HEV appears to exist as a single serotype, with experimental vaccines providing strong cross-genotype protection (102, 118). Similarly, the identification of conserved, immunodominant epitopes has resulted in greatly improved diagnostic tests that appear to detect all HEV strains (5, 20, 39, 42, 47, 66, 72, 113, 115). A candidate HEV vaccine, based on a truncated HEV ORF2 protein (amino acids 112 to 607) expressed in insect cells, has been the subject of extensive preclinical studies (33, 78, 102, 117, 120), and a phase III trial was concluded in Nepal during 2003, with analysis ongoing.

EPIDEMIOLOGY AND TRANSMISSION

Despite sharing the primary mode of transmission via human feces, HAV and HEV have very different distributions of both disease and infection worldwide. These differences appear to be linked at least partly to the efficiency of virus transmission, with HAV being excreted in very high titers allowing both waterborne and person-to-person spread, whereas HEV is more commonly seen in waterborne outbreaks. However, unresolved questions remain regarding the prevalence of HEV infection in areas in which HEV is highly endemic, such as Nepal, where infection with HAV is almost universal and yet less than 50% of the population shows evidence of exposure to HEV (5, 23).

HAV

Transmission

The generalized course of infection and serological responses for both HAV and HEV are shown in Fig. 2. Due to its enteric route of transmission, HAV has been widely assumed to infect cells lining the alimentary tract. However, studies in Caco-2 cells suggest that progeny HAV from intestinal epithelial cells is released back into the lumen of the gut rather than into the circulation (16), and the virus is around 10,000-fold more infectious when administered by the intravenous route rather than the oral route (79), consistent with early studies that suggested the liver as the primary site of replication, with amplification in the alimentary tract making little or no contribution (56). During the incubation period of 4 to 6 weeks after first exposure to the virus, HAV spreads throughout the liver without causing any cytolytic effects, but liver damage eventually results and is most likely mediated by the cellular immune response to the virus, with infections in young children generally following a benign course. Virus is excreted through bile to the gut, and very high titers of infectious HAV will be present in most patients at the onset of illness. Virus is generally cleared within several weeks, although prolonged excretion of HAV has been reported in a significant proportion of cases. In general, titers of excreted virus will be highest before the onset of obvious symptoms.

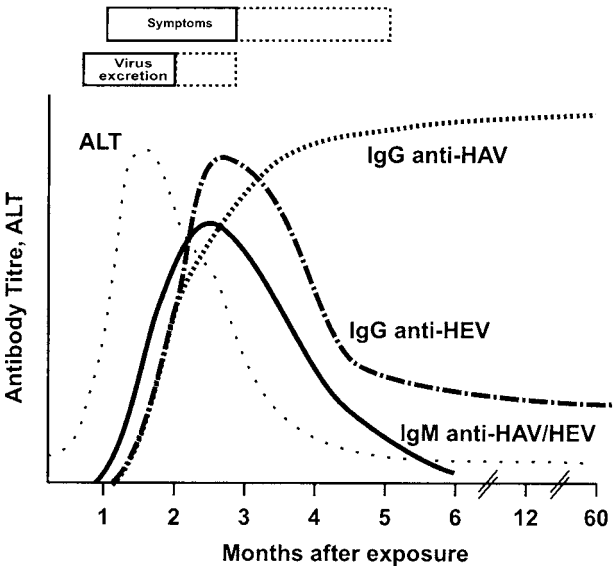


FIGURE 2 Serological and virological courses of infection with HAV or HEV. For HAV, the serological responses shown are typical of those detected with numerous commercially available assays. For HEV, the serological responses shown are those that probably occur in most patients, but the detection of these responses will vary widely depending on the assays used. High levels of HAV-specific IgG provide lifelong protection from reinfection, but HEV-specific IgG declines rapidly during the first 6 months and might not persist at protective levels for life. Modified from reference 4.

Although the vast majority of progeny virus is excreted in the feces, HAV patients display a viremia that may last for some weeks around the time of clinical presentation. Transmission of HAV via blood products has been demonstrated (71), and inactivation procedures to eliminate HAV are clearly required in the manufacture of blood products (58). Fortunately, such processes have now been validated for HAV, and further transmission is unlikely (1, 53, 59).

Epidemiology—High, Low, and Intermediate Rates of Infection

HAV is found worldwide, but with widely varying epidemiological patterns that largely reflect sanitation standards (14). In countries or areas with poor sanitation, high levels of HAV exposure result in most individuals becoming infected at an early age, but in these circumstances HAV is not a major public health problem because the symptoms tend to be mild or absent in children and very few members of the adult population are susceptible. However, HAV can certainly cause clinical disease in children, most obviously in foodborne outbreaks in schools of developed countries (51, 80) but also in endemic populations in countries such as Nepal (I. L. Shrestha, personal communication) and Turkey, where HAV was the most commonly identified cause of fulminant hepatic failure in children from 1 month to 17 years of age (12).

Conversely, in countries or populations with high standards of personal and public sanitation, relatively few individuals are exposed at an early age. In many countries of Western Europe, uniformly high standards of sanitation result in very low rates of HAV disease even though the vast majority of the population is susceptible. In the United States, Australia, and other countries with generally high sanitation levels but a wide diversity of socioeconomic circumstances, sporadic HAV infection continues at a low level, especially in the very young and even more so in disadvantaged communities where standards of personal hygiene are low. Combined with high transmissibility, this focal circulation of virus in some populations and geographic areas provides an ongoing source of virus for sustained transmission throughout the broader community.

Household or sexual contact with a person known to have hepatitis A was the most commonly identified risk factor for disease during the period from 1990 to 2000 in the United States (14%), but 46% of cases had no identified risk factor (15). Children in day care centers were identified as a risk factor in 8% of cases, but children are likely to be a factor in a large proportion of the cases where no risk factor was identified (94). An important aspect in the epidemiology and transmission of HAV is the high titer of virus produced, combined with the very great physical stability of the viral particle, which is relatively insensitive to extremes of heat: infectious virus is readily recovered after heating for 10 min at 60°C, and as much as 78°C when in the presence of 1 M $MgCl_2$ (3).

HAV disease burden in the United States was estimated at more than 140,000 cases and 80 deaths annually in 1990 (43), but it declined rapidly over the next 10 years (see reference 15 for a comprehensive review). This decline was most pronounced in children, from a peak incidence of 39 cases/100,000 in 1990 to around 2 cases/100,000 in 2002, coincident with the introduction of targeted immunization of children from high-risk ethnic and racial groups and geographic areas, but the rate in adults (>18 years) also fell, from around 17 cases/100,000 to 2 cases/100,000, during the same period (15).

The public health problem of HAV in the United States and other countries with low-level HAV endemicity is compounded by the potential for large outbreaks of HAV spread by contaminated foods. Food- and waterborne infection with HAV was identified as a factor in 4% of cases from 1990 to 2000 (15), but this proportion is likely to increase because such cases will not be prevented by targeted immunization strategies.

In the past, such outbreaks were largely confined to shellfish, which concentrate viruses such as HAV from large volumes of water and which are generally eaten raw or only lightly cooked. Examples are provided by outbreaks seen in Shanghai, China, in 1988 (44), the United States in 1991 (31), and Australia in 1997 (24). However, the increased trade in foodstuffs between countries now creates additional sources of infection, such as a number of outbreaks within the United States which have been traced to fresh and frozen foods imported from Mexico (29, 51, 80, 111), where they had presumably been exposed to contaminated water during production or processing.

Intravenous and other illicit drug use is also positively associated with a higher risk of HAV infection, and may play a major role in the epidemiology of HAV in some societies (90). Men who have sex with men are at increased risk, and outbreaks in this population in different countries have been described in recent years (15).

Residents from developed countries who travel to regions of endemicity, especially those visiting friends and relatives in regions of endemicity, are at greatly increased risk of HAV infection (8). The relative contribution of travel to the HAV disease burden may be underestimated, because travel history is often overlooked in diagnosis.

The greatest disease burden for HAV worldwide falls on countries that have previously had high rates of HAV infection but are now undergoing rapid economic growth and improvements in public sanitation and infrastructure, resulting in intermediate rates of HAV infection. Improved sanitation has the effect of sparing many young children from infection, but this results in a much larger pool of susceptible older children and young adults in whom disease is more pronounced. HAV may continue to circulate widely through household and close contact, resulting in high rates of clinical disease. Universal immunization programs may be required to reduce disease burden in these populations, but this is unlikely to be feasible for most countries in the near future.

HEV

Transmission

Hepatitis E is primarily transmitted via the fecal-oral route. HEV RNA is detectable in the feces of 50% of patients 2 weeks after onset (22), and in one case has been detected at 52 days after onset (73). Consumption of contaminated water is by far the most common route of transmission for clinical cases of HEV in countries where HEV is endemic, with household contact being much less important than for HAV.

Travel to HEV-endemic areas is the most common source of HEV infections in areas of nonendemicity such as the United States and Western Europe, but locally acquired HEV infections also occur, representing zoonotic infections from pigs or other unidentified hosts (45, 48, 69, 85). Genotyping has demonstrated close relationships between viruses from human cases of HEV and local swine HEV strains in many developed countries (for an example, see reference 48), and cross-species infection has been experimentally established for swine and human HEV (68), although other strains are unable to cross species (67). The route of transmission for these HEV cases is unknown, as patients with clinical cases generally have no

evidence of contact with swine. In Japan, a number of reports have demonstrated HEV infection following the consumption of raw or partially cooked liver or meat from swine or deer harboring corresponding strains of HEV (70, 75, 76, 93, 96, 114), but this is unlikely to be a significant source of infection in other countries.

HEV leads to a significant viremia, with the majority of patients being positive by reverse transcriptase PCR (RT-PCR) at the time of disease onset, and there have been several reported cases of hepatitis E associated with transfusion both in countries where HEV is endemic and in countries where it is not (10, 65).

Epidemiology—HEV-Endemic and Non-HEV-Endemic Countries

In countries where HEV is endemic, sporadic HEV infection is often the most common form of viral hepatitis on an annual basis, accounting for around 70% of cases in Kathmandu, Nepal (7), but above this background major epidemics also occur with a period of around 7 to 10 years. For example, HEV was shown retrospectively to be responsible for 16 of 17 epidemics of enterically transmitted hepatitis in India (9), but HEV is also responsible for at least 25% of cases of sporadic hepatitis between epidemics (54). Epidemics are most often associated with the wet season; however, one study has shown an increased rate of infection associated with a period of unusually low rainfall, presumably by concentrating human wastes in a riverine ecology (25).

Clinical HEV infection in HEV-endemic countries is most common in adolescents and young adults, but occurs to a lesser extent from childhood (52) to late ages. There appear to be considerable differences in exposure rates among countries where HEV is considered endemic: in Egypt around 60% of children were exposed to HEV by the age of 10, and this rate did not increase further with age (26), whereas in Nepal only 16% of 12-year-olds had evidence of exposure to HEV, peaking at 31% later in life (23).

It is likely that the risk of clinical disease increases with age, as for hepatitis A, but while clinical HAV infection is uncommon in many developing countries because of high exposure rates in children, HEV exposure rates are never as high as for HAV and a large proportion of the population remains susceptible to adulthood.

In developing countries, exposure to water contaminated with human waste is a clear risk factor for disease, and close contact with domestic animals may also be a risk. Importantly, boiling of drinking water appears to be protective (46), but while this will not be practical for all residents in many developing countries, it is advisable for pregnant women because of the high risk of fulminant hepatitis E in this population (see below). The low rate of person-to-person spread of HEV is likely to be due to a combination of lower particle stability (32) and lower virus titers in excreta than for HAV.

Reported seroprevalence rates for immunoglobulin G (IgG) anti-HEV in presumed non-HEV-endemic countries vary widely, at least partly due to the different assays used, which show very poor concordance (64). Observed rates of antibody between 1 and 5% are most common, at least some of which can be considered to represent true anti-HEV reactivity on the basis of neutralization tests (119). Experimental infections of macaques have demonstrated a strong dose response for the development of clinical HEV disease (104), and the unexpectedly high rate of HEV seroprevalence in countries where clinical HEV is very rare may represent subclinical infection with low doses of zoonotic strains of HEV. There is some evidence for increased exposure to HEV in groups with occupational exposure to swine (55). Travel to countries where HEV is endemic remains the greatest risk factor for clinical HEV infection in residents of non-HEV-endemic (developed) countries (88). This risk can be assumed to be especially great for travelers visiting friends and relatives, as for HAV (8).

CLINICAL SIGNIFICANCE

Clinical Presentation and Course

Acute infections with any of the hepatitis viruses cannot be distinguished on clinical characteristics or pathological examinations, and the diseases caused by HAV and HEV are considered together here. However, HEV is unique among the hepatitis viruses in being associated with a high mortality during pregnancy due to fulminant hepatitis with a very rapid onset, approaching 30% in the third trimester, and the rate of fulminant hepatitis for HEV outside of pregnancy is probably around 10-fold higher than for HAV (1 versus 0.1%) (13). The reasons for such severe outcomes of HEV infection are not known, but it is clear that women should take all possible precautions to avoid exposure to HEV during pregnancy. A major risk factor in this regard is travel from non-HEV-endemic countries to HEV-endemic areas such as India and Pakistan.

Infection with HAV or HEV can result in a broad range of clinical outcomes, from subclinical infections (especially in children, or with low doses of HEV) to fulminant hepatitis. Disease severity shows a general increase with age; however, clinical and even fulminant hepatitis A and E do occur in children, and there is no reason to exclude the enterically transmitted hepatitis viruses from diagnostic consideration on the basis of patient age. A retrospective study in Turkey has shown HAV to be the most common identifiable cause of fulminant hepatitis in children (12).

Clinical presentation of acute viral hepatitis commonly begins with nonspecific, "flu-like" symptoms such as fever, headache, anorexia, nausea, and abdominal discomfort. Physical examination will usually reveal an enlarged, tender liver. The first distinctive sign of hepatitis is usually dark urine (excretion of conjugated bilirubin), followed by pale feces and jaundice (yellow discoloration of the skin and sclera), but many patients will not show visible signs of jaundice despite severe symptoms, even when raised levels of serum bilirubin are present. However, it must be stressed that these nonspecific symptoms alone are not sufficient to infer acute viral hepatitis in a patient without jaundice, and liver function tests are an important adjunct to diagnosis (19), with raised levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) detectable at the time of onset. Unless there are strong epidemiological grounds for suspicion, hepatitis A or E should not be considered in the absence of one of these biochemical markers or jaundice.

In most developed countries, hepatitis A is much more likely than hepatitis E unless there are specific indications to suggest HEV, such as past HAV infection or recent travel to HEV-endemic areas combined with a history of receiving inactivated HAV vaccine. Patients with proven evidence of past HAV infection will not have HAV, other than those cases that represent relapses after recent HAV infection (92). In most developing countries HEV is more likely than HAV, although HEV appears to be relatively infrequent in many parts of South America.

Serum aminotransferase levels usually resolve after a period of 3 to 4 weeks. Normalization of liver enzymes usually marks complete recovery; however, many patients will report an intolerance to fatty foods which may last for years. Relapses are rare for hepatitis A, occurring in around 7 to 12% of

patients beyond 1 month (92, 98). Protracted hepatitis A has been associated with a particular HLA allele (37). Prolonged disease with hepatitis E has also been observed in some areas of endemicity and is frequently characterized by cholestasis with jaundice and itching. Cholestasis can occur with both hepatitis A and hepatitis E.

While both HAV and HEV (outside pregnancy) have low overall rates of fulminant hepatitis, the onset of encephalopathy can be quite rapid (around 7 days from onset of dark urine for hepatitis A). HEV has been found to be responsible for around 62% of cases of fulminant hepatitis in studies from India (74) and Bangladesh (91), where the virus is highly endemic. Acute HAV infection on a background of chronic hepatitis C or hepatitis B is associated with more severe outcomes (107).

Dose Response to Infection

HAV and HEV differ markedly in the relationship between infecting dose and the course of infection and disease. For HAV, studies in tamarins have shown a very clear correlation between higher infectious doses and reduced incubation period to develop disease, but no correlation with disease severity (79). This is consistent with the high secondary clinical attack rates seen for HAV. Low-dose infections of cynomolgus macaques with HEV have also demonstrated an increased time to seroconversion, but HEV disease severity showed a positive correlation with high infectious doses (2, 104). This attenuation of low-dose infections may help to explain the low secondary attack rates of HEV, and the rarity of clinical HEV disease in non-HEV-endemic countries despite a seroprevalence of around 2%.

Vaccines and Antiviral Agents

Passive immunization with human gamma globulin has been used for many years to provide short-term protection against hepatitis A, and it is still used for postexposure prophylaxis (<2 weeks postexposure) and for infants less than 2 years of age. As noted above, there are now a number of highly efficacious inactivated vaccines available against hepatitis A (Havrix and Vaqta in the United States, plus Avaxim and Epaxal worldwide) or hepatitis A and B (Twinrix), and these have replaced the use of gamma globulin for protection of individuals with future risk of HAV infection. Active immunization against hepatitis A is recommended for children (>2 years) in communities with elevated rates of hepatitis A, travelers to countries where standards of hygiene and sanitation are low, men who have sex with men, recipients of clotting factors, injecting drug users, and patients with chronic hepatitis (in whom disease severity is exacerbated) (19). Targeted HAV immunization has contributed to the rapid decline in HAV disease rates from 1990 to 2002.

No vaccines are yet available for protection against hepatitis E, but the prospects for these are very good. Studies by Purcell, Emerson, and colleagues at the NIH have demonstrated that antibody to PORF2 is sufficient to protect against hepatitis E (101) and that active immunization of macaques with 56-kDa subunit antigen (amino acids 112 to 607) expressed in the baculovirus system has conferred protection against viral challenge with both homologous and heterologous HEV strains (33, 66, 78, 101–103, 117, 120). A phase III clinical trial was completed for this candidate vaccine in Nepal in 2003, and results should be available in the near future. It is likely that this vaccine would be most useful for travelers (including the military, Peace Corps volunteers, etc.) and for residents of countries where HEV is endemic.

No antivirals are available for treatment of hepatitis A or E, and it is unlikely that antivirals would be useful because the acute disease is largely immunopathogenic. Experimental inhibitors have been developed that are active against the 3C protease of HAV, but clinical development of these is unlikely.

No specific symptomatic or anti-inflammatory treatments can be advised for hepatitis A or E. Bed rest and attention to diet (avoiding fatty foods) are commonly recommended to minimize symptoms and speed recovery; alcohol intake should be minimized. Pruritus is a feature of cholestatic hepatitis that may justify the cautious use of corticosteroids.

Intensive supportive medical care, as for acute liver failure due to other causes, is required in cases of fulminant hepatitis A or E. Fulminant hepatitis A has a better prognosis for spontaneous recovery than hepatitis B (69 versus 19% [84]). Liver transplantation is less frequently performed, and there is some risk of graft reinfection (41).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Standard methods for collection, transport, and storage of sera or plasma are adequate for the detection of both IgG and IgM antibodies to HAV and HEV, which are the only standard diagnostic tests performed. Sera or plasma can be stored at 4°C for weeks but should be frozen under other circumstances (−20 or −70°C for storage and dry ice for shipping). Shipping is most often an issue with HEV testing, which is not available in many routine diagnostic laboratories. IgM is more sensitive to freezing and thawing than is IgG, and repetitive cycles should be avoided. Feces may be collected for epidemiological studies of HAV (genotyping of strains) and are stable as a solid mass or slurries in phosphate-buffered saline or water for months or years at −70°C. Serum is generally preferable for this purpose. HEV is difficult to detect in feces, and serum is preferred for RT-PCR and genotyping. Because HEV is more labile than HAV, sera for HEV RT-PCR studies must be tested within 24 h or frozen and shipped at −70°C.

Rapid, point-of-care (RPOC) tests for HEV-specific IgM are now available outside the United States (20, 72), which can overcome the need for collection and shipping of venous blood. These tests can be used either for fresh whole blood or for serum and plasma as for laboratory assays.

DIRECT DETECTION

Electron Microscopy

Electron microscopy and especially immunoelectron microscopy are of historical importance for their roles in identifying HAV (40) and HEV (17) particles before the characterization and molecular cloning of the viruses and development of specific diagnostic tests, but they are insensitive and technically difficult and have no diagnostic value.

Antigens

HAV

HAV antigen (HAAg) is very stable and is readily detected in acute-phase fecal samples collected from many patients. Viral antigen is detected by enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies, and may be present in very high titers in some patients, but patients almost invariably have IgM anti-HAV present by the time of clinical presentation and antigen detection provides no diagnostic advantage over serology. There are no commercially available tests for HAAg.

HEV

HEV antigen is less stable than HAAg, and no reliable tests are available (either commercially or on a research-only basis) for the detection of antigen in clinical samples. It is unlikely that antigen detection would have any clinical value relative to serology or RT-PCR performed on sera.

Nucleic Acids

HAV

HAV patients are viremic in the prodromal phase, and one recent study from Brazil has reported around 12% of children during a common-source outbreak to have been RT-PCR positive but IgM negative ("window period") at the time of testing, together with 26% of patients initially classified as acute, sporadic non-A-C hepatitis (30). The high proportion of window period cases found in this study may be related to high-dose infections with short incubation periods, as all patients available to follow-up seroconverted. Serum is the preferred specimen for nucleic acid detection, but plasma is acceptable provided that anticoagulants do not interfere in the reactions; feces are least suitable, with variable levels of virus and high levels of inhibitors. RT-PCR is not currently recommended for routine diagnostic testing of possible acute hepatitis A, but further studies may be warranted. The blood supply is not currently tested for HAV RNA by RT-PCR; conversely, many manufacturers now routinely conduct HAV RT-PCR screens on pools of source plasma for blood products. HAV RNA can also be used for genotyping of virus for research purposes using RT-PCR primers in the region of VP1-2A (Fig. 1) (11, 21).

HEV

RT-PCR remains the "gold standard" for specificity in the diagnosis of acute HEV infection. However, levels of HEV RNA are generally low and the sensitivity of RT-PCR for diagnosis is very dependent on (i) patient presentation early in disease, (ii) rapid and careful processing of serum or plasma samples and appropriate transport at –70°C, and (iii) reference laboratories for testing and quality control. Serological tests will be preferable for routine diagnosis, and improved tests are now becoming available (see below). RT-PCR has provided the vast majority of information on zoonotic infections with HEV around the world (49, 57, 65, 86, 109, 114, 116), and will remain useful for confirmatory testing in countries of low endemicity such as the United States.

VIRAL ISOLATION

Many isolates of HAV have been adapted to cell culture, but virus isolation is insensitive and generally requires multiple passages over a period of many months, and it is not useful in diagnosis or epidemiological studies. HEV replication has been demonstrated in primary macaque hepatocytes (97), but the virus has proven refractory to propagation in standard cell cultures and virus isolation is not useful.

VIRAL IDENTIFICATION AND TYPING SYSTEMS

As described above, the direct detection of HAV and HEV RNA by RT-PCR and sequence-based genotyping has been used for the identification of potential sources of sporadic cases of HEV in non-HEV-endemic countries, and for the investigation of common-source outbreaks of HAV. HEV genotyping is usually based on the ORF1 region and to a lesser extent on the ORF2 region (49, 57, 86, 109), while HAV strains and genotypes are determined by sequencing in the VP1-2A region of the genome (11, 21).

SEROLOGICAL TESTS

Despite the very good specificity of most serological tests for HAV and HEV, their appropriate use and interpretation are dependent on patients meeting the clinical criteria for acute hepatitis, particularly in areas where the viruses have low incidence, such as the United States and other developed countries. The clinical criteria for the HAV surveillance case definition in the United States consist of "an acute illness with discrete onset of symptoms (e.g., fatigue, abdominal pain, loss of appetite, intermittent nausea, and vomiting) and jaundice or elevated serum aminotransferase levels" (19). Serological testing should be limited to persons who meet the clinical criteria or to persons likely to have been exposed to HAV (19), and in light of the nonspecific nature of the symptoms, the importance of jaundice or elevated ALT or AST in meeting the case definition must be stressed. In the absence of these clinical criteria for patients in regions of nonendemicity, the positive predictive value of the tests will be severely compromised.

HAV

Tests for HAV-specific IgM are commercially available from numerous sources in a variety of formats (ELISA, enzyme immunoassay, and IMx). The technology for detection of HAV-specific IgM is sufficiently well established that there are no significant differences between the diagnostic performances (sensitivities and specificities) of the laboratory-based testing platforms. Choices of diagnostic assays for HAV may reasonably be made on the basis of convenience for the testing laboratory, with a range from single-strip ELISAs for small numbers of specimens to completely automated systems for high-volume laboratories.

Test principles are either IgM class capture or indirect ELISA. In the IgM class capture assays, plates coated with anti-human IgM are used to capture IgM from patient samples, with HAV-specific IgM being detected by binding of labeled HAAg (inactivated HAV derived from cell culture). Indirect ELISAs use plates coated with HAAg, to which HAV-specific IgM will bind, and bound IgM is then detected using anti-human IgM conjugates. In both cases, assay reactivity and cutoffs are determined from internal controls supplied with the kits. Because HAAg binding to plates is generally inefficient, monoclonal antibodies are sometimes used to facilitate the binding of antigen to plates. All commercially available HAV serological assays use inactivated, whole HAV derived from cell culture and thus detect the same antibody specificities.

Total or IgG anti-HAV does not have diagnostic value because antibody persists for life, although a rising titer of IgG (greater than fourfold in specimens taken 2 weeks apart) is indicative of acute HAV. Total HAV-specific antibody is a marker of immunity and can be used for prevaccination screening to identify those who require immunization, but the cost-effectiveness of screening depends on many factors (106). Postvaccination tests are not required due to the very high efficacy of available HAV vaccines. Tests for the measurement of total anti-HAV use a competitive ELISA format, where HAAg is used to coat plates and specific antibody in patient serum binds to the antigen, resulting in a proportionately reduced assay signal from the binding of labeled monoclonal antibodies to HAAg.

In addition to serum or plasma, saliva can be used for detection of anti-HAV IgM or total antibody (99, 108), but

the use of saliva or other samples, such as urine, falls outside the manufacturers' recommendations for current tests. These samples can be useful for research and investigational purposes but require internal validation.

RPOC tests for HAV-specific IgM have been developed (4) but are not yet commercially available. In common with the RPOC test for HEV-specific IgM (20, 72) (see below), these tests may prove especially useful in outbreak investigations.

HEV

A number of research and commercial immunoassays for HEV-specific IgG and IgM are available in various countries, but with major differences in sensitivity and specificity (see below). Only IgG ELISAs are currently available in the United States. The appropriate use and interpretation of current serological assays for HEV infection must take into account differences in assay sensitivity and specificity, the pattern of serological responses (IgG and IgM) to various antigens, and the widely varying rates of prevalence of clinical HEV infection worldwide.

Laboratory-Based HEV Serological Tests

Many recombinant and synthetic peptide antigens have been used in diagnostics for HEV. Antigens included in early HEV diagnostic tests were notable for the transient IgG response observed in most patients (27, 28), which provided some specificity for acute infection but at the cost of sensitivity (7). This variability in IgG responses also contributes to the uncertainty regarding seroprevalence rates around the world, as studies using different antigens cannot be compared. Using more advanced antigens (such as the truncated forms of PORF2 expressed in insect cells, and ORF2.1 expressed in *Escherichia coli*), IgG levels appear to persist for many years (Fig. 2). High titers of IgG in patients are suggestive of acute infection, but this correlation is imperfect (5, 42) and the detection of IgG antibody is thus of little use for diagnosis of acute infection, especially in developing countries where HEV is endemic and large numbers of patients will have antibody from past infections.

HEV-specific IgM is therefore preferable for diagnosis. As in the case of IgG, antigens vary significantly in their rates of reactivity with HEV-specific IgM. Second-generation commercial tests use a cocktail of three antigens (113), each of which has IgM reactivity with a proportion of HEV patient sera; however, positive sera show widely variable reactivities, presumably related to the number of individual antigens recognized by each patient, and the total reactivity rate is still around 60% (112). An artificial recombinant mosaic protein, incorporating numerous linear peptide epitopes, has also been used in research settings (39) but suffers from a similar variability in patient reactivity which decreases assay performance.

Improved IgM ELISAs have now been developed, based on highly conserved and immunodominant antigens derived from the capsid protein, PORF2. The ORF2.1 antigen, representing amino acids 394 to 660 of PORF2 expressed in *E. coli*, was originally shown to be highly conserved and immunodominant in the IgG response to HEV infection (61–63) and has subsequently been developed into an ELISA for HEV-specific IgM (20). This test has an overall sensitivity of 98.7% (149 positive of 151 confirmed HEV patients) and a specificity of 97.6% (203 negative of 208 patients with other forms of viral hepatitis or other diseases) (20), and it is commercially available outside the United States.

Sensitive and specific tests for HEV-specific IgM have also been developed using the truncated r55k antigen, representing amino acids 112 to 607 of PORF2 expressed in insect cells, in both indirect ELISA and IgM class capture ELISA formats (89, 115), but these are not commercially available.

RPOC HEV Serological Tests

The major disease burden of HEV falls in developing countries and is especially severe among displaced populations such as refugees, with major epidemics observed from 2004 to 2005 in the Greater Darfur region of Sudan. Timely access to sophisticated laboratory facilities is not available in these situations, highlighting the need for rapid diagnostic assays that can provide reliable diagnosis at the point of care, with minimal training or infrastructure. RPOC tests for HEV-specific IgM have now been developed using the ORF2.1 antigen and corresponding monoclonal antibodies in a novel format of immunochromatographic test (Fig. 3) (20, 72), and they will be commercially available in many countries (outside the United States) in the near future.

The HEV IgM RPOC test has been evaluated in two separate studies. In the first study (20), the RPOC test showed a sensitivity of 96.7% (146 of 151) and an overall specificity of between 98.6% (205 of 208) and 96.9% (220 of 227) depending on exclusion or inclusion of sera having high levels of rheumatoid factor (RF), a common source of false-positive reactivity in rapid assays. In the second study (72), the RPOC test showed a slightly lower sensitivity, of 93% (186 of 200), but a specificity of 99.7% (320 of 321) against a panel including RF-positive sera and sera from patients with acute Epstein-Barr virus infection, a further source of problems in many RPOC tests. The positive and negative predictive values of the test were estimated at 98.0 to 99.5% and 97.6 to 95.0%, respectively (20, 72). These results suggest that RPOC tests will certainly prove useful for the diagnosis of HEV in field situations in regions of endemicity and may also be considered as an alternative to ELISAs for laboratory use in both non-HEV-endemic and HEV-endemic regions.

These RPOC tests offer the further benefit of reduced collection of venous blood, which is difficult in small children and not well tolerated in some cultural settings.

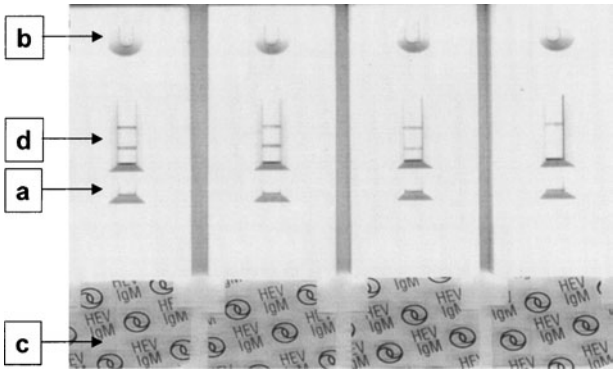


FIGURE 3 RPOC tests for HEV-specific IgM. Undiluted serum (25 μl) was added to the specimen window (a) and allowed to migrate through part of the membrane. Approximately 30 s later, 3 drops of buffer were added to the buffer window (b), the separator was removed by pulling the protruding end (c), and 1 drop of wash buffer was added to the specimen window. Results were visible through the viewing window (d) after 8 min. Samples are considered positive for IgM anti-HEV if two colored lines appear in the viewing window and negative for IgM anti-HEV if a colored line appears only at the control line. Test kits were as described in references 20 and 72 and were kindly provided by Genelabs Diagnostics. Data are from my laboratory.

Serological Diagnosis of HEV Infection in Areas of Low Prevalence

In areas with a low incidence of clinical HEV infection, such as the United States and Western Europe, assay specificity will have a very large impact on the predictive value of HEV serological tests. In order to be useful, HEV diagnostic assays will need to demonstrate very high specificity, high sensitivity, and the ability to detect infections caused by the diverse range of HEV strains (including potentially zoonotic strains) that are present worldwide. Diagnostic assays for the detection of HEV-specific IgG have considerable value for the diagnosis of acute hepatitis in travelers returned from areas of endemicity, among whom the incidence may be much higher than the approximate 2% prevalence in the healthy population. However, with the recognition that HEV should be considered in the diagnosis of sporadic acute hepatitis *without* a travel history, the need for more specific tests becomes evident. For example, if the incidence of HEV infection among acute hepatitis patients in the United States was 0.2%, then only 1 in 10 patients with samples reactive in a test for HEV-specific IgG would be a true positive, leading to an unacceptable positive predictive value.

The detection of HEV-specific IgM should therefore become the method of choice for diagnosis of acute HEV infection in areas of low prevalence. Early assays for HEV-specific IgM showed false-positive reactivity in 3% of U.S. blood donors (technical bulletin; Genelabs Diagnostics, Singapore, Singapore, 1998), roughly equivalent to the rate of IgG reactivity, which limited their usefulness in areas of nonendemicity. A prototype IgM ELISA based on the ORF2.1 antigen was positive for only 1 of 500 Australian blood donors (specificity of 99.8%; D. Anderson, M. Riddell, F. Li, I. Shrestha, T. Howard, G. Perry, and G. Mearns, *Abstr. 10th Int. Symp. Viral Hepatitis Liver Dis.,* abstr. E001, 2000). A modified version of this test demonstrated a higher level of false-positive reactivity (2.4%) in studies to evaluate the RPOC test (20), but the commercial version of the ELISA has not been fully evaluated to date. The quantitative indirect IgM ELISA (89) and the IgM class capture assay (115) using r55k antigen have reported specificities of around 100%. Notably, the RPOC assay is also IgM class capture and has a specificity between 98.6 and 99.7% in different studies (20, 72). Many of these new tests should be suitable for use in regions of nonendemicity.

Serological Diagnosis of HEV Infection in Areas of High Prevalence

Although the titer of HEV-specific IgG tends to decline markedly in the first year after infection (Fig. 2), this relationship is not reliable enough to form the basis of differential diagnosis (5, 42). The detection of HEV-specific IgG is therefore of little use for diagnosis of acute infection in developing countries where HEV is endemic and large numbers of patients will have antibody from past infections. Detection of HEV-specific IgM must therefore be the method of choice in areas of endemicity.

As HEV accounts for as much as 70% of the cases of acute sporadic hepatitis in countries where HEV is endemic, the specificity of assays is less critical in these settings, and assay sensitivity is of primary concern. The HEV IgM assays based on recombinant antigens expressed using the baculovirus system (89, 115), or the ORF2.1 antigen expressed in *E. coli* (5, 20) all appear to have sufficient sensitivity to allow their use in settings of endemicity. The robustness of assays should also be considered. For example, assays will sometimes be processed and interpreted manually where equipment such as ELISA washers and readers is not available. The ORF2.1 IgM ELISA has been evaluated in routine clinical laboratories based in regions of endemicity, allowing the identification of acute sporadic hepatitis E in a large proportion of patients in Nepal (7).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

In many developed countries, including the United States, the rarity of HEV infection and declining rates of HAV infection, coupled with increasing rates and recognition of hepatitis C and hepatitis B virus infections, provide a challenge in the correct diagnostic test selection for individual patients. In particular, testing for HAV IgM in the absence of clinical or epidemiological features consistent with acute hepatitis A is likely to result in false-positive results (19), and the same is true of HEV. Close attention should be paid to the clinical characteristics and patient history, including overseas travel, in interpretation of test results.

Hepatitis A is a reportable disease in the United States, Australia, and many other developed countries, where patient contacts are generally managed by active or passive immunoprophylaxis. In routine laboratories, interpretation of HAV and HEV IgM serological tests is based on reference to internal test controls according to manufacturers' specifications. Many laboratories test for total HAV Ig and if positive retest for HAV IgM, which may be more convenient for sample throughput in areas of low endemicity. There are no standard confirmatory tests for HAV and HEV, again highlighting the need for test requests to be justified on clinical and/or epidemiological grounds in order to reduce false-positive results. False-negative results are uncommon for HAV and should be uncommon with the more recently developed HEV tests, because patients almost always have IgM present by the time of clinical presentation and the window period for seroconversion is very narrow. When false negatives are strongly suspected, for example, a negative HAV IgM test for a patient with raised ALTs and contact with a person with a confirmed case of HAV, it may be appropriate to request a second specimen for retesting.

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Hepatitis C and G Viruses*

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HEPATITIS C VIRUS

Taxonomy

Hepatitis C virus (HCV) is classified in the *Flaviviridae* family, in the genus *Hepacivirus*. It is distantly related to arthropod-borne flaviviruses such as yellow fever virus, West Nile virus, and dengue virus but is more closely related to GB virus (GBV) type C (also known as hepatitis G virus) and pestiviruses.

Description of the Agent

HCV is an enveloped, single-stranded, positive-sense RNA virus which is 30 to 60 nm in diameter (121). Figure 1 displays the organization of the HCV genome, approximately 9,600 nucleotides in length. It consists of highly conserved 5′ and 3′ noncoding regions with a single open reading frame region producing a 3,011-amino-acid polyprotein. After posttranslational processing, the polyprotein is cleaved into structural and nonstructural proteins. The four structural proteins consist of the core (C), envelope 1 (E1), envelope 2 (E2), and transmembrane (p7) proteins. The six nonstructural proteins (NS2 to NS5B) are essential to viral replication and growth and therefore are attractive targets for antiviral therapy. The highly conserved NS5B protein produces the RNA-dependent RNA polymerase. Viral kinetic studies with patients receiving antiviral treatment suggest that HCV replicates at a prolific rate, producing 10^{12} virions/day, with a half-life of 2 to 3 h (83).

Hepatitis C virus infects primarily humans, and the vast majority of human infections are transmitted by other humans, usually through some blood exposure. Although persistent infection occurs in the chimpanzee, *Pan troglodytes*, the animal is not a significant reservoir for HCV.

One of the major hurdles in hepatitis C research has been the inability to grow the virus in cell culture. In recent years, the replicon system was developed to allow limited in vitro study of the virus. This system was developed by inserting part of the HCV genome containing the 5′ untranslated region (5′ UTR), along with the neomycin resistance gene and heterologous viral regulatory sequences into hepatoma cells (69). The replicon produced abundant proteins and viral RNAs de novo in hepatoma cell lines, as well as B cell and epithelial cell lines (118, 142), but did not produce infectious virus. Production of infectious HCV particles was recently facilitated by the discovery of a wild-type strain taken from a patient with fulminant hepatitis, JFH1, which was genetically altered to create an infectious chimeric HCV. By using the nonstructural region of the JFH1 strain in combination with RNA from a different HCV strain, infectious virus was grown and passaged in an optimized cell culture milieu, representing a major breakthrough in the study of hepatitis C virus (67, 132, 141).

HCV has been classified into six major genotypes, which differ in nucleotide sequence by as much as 30%, and more than 80 subtypes (32). These genotypes, numbered 1 through 6, have unique geographical niches and important clinical implications. Genotype 1 is most common in the United States and Europe, while genotypes 4, 5, and 6 are common in Egypt, South Africa, and Southeast Asia, respectively. In the United States, genotype 1 accounts for approximately 71.5%, genotype 2 for 13.5%, genotype 3 for 5.5%, and genotype 4 for 1.1% of the total HCV infections (57). While there is little difference in the transmission and natural history among the various genotypes, the genotype is the strongest predictor of response to peginterferon and ribavirin therapy and thus is routinely determined during the evaluation of patients. Previously, researchers believed that patients were infected with only one genotype (129); however, with new serotyping methods, it appears that sequential infections with different HCV isolates may occur more frequently than previously recognized, although simultaneous viremia with more than one HCV isolate is rarely observed (41, 101, 126). Finally, genotype 3 infections seem to be more commonly associated with hepatic steatosis, although the exact mechanism is undetermined (1).

Within an infected host, there are closely related, but genetically distinct, HCV variants known as quasispecies. Because hepatitis C is an RNA virus which lacks a proofreading RNA polymerase, it is able to generate a large population of different viral variants. HCV quasispecies diversity is thought to be a result of host immune pressure and is maximally observed in the hypervariable region 1 (HVR-1) of the second envelope gene (E2) (135). Conversely, in chronically infected patients, a higher pretreatment genetic diversity correlates with treatment failure (89).

Epidemiology and Transmission

HCV infection is a growing major health problem in the United States and worldwide, afflicting an estimated 3.8 million people

* This chapter contains information presented in chapter 97 by Margaret C. Shuhart and David R. Gretch in the eighth edition of this Manual.

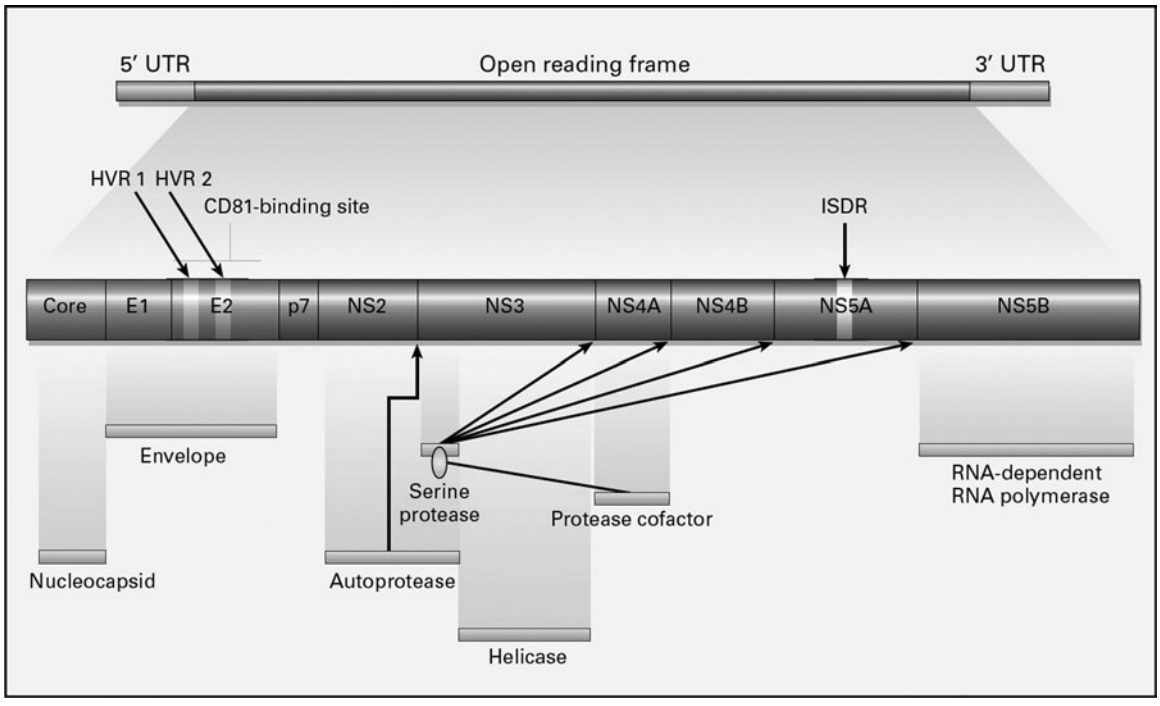


FIGURE 1 The HCV genome and expressed polyprotein. HCV, a single-stranded RNA virus of 9.5 kb, consists of a single open reading frame and two untranslated regions (UTRs). It encodes a polyprotein of approximately 3,000 amino acids, which is cleaved into single proteins by a host signal peptidase in the structural region and the HCV-encoded proteases in the nonstructural (NS) region. The structural region contains the core protein and two envelope proteins (E1 and E2). Two regions in E2, called hypervariable regions 1 and 2 (HVR 1 and HVR 2), show extreme sequence variability, which is thought to be the result of selective pressure by virus-specific antibodies. E2 also contains the binding site for CD81, the putative HCV receptor or coreceptor. The nonstructural proteins have been assigned functions as proteases (in the case of NS2, NS3, and NS4A), helicase (in the case of NS3), and RNA-dependent RNA polymerase (NS5B). Although the crystal structure of NS3 and NS5 is known, the function and properties of the other proteins (such as p7) are less well characterized. A region in NS5A has been linked to the response to alpha interferon therapy and is therefore called the interferon sensitivity-determining region (ISDR). However, the relevance and importance of this region are still unclear. (Reprinted with permission of B. Walker and the *N. Engl. J. Med.* **345:**42, copyright 2001 Massachusetts Medical Society, all rights reserved.)

in the United States (1.6%) and 170 million persons worldwide (4, 8). Over 10,000 persons die each year in the United States from HCV-related disease, and it ranks as the leading cause of liver failure requiring transplantation (4, 60). Death from chronic liver disease is the tenth leading cause of death in Americans; hepatitis C-related deaths from chronic liver disease increased by 220% from 1990 to 1998 (130). The incidence of HCV climbed steadily until the late 1980s, peaking at 230,000 cases per year (20). Since the institution of HCV screening and a greater awareness of the transmission routes, this rate has declined to 36,000 per year. In the United States, HCV infection is more common in men, blacks, and middle-aged persons (8).

HCV is spread predominantly through contaminated blood via percutaneous exposures. Injection drug use (IDU) is the most common route of transmission, accounting for 55 to 65% of all infections (35). Receipt of infected blood products or organs represented an important cause of HCV infection prior to 1992; however, since initiation of screening for HCV, this is an uncommon source of infection. With later-generation antibody screening, the risk of acquiring HCV through a blood transfusion was reduced to 1 in 103,000 (100) and has been further reduced to 1 in 2 million by minipool testing of blood donations by nucleic acid tests (114). In contrast to human immunodeficiency virus (HIV) and hepatitis B virus, HCV is not efficiently transmitted through sexual intercourse; the estimated annual risk of transmission in long-term monogamous partnerships is 0 to 0.6% (120). Intranasal cocaine use, exposure to contaminated medical equipment, needlestick exposures, and vertical transmission all contribute to the spread of HCV infection. HCV can be detected in saliva, but casual contact is thought to be a very uncommon route of transmission. For occupational exposures, the risk of HCV transmission from a hollow-bore needlestick injury is 1.8% (20). Finally, a small proportion (10%) of cases does not have an identifiable transmission source.

Clinical Significance

Although HCV infection is the most common bloodborne infection in the United States, the natural history tends to be mild for the vast majority of patients (50, 104). Between 15 and 40% of patients exposed to HCV clear the infection in the acute setting (4, 47). The remaining 60 to 85% are

chronically infected, although spontaneous clearance infrequently occurs (134). Approximately 20% of HCV-infected patients develop cirrhosis, a process which takes over 2 decades on average. Progression to cirrhosis is accelerated by concurrent heavy alcohol use, HIV infection, male gender, and acquisition after age 40 (90, 98). Patients with cirrhosis are then susceptible to end-stage liver disease (30% over 10 years) and hepatocellular carcinoma (1 to 4% per year) (33).

The clinical presentation of HCV infection is indolent and often asymptomatic. Fatigue is the most common complaint. A small percentage (10 to 30%) of patients with acute HCV infection develop the classical symptoms of viral hepatitis: jaundice, nausea, vomiting, abdominal pain, lethargy, and myalgias (84). Hepatitis C is frequently diagnosed after routine laboratory testing results for hepatic transaminases are abnormal. If there is HCV-related cirrhosis, patients may complain of abdominal distention, edema, jaundice, easy bruising, or clay-colored stools. In addition, HCV is associated with extrahepatic manifestations, mainly through immune-mediated mechanisms, including type II cryoglobulinemia, membranoproliferative glomerulonephritis, porphyria cutanea tarda, lichen planus, and non-Hodgkin's lymphoma (30).

The results of several large clinical trials have established the benefit of pegylated alpha interferon (peginterferon-α) and ribavirin for treatment of chronic HCV infection (37, 72), with sustained virologic response (SVR) rates of 56% (peginterferon-α2a[Pegasys]) and 54% (peginterferon-α 2b [PEG-Intron]). Infection with genotypes 2 or 3 had the greatest influence on the likelihood of achieving SVR; patients with genotype 2 or 3 had response rates of 76 to 82% compared to 42 to 46% for genotype 1 (37, 72). Furthermore, the SVR rates in genotype 2 or 3 infection are equivalent for 24 and 48 weeks of therapy (44), allowing for a shorter course of therapy without compromising efficacy. While high-dose ribavirin (1,000 to 1,200 mg/day) is equivalent to low-dose ribavirin (800 mg/day) in genotype 2 or 3 infection, the higher dose is necessary for genotype 1 infection (113). Thus, the current treatment recommendations are (i) 48 weeks of peginterferon plus high-dose ribavirin for genotype 1 infection or (ii) 24 weeks of peginterferon plus low-dose ribavirin for genotype 2 or 3 infection. Because of the significant cost and side effects associated with treatment, treatment is usually recommended for patients with significant fibrosis on liver biopsy specimens, compensated liver disease, normal hematologic and biochemical indices, no or stable depression, and no autoimmune disease (113).

Although hampered by the lack of a cell culture system, development of new treatments for HCV is brisk and many therapies are in initial clinical trials. These therapies are categorized into life cycle inhibitors, virus attachment and cell entry blockers, immunotherapies, therapeutic vaccination, and antifibrotics (74). Various methods to improve the efficacy of interferon are under way, including conjugation to albumin, different types of interferon, and oral interferon inducers. Minimizing side effects of current therapy, such as hemolysis with ribavirin, is the impetus behind expanded-spectrum nucleoside analogs (like Viramidine) and inosine 5′-monophosphate dehydrogenase (IMPDH) inhibitors (like VX-497).

Currently, there is no FDA–licensed vaccine available for prevention of HCV infection. Vaccines in development include protein subunit, DNA, and viral vector vaccines. An effective vaccine must elicit a durable, high-titer, and cross-reactive anti-E2 response as well as a multispecific cell-mediated immune response (49).

Collection, Transport, and Storage of Specimens

Serologic testing can be performed on serum or plasma after routine processing. For viral RNA quantification, serum is preferred, but plasma is acceptable if collected in tubes containing EDTA or citrate. Collection tubes with heparin should be avoided because it binds *Taq* polymerase, a necessary enzyme for PCRs (133). After collection, blood specimens should be promptly transported to the laboratory; transportation of specimens at room temperature is acceptable. For serum samples, the blood should be allowed to clot and then centrifuged within 4 h (Bayer bDNA 3.0) or 6 h (Amplicor/Amplicor Monitor 2.0). After centrifugation, the serum should be removed aseptically. Plasma samples are handled in a similar fashion, with removal of the plasma after centrifugation. For PCR-based detection, transportation and processing within 2 h is preferred; there is a slight but insignificant decrease in viral RNA levels if the specimens are stored at 25°C for 72 h (42). Storage of processed specimens at −70°C is optimal; however, one study found no decay in HCV RNA levels for specimens stored at −20°C for over 2 years and tested by reverse transcription-PCR (RT-PCR) and bDNA (48). Manufacturer instructions state that specimens may be stored at 2 to 8°C (Amplicor) or −20°C (bDNA) up to 72 h. Repeated freeze-thaw cycles should be avoided because they may lead to loss of HCV RNA; specimens tested by the Amplicor and bDNA assays may be frozen and thawed up to three and four times, respectively. For similar reasons, thawed specimens should be kept cold.

Specimen processing should be performed in an approved biosafety level two laboratory because the virus is potentially infectious, especially with blood-blood exposures, such as a needlestick injury.

Direct Detection

Microscopy

Traditional electron microscopy is rarely used as a diagnostic tool because it is expensive and unable to detect the low levels of viral particles in serum. Employed in research situations, electron microscopy studies describe HCV as spherical, approximately 40 nm in size, and of a relatively smooth contour (88).

Antigen Detection

An assay for antigen detection of the core protein has been developed (Total HCV Core AG assay; Ortho-Clinical Diagnostics, Raritan, N.J.) using an enzyme-linked immunosorbent assay (ELISA) method for detection. It appears to be accurate and specific, but lacking in sensitivity (lower limit of detection, 20,000 IU/ml) (15), and is not currently licensed by the FDA.

Nucleic Acid Tests

Nucleic acid tests (NAT) differ from serologic assays in several ways. They directly detect the presence of HCV RNA, and thus a positive NAT nearly always means that a patient is HCV infected. HCV RNA can be detected in serum 1 to 3 weeks after exposure, several weeks earlier than by detection of antibodies (113). NAT can be classified into qualitative methods (RT-PCR and transcription-mediated amplification [TMA]) and quantitative methods (bDNA and quantitative RT-PCR). An expert panel recommends testing with a sensitive NAT for patients who are (i) serologically positive, or (ii) serologically negative but have unexplained liver disease, or (iii) serologically negative and immunocompromised, or (iv) suspected of having acute HCV infection (113).

Qualitative Tests

The FDA has approved three qualitative RT-PCR diagnostic kits: AMPLICOR 2.0 (Roche Diagnostics, Indianapolis, Ind.); Ampliscreen 2.0 (Roche Diagnostics); and UltraQual (National Genetics Institute, Los Angeles, Calif.) (Table 1). Adaptation of the complete bioanalytical system (COBAS) for AMPLICOR test has allowed for semiautomation, simplified extraction, and a single-tube reaction for reverse transcription and amplification. With the COBAS instrument, sensitivity and specificity remained excellent (96 and 100%, respectively, using antibody status and elevated alanine aminotransferase [ALT] tests as the "gold standard") while reducing technician "hands-on" time (2). It has a very low limit of detection, 50 IU/ml (82), although recent refinements in the extraction method and concentration of specimens suggest that detection of viremia <12 IU/ml may be possible (36).

Qualitative NATs are useful, then, in cases where one is trying to confirm viremia or where low-level viremia is possible. Although the qualitative assay is quite sensitive, it is possible that viremia below the limit of detection and/or intermittent viremia occurs (19, 36). Therefore, follow-up HCV RNA testing is indicated 1 year after exposure with documented seroconversion even if initial RNA tests were negative, and in high-risk populations whenever ALT elevation occurs even if RNA tests were previously negative. As with any PCR-based test, contamination may lead to false-positive results. One source of contamination is amplicon carryover, although this is less common than in the past and can be reduced by the addition of uracil-*N*-glycosylase (UNG) to the PCR mixture. However, in the 1996 EUROHEP study of 86 laboratories, there were 5 laboratories that generated false-positive results in six HCV RNA-negative samples, despite the use of UNG (23). Contamination from positive clinical samples can occur, and this source of contamination is not affected by the addition of UNG. This finding emphasizes the need for careful controls in all assays and ongoing proficiency testing by all laboratories using PCR-based assays for molecular diagnosis.

TMA tests are also available. VERSANT HCV RNA Qualitative Assay (Bayer Diagnostics, Emeryville, Calif.) has a lower detectable limit of 5 IU/ml and sensitivity of >98% compared to serially diluted specimens quantified by bDNA (96). Approved by the FDA for screening blood and organ donations, the VERSANT and the Procleix HIV-1/HCV nucleic acid (TMA) tests, (Gen-Probe, San Diego, Calif.) can detect early or immunologically silent infections, thus improving the safety of blood transfusions. Additionally, TMA may be useful in predicting which patients are at risk for virologic relapse upon cessation of interferon and ribavirin therapy, defining treatment response and determining acute infection (25, 99).

Quantitative Tests

HCV quantitative viral tests have several important clinical applications (Table 1). For example, they are important tools in predicting response to antiviral treatment. In one study using the SuperQuant quantitative PCR assay (National Genetics Institute) to quantify HCV RNA, patients with a baseline HCV RNA count of >2 million copies/ml had a 9% lower sustained virologic response rate than those with <2 million copies/ml (37). (Subsequent to this report, HCV RNA quantification was standardized using a WHO standard in international units, and it was determined that 800,000 IU/ml corresponds to 2 million copies/ml in the SuperQuant assay [85]). For patients with genotype 1 infections who have a <2-log drop in HCV RNA levels after 12 weeks of therapy, it is unlikely that they will have an SVR and treatment is usually stopped in these situations (37, 72, 124). This early cessation spares patients from the high cost and toxicity associated with year-long therapy. Similarly, recent data suggest that patients with genotype 2 or 3 who have an undetectable HCV RNA level after 4 weeks of treatment may receive a shortened course (12 weeks total) of therapy without any compromise in efficacy compared to the standard 24-week course (71), although more studies are needed before these findings can be translated into clinical recommendations.

Two competing methods were introduced for HCV quantification: the quantitative RT-PCR (MONITOR 2.0; Roche Diagnostics) and the branched-chain DNA assay (VERSANT HCV RNA assay 3.0; Bayer Diagnostics). In the clinical diagnostic setting, we reported that 97.6 and 95.2% of all viremic specimens (defined as positive for HCV RNA by qualitative RT-PCR) were positive using these quantitative HCV RNA tests (MONITOR 2.0 and VERSANT 3.0, respectively) (77), which suggests that these assays may be useful for initial screening of specimens with a high likelihood of being positive for HCV RNA.

Analytical studies have also found the MONITOR 2.0 assay to be only slightly more sensitive than the VERSANT bDNA 3.0 test (62, 77), with a 95% detection rate at 732 IU/ml for the MONITOR 2.0 test versus 817 IU/ml for the bDNA 3.0 test in our study (77). Importantly, the former test suffered from increased variability and a limited dynamic range above 500,000 IU/ml, while the bDNA test was linear to 7.7 million IU/ml (62, 77). Furthermore, a subset of specimens gave nonlinear results between 200,000 and 500,000 IU/ml in the MONITOR 2.0 test, leading to the recommendation that all specimens be diluted 1:50 or 1:100 in healthy human serum prior to testing in the MONITOR assay if diagnosis is likely (77); negative specimens should then be retested undiluted to achieve the best assay linearity (55, 77). Neither the MONITOR 2.0 nor the in-house proprietary SuperQuant quantitative PCR assay (National Genetics Institute) is FDA approved for HCV quantification. The latter test is mentioned here because it has been used in several key interferon clinical trials (73, 91) and levels are tightly correlated to those of the MONITOR 2.0 (55).

In studies comparing the bDNA 3.0 to the MONITOR 2.0, the overall concordance was high (R^2 range, 0.86 to 0.95) (11, 77). For patients coinfected with HIV and HCV, there was significant variability between the assays in the classification of high (≥800,000 IU/ml) or low (<800,000 IU/ml) viral level (107). Because of this variability and difficulties in comparing the two assays for patients with high viral levels, it is recommended that clinicians use the same assay to follow patients' response to treatment (107).

The bDNA 3.0 method is more precise and reproducible than that of the MONITOR 2.0 and has achieved similar sensitivity (29). The bDNA assay has undergone three iterations, with improved sensitivity and linearity and equivalent quantification of all genotypes (75). The third-generation assay (Versant bDNA 3.0; Bayer Corporation, Tarrytown, N.Y.) has a lower limit of detection of 615 IU/ml and a reduced upper range of 7.7 million IU/ml (29), improvements that better reflect the HCV viral loads seen clinically. Although not as sensitive as the qualitative NAT, it does provide quantitative results, which can be helpful in predicting and monitoring treatment response. The specificity is good, ranging from 96 to 98.8%, in specimens from seronegative blood donors (11). The assay is highly reproducible (127); infected patients not on treatment should not vary more than 2.6-fold (29). High levels of unconjugated bilirubin (>20 mg/dl) and total protein (>9 g/dl) interfered with

TABLE 1 Diagnostic assays for HCV RNA detection and quantification

Assay	Method	Manufacturer[a]	Stated sensitivity (IU/ml)	Upper range (IU/ml)	Use	Comments
Qualitative						
Ultra-Qual	RT-PCR	National Genetics Institute	40–50	NA[b]	Blood donation screening	
AMPLICOR (v2.0)	RT-PCR	Roche	50	NA	Evidence of active infection, defining treatment response	
Ampliscreen (v2.0)	RT-PCR	Roche	50	NA	Blood donation screening	
Procleix HIV-1/ HCV assay	TMA	Gen-Probe	5–10	NA	Blood and organ donation screening	
Versant	TMA	Bayer	5–10	NA	Evidence of active infection, defining treatment response	
Quantitative						
Monitor (v2.0)	RT-PCR	Roche	600	500,000	Pretreatment quantification, determination of response to treatment	Sensitive, but unreliable at higher viral loads
Quantiplex bDNA (v3.0)	Branched chain amplification	Bayer	615	7.7 million	Pretreatment quantification, determination of response to treatment	No extraction or clean room required, broader linear range than MONITOR
TaqMan real-time PCR	RT-PCR	Roche	10–71	100 million	Pretreatment quantification, determination of response to treatment	Very sensitive, wide dynamic range, automated
TaqMan real-time PCR	RT-PCR	Abbott	10	At least 10 million	Pretreatment quantification, determination of response to treatment	Very sensitive, wide dynamic range, automated

[a] Roche, Roche Diagnostics; Bayer, Bayer Diagnostics; Gen-Probe, Gen-Probe Inc. (marketed by Chiron), Abbott, Abbott Molecular.
[b] NA, not applicable.

HCV RNA quantification (29), but these are relatively uncommon clinical situations. Additionally, it is important that the specimen be homogeneous and well mixed prior to addition to the microwell. Otherwise, the protein matrix is not formed and there is no blocking agent for the hybridization step, leading to a high background and false positives (29).

Two real-time PCR methods use TaqMan technology (Roche Diagnostics and Abbott Molecular, Des Plaines, Ill.) yielding quantitative results with comparable sensitivity to the qualitative test and a broad linear range (>6 $\log_{10}$) (9, 56). Real-time methods are faster, more automated, reliable, and cost-effective and thus represent real progress in HCV RNA quantification. Both Roche and Abbott make TaqMan HCV RNA analyte-specific reagents (ASR); the Abbott assay is run on the ABI 7000 series sequence detector, and the Roche assay employs the TaqMan 48 instrument. Both assays use internal controls. The Roche ASR has a limit of detection between 10 and 71 IU/ml with a linear range between 1.5 and 6.5 $\log_{10}$ (36, 56), while the Abbott ASR has a limit of detection of 10 IU/ml and is linear to at least 10 million IU/ml (18a).

The LightCycler is a real-time instrument which uses glass capillaries to allow for more rapid heating and cooling, thus shortening the assay time after RNA isolation to 70 minutes (54). An alternative real-time method, which has been used for HCV quantification on the LightCycler instrument, employs SYBR green I dye to bind the intermediate, double-stranded HCV RNA (93) and obviates the use of a probe, thus reducing the variation in quantification between different genotypes (53). Specificity is determined by amplicon melting temperature.

Many of the initial quantitative HCV RNA tests used different units, making direct comparisons difficult. To correct this problem, the National Institute for Biological Standards and Controls established in 1997 the first WHO international standard, taken from a high-titer genotype 1a sample and diluted with pooled serum to achieve a concentration of 10^5 IU/ml (97). The second international standard was created from the same lot as the original and will have the same unitage. These standards allow for conversion of the various quantitative tests into a common unit. With the SuperQuant PCR (National Genetics Institute), 1 IU/ml is equal to 2.5 copies/ml (85). For the MONITOR 2.0, 1 IU/ml equals 2.7 copies/ml. The bDNA v2.0 reported output in equivalents; 1 IU/ml equals 6.3 equivalents/ml. By contrast, the bDNA v3.0 gave results in copies/ml; 1 IU/ml equals 5.2 copies/ml (11).

It is important to emphasize that the stated sensitivities of the various assays are not always reproducible in the clinical setting. In College of America Pathologists (CAP) proficiency testing, it is not uncommon to see "less sensitive" tests detecting HCV RNA whereas "more sensitive" tests report as negative. In addition, given the extensive genetic diversity of HCV, precise determination and quantification of different HCV genotypes have been problematic for quantitative tests in the past. This issue will persist until there is accurate proficiency testing specific for different HCV genotypes.

Isolation Procedures and Identification

No routine culture method is available for HCV.

Genotyping

As mentioned previously, knowledge of a patient's genotype is important because it directs the duration and dosage of therapy and is the most powerful predictor of response. The gold standard for genotyping is sequencing, whereby the viral nucleic acid is amplified and then a specific region is compared by computer analysis to the established genotypes. Sequencing of the core, E1, NS3, and NS5 genes can each determine genotype and produce equivalent results (70). While sequencing of these HCV genetic regions represents the most definitive test for defining HCV genotypes, the approach is presently too expensive and time-consuming for large-scale diagnostic use although with increasing automation the costs may decline (6).

At present, most commercially available genotyping tests target the highly conserved 5′noncoding region (5′NCR) of HCV. All tests which target the 5′NCR do not accurately subtype. Since this region is also used for most qualitative and quantitative HCV RNA tests, it is the most convenient and least costly region to assay. However, mixtures of 5′NCR mutations within the same clinical isolate lead to erroneous results interpreted as mixed genotype infections in approximately 5% of specimens, results that have not been confirmed using more sensitive and definitive molecular approaches capable of detecting a second HCV genotype at levels below 1:100 relative to a primary genotype (D. Gretch, unpublished data). Despite this limitation, rapid tests for 5′NCR-based genotyping of HCV clinical isolates have been developed and implemented in many laboratories. A commercially available sequencing kit (Trugene 5′ NC, Bayer) targets this region and is accurate in detecting all six genotypes, yet is still susceptible to misclassification based on spurious mutations occurring in vivo. The technique requires a processing time (5 h) and incurs costs similar to those of another popular technique, the line probe assay (45). Like the line probe assay, the Trugene assay is less accurate in detecting subtypes because of the minor nucleotide changes between subtypes in the 5′NCR.

Recently, the real-time PCR method was adapted to detect genotypes (18, 102). This method uses melting curve analysis with fluorescent resonant energy transfer (FRET) probes targeted to the 5′NCR. Similar to other methods which use a 5′NCR target, subtyping is not reliable. However, when compared to the reference standard of NS5B sequencing, the method detected 99% of genotype 1, 94% of genotype 2, 91% of genotype 3, and 100% of genotype 4 isolates (95). The method has excellent concordance with the line probe assay but takes 60% less time, and the reagents cost less. Other tests using real-time PCR to genotype HCV are also under development.

The Invader assay (Third Wave, Madison, Wis.) is a commercially available ASR which can be performed directly on amplified products without purification. It uses a microplate format and takes 2 h to perform; however, it is unable to subtype (40).

Restriction fragment length polymorphism (RFLP) analysis on the amplified 5′NCR and NS5 detects point mutations associated with specific genotypes. Using endonucleases to cleave at genotype-specific sites, RFLP produces unique banding patterns on ethidium bromide gels. The technique has a sensitivity of 96% but is hampered by partial digestions and indeterminate results.

The line probe assay mentioned above (INNO-LiPA HCV II; Bayer) is a DNA hybridization test which uses the biotinylated PCR products of the 5′NCR to hybridize to type-specific probes on a nitrocellulose strip (115). It is as accurate as any test using the 5′NCR and able to detect the six major genotypes (116). The LiPA II has a near 100% concordance to the Trugene sequencing test but an 83 to 84% concordance with "home-brew" sequencing techniques and may be less accurate for RNA levels less than 20,000 IU/ml (6, 76). Mistyping is possible due to spurious mutations; these novel line probe

patterns have been described (76). In a comparison of 5′NCR sequencing to the LiPA II tests, 4/50 (8%) isolates had a genotype 1a isolate by sequencing but a genotype 2a isolate on LiPA, a difference which has significant implications for treatment decisions and outcomes (6). Furthermore, the line probe and RFLP methods detected mixed genotype infections in 5 to 10% of specimens. As mentioned above, definitive confirmatory testing has not supported conclusions drawn from these screening assays about mixed genotype HCV infections in humans.

Two serologic methods, recombinant immunoblotting (Chiron RIBA and in-house NS4 IBA) and ELISA (Abbott Murex), have also been used to detect genotype-specific antibodies to the NS4 or core regions (27, 39, 86, 103). Because they detect antibodies, rather than HCV RNA, they are simpler, cheaper, and more rapid and can provide a serotype in the absence of detectable RNA. Furthermore, they can detect exposure to multiple genotypes (101). However, antibody response is diminished in HIV-infected persons (10). Fewer serotypes (three or four instead of six) are detected by the immunoblot assays (10, 27, 101, 103). The current ELISA assay (HC03; Abbott Murex) detects all six serotypes and has improved sensitivity and specificity over earlier versions (39, 58, 86). Concordance between serotyping, line probe, and sequencing methods has been good in various studies (58, 86, 103).

Serologic Tests

Screening for hepatitis C is most often performed with an enzyme immunoassay (EIA) because it is inexpensive, easy to use, reproducible, automated, and now quite accurate. The first-generation EIAs (EIA 1.0) were licensed by the FDA in 1990 and detected antibodies to the c100-3 epitope of NS4. Screening of the blood donations significantly reduced transfusion transmission of HCV (28); however, this test suffered from low sensitivity (80% in high-risk populations) and low specificity (30% in the blood-donor setting) (43). The second-generation EIAs (EIA 2.0) were introduced in 1992 and improved both the sensitivity and the specificity (to 92 to 95% and 88 to 95%, respectively) by incorporating additional antigens from the core and nonstructural regions (43, 94). Furthermore, the mean window period of seroconversion was reduced from 16 weeks to 10 weeks (43). In 1996, the third-generation test (Ortho ELISA 3.0; Ortho-Clinical Diagnostics, Inc.) was licensed and incorporated a fourth antigen (NS5), as well as reconfigured core and NS3 antigens (Fig. 2).

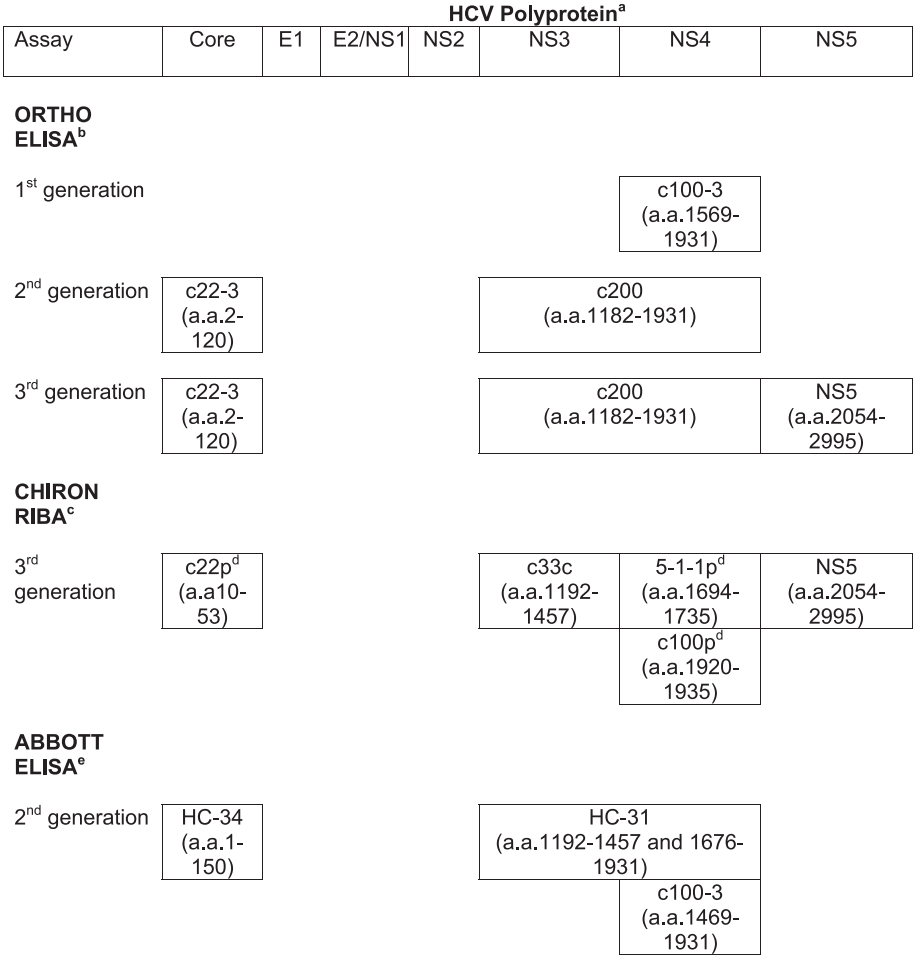


FIGURE 2 HCV antigens used for serologic assays. Abbreviations for superscript a: E, envelope; NS, nonstructural protein; a.a., amino acid sequence of recombinant protein or synthetic peptide antigen. Superscript b, Ortho HCV ELISA (version 3.0; Ortho-Clinical Diagnostics, Inc.). Superscript c, Chiron RIBA HCV strip immunoassay (SIA; version 3.0; Chiron Corporation). Superscript d: p, synthetic peptide. Superscript e, Abbott HCV EIA (version 2.0; Abbott Laboratories). (Used with permission from S. Richter, *J. Clin. Microbiol.* **40:**4408, 2002.)

It is a highly sensitive (97 to 99%) and specific (≥99%) assay (21, 131), although false negatives can occur in the immunosuppressed (i.e., HIV-positive persons and transplant recipients) and hemodialysis populations (5, 43, 82). This false-negative rate can be significant; in one study, 19/100 (19%) of HIV-infected patients were serologically negative but HCV RNA positive. If the test is used in low-prevalence populations (<10%) such as volunteer blood donors or active military personnel, the false-positive rate is estimated to be 35% (95% confidence interval, 16 to 60%), illustrating the need for confirmatory testing (5). Additionally, children born to HCV-infected women may test positive for anti-HCV antibodies for the first year of life (94).

The current FDA-approved HCV antibody assays are the Ortho ELISA 3.0 and the Abbott EIA 2.0 (Abbott Laboratories, Abbott Park, Ill.). The ELISA 3.0, when compared to EIA 2.0, is better able to detect acute infection and remote nonviremic HCV infections (38, 123). Nevertheless, antibodies still require 4 to 10 weeks (average, 8 weeks) to develop so this assay is of limited use in detecting acute HCV infection (113).

The recombinant immunoblot assay (RIBA) (RIBA v 3.0 Strip Immunoblot Assay; Chiron Corporation, Emeryville, Calif.) is another serologic test which detects recombinant HCV antigens derived from different regions of the HCV genome and identifies responses to individual HCV proteins. Human superoxide dismutase (hSOD) is included as a control to detect nonspecific antibodies. Results are reported as positive (detection of two or more antigens at 1+ or greater reactivity, no reactivity to hSOD), indeterminate (one antigen detected or a reaction to hSOD and one or more HCV antigens), or negative (no antigens detected). The test can be helpful in discerning false-positive EIA results from resolved infections (the latter will be RIBA+) and therefore may be used as confirmation in low-risk patients (healthy blood donors) and if a high-risk patient is HCV RNA negative (94).

In an attempt to predict which supplemental tests will be positive in patients screening positive by EIA, the Centers for Disease Control and Prevention (CDC) conducted a study of the signal to cutoff ratio (S/Co). In the EIA test, samples are compared against a standard and an optical density reading is generated for laboratory use. The S/Co is the sample absorbance compared to the predetermined cutoff absorbance and serves to distinguish positive from negative readings. An S/Co of ≥1 is reported as positive, while a ratio of less than 1 is reported as negative. Samples which are just over 1 are more likely to be false positives (seen in patients with autoimmune diseases). The CDC study found that an S/Co greater than 3.8 provides positive confirmatory RIBA tests 95% of the time (5); thus, routine RIBA testing of specimens with S/Co greater than 3.8 is not needed routinely. The S/Co is incorporated into the recommended laboratory algorithm for anti-HCV testing and result reporting (Fig. 3).

A similar S/Co study of the enhanced chemiluminescence assays (VITROS; Ortho-Clinical Diagnostics) found that an S/Co of ≥8 predicted RIBA positivity in 95 to 98% of chemiluminescence assay-positive samples (5). This microparticle-based test detects HCV core antigen and may detect antibodies more quickly in acute infection but more frequently has indeterminate results and false positives, thus limiting its use as a screening test (51, 78).

Antiviral Susceptibility

Although HCV is commonly refractory to interferon and ribavirin, testing for antiviral susceptibility is not available

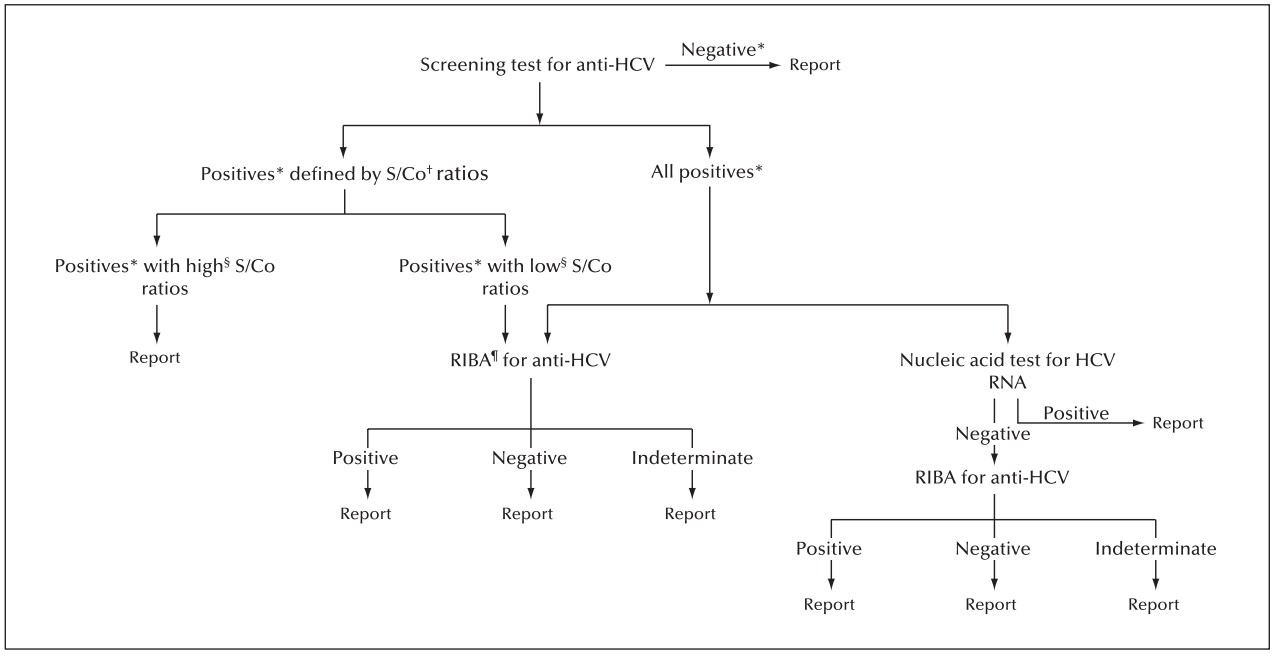


FIGURE 3 Laboratory algorithm for antibody to HCV (anti-HCV) testing and result reporting. Symbols: *, interpretation of screening immunoassay test results based on criteria provided by the manufacturer; †, signal to cutoff; §, screening-test-positive results are classified as having high S/Co ratios if their ratios are at or above a predetermined value that predicts a supplemental test-positive result ≥95% of the time among all populations tested; screening-test-positive results are classified as having low S/Co ratios if their ratios are below this value; ¶, recombinant immunoblot assay. (Used with permission from M. Alter, *Morb. Mortal. Wkly. Rep.* **50:**1–15, 2003.)

currently because clinical isolates cannot be cultured for antiviral susceptibility. An early study suggested that a large number of mutations in the NS5A region (the interferon sensitivity determining region) correlated with the poorer treatment response in genotype 1-infected patients (31), but the significance of this association has not been confirmed in either clinical or molecular studies in Western countries (46). More recently, researchers have overcome the hurdles necessary to developing in vitro replicon cell lines that are resistant to interferon and have highlighted the importance of cellular factors and interferon signaling pathways in the response to interferon (81).

Evaluation, Interpretation, and Reporting of Results

Patients at risk for HCV should be screened according to CDC guidelines (Table 2). An anti-HCV high positive result in a patient with hepatitis is highly suggestive for HCV infection. However, confirmatory testing, usually with a sensitive nucleic amplification test (i.e., Amplicor, TMA, or real-time PCR), is required because 15 to 40% of patients resolve their HCV infection acutely and there may be other explanations for the liver enzyme abnormalities (i.e., alcohol abuse, hepatitis B, or medications). A single negative confirmatory NAT is usually indicative of resolved infection, but it is possible that a patient may have low-level or intermittent viremia. For this reason, a second NAT is usually performed within 6 to 12 months. A high positive EIA test indicates past or current infection in over 95% of cases. A negative EIA test for a low-risk, immunocompetent patient who has not had any recent potential exposures to HCV can be interpreted as no evidence of infection. A negative HCV EIA is not as reliable for acute infections (within 3 months) and for immunosuppressed persons (HIV positive or with chronic renal disease). Therefore, for suspected acute infections, as in occupational exposures, one should assess HCV by EIA at the time of exposure, 4 to 6 weeks later, and then 4 to 6 months after exposure (117). HCV RNA testing should be performed at 4 to 6 weeks and 4 to 6 months and to confirm any positive HCV EIA. Also, immunosuppressed patients with risk factors for HCV acquisition and/or elevated ALT should be tested for HCV RNA. Other situations and the associated interpretations are listed in Table 3. Guidelines for the reporting of results are detailed in Fig. 3.

The rarely used RIBA test is reserved for confirming infections in low-risk EIA-positive patients and in patients who are high risk but NAT negative. Low-risk patients testing EIA positive but RIBA negative are likely not infected, but instead have a false-positive EIA. In contrast, RIBA-positive, NAT-negative patients with risk factors for HCV may have intermittent viremia and follow-up NAT is recommended in the subsequent 6 to 12 months.

In general, HCV genotype and quantitative tests should be performed only for patients for whom HCV treatment is being considered. Although quantitative HCV RNA assays are standardized into international units per milliliter, there is substantial variability between the quantitative tests, so the same assay should be used prior to and during treatment (107).

HEPATITIS G VIRUS/GB VIRUS TYPE C

Taxonomy

Although hepatitis G virus (HGV) is the most closely related virus to hepatitis C in the *Flaviviridae* family, it shares only 27% genetic homology (68, 108) and therefore is not classified in the *Hepacivirus* genus, but rather in an unassigned genus, group GB. It was discovered concurrently by two independent groups: one group isolated the virus from a patient with cryptogenic hepatitis (non-A to non-E) and gave the virus the name of hepatitis G (68). The second group employed subtractive cloning to isolate the virus from patients at high risk for hepatitis (IDUs and persons of West African origin) and volunteer blood donors (108). The second group had previously isolated two viral strains from a 34-year-old surgeon (whose initials were GB) who had post-transfusion non-A, non-B hepatitis (24), and thus the group named the virus GB virus, type C (GBV-C), based on genetic similarities. These viruses were later shown to have 96% homology and are essentially the same virus (61). Neither name is accurate, however, because studies over the ensuing decade demonstrated that the virus is not associated with hepatitis, and the virus was not isolated from the patient with the initials GB. For the sake of simplicity, GBV-C is used here to designate this virus, as others have done (111).

Description of the Agent

GBV-C is a single-stranded, positive-sense, enveloped RNA virus with a genome of 9,392 nucleotides (68). Organized similarly to other flaviviruses, GBV-C has a single, long open reading frame encoding a single large polyprotein with structural proteins at the 5′ end and nonstructural proteins at the 3′ end. The structural proteins include two enveloped proteins,

TABLE 2 Persons for whom HCV testing is recommended[a]

Persons who have injected illicit drugs in the recent and remote past, including those who injected only once and do not consider themselves to be drug users
Persons with conditions with a high prevalence of HCV infection, including:
Persons with HIV infection
Persons with hemophilia who received clotting factor concentrates before 1987
Persons who were ever on hemodialysis
Persons with unexplained abnormal aminotransferase levels
Prior recipients of transfusions or organ transplants, including:
Persons who were notified that they had received blood from a donor who later tested positive for HCV infection
Persons who received a transfusion of blood or blood products before July 1992
Persons who received an organ transplant before July 1992
Children born to HCV-infected mothers
Health care, emergency medical, and public safety workers after a needlestick injury or mucosal exposure to HCV-positive blood
Current sexual partners of HCV-infected persons

[a] Table adapted from M. Alter, *MMWR Recomm. Rep.* **47**(RR-19)**:**1–39, 1998.

TABLE 3 Recommendations for reporting results of testing for antibody to HCV (anti-HCV) by type of reflex supplemental testing performed[a]

Anti-HCV screening test results	Supplemental test results	Interpretation	Comments
Screening-test negative	Not applicable	Anti-HCV negative	Not infected with HCV, unless recent infection is suspected or other evidence exists to indicate HCV infection; false negatives can occur in immunocompromised persons
Screening-test positive with high S/Co ratio	Not done	Anti-HCV positive	Probably indicates past or present HCV infection; supplemental serologic testing not performed. Samples with high S/Co ratios usually (≥95%) confirm positive; however, if patient is asymptomatic and lacks risk factors, a false-positive test is possible and supplemental testing is recommended.
Screening-test positive with low S/Co ratio	Not done	Anti-HCV positive or negative	More likely to be a false positive; confirmatory testing with RIBA or NAT recommended
Screening-test positive	RIBA positive	Anti-HCV positive	Indicates past or present HCV infection
	RIBA negative	Anti-HCV negative	Not infected with HCV, unless recent infection is suspected or other evidence exists to indicate HCV infection
	RIBA indeterminate	Anti-HCV indeterminate	HCV antibody and infection status cannot be determined; another sample should be collected for repeat anti-HCV testing (>1 month) or for HCV RNA testing.
	NAT positive	Anti-HCV positive, HCV RNA positive	Indicates active infection
	NAT negative, RIBA positive	Anti-HCV positive, HCV RNA negative	The presence of anti-HCV indicates past or present infection; a single negative HCV RNA result does not rule out active infection.
	NAT negative, RIBA negative	Anti-HCV negative, HCV RNA negative	Not infected with HCV
	NAT negative, RIBA indeterminate	Anti-HCV indeterminate, HCV RNA negative	Screening test with anti-HCV result probably a false positive, which indicates no HCV infection. Alternatively, it may represent a resolved infection with waning antibody titers.

[a] Table adapted from M. Alter, *Morb. Mortal. Wkly. Rep.* **52:**1–15, 2003.

E1 and E2, which are less heavily glycosylated than in hepatitis C, perhaps explaining the greater frequency of viral escape and viral persistence seen in HCV infections (61). Although the core protein was difficult to identify, electron microscopic images showed the existence of a nucleocapsid and it is now believed to exist in a truncated form proximal to E1 (140). The nonstructural proteins are similar to those in HCV: a thiol protease in NS2 region, a serine protease and helicase in NS3 region, a protease cofactor in NS4, an interferon sensitivity determining region (ISDR) in NS5a region, and an RNA-dependent, RNA-polymerase in NS5b region (111). In addition, a portion of the 5′ NCR serves as an internal ribosomal entry site (IRES), but its activity is very weak compared to the IRES in HCV. There are four different genotypes, which are strongly linked to particular geographic locations but do not have any important clinical implications (109).

GBV-C has a density between 1.07 and 1.18 g/ml (140). The virus is found primarily in humans, although monkeys can also harbor the virus. Both in vivo and in vitro studies suggested that the virus is hepatotropic (105, 106). However, other studies contradicted this finding, instead suggesting that the virus is primarily lymphotropic and may replicate in $CD4^+$ T cells (92, 140). These differences may be due to poorly specific strand-specific RNA detection (i.e., self-annealing primers) or serum contamination of hepatocytes. A further study found a low liver:serum ratio of GBV-C virus, but a high liver:serum ratio of HCV (87), weakening the evidence for hepatotropism. Thus, it remains controversial whether the agent replicates in hepatocytes.

Epidemiology and Transmission

Exposure to, and infection with, GBV-C is very common worldwide. Using GBV-C viremia as a marker of infection, the prevalence of GBV-C in healthy blood donors is especially high in South and West Africa (10 to 14%), moderately high in Southeast Asia (4 to 6%), and low in Japan, the United States, and China (0.6 to 2%) (52). In persons living in the United States who had no exposure to blood products, the prevalence of viremia ranged between 0.8 and 4.2% (34, 68, 111). This prevalence rate was even greater, 13 to 38%, in patients with frequent exposure to blood products, such as IDUs and patients receiving frequent blood transfusions. Serological studies showed that *exposure* to GBV-C was still more common, with rates three to six times greater than in

viremia studies. Using an assay to detect antibodies to E2, researchers found that 9 to 16% of blood donors and 41 to 65% of IDUs were seroreactive. Not surprisingly, coinfection with HIV and HCV also occurs frequently: 29 to 46% of HIV-positive and 37% of HCV-positive patients were seroreactive to GBV-C (34, 136). Between 10 and 20% of patients with HCV also have detectable GBV-C RNA (16, 68).

GBV-C is transmitted predominantly through exposure to contaminated blood (3) but may also be spread sexually and vertically during childbirth. Therefore, GBV-C resembles more closely the transmission routes of HIV, rather than HCV, which is primarily bloodborne (111). For example, the rates of GBV-C viremia are similar in HIV-positive patients who acquired HIV parenterally and in those who acquired it sexually (139). Of infants born to HIV-negative, HCV-negative women with GBV-C viremia, 89% have detectable viremia at 3 to 9 months of age (66).

Clinical Significance

Despite being a very common infection, GBV-C does not have any direct association with disease in humans and is believed to be nonpathogenic. The initial identification of the agent came from isolated cases of cryptogenic hepatitis (68, 108); however, subsequent studies using reliable controls determined that there was no causal relationship with hepatitis (3). Furthermore, there is a poor correlation between viremia and ALT elevation (3, 68), in contrast to HCV and HBV viremia. Studies of patients coinfected with HCV and GBV-C found no difference in the rate and severity of their liver disease compared to those of patients with isolated HCV infection (3).

The majority of infections are subclinical, persist for years at a constant high level, and then clear without a preceding decline in viral load (63). Clearance seems to occur when patients develop E2 antibodies, suggesting that these antibodies have neutralizing activity (111, 119). In contrast to HCV, clearance of GBV-C occurs in the majority of cases (70 to 80% of the time) and immunity is lifelong (34, 111, 121).

Perhaps the most important clinical aspect of GBV-C is its apparent beneficial effect on HIV infection in patients infected with both viruses. Several studies have found that patients with GBV-C viremia have lower HIV viral levels, greater CD4 counts, and increased survival (65, 125). In one study, GBV-C RNA-negative patients were 3.7 times more likely to die than patients with detectable GBV-C RNA (112). The apparent protection of GBV-C viremia persists even in the era of highly active antiretroviral therapy; GBV-C levels were inversely proportional to HIV RNA levels (122). Two studies, however, did not find an association between GBV-C infection and better HIV outcomes, but they studied patients with greater CD4 counts and classified GBV-C on the basis of a single time point, possibly introducing misclassification of viremia (14, 17). Several authors have speculated that GBV-C has a direct inhibitory effect on HIV replication (137), perhaps by induction of HIV-inhibitory cytokines and down-regulated expression of HIV-coreceptor CCR5 (138). More recently, other researchers have found that *loss* of GBV-C RNA is more predictive of HIV progression, perhaps by an associated decline in CD4 count (128, 136).

GBV-C appears to be sensitive to interferon and, to a lesser extent, ribavirin, but viremia recrudesces after cessation of treatment (59). Coinfection with HCV and GBV-C does not impact the efficacy of interferon/ribavirin therapy for HCV infection (16). Finally, GBV-C-infected patients receiving liver transplants do not have poorer posttransplant outcomes than do GBV-C-uninfected patients (16, 22).

Collection, Transport, and Storage of Specimens

No studies have examined the stability of GBV-C; however, because of its similarity to HCV, the methods used for handling and storage of HCV samples should be sufficient. Although GBV-C appears to be nonpathogenic, it is prudent to use a biosafety level two cabinet when working directly with the virus.

Direct Detection

A variety of nucleic acid amplification techniques have been used to directly detect GBV-C, including real-time PCR and bDNA (87, 111). Initial PCR assays used the NS3 region as an amplification target, but the 5′ untranslated region (5′ UTR) is more highly conserved than either the NS3 or NS5 regions and thus provides better sensitivity. Combining the 5′ UTR and NS5a amplification targets, PCR has demonstrated a sensitivity of >90% (7, 13). In comparison to laboratory-developed PCR assays, a commercial assay from Abbott (LCx) showed better sensitivity, probably because it targeted the 5′UTR region (12). However, there was a great amount of heterogeneity in the detection of GBV-C RNA from 18 specialty laboratories, even those which used a commercial assay (64). Interestingly, GBV-C exhibits a much a wider range of viremia than does HCV, from 25,000 to 250,000,000 genome equivalents/ml (111).

Isolation Procedures and Identification

Infectious GBV-C clones have been generated in vitro, but routine culture methods are not available for clinical use (140).

Genotyping

There are at least five different genotypes of GBV-C as detected using RFLP, type-specific PCR, and phylogenetic analysis (79, 80). Genotype 1 predominates in West Africa, genotype 2 in the United States and Europe, genotype 3 in Asia and South America, genotype 4 in Southeast Asia, and genotype 5 in southern Africa (80, 110).

Serologic Tests

By cloning the E2 protein and expressing in Chinese hamster ovary cells or *Escherichia coli*, an ELISA using anti-E2 antibodies has been developed (26, 119). As mentioned previously, the development of antibodies to E2 is associated with resolution of GBV-C viremia (119). A strip immunoblot assay detecting core, NS3, and NS4 proteins is also available (34).

Evaluation, Interpretation, and Reporting of Results

GBV-C does not appear to cause disease in humans. Therefore, testing for GBV-C antibodies or GBV-C RNA in patients with cryptogenic cirrhosis or abnormal liver function tests is not warranted. There are no FDA-approved tests for GBV-C, but a PCR assay from Abbott (LCx) is available. An antibody response to E2 is indicative of exposure to GBV-C and clearance, whereas a positive GBV-C RNA test is indicative of acute or chronic infection.

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Rotaviruses, Caliciviruses, Astroviruses, Enteric Adenoviruses, and Other Diarrheic Viruses*

TIBOR FARKAS AND XI JIANG

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Common causes of infectious gastroenteritis include bacterial, parasitic, and viral pathogens. In comparison, the study of viral gastroenteritis, which started with the discovery of many prototype viruses in the early 1970s, in general has been limited by the inability to grow most of these pathogens in cell culture, the lack of efficient animal models, and the high genetic and antigenic variation of these viruses. The disease burden of viral gastroenteritis, however, has been increasingly recognized, particularly in the developed countries, where improved sanitation has led to a significant decrease in bacterial and parasitic gastroenteritis but has had little effect on viral gastroenteritis. Viruses associated with gastroenteritis diseases in humans include rotavirus, calicivirus (norovirus and sapovirus), astrovirus, and enteric adenovirus. Rotaviruses are the most important cause of severe acute gastroenteritis in young children. Noroviruses have been recognized as the most important cause of nonbacterial epidemics of gastroenteritis. Sapoviruses, astroviruses, and enteric adenoviruses are important causes of pediatric gastroenteritis. Other viruses, such as coronavirus, torovirus, Aichi virus, and picobirnavirus, have been implicated in human gastroenteritis, but their etiologic role is not well established.

TAXONOMY

Rotaviruses

Rotaviruses are classified as members of the *Rotavirus* genus within the family *Reoviridae,* which contains 10 other distinct genera. Based on group-specific antigens of the major viral structural protein VP6, rotaviruses are serologically classified into seven groups (A to G) (32). Group-specific epitopes are also found on other structural proteins as well as on some nonstructural proteins. Groups A to C rotaviruses infect both humans and animals, with the group A rotaviruses infecting humans most frequently and causing disease mainly in young children. Groups D to G rotaviruses are mainly animal pathogens. Within each group, rotaviruses are further classified into serotypes based on neutralization assays using antibodies against two major outer capsid proteins, VP4 and VP7. VP4 is a spike protein on the capsid surface which is sensitive to protease cleavage; therefore, the types based on the VP4 protein are also called P types (protease sensitive). VP7 is a glycoprotein, and the types based on this protein are also called G types. There are 14 VP7 (G) serotypes of rotaviruses, and strains of animal and human origin may fall within the same serotypes. A correlation between genotype and serotype has been observed for VP7 (G) types, but this correlation is less clear for VP4 (P) types. Recently a new G genotype (G genotype 15) has been described from a cattle farm in India; however, serotype determination of this strain has not been reported yet. Due to a lack of readily available typing serum or monoclonal antibodies for VP4 (P) serotypes, the classification of VP4 (P) types has relied mostly on sequencing of the VP4 gene (P genotype). Numbers assigned for P serotypes and genotypes are different, and 14 P serotypes and 23 P genotypes of VP4 have been described.

Caliciviruses

The *Caliciviridae* family includes four genera from which *Norovirus* and *Sapovirus,* also called human caliciviruses (HuCVs), mainly cause gastroenteritis in humans (47, 101). These two genera were previously called "Norwalk-like viruses" and "Sapporo-like viruses," named after their prototype strains, the Norwalk and the Sapporo viruses, respectively. Classification of HuCVs is based on genetic analysis of the viral genomes. Within each genus, strains are further grouped into genogroups (GG) and genetic types or clusters. Noroviruses can be classified into five genogroups with GG I, II, and IV containing the human isolates, GG III containing the bovine strains, and GG V containing the recently discovered murine norovirus (7, 80, 149). Noroviruses from pigs closely related to GG II human isolates also have been described. There are over 20 established norovirus genetic clusters within the five genogroups; with the continuous discovery of new strains, the number is growing. According to the most recent classification scheme, sapoviruses are classified into nine genetic clusters within five genogroups. GG I, II, IV, and V contain human isolates, and GG III contains the porcine sapoviruses (38).

The antigenic relationships among different HuCVs have been studied mainly based on recombinant virus-like particles (VLPs) and antibodies raised against them in laboratory animals. Since HuCVs are genetically and antigenically diverse and the currently available assays do not cover all types, these results remain preliminary. Overall the antigenic types correlate with the genetic types: noroviruses and sapoviruses are

* This chapter contains information presented in chapter 94 by Martin Petric and Raymond Tellier in the eighth edition of this Manual.

antigenically distinct; limited cross-antigenic reactivity can be detected within genera and between genogroups, and the cross-reactivity increases among strains within the same genogroup. The relationship of the antigenic types with the serotypes of HuCVs remains unknown because a virus neutralization assay remains unavailable for HuCVs.

Astroviruses

The family *Astroviridae* contains human and animal viruses with a characteristic star-like (*astron* means star in Greek) surface structure. Astroviruses were discovered in stools of infants with vomiting and diarrhea in 1975 (97) and later isolated from numerous wild and domestic animal species. Recently astroviruses have been grouped into two genera, *Mamastrovirus* and *Avastrovirus*, for their major host species (152).

Eight serotypes of human astrovirus have been distinguished by immunofluorescence, neutralization, immune electron microscopy (IEM), or genotyping (11, 62, 88). Complete or partial sequences of the viral genome of the prototype strains have been reported, and the reagents for typing and the reference strains of each type are available from the Oxford Group in the United Kingdom. The genetic sequence of the major human astrovirus serotypes allowed molecular classification of the viral family, and available data indicated a good correlation between the serotyping and genotyping (11, 116). Astroviruses seem to cause disease in a species-specific manner, and viruses isolated from different species are serologically unrelated.

Enteric Adenoviruses

The *Adenoviridae* family contains two genera, *Mastadenovirus* and *Aviadenovirus*. There are 51 different human adenovirus serotypes, which are grouped into six subgroups (A to F) based on their ability to agglutinate red blood cells and the size, composition, homology, and organization of their genome. Many adenoviruses are readily isolated from human stools, but their role in acute gastroenteritis is unclear. There are two serotypes, however, Ad40 and Ad41, in subgroup F, that have been clearly associated with infantile diarrhea (25, 40, 143, 160) and are referred as "enteric adenoviruses." These enteric adenoviruses are also called "fastidious" adenoviruses, due to their difficulty to grow in cell culture.

Coronaviruses and Toroviruses

Coronavirus and *Torovirus* represent two genera of the *Coronaviridae* family, which, with two other families, *Arteriviridae* and *Roniviridae*, forms the *Nidovirales* order, a group of enveloped, positive-sense RNA viruses that all synthesize a 3′ coterminal set of subgenomic mRNAs in infected cells (56).

Aichi Virus

According to genetic analysis, Aichi virus has been classified as a new genus, *Kobuvirus*, in the *Picornaviridae* family (144). Recently a new species, the bovine kobuvirus, has been isolated from HeLa cell cultures that possibly had been contaminated with this virus from the calf serum used to maintain the cells.

Picobirnaviruses

The name *Picobirnavirus* was proposed for a new genus of viruses that are different from the existing members of the *Birnaviridae* family. The name picobirna reflects their small size (pico-) and bisegmented double-stranded RNA (dsRNA) genome.

DESCRIPTION OF THE AGENTS

Rotaviruses

Viruses described as ~70-nm particles with a wheel-like appearance were described from a number of animal species during the 1960s. Similar viruses of humans were first identified in 1973 by thin-section electron microscopy (EM) of the duodenal mucosa of children with acute gastroenteritis. Later these viruses were designated rotaviruses (*rota* means wheel in Latin). Mature rotavirus particles are about 70 nm in diameter and nonenveloped (Fig. 1) and possess a triple-layered icosahedral protein capsid composed of an outer layer, an intermediate layer, and an inner core. The rotavirus genome contains 11 segments of dsRNA, ranging in size from ~660 bp (segment 11) to ~3,300 bp (segment 1). The gene coding assignment of the 11 genome segments has been determined and includes six major structural proteins (VP1, VP2, VP3, VP4, VP6, and VP7) that appear on the matured viral particles and six nonstructural proteins (NSP1 to NSP6) that are expressed in infected cells and play important roles in the viral genome replication, protein synthesis, and viral capsid assembly and maturation. One of the nonstructural proteins, NSP4, has been found to be a viral enterotoxin (9, 33).

Caliciviruses

Caliciviruses are small (30 to 38 nm), round, and nonenveloped (Fig. 1). Sapoviruses have the typical calicivirus morphology with the "Star of David" appearance (Fig. 1). Noroviruses reveal a smoother surface structure and therefore were also called "small round structured viruses" (SRSV). Cryoelectron microscopy and X-ray crystallography analysis of recombinant VLPs revealed that noroviruses possess a T = 3 icosahedral capsid, composed of 180 capsid proteins that form 90 dimeric capsomers. Each capsid protein possesses two major domains, the shell (S) and the protrusion (P) domains. The P domain consists of two subdomains (P1 and P2). The P2 subdomain is the most variable region of the capsid protein and is responsible for the receptor binding and possibly for the antigenic properties of noroviruses.

Noroviruses contain a single-stranded, positive-sense, poly(A)-tailed RNA genome of ~7.5 kb (67, 70, 87). It is organized into three open reading frames (ORFs), with ORF1 encoding the nonstructural proteins, ORF2 encoding the capsid protein, and ORF3 encoding a minor structural protein. The sapovirus genome is slightly different in that the nonstructural and capsid protein encoding sequences are fused into one large ORF.

Astroviruses

Astroviruses are small (28 to 30 nm in diameter), round, nonenveloped viruses (Fig. 1). They reveal a star-like appearance, but the typical star-like morphology may be visible on only 10 to 15% of the virions in negatively stained EM preparations. Cryoelectron microscopy images of human astrovirus serotype 1 revealed a rippled solid capsid shell with 30 dimeric spikes and an icosahedral symmetry (100).

Astroviruses contain a single-stranded, positive-sense RNA genome of about 6.8 kb excluding the poly(A) tail. The viral genome contains three ORFs. ORF-1a and ORF-1b encode the nonstructural proteins; ORF-1a contains a protease motif, transmembrane helices and nuclear localization signals, and a ribosomal frame shifting signal, while ORF-1b contains an RNA polymerase motif. ORF 2 encodes the capsid precursor protein (~87 kDa) that is intracellularly cleaved to a 70-kDa protein that forms the capsid and later cleaved by

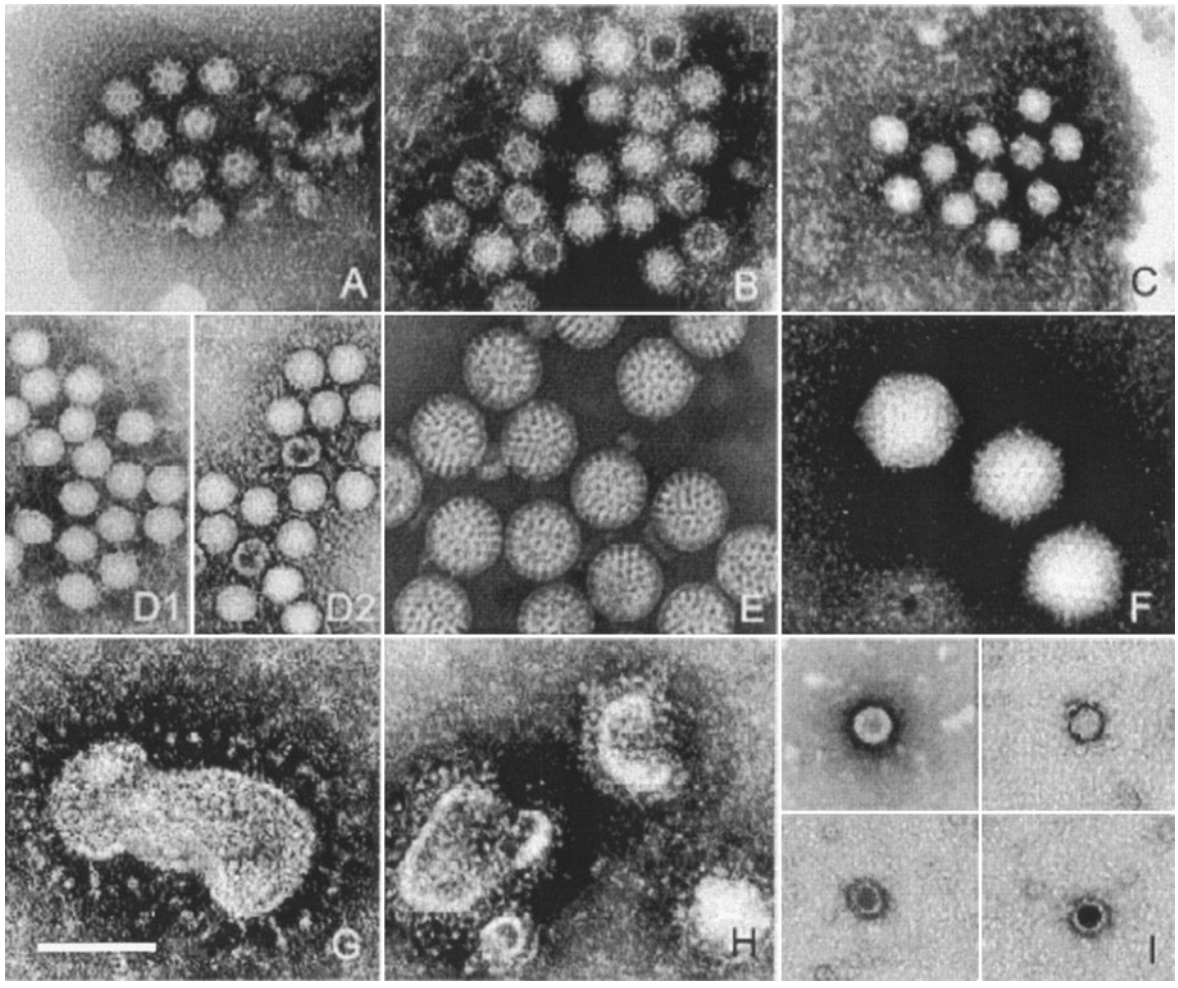


FIGURE 1 Electron micrographs of gastroenteritis viruses. (A) Sapoviruses; (B) noroviruses; (C) astroviruses; (D1) Aichi virus; (D2) picornaviruses; (E) rotaviruses; (F) adenoviruses; (G) coronaviruses; (H) torovirus-like particles; (I) picobirnaviruses. Bar, 100 nm. Modified from Fig. 2 of chapter 94 by Martin Petric and Raymond Tellier in the 8th edition of this Manual. Panel D1 was provided by Teruo Yamashita, and panels E and I were provided by Charles Humphrey.

trypsin to smaller peptides of 25 to 34 kDa, resulting in infectious virions.

Enteric Adenoviruses

The name adenovirus originates from the Greek word *aden,* which means gland, because the first human adenoviruses were isolated from adenoid tissue in 1953. Since then, human adenoviruses have been linked with a number of diseases including respiratory illness, conjunctivitis, and diarrhea (57). Adenoviruses are nonenveloped, icosahedral particles 70 to 100 nm in diameter (Fig. 1). Mature virions consist of a DNA-containing core surrounded by a protein shell. The genome is a linear double-stranded DNA of 30 to 38 kb (the size varies from group to group). The protein shell (capsid) is composed of 252 capsomeres, of which 240 are hexons and 12 are pentons. There are a total of 11 viral structural proteins from which three coat proteins (hexon, penton base, and fiber) are of important clinical significance. These proteins are involved in viral entry (fiber) and intracellular transportation, and the hexon and fiber contain the major neutralization epitopes.

Coronaviruses and Toroviruses

Coronaviruses and toroviruses are pleomorphic, enveloped positive-stranded RNA viruses. The genomic RNA is 25 to 31 kb, making them the largest RNA viruses. The RNA is surrounded by nucleoprotein (N); the nucleocapsid is enclosed in an envelope composed of membrane protein (M) and a host cell-derived lipid bilayer containing inserted S (spike), HE (hemagglutinin-esterase), and other surface proteins (56).

Aichi Virus

The Aichi viruses are ~30 nm in diameter, a size which is indistinguishable from that of other picornaviruses (Fig. 1). They encapsidate a genome of ~8.2-kb, positive-sensed RNA with a poly(A) tail. The genome contains a single large ORF that encodes a polyprotein which is processed by protease cleavage to individual functional proteins. The virion is composed of three structural proteins (VP0, VP1, and VP3) from which VP0 strongly reacts with convalescence phase patient sera (157).

Picobirnaviruses

The picobirnaviruses are nonenveloped, 35- to 41-nm-diameter, round particles with a smooth surface (Fig. 1) and an icosahedral T = 3 symmetry. The sizes of the two genomic segments vary, ranging from 2.3 to 2.6 kb for genomic segment 1 and 1.5 to 1.9 kb for genomic segment 2 (19). The full-length genomic segment 2 sequences of two human isolates (3-GA-91 and 1-CHN-97) have been determined, and both sequences contained a single ORF with RNA-dependent RNA polymerase motifs that are common among many RNA viruses.

EPIDEMIOLOGY AND TRANSMISSION

Gastroenteritis viruses are transmitted by the fecal-oral pathway and through person-to-person and environmental surface contact (Table 1). These viruses also can be transmitted by contaminated water and food, which usually results in large outbreaks. Most viral gastroenteritis remains endemic and represents a significant disease burden in both developing and developed countries. In temperate countries, viral gastroenteritis does not have the typical summer peak of most bacterial and parasite-caused gastroenteritis; instead, viral gastroenteritis is more common in colder seasons, with rotavirus diarrhea having a typical winter-spring peak.

Rotaviruses

Rotaviruses usually cause severe dehydration in infants and children under 2 years of age, which makes rotaviruses a major cause of childhood deaths in the developing countries. Rotavirus mortality is much lower, but the disease burden remains high, in the developed countries, representing approximately 2.7 million diarrheal episodes each year in the United States with significant numbers of physician visits and hospitalizations and high medical and societal costs.

The source of infection for young infants is likely acquired from older siblings or parents with subclinical infection. Shedding of rotavirus from the intestinal tract prior to onset of diarrhea or following cessation of diarrhea has been well documented. Rotaviruses are able to survive on the environmental surfaces at ambient temperature and are resistant to physical inactivation, which may contribute to their efficient transmission (1, 131). Cross-species transmission of rotaviruses has been demonstrated, and comparison of genetic sequences of human and animal rotaviruses often reveals close identity, indicating a zoonotic potential of rotavirus disease. In temperate countries rotavirus diarrhea usually exhibits a typical winter/spring peak, during which over one-third of the diarrhea cases seen in the emergency room and regular clinics are caused by rotaviruses. This seasonal pattern of rotavirus infection observed in temperate climates does not occur uniformly in tropical countries.

Caliciviruses

HuCVs are highly contagious and environmentally stable, characteristics which are particularly important for transmission through contaminated surfaces, food, and water (30). Noroviruses have been recognized as the most important causes of nonbacterial acute gastroenteritis in all ages in both developing and developed countries (34). Sapoviruses cause disease mainly in children; however, outbreaks among adults also have been reported (20, 73, 117). Because of their ability to cause large outbreaks, incapacitating the affected individuals and causing public panic, noroviruses are listed as category B agents by the National Institute of Allergy and Infectious Diseases (NIAID) Biodefense Research Agenda. Such outbreaks are often described as occurring in hospitals, educational institutions, nursing homes for the elderly, military settings, and cruise ships. Seroprevalence studies showed

TABLE 1 Epidemiology, transmission, and clinical significance of gastroenteritis viruses

Virus	Mode of transmission	Target population	Clinical significance	Disease control and prevention
Rotavirus	Fecal-oral	Children <5 yrs	Acute severe gastroenteritis, mostly sporadic, nosocomial infection can occur	Rehydration in severe cases, vaccines have been developed
Norovirus	Fecal-oral, close contact, water and food	All age groups	Moderate-severe gastroenteritis, commonly causes outbreaks in variety of settings	Personal hygiene, hand washing, avoid crowds, control food handlers
Sapovirus	Fecal-oral, close contact	Mainly children	Mild-moderate gastroenteritis, subclinical infection occurs, sporadic but outbreaks occur	Personal hygiene, hand washing
Astrovirus	Fecal-oral	Mainly children	Mild-moderate gastroenteritis, subclinical infection occurs, sporadic but outbreaks occur	Personal hygiene, hand washing
Adenovirus	Fecal-oral	Mainly children <5 yrs	Moderate-severe gastroenteritis, sporadic, nosocomial infection, persists in immunocompromised patients	Personal hygiene, hand washing
Picobirnavirus	Unknown, possibly fecal-oral	Children and adults	Possibly sporadic mild-moderate gastroenteritis	Personal hygiene, hand washing
Aichi virus	Unknown, possibly fecal-oral	Children and adults	Possibly sporadic mild-moderate gastroenteritis	Personal hygiene, hand washing
Coronavirus	Unknown, possibly fecal-oral	Possibly all age groups	Possibly sporadic mild-moderate gastroenteritis	Personal hygiene, hand washing
Torovirus	Unknown, possibly fecal-oral	Possibly all age groups	Possibly sporadic mild-moderate gastroenteritis	Personal hygiene, hand washing

that both noroviruses and sapoviruses have a worldwide distribution with 70 to 100% of individuals >5 years of age possessing antibodies against them (71, 72, 112, 113). Recently human histo-blood group antigens including the ABO, Lewis, and secretor/nonsecretor types were identified as the receptors for noroviruses (63, 92, 98, 137). Different norovirus strains recognize different histo-blood group antigens, resulting in a variation of natural resistance/susceptibility between the human host and the different norovirus types (60, 61, 137). The discovery of enteric caliciviruses from animals that are genetically closely related to human strains raised the question about calicivirus gastroenteritis as a zoonotic disease and animals as possible reservoirs for human illness (54, 80, 94, 135). Direct evidence for interspecies transmission of enteric caliciviruses is still missing.

Astroviruses

Mammalian astroviruses are generally associated with gastroenteritis, whereas avian astroviruses can cause both intestinal and extraintestinal diseases such as nephritis, hepatitis, or immunodeficiency. Human astroviruses are associated mainly with pediatric gastroenteritis, although cases among caretakers of sick children or immunocompromised adults (50) and outbreaks among military troops (12) or in nursing homes (90, 103) have been described. Astrovirus infection was linked to 2 to 10% of pediatric gastroenteritis cases worldwide depending on the settings and diagnostic tests used. Asymptomatic infections are common in all ages. Among school-age children there is a 70 to 90% seroprevalence to astroviruses, indicating frequent infections during childhood.

Enteric Adenoviruses

Although the role of enteric adenoviruses in acute gastroenteritis has been definitively established, the incidence of adenovirus-related gastroenteritis differs considerably among various studies and locations. Outbreaks of enteric adenovirus diarrhea have been reported in hospitals and more often in day care centers. Enteric adenoviruses are associated with 5 to 20% of cases of pediatric diarrhea (142). It occurs most often in children younger than 4 years of age, and symptoms last for 5 to 12 days, which is longer than the duration of other viral diarrhea. Like other enteric pathogens, enteric adenoviruses are frequently detected in stool specimens of children with asymptomatic infection. Prolonged or chronic diarrhea of immunocompromised patients linked to enteric adenoviruses also has been reported (132, 141).

Coronaviruses, Toroviruses, Aichi Virus, and Picobirnaviruses

The role and significance of these viruses in human diarrhea are not yet firmly established. Coronaviruses infect the respiratory and gastrointestinal tracts, and they are a recognized cause of common cold in humans. Although coronaviruses, such as transmissible gastroenteritis virus of pigs and canine coronavirus, are major gastrointestinal pathogens of animals, agents resembling these viruses have been only sporadically reported in cases of human gastrointestinal illness, particularly in newborns. The evidence for human enterotropic coronaviruses depends on visualization by EM of coronavirus-like particles in stools of infants with diarrhea, serologic studies of outbreaks of infant diarrhea, and infrequent isolation of coronaviruses from stools of infants and children with diarrhea.

Toroviruses seem to be associated with asymptomatic enteric infections in swine and with severe diarrheal disease in horses (Berne virus) and cattle (Breda virus) and were also implicated in human gastroenteritis (10, 65, 85, 86). The detection of human torovirus-like particles in gastroenteritis was first reported in 1984, and these agents were shown to be associated with acute and persistent diarrhea in children and to be potentially important nosocomial pathogens. Seroconversion following infection has been demonstrated by IEM and hemagglutination inhibition assays with virus purified from stool specimens (29).

Aichi virus was first isolated from stool samples of patients during an investigation of oyster-associated gastroenteritis outbreaks that occurred in 1989 in Japan. The antibody prevalence to Aichi virus was 7.2% in children less than 4 years of age, and it increased with age to around 85% by 35 years of age, indicating the circulation of these viruses in the human population (158).

Picobirnaviruses were first identified by analysis of stool samples of humans and rats by polyacrylamide gel electrophoresis (PAGE) in 1988. Since their original description, they have been detected in fecal samples of a wide range of domestic and wild animals. The frequency of picobirnaviruses in human stool samples (normal or with diarrhea) has been investigated in several countries mainly by PAGE detection of viral dsRNA. The detection rate was ~20% in outbreaks of human gastroenteritis and 9 to 13% in inpatients with or without diarrhea in various age groups. A much lower detection rate (~0.5%) also has been reported in children. Picobirnaviruses are more frequently detected in immunocompromised patients (42, 50).

CLINICAL SIGNIFICANCE

Clinical symptoms of viral gastroenteritis include watery diarrhea, vomiting, anorexia, abdominal pain, and fever, with rotavirus gastroenteritis being the most severe. The disease in most cases lasts for 2 to 3 days; however, viral shedding can continue for several weeks. Due to the lack of effective antiviral drugs against gastroenteritis viruses, treatment of patients with viral gastroenteritis has been focusing mainly on the relief of the clinical symptoms. Rehydration therapy is the most important treatment for severe cases and for reducing mortality in the developing countries. A prompt diagnosis of viral gastroenteritis is important; although it may not help to cure the disease, it can help avoid unnecessary use of antibiotics.

Rotaviruses

Although, as previously described, rotaviruses cause mainly severe gastroenteritis in infants, rotavirus infection also has been implicated in biliary atresia and central nervous system disease. The lack of antiviral drugs against rotaviruses and the significant disease burden necessitate a vaccine for rotaviruses. The first licensed rotavirus vaccine (Rotashield; Wyeth) was a tetravalent vaccine that contained reassortants of four major serotypes in a simian rhesus rotavirus background. Rotashield went on the market in 1998 in the United States, but it was withdrawn in 1999 due to concern that it caused intussusception. A second rotavirus vaccine (LLR; Lanzhou Institute of Biological Products), based on an attenuated strain of a lamb rotavirus, was licensed in China in 2000, and a third rotavirus vaccine (Rotarix; GlaxoSmithKline), based on an attenuated single human strain, was licensed in Mexico in 2004. The newest rotavirus vaccine, a live, oral, pentavalent human-bovine (WC3) reassortant vaccine (Rotateq; Merck & Co., Inc.), was licensed by the Food and Drug Administration (FDA) in early 2006.

Caliciviruses

Since noroviruses can be linked to the great majority of viral gastroenteritis epidemics, the need for vaccines or antivirals is apparent. At present, preventive strategies for calicivirus infection target the elimination of the source of infection by increased personal hygiene, cleaning and disinfecting of environmental surfaces, and identification of sick food-handlers and contaminated food or water. Several approaches to norovirus vaccine development have been investigated, such as transgenic plants expressing norovirus proteins as an edible vaccine and VLPs produced in baculovirus as a subviral vaccine. The major challenges of vaccine development for HuCV gastroenteritis are the tremendous genetic and antigenic diversity of these viruses and the lack of a suitable animal model for efficacy studies.

Astroviruses and Enteric Adenoviruses

Acquired immunity might be protective against astrovirus and adenovirus gastroenteritis, since symptomatic infections occur mainly in young children. In both cases the severity of gastroenteritis is usually mild and self-limiting and rarely requires rehydration therapy. Interruption of transmission should be the main focus of prevention by increased personal hygienic measures and disinfection of contaminated environment. At present there are no vaccines or antivirals available.

Coronaviruses, Toroviruses, Aichi Virus, and Picobirnaviruses

As mentioned previously, the real significance of these viruses in human gastroenteritis is not yet well established.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Gastroenteritis viruses replicate and cause disease mainly in the intestine. Therefore, stool specimens are the major source of specimens for laboratory diagnosis, although vomitus can also be used. Specimens are preferably collected within the first 48 h of illness. Specimens collected in later stages have a lower detection rate, although prolonged shedding of some gastroenteritis viruses up to several weeks after onset of illness has been reported. Stool specimens of a few grams are sufficient for detection by EM or antigen or nucleic acid detection methods. The samples can be collected by bedpan and then transferred into smaller containers or test tubes. Specimens can be recovered from a diaper with a wooden tongue depressor. Alternatively, a portion of the diaper can either be lined with plastic wrap to capture watery stool or cut out and soaked with sterile water or phosphate-buffered saline (PBS) to extract diagnostic material. Rectal swabs may be easy to obtain, but they are poor specimens for viral diagnosis and should be discouraged.

In general, the best results are obtained by testing the samples fresh, particularly for good viral morphology by EM. Stool samples can be stored at 4°C for weeks if they are not tested immediately after collection or need to be transported to the diagnostic laboratory. For prolonged storage, specimens should be kept frozen, preferably at −70°C. If multiple tests are planned for multiple pathogens or for confirmation of the results, aliquots of the samples are usually prepared to avoid repeated freezing and thawing of the bulk samples, which usually destroys the viral structures and results in decreased detection rates. Stool samples can be shipped on ice or dry ice depending on their storage conditions (e.g., short- or long-term storage), with care being taken to avoid unnecessary freezing and thawing between shipment and storage.

Serum samples are used for detection of antibodies against gastroenteritis viruses. Documenting seroconversion between acute- and convalescent-phase serum samples has been particularly useful for diagnosis of viral infection in outbreak investigations, but it is used mainly in research laboratories. An acute-phase serum is usually collected within a few days of the onset of illness, and a convalescent-phase serum is obtained 2 to 3 weeks later. Blood and serum samples should be handled with precautions for transmission of bloodborne pathogens. For long-term storage, the samples are preferred to be kept at −20 or −70°C in aliquots.

Drinking water, food, or other environmental specimens are used in research on viral (mainly norovirus) gastroenteritis outbreaks. If a food or water item is suspected as the source of an outbreak, a sample should be obtained as early as possible and stored at 4°C. Since testing of these items for the presence of viruses is not routinely done in most of the diagnostic laboratories and often requires special methodology (e.g., filter concentration of 5 to 100 liters of water and processing of food to remove food debris), a laboratory with the capacity to test these specimens should be contacted (e.g., local health departments or CDC).

LABORATORY DIAGNOSIS OF VIRAL GASTROENTERITIS

Diagnostic assays commonly used for viral gastroenteritis include direct visualization of viruses in clinical samples (EM, IEM, or solid phase-IEM [SPIEM]), detection of viral antigens (enzyme-linked immunosorbent assay [ELISA] or enzyme immunoassay [EIA], latex agglutination), detection of viral nucleic acids (RNA-PAGE, DNA-agarose gel, dot blot hybridization assay, PCR, reverse transcription-PCR (RT-PCR), real-time PCR, and nucleic acid sequence-based amplification [NASBA]), and virus isolation in cell culture. Due to the lack of reagents for some viral families described above, many diagnostic methods are not commercially available yet or are under development. The principles of these methods have been described in chapter 18. In this chapter, special emphasis has been placed on the detection of viruses or viral components in stool specimens, which is difficult because of the low viral load of many gastroenteritis viruses and the presence of unknown factors in stools that can interfere with the assays.

Direct Detection

Direct EM

Direct EM is used to visualize the negatively stained viral particles on a grid without the additional steps of grid preparation (121). It is fast, does not require specific reagents, and can be used for any viruses, but it is relatively less sensitive (minimum of $\sim 10^6$ viral particles per ml) than other techniques and requires expensive equipment. Liquid stool samples can be applied to the grid following a low-speed centrifugation (4,000 × *g* for 10 min) to remove large debris. For solid stool samples, a 10 to 20% stool suspension in either distilled water or a 1% ammonium acetate solution is made. One drop (5 to 10 μl) of the sample is then applied to a copper EM grid (300- to 400-mesh size) which is coated with formar (polyvinyl formol) reinforced by carbon shadowing (commercially available). The sample drop also can be placed on a piece of Parafilm, and then the grid is floated on the top of the drop. After incubation for approximately 1 min, excess

specimen is removed from the grid with a filter paper. Longer incubation may be used but without letting the samples dry out. If the resulting grid is too thick, the sample may be diluted or the grid may be washed with a drop of distilled water. The grid is then stained with a drop of 2% phosphotungstic acid (pH 7) for 10 to 30 s and then blotted with a filter paper. The dried grid is ready for EM examination.

For specimens containing a low concentration of viruses, a step of concentration by ultracentrifugation may be used. The Beckman Airfuge with an EM-90 rotor is useful to deposit the specimens on the EM grid for small volume samples. A centrifugation at 100,000 × *g* for 1 h is used to pellet most of the viral particles. Following the centrifugation, the pellet is resuspended in distilled water and processed for making the grids as outlined above. The samples may be further diluted if the sample layer on the grid is too thick. A step of low-speed centrifugation to remove large aggregates also may help to avoid overloading.

The stool suspension can also be subjected to agar diffusion to concentrate the virus particles. Briefly, the stool specimen is placed on a small block of 1% agar made up in distilled water, and the grid is floated on the surface of the drop. After incubation at room temperature until the drop is absorbed into the agar, the grid is removed and processed for EM as described above. This procedure not only concentrates the viruses but also removes excessive quantities of salts which may precipitate on the grid, obscuring the viruses.

Under EM, negatively stained virus particles appear as lighter structures surrounded by a dark background (Fig. 1) (121). The most definitive of the spherical structures are the adenoviruses and rotaviruses, whose icosahedral capsids are readily discerned (Fig. 1). Each of the smaller round viruses, including the norovirus, sapovirus, astrovirus, and Aichi virus, although with similar shape (round) and size (30 to 40 nm in diameter), has its unique surface appearance: sapovirus reveals a rigid surface structure with cup-like depressions of a typical "Star of David" appearance; norovirus has a less rigid surface appearance and does not have the Star of David structure; astrovirus reveals a distinct five-point star appearance; and Aichi virus reveals a smooth surface indistinguishable from that of other picornaviruses (Fig. 1). However, viral particles lacking well-defined features are commonly seen, which can pose diagnostic dilemmas, particularly when they are seen as individual particles. Thus, a confident diagnosis relies on a repeated observation of identical particles in more than one EM field with some particles revealing typical morphology.

Among the nonspherical particles, the coronavirus forms pleomorphic structures with well-delineated dumbbell-shaped peplomers or spikes. Generally, the peplomers form a dark halo around the enveloped capsid. Torovirus-like particles have a less well defined peplomer fringe. A proportion of them are kidney shaped, while others have well-established darker-staining regions in the center of the particle (Fig. 1).

In addition to the above well-recognized virus particles, there are a number of other structures visualized in stool specimens. These include the obvious flagella, pili, and bacteria, as well as cell wall components, cell membrane blebs, and bacteriophages. The bacteriophage may pose problems since they may be confused with small gastroenteritis viruses, especially if their tails are absent. Membrane blebs can be confused with toroviruses.

IEM

IEM technique has played an important role in the discovery of a number of viral pathogens including the prototype Norwalk virus (79, 121). The principle of the technique is to form virus-antibody aggregates by incubation of the samples with specific antibodies, and these aggregates are much easier to identify under EM, which may improve the sensitivity up to 100-fold. This technique also is used to measure antibody against a virus, by comparison of the numbers and sizes of the viral aggregates at different concentrations of serum samples used. For detection of viruses in stool samples, antibodies from different sources (serum samples of patients, hyperimmune antibodies from animals, or monoclonal antibodies) can be used. The dilution of antibodies used should be determined empirically in each case. For convalescent-phase sera from patients, a ratio of 1:1 of the sera and stool suspensions may be used. Hyperimmune antisera from animals may be used in a much higher dilution. Using too much antibody may result in overcoating of the viral particles, making it difficult to recognize fine surface structures.

After incubation for 1 h at 37°C, the samples are centrifuged for 30 min at 15,000 × *g*. The resulting pellet that contains the virus-antibody complexes is then processed for making the grid directly or by the agar block technique described above. The antibodies used in the IEM technique can also be labeled with colloidal gold, which may help the detection of viruses on the grids. A further modified method is the solid-phase IEM, which is designed to capture the viruses by specific antibodies with which the EM grid is precoated (89). This method may not result in typical virus clumps but leaves less debris on the grid.

Antigen Detection Assays

Antigen detection assays can be highly sensitive and specific if hyperimmune or high-titered monoclonal antibodies are used. Common formats include ELISA or EIA and latex agglutination and rapid membrane-based assays which are simple, fast, and suitable for clinical diagnosis. Because of the lack of reagents due to the difficulty to propagate many gastroenteritis viruses, reliable antigen detection assays remain unavailable for many viral families. In addition, due to the high degrees of genetic and antigenic variations, some existing assays need to be improved for better sensitivity and specificity.

Commercial ELISA or EIA and latex- and rapid membrane-based assays are available for rotaviruses and enteric adenoviruses (Tables 2 and 3) but are limited or not yet available for other gastroenteritis viruses. Several companies have developed and commercialized antigen detection tests for astroviruses and noroviruses (e.g., R-Biopharm AG and Dako), but at present these tests are available only for research and not used in clinical diagnostic laboratories in the United States. Some commercially available antigen detection tests are listed in Table 2 and recommended mainly for rotavirus and enteric adenovirus diagnosis.

ELISAs

Several antigen detection ELISAs for the diagnosis of astroviruses and human caliciviruses utilizing monoclonal antibodies or hyperimmune polyclonal antibodies have been described (55, 69, 71, 82, 106, 119), and commercial kits based on these reagents are under development. Finally, ELISAs have been developed and used in research for the detection of human toroviruses and Aichi virus.

In commercial ELISA kits, 96-well plates with breakaway strips are commonly used, especially for diagnosis of rotaviruses in clinical laboratories. Stool suspensions (10 to 20%) are prepared in PBS, pH 7.4, or sodium carbonate buffer, pH 9.5. The whole assay can be completed within 2 h and has a sensitivity equal to or greater than that of EM.

TABLE 2 Commercial antigen detection tests available for diagnosis of viral gastroenteritis[a]

Company (website)	Organism(s) detected (product trademark name)
Inverness Medical Professional Diagnostics (http://www.invernessmedicalpd.com)	Rotavirus (Virogen Rotatest)
Bio-Rad Laboratories (http://www.bio-rad.com)	Rotavirus (Pastorex Rotavirus, Pathfinder Rotavirus)
Meridian Bioscience (http://www.meridianbioscience.com)	Rotavirus, adenovirus (ImmunoCard STAT! Rotavirus, ImmunoCard STAT! Adenovirus, Premier Rotaclone, Premier Adenoclone—Type 40/41, Rapid Strip Rota-Adeno)
bioMerieux (http://www.biomerieux.com)	Rotavirus, adenovirus (Slidex Rota-Kit 2, Slidex Rota-Adeno Kit, VIKIA Rota-Adeno)
Orion Diagnostica (http://www.oriondiagnostica.fi)	Rotavirus, adenovirus (Rotalex, Adenolex, Diarlex, Diarlex MB)
ANI Biotech (http://www.anibiotech.fi)	Rotavirus, adenovirus (BIOCARD Rota, BIOCARD Adeno, ANI Rotatest, BIOCARD Rota Stick, BIOCARD Adeno Stick)
Dako (http://www.dako.com)	Rotavirus, adenovirus, astrovirus, norovirus (IDEIA Rotavirus, IDEIA Adenovirus, IDEIA Astrovirus, IDEIA Norovirus)
R Biopharm AG (http://www.r-biopharm.com)	Rotavirus, adenovirus, astrovirus, norovirus (RIDASCREEN Rotavirus, RIDASCREEN Adenovirus, RIDASCREEN Astrovirus, RIDASCREEN Norovirus)

[a] This list is based on a search of the Internet and the literature with the best knowledge of the authors. Many of the assays listed in the previous editions of this chapter cannot be found, possibly due to name or brand change, product withdrawal, or reorganization or merge of manufacturers. Some of the kits are available only for research use in the United States or other countries. Requesting information of FDA approval of a test kit from the vendor is necessary before it is used in clinical diagnostic laboratories in the United States.

ELISAs also have been used for typing viruses such as the G typing of the group A rotaviruses based on type-specific monoclonal antibodies (124). ELISA also can be used to detect antibodies by coating the plate with specific viral antigens. Such assays have been used in the serosurveillance or outbreak investigations for rotaviruses and caliciviruses (37, 134). With the cloning and expression of viral genomes, similar assays are expected to be developed for other viral families.

Rapid membrane-based ELISA or EIAs are valuable for small laboratories with sporadic demand for diagnosis of viral gastroenteritis (28). Kits for these tests include all reagents and require minimal equipment, as the test results can be read visually. The tests generally include built-in positive and negative controls to ensure that it has been performed appropriately. These tests can be completed within 30 min. Because of their speed and simplicity, these kits are amenable to point-of-care testing.

Latex Agglutination Assays

The latex agglutination tests are based on the agglutination of latex beads coated with virus-specific immunoglobulins by the virus particles or viral antigens present in the specimens. Since some fecal specimens can cause nonspecific agglutination, negative-control latex beads coated with nonspecific immunoglobulins are also included in the test. Usually a 10% stool suspension is prepared in PBS, large particles are removed by low-speed centrifugation, and the supernatant is mixed with the latex reagent. A positive result is indicated by visible clumping (agglutination) of the latex particles, which usually occurs within minutes. The assays are generally less sensitive than ELISA but are useful in small diagnostic laboratories.

Nucleic Acid Detection Assays

Diagnosis based on detection of viral genome includes detection of viral DNA or RNA by electrophoresis, by hybridization, or following amplification of the viral DNA or RNA by PCR or RT-PCR. The general principle of each technique has been described in chapter 16. The selection of methods depends on the nature of the viral genomes, e.g., DNA or RNA, single or double strands, single or segmented genome, and sizes of the genomes (Table 4). The selection of methods also depends on the purpose of the study: rapid diagnosis (direct detection methods and PCR or RT-PCR), typing (PCR or RT-PCR by type-specific primers), or research (PCR or RT-PCR followed by sequencing).

Direct Staining following Electrophoresis

Direct staining of viral RNA or DNA by ethidium bromide is not widely used for diagnosis of viral gastroenteritis because of the insensitivity of the methods.

Detection of Enteric Adenoviral DNA following Restriction Enzyme Digestion

Direct detection of enteric adenoviral DNA by electrophoresis requires a step of restriction enzyme digestion because of the large size of adenoviral genomic DNA. In addition, the resulting DNA fragments reveal unique patterns by specially selected restriction enzymes, which are useful for genetic identification of the strains. Among the restriction endonucleases, SmaI proved effective in identifying adenoviruses 40 and 41 (17).

Electropherotyping of Double-Stranded, Fragmented RNA Genome of Rotaviruses

The segmented, dsRNA genome of the rotaviruses has traditionally been the target for detection and typing (109). The viral RNA is extracted directly from stool specimens and then subjected to electrophoresis followed by detection with silver staining. This method is as sensitive as EM. It is also used in the classification of rotaviruses, in which the appearance of an unusual electropherotype could denote a novel strain or group of rotavirus. Schematic diagrams of electropherotypes of group A to G rotaviruses have been compiled and serve as a reference. Similar methods of electrophoresis,

TABLE 3 Diagnostic methods used for viruses associated with human gastroenteritis

Virus	Diagnostic methods				References for commercial tests
	EM	Antigen detection	Nucleic acid detection	Virus isolation	
Rotavirus	Readily detected but not commonly used	Commercial kits available	Good for genotyping	Difficult but adaptable	22, 26, 27, 28, 31, 41, 93, 139
Norovirus	Low sensitivity, IEM used in research	Recombinant-antigen-based assays used in research laboratories[a]	For detection and molecular epidemiology in research laboratories	NA[b]	18, 126
Sapovirus	Low sensitivity, typical calicivirus morphology	Recombinant-antigen-based assays under development	For detection and molecular epidemiology in research laboratories	One porcine strain was adapted, not for human strains	NA
Astrovirus	Low sensitivity, difficult to see typical star-like morphology	Monoclonal antibody-based assays, good for typing	For detection and molecular epidemiology in research laboratories	Adapted in cell culture	23, 102, 122
Adenovirus	Readily detected, but not specific for Ad 40 and 41	Commercial assays available	For typing and detection	Difficult to grow in cell culture	108, 140, 151
Picobirnavirus	Difficult to detect	NA	For detection	NA	NA
Aichi virus	Cannot differentiate from other picornaviruses	Limited tests available in research laboratory	For detection	Readily grows in cell culture	NA
Coronavirus	Atypical morphology	Limited tests available in research laboratory	For detection	NA	NA
Torovirus	Atypical morphology	Limited tests available in research laboratory	For detection	NA	NA

[a] Commercial kits are available for research purposes, not for clinical diagnostic laboratories in the United States.
[b] NA, not available.

using silver staining instead of ethidium, have been used for the detection of picobirnaviruses.

Dot Blot Hybridization

In general, dot blot hybridization is more sensitive than direct staining methods and is comparable to most antigenic detection methods. Dot blot hybridization has been used for the detection of rotaviruses, caliciviruses, astroviruses, and enteric adenoviruses but has been less frequently used since the introduction of PCR or RT-PCR because of the higher sensitivity of the last two methods. In addition, dot blot hybridization requires long prehybridization and hybridization incubations, which are time-consuming. However, hybridization has been used in combination with PCR or RT-PCR to improve the sensitivity and specificity of the methods. Dot blot hybridization has been used particularly for detection of toroviruses in stool specimens because detection of these viruses by EM is difficult. In general, dot blot hybridization is used mainly in research laboratories.

PCR and RT-PCR

PCR and RT-PCR are the most sensitive methods used for the diagnosis of gastroenteritis viruses. They are particularly useful in combination with sequencing for molecular epidemiology studies of these viruses (34, 38, 104, 148). For example, RT-PCR assays have been used for the G and P typing of the group A rotaviruses, in which type-specific primers targeting the unique regions of the VP7 and VP4 genes have been used to differentiate the G and P types, respectively, by DNA electrophoresis analysis following the RT-PCR amplification of the viral RNA (43, 111). RT-PCR is also used for studying the genetic variation and genotyping of caliciviruses and astroviruses. In these studies, primers targeting the highly conserved regions of the viral genome, usually in the viral nonstructural protein regions, such as the RNA polymerase region, are used for broad detection followed by sequencing of the RT-PCR products. Primers targeting the less conserved viral capsid gene also have been used, which is believed to be biologically more relevant. Simultaneous analysis of both the RNA polymerase and capsid regions led to the description of several recombinant calicivirus strains.

Advanced technologies such as real-time PCR (53, 75, 127) using automated processes have been developed for rapid diagnosis of gastroenteritis. The particular importance of these techniques is the quantitative measurement of the

TABLE 4 Taxonomy and virologic properties of gastroenteritis viruses

Virus	Family	Size (nm)	Appearance under EM	Viral genome	Genetic or antigenic types
Rotavirus	*Reoviridae*	70	Wheel-shaped, triple-layered capsid	dsRNA	7 antigenic groups (A–F) and 14 G and 23 P types
Norovirus	*Caliciviridae*	30–38	SRSV[a]	ss(+)RNA[b]	5 genogroups and >20 genetic types (clusters)
Sapovirus	*Caliciviridae*	30–38	SRSV, with Star of David appearance	ss(+)RNA	5 genogroups and >9 genetic types (clusters)
Astrovirus	*Astroviridae*	28–30	SRSV, star shaped	ss(+)RNA	8 serotypes
Adenovirus	*Adenoviridae*	70–100	Icosahedral capsid	dsDNA	Ad40 and Ad41 linked to gastroenteritis
Picobirnavirus	*Birnaviridae*	35	Small round virus	dsRNA	Unknown, genetic variation found
Aichi virus	*Picornaviridae*	30	Small round virus	ss(+)RNA	Unknown
Coronavirus	*Coronaviridae*	60–200	Pleomorphic with club-shaped projections	ss(+)RNA	Unknown
Torovirus	*Coronaviridae*	100–150	Pleomorphic with torus-shaped core	ss(+)RNA	Unknown

[a] SRSV, small round structured virus.
[b] ss, single stranded.

PCR products in a timely fashion, which makes possible measuring viral load for viruses that do not grow in tissue culture. Multiplex PCR and RT-PCR assays with pooled primers have been used for broad detection of viruses from different families (15, 110, 128). Finally, microarray and gene chip technology has been explored for diagnosis of viral gastroenteritis (21, 96) but is not widely used yet.

One of the big challenges of virus detection in stool specimens by PCR or RT-PCR is the potential presence of inhibitors to the assays. Inhibitory effects can be monitored by including internal nucleic acid controls to the assays. Approaches to reduce such inhibitors include dilution of the extracted RNA or addition of detergents or denaturing chemicals during RNA extraction (125). Among many reagents, guanidinium thiocyanate is the most frequently used agent for a number of commercial methods for extraction of nucleic acid from different clinical specimens including stools (e.g., RNAzol and TRIzol). The extracted viral DNA or RNA is recovered by phenol choloroform extraction followed by ethanol precipitation or by adsorption onto silicon particles or membranes. Stool specimens often contain different microorganisms as well as host cells, which could release a large amount of nucleic acids that could be nonspecifically amplified by the virus-specific primers. Sometimes these nonspecific PCR products cannot be avoided even under high-stringency conditions of the assays. In this case, hybridization with virus-specific probes usually is required to confirm the results of the assay (133).

Most viral families causing gastroenteritis are genetically diverse, which imposes a further challenge in the application of PCR and RT-PCR for clinical diagnosis. In the example of human noroviruses, there are over 20 genetic clusters within two or three genogroups (46, 47, 81). By using primers targeting highly conserved regions of the genomes, mainly in the RNA-dependent RNA polymerase (RdRp) region, the majority of known human noroviruses are detected (5, 16, 68). However, due to the high diversity of the norovirus genus, there is probably no single primer pair that can detect all strains. A similar situation also is true for the sapoviruses and astroviruses. For better diagnosis, multiple primer sets targeting different regions of the genome have been used to increase the chance of detection of genetically distantly related strains (147, 150). In addition, degenerate primers based on sequence variation of known family members also have been used (34, 35, 38, 76). It should be pointed out that even with the improved primers these assays may still miss some strains and continued efforts for the design and evaluation of new broadly reactive primers are necessary. Some of the commonly used primers for the detection of astroviruses, noroviruses, and sapoviruses are listed in Table 5.

Because there are readily available antigenic diagnostic methods, RT-PCR has not been as widely used for diagnosis of rotavirus-associated gastroenteritis, although it has been specially used for genotyping of rotaviruses. On the other hand, due to the lack of readily available and reliable antigenic assays for many other enteric viruses, RT-PCR plays an important role in their diagnosis, including noroviruses, sapoviruses, and astroviruses (36, 38, 153), and a commercial RT-PCR kit for the detection of astroviruses and noroviruses has been recently developed (Calici/Astrovirus Consensus kit; Argene). PCR and RT-PCR also have been developed and increasingly used in the clinical and basic research of enteric adenoviruses, coronaviruses, toroviruses, picobirnaviruses, and Aichi virus (3, 8, 29, 129, 156, 158). Further improvement by standardization and automation of these methods may help their wider application in the diagnosis of viral gastroenteritis in general clinical laboratories.

NASBA

Nucleic acid sequence-based amplification (NASBA) of RNA targets utilizes a multienzyme reaction modeled after retroviral replication. It is based on the simultaneous activities of reverse transcriptase, RNase H, and T7 RNA polymerase. The NASBA reagents are commercially available in an easy-to-use format (NucliSens Basic kit; bioMerieux Inc., Durham, N.C.), and only the target-specific primers and the capture probe need to be provided by the investigator. It is an isothermal reaction conducted at 41°C, which does not require expensive thermocyclers and generates the same number of copies as RT-PCR in a shorter time. Detection methods utilizing NASBA have been described for rotavirus (66), astrovirus (136), and norovirus (49, 107).

Isolation in Cell Culture

While growth in cell culture has a major impact on the investigation of a virus, it is generally time-consuming and is not considered sufficiently rapid to contribute to the meaningful management of disease. Most of the enteric viruses causing human gastroenteritis are fastidious in cell culture.

TABLE 5 Commonly used RT-PCR primers for astro-, noro- and sapovirus detection

Virus	Primers	Genomic regions	Length (bp)	Reference
Astrovirus	Mon340/Mon348	ORF1a	289	Belliot et al., 1997 (11)
	A1/A2	ORF1a	192–237	Guix et al., 2002 (52)
	Mon343/Mon344	ORF1b	316	Belliot et al., 1997 (11)
	PreCAP1/82b	ORF2	719	Yan et al., 2003 (159)
	Mon244/Mon245	ORF2	413	Noel et al., 1995 (116)
	Mon269/Mon270	ORF2	449	Noel et al., 1995 (116)
	prBEG/Mon2	ORF2/3′UTR[b]	296–324	Saito et al., 1995 (130)
	DM4/Mon2	ORF2/3′UTR	1,200	Walter et al., 2001 (153)
	Mon2/Mon69	3′UTR	89	Mitchell et al., 1995 (105)
Norovirus[a]	NV35/NV36	ORF1 (region A)	470	Wang et al., 1994 (154)
	SR33/SR46	ORF1 (region A)	123	Ando et al., 1995 (6)
	SR33/SR48,50,52	ORF1 (region A)	123	Ando et al., 1995 (6)
	NI/E3	ORF1 (region A)	113	Green et al., 1995 (45)
	p289/p290	ORF1 (region A)	319	Jiang et al., 1999 (68)
	P289H,I/P290H-K	ORF1 (region A)	319	Farkas et al., 2004 (38)
	JV12/JV13	ORF1 (region A)	327	Vinje et al., 1996 (148)
	JV12Y/JV13I	ORF1 (region A)	327	Vennema et al., 2002 (145)
	Mon432/Mon434	ORF1 (region B)	213	Fankhauser et al., 2002 (34)
	Mon431/Mon433	ORF1 (region B)	213	Anderson et al., 2001 (4)
	Mon381/Mon383	ORF2 (region C)	322	Noel et al., 1997 (115)
	G1SKF/G1SKR	ORF2 (region C)	330	Kojima et al., 2002 (84)
	G2SKF/G2SKR	ORF2 (region C)	344	Kojima et al., 2002 (84)
	CapA/CapB1, B2	ORF2 (region D)	177	Vinje et al., 2004 (147)
	CapC/CapD1, D3	ORF2 (region D)	253	Vinje et al., 2004 (147)
	COG1F/COG1R	ORF1/ORF2	85	Kageyama et al., 2003 (76)
	COG2F/COG2R	ORF1/ORF2	98	Kageyama et al., 2003 (76)
Sapovirus	Sapp36/Sapp35	ORF1 (RdRp)	487	Matson et al., 1995 (99); Berke et al., 1997 (14)
	p289/p290	ORF1 (RdRp)	331	Jiang et al., 1999 (68)
	P289H,I/P290H-K	ORF1 (RdRp)	331	Farkas et al., 2004 (38)
	SR80/JV33	ORF1 (RdRp)	320	Vinje et al., 2000 (146)
	SLV5317/SLV5749	ORF1 (Cap)	434	Yan et al., 2003 (159)
	SV-F11/SV-R1	ORF1 (Cap)	780	Okada et al., 2002 (118)
	SV-F21/SV-R2	Nested	434	Okada et al., 2002 (118)

[a] Norovirus regions A and B are in the RNA-dependent RNA polymerase (RdRp) region, and regions C and D are in the capsid gene (Cap).
[b] UTR, untranslated region.

Even for rotaviruses, although many strains have been successfully adapted to cell culture, the primary isolation of these strains usually requires multiple passages. Cell lines for isolation of rotaviruses include MDBK, PK-15, BSC-1, LLC-MK2, MA104, CaCo-2, and HRT-29. To grow them in cell culture, the medium must be supplemented with proteases such as trypsin or pancreatin. This approach can be adapted for titration of viruses by plaque assay and serotyping by virus neutralization.

The enteric adenovirus types 40 and 41 grow best in a Graham 293 human embryonic kidney cell line that has been transformed by adenovirus type 5 DNA. Isolation of astroviruses from clinical samples is also difficult, although most of the astrovirus serotypes have been adapted in HEK or LLC-MK2 cell culture (138). Cell culture propagation of astroviruses requires the presence of trypsin in the culture medium.

Propagation of human caliciviruses, human coronaviruses, toroviruses, and picobirnaviruses in cell culture has not been achieved yet. The porcine enteric calicivirus, a sapovirus, has been successfully adapted to grow in porcine kidney cell lines but only in the presence of intestinal contents of pigs as a supplement in the culture medium. The murine norovirus has been reported to replicate in primary dendritic cells and macrophages (155). The only gastroenteritis virus to be readily cultivated in cell culture is the Aichi virus, which grows well in monkey kidney cell lines (BSC-1 or Vero).

IDENTIFICATION

Typing Systems

The typing of gastroenteritis viruses includes antigenic (serotype) and genetic typing. Antigenic typing methods require type-specific antibodies, usually monoclonal antibodies against specific antigenic epitopes of the viruses. If the antibodies are known to be specific for a neutralization epitope, they may be used for typing by a regular solid-phase format of ELISAs. However, for serotyping, tissue culture-based neutralization tests using type-specific antibodies are commonly used. Due to the lack of a cell culture for many

gastroenteritis viruses, such assays remain unavailable for most gastroenteritis viral families. The genotyping methods are usually based on the genomic sequence variation determined by typing PCR or RT-PCR using specific primers, or by regular PCR or RT-PCR followed by sequencing or probe hybridization.

Both antigenic typing and genetic typing of gastroenteritis viruses are important in understanding the classification and epidemiology of these pathogens and for developing preventive strategies against viral gastroenteritis. Typing does not affect clinical treatment. Therefore, typing of gastroenteritis viruses is used mainly in research laboratories.

Rotaviruses

Monoclonal antibody-based typing assays have been developed for typing the G and P types of rotaviruses. However, due to the limited supply of monoclonal antibodies representing all serotypes, genotyping methods have been used more frequently. Genotyping of group A rotaviruses is performed by RT-PCR using type-specific primers targeting the gene segment coding for VP7 (G-genotype) or VP4 (P-genotype). Two common primer sets are currently used in rotavirus G-typing surveillance programs (24, 43); however, it was shown that point mutations in genotype-specific primer regions can lead to mistyping or failure to genotype, indicating that primer usage requires close monitoring and updating (64, 123).

Caliciviruses

Because of the lack of a cell cuture system, serotyping of HuCVs has not yet been achieved. Genetic typing of HuCVs is based on probe hybridization or sequencing of RT-PCR products targeting the RdRp or capsid encoding regions (6, 39, 149).

Astroviruses

For typing of astroviruses, immunologic assays such as IEM and typing ELISA have been described but are not commercially available (88, 114, 116). The most commonly used RT-PCR-based typing methods for astroviruses are summarized in a recent review by Guix et al. (51).

Adenoviruses

Typing of adenovirus types 40 and 41 can be established by a variety of methods. Adenovirus genomic DNA restriction enzyme analysis has been used in the past for the differentiation of enteric adenoviruses. Recently, PCR by itself or in combination with restriction enzyme analysis has become a convenient tool for type-specific identification of adenoviruses (2, 156) and a quantitative real-time PCR assay also has been described (74). With the development of monoclonal antibodies that are able to detect and distinguish the enteric adenoviruses from each other and from the nonenteric adenoviruses, commercially available EIAs have become more widely used (13, 91, 108).

Serologic Tests

Neutralization tests based on plaque reduction or based on epitope-blocking assays using type-specific monoclonal antibodies have been described for rotaviruses. Other methods have also been developed for seroepidemiology study of rotavirus; more information about these methods can be found in several review articles (58, 59, 77, 78). There are no neutralization-based serologic tests available for the diagnosis of other viral gastroenteritis. However, antibody detection ELISAs based on specific viral proteins as the capture antigens have been used in epidemiology studies of gastroenteritis viruses. Recombinant viral capsid proteins of caliciviruses and astroviruses generated in the baculovirus and other systems have been an excellent source of viral antigens for these studies (44, 48, 71). Application of these assays in serosurveillance against gastroenteritis viruses has played an important role in an understanding of the importance of these viruses in different populations. Monitoring seroconversion based on the collection of paired acute- and convalescent-phase sera has been used in outbreak investigations.

ANTIVIRAL SUSCEPTIBILITIES

There are no effective antiviral agents available for the prevention or treatment of viral gastroenteritis. Specific antivirials have been tested in animal models or in vitro but are not used for human treatment.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Diagnosis of viral gastroenteritis remains difficult because of the lack of readily available commercial kits for many viral families causing acute gastroenteritis (Table 3). The fact that a variety of microbial pathogens, including bacterial and parasitic agents in addition to viruses, as well as noninfectious causes, cause acute gastroenteritis in humans further complicates the situation. Furthermore, many viral pathogens are known to cause subclinical infection, and in some cases, more than one pathogen is detected in the same clinical sample or same episode of clinical illness, which may further complicate the diagnosis for true etiology. Thus, diagnosis of viral gastroenteritis largely relies on the clinical information and sometimes on exclusion of other causes with available tests.

Generally, the EM technique remains the most simple and rapid method for those clinical laboratories that have access to the equipment. Although it is relatively less sensitive, EM still serves as the "gold standard" for other methods in clinical diagnosis of many gastroenteritis viruses. The method requires experienced individuals who are familiar with the morphologies of different gastroenteritis viruses, including differentiation of atypical viral particles from many unrelated cellular and microbial debris and structures commonly seen in the stool specimens. The use of reference grids (121) with typical viral morphology of gastroenteritis viruses is helpful for a person who is new to the field to gain such experience.

The antigenic methods are highly sensitive and specific, and, if available, are the best choice for clinical diagnosis. They are also simple and relatively free of problems and are suitable to be commercialized. Good examples include the assays for rotaviruses and enteric adenoviruses (Table 2).

The nucleic acid-based assays, particularly the PCR and RT-PCR assays, are most sensitive and specific, and therefore of great potential for clinical diagnosis. The combination of these assays with hybridization and other technology, such as real-time PCR, further increases specificity, sensitivity, and speed. A further application of great potential for PCR and RT-PCR is the detection of viruses in environmental samples to assess the burden of viral gastroenteritis originating from food and water sources and to better understand the epidemiology of these viruses (83, 95, 120). However, due to the expertise required and the lack of commercial kits, PCR and RT-PCR for gastroenteritis viruses remain largely confined to research or public health laboratories. The genetic diversity of individual virus families poses further challenges for the use of these assays in clinical laboratories. Despite these challenges, commercial RT-PCR kits for the detection of these viruses will hopefully be available in the near future.

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Rabies Virus*

LILLIAN A. ORCIARI AND CHARLES E. RUPPRECHT

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TAXONOMY

The etiologic agents responsible for the acute, progressive viral encephalomyelitis known as rabies belong to the order *Mononegavirales*. These single-stranded, bullet-shaped RNA viruses are in the family *Rhabdoviridae* and the genus *Lyssavirus* and contain nonsegmented genomes of approximately 12 kb (13). Included in the genus are the prototype species, *Rabies virus;* the less commonly known species of nonrabies lyssaviruses, *Mokola virus, Lagos bat virus, Duvenhage virus, European bat virus 1, European bat virus 2,* and *Australian bat virus;* and four proposed new lyssavirus species, *Aravan virus, Khujand virus, Irkut virus,* and *West Caucasian bat virus* (1, 22, 24). Although rabies viruses are endemic to five of seven continents, the geographic distributions of the other lyssaviruses are more localized (2, 11, 27, 30, 47). Differences among lyssaviruses were first noticed by serologic cross-neutralization studies and were used to divide them into serogroups according to these phenotypic differences from *Rabies virus* serogroup 1 (51). Later, differences among these species were based on nucleotide sequence analysis, and viruses were grouped as different species or by genotypes (1 through 7). Current commercial human and animal vaccines are based upon rabies virus and provide adequate cross-protection against most other lyssaviruses, with the exception of *Mokola virus, Lagos bat virus,* and *West Caucasian bat virus*.

DESCRIPTION OF THE AGENT

The virions are rod shaped, approximately 180 by 75 nm, consisting of five structural proteins: the glycoprotein (G), matrix protein (M), nucleoprotein (N), phosphoprotein (P), and large polymerase protein (L). The outer surface of the virus is contained in an envelope bilayer derived from the host cell cytoplasmic membrane during budding (Fig. 1). Peplomers, viral G protein trimeric spikes of approximately 10 nm, are inserted within the surface of the envelope. The rabies virus G protein binds with host cell surface receptors (adsorption), thus initiating a cascade in the infectious cycle and replication. The inner surface lining of the envelope is formed by the M protein, which binds with the G protein and envelope and the ribonucleoprotein (RNP) core.

The RNP is composed of tightly wound RNA, encapsidated by the phosphorylated N protein, and associated with the P and L polymerase proteins. The RNA-nucleoprotein complex is responsible for viral replication. The virus-encoded polymerase (L gene) transcribes the genomic strand of rabies virus RNA into leader RNA and five capped and polyadenylated mRNAs, which are translated into proteins. Translation, which involves the synthesis of the N, P, M, G, and L proteins, occurs on free ribosomes in the cytoplasm. Although G protein synthesis is initiated on free ribosomes, completion of synthesis and glycosylation (processing of the G protein) occurs in the endoplasmic reticulum and Golgi apparatus. In part, the intracellular ratio of leader RNA to N protein regulates the switch from transcription to replication, and when this switch is activated, replication of the viral genome begins. The first step in viral replication is synthesis of full-length copies (positive strands) of the viral genome. When the switch to replication occurs, RNA transcription becomes "nonstop" and stop codons are ignored. The viral polymerase enters a single site on the 3′ end of the genome and proceeds to synthesize full-length copies of the genome. These positive strands of rabies virus RNA serve as templates for synthesis of full-length negative strands of the viral genome. During the assembly process, the N-P-L complex encapsidates negative-stranded genomic RNA to form the RNP core, and the M protein forms a capsule, or matrix, around the RNP. The RNP-M complex migrates to an area of the plasma membrane containing G protein inserts, and the M protein initiates coiling. During assembly, the M-RNP complex binds with the G protein, and the completed virus buds from the plasma membrane (44, 54, 55).

From a diagnostic viewpoint, the N and G proteins have been the focus of most laboratory research evaluations. Rabies diagnosis is based on the ability to detect intracellular viral inclusions, which are collections of RNP. Since the rabies virus G protein and host cell receptor interactions initiate the infection cycle, the G protein is directly involved in pathogenesis and virulence, induction of immune response, and binding of neutralizing antibodies (10, 12). Vaccines prepared from whole virions, purified rabies virus G proteins, or recombinant viral vaccines encoding rabies virus G proteins have been used to successfully immunize and protect animals from rabies virus infections (34, 52). Additionally, antibodies to the G protein have been used as a method to evaluate vaccine potency.

Detailed reviews of rabies virus replication and virus pathogenesis have been published recently (12, 14).

* This chapter contains information presented in chapter 102 by Jean S. Smith in the eighth edition of this Manual.

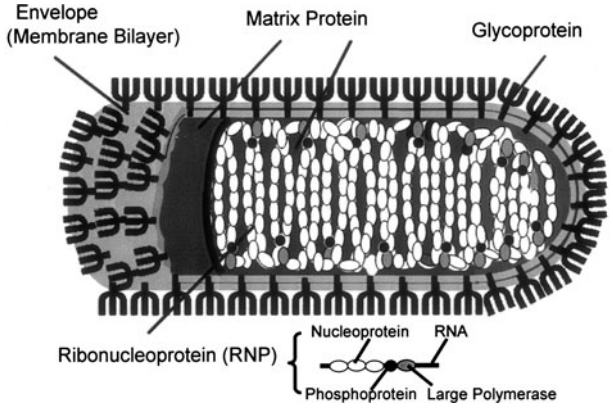


FIGURE 1 Rabies virus structure.

As an enveloped RNA virus, the rabies virus is sensitive to lipid solvents (detergent solutions, ethers, and chloroform), alcohols (70% ethanol or isopropanol), iodine preparations, and quaternary ammonium compounds. Despite susceptibility to lipid solvents, acetone does not inactivate virus completely after routine fixation times of 1 h. However, the rabies virus is sensitive to drying and is inactivated by pasteurization and UV light (7, 20).

EPIDEMIOLOGY AND TRANSMISSION

Nearly 7,000 rabies cases were reported in animals from the United States during 2004. The majority of these cases were reported in wildlife, accounting for 92% of all reported rabies cases. Among these, raccoons were the predominant host species, with more than 2,500 cases. There are seven predominant patterns among mesocarnivores in defined geographic regions of the United States (Fig. 2). Among these are raccoons in the eastern United States, skunks in the north central, south central, and California regions, Arctic and red foxes in Alaska, gray foxes in Texas and Arizona, and mongoose in Puerto Rico. Although dogs represent a major reservoir species in most developing nations, enzootic canine rabies has been eliminated in the United States and other developed countries. Due to canine vaccination programs, rabies cases in dogs in the United States have declined over the last 60 years from >6,000 cases annually to only 94 in 2004 (all due to spillover infections from rabid wildlife). Domestic animal rabies represented only 8% of the total reports. Rabid bats (19.9%) were reported from all parts of the mainland United States. Of the human rabies cases detected in the United States during 2000–2004, 15 of 21 were associated with rabies virus variants of bat origin (four due to transplanted organs or tissues from a donor exposed to a bat). The remaining six human cases were related to terrestrial variants, four from dogs acquired outside the United States (Africa, Philippines, Haiti, and El Salvador), one case related to a raccoon variant, and one case from Puerto Rico, associated with a virus found in mongooses and dogs (21).

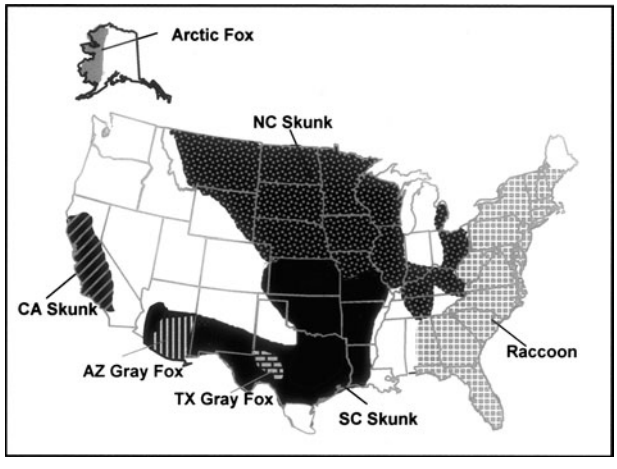


FIGURE 2 Distribution of major terrestrial rabies reservoirs in the United States.

Rabies viruses are usually transmitted in infected saliva via the bite of a rabid animal. Human rabies cases due to nonbite exposures are extremely rare. Nonbite exposures include scratches, open wounds, and mucous membranes contaminated with a source of rabies virus, such as infected saliva or central nervous system (CNS) tissues from a rabid animal. Inhalation of aerosols is another source of nonbite exposure. Two human rabies cases were related to exposures with aerosolized high-titered rabies viruses, and two cases occurred in bat cave researchers without clearly defined exposure histories (5, 53). Immediate wound cleansing with soap and water should be performed if an exposure is suspected, and medical care should be obtained. The local health department should be consulted if there are questions regarding prophylaxis, based on the recommendations of the Advisory Committee on Immunization Practices (ACIP) for Rabies Prevention in Humans. Persons who have been bitten by an animal suspected or proven to be rabid by laboratory diagnosis should receive prompt postexposure prophylaxis. Unvaccinated individuals exposed to a rabid animal should receive human rabies immunoglobulin (RIG) at a dosage of 20 IU/kg of body weight. The RIG should be infiltrated around the wound, and any remaining amount should be given at a site distant from where the vaccine was administered. In addition, five doses of a human rabies vaccine licensed for use in the United States should be administered on days 0, 3, 7, 14, and 28 postexposure. Individuals who have received preexposure vaccinations require only two doses of vaccine on days 0 and 3, regardless of the preexposure antibody titer (6).

CLINICAL SIGNIFICANCE

Rabies may be misdiagnosed because of the nonspecific symptoms associated with the prodromal period, which appear as flu-like symptoms. The usual incubation period is approximately 20 to 90 days after exposure. Following the prodromal period, an acute neurological phase occurs. The majority of rabies cases manifest as an encephalitic form, and fewer than 20% of cases are observed as a paralytic presentation. Among patients exhibiting classical encephalitic symptoms, generalized arousal or hyperexcitability with intermittent lucid periods may appear, including periods of confusion, hallucinations, agitation or aggressive behavior, autonomic dysfunction, difficulty swallowing, hypersalivation, lacrimation, sweating, and dilated pupils. Cranial nerve deficits include anisocoria and facial or tongue weakness. Hydrophobic and aerophobic spasms can be dramatic. Finally, disease presentation includes cardiopulmonary problems and instability leading to coma, cardiac arrest, and death.

The paralytic form of presentation includes flaccid muscle weakness, frequently spreading in an ascending pattern to the

other extremities. In some cases there is laryngeal and facial muscle weakness or bilateral deafness. These cases may resemble Guillain-Barré syndrome. Other symptoms include urinary incontinence. Hydrophobia is less common. Mild inspiratory spasms may be present. Cardiopulmonary complications and instability result in cardiac arrest and death (16, 17, 19).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

All samples collected for rabies virus testing may contain infectious virus. Appropriate handling and shipping precautions should be maintained. Acetone-fixed brain impressions and infected cell culture slides should be manipulated with forceps or gloves, because acetone fixation for 1 h does not fully inactivate rabies virus. Minimum personal protective equipment for routine work should include a gown or lab coat with sleeve protectors, double gloves, safety glasses, and a biological safety cabinet. The addition of heavy rubber gloves and a face shield is required when removing brains from animals. Although rabies virus is a biological safety level 2 agent in diagnostic laboratories, if large quantities of the virus are handled in a high-volume research laboratory or if nonrabies lyssaviruses are evaluated, biological safety level 3 precautions are recommended. The virus is susceptible to a number of disinfectants (e.g., 1:256 quaternary ammonium compound, 0.5% sodium hypochlorite, 70% isopropanol, and 10% iodinophor). All laboratory surfaces need to be decontaminated after use with one of the above disinfectants. All contaminated waste and instruments should be decontaminated by autoclaving for a minimum of 60 min at 15 lb of pressure (7, 20).

Preexposure rabies vaccination is recommended for persons at risk of exposure, including veterinarians, animal control workers, field biologists, and rabies laboratorians. For all individuals working in a routine rabies diagnostic laboratory, periodic determination of rabies virus neutralizing antibody (VNA) titer is recommended every 2 years. Individuals working in high-volume rabies research facilities with continuous exposures should check VNA levels every 6 months. Preexposure vaccination does not equate with protection. All potential rabies virus exposures need to be evaluated on a case-by-case basis, and true exposures should be treated regardless of prior vaccination, as recommended in current ACIP guidelines (immediate wound cleansing and booster vaccinations on day 0 and day 3) (6).

The latest information on the collection, transport, and storage of specimens for rabies diagnosis may be obtained from the CDC rabies website resources. For information on collection of (unfixed) postmortem brain tissues for rabies diagnosis, refer to http://www.cdc.gov/ncidod/dvrd/rabies/Professional/publications/DFA_diagnosis/DFA_protocol-b.htm#IV; for information regarding diagnosis of rabies from formalin-fixed brain tissues from animals or humans, refer to http://www.cdc.gov/ncidod/dvrd/rabies/Professional/Prof.forms/formalin.htm; and for antemortem diagnosis of human rabies cases, refer to http://www.cdc.gov/ncidod/dvrd/rabies/Professional/Prof.forms/antem.htm. Submission forms must accompany each sample and may be obtained at http://www.cdc.gov/ncidod/dvrd/rabies/Professional/Prof.forms/patienth.htm. The CDC should be consulted before samples are submitted for primary rabies diagnosis or confirmation (404-639-1050). A summary of the routine laboratory tests for rabies is given in Table 1. Samples for antemortem and postmortem diagnosis of rabies in humans should be transported to the laboratory on dry ice within 24 h. Samples should be shipped in approved leak-proof containers inside an outer container according to the current International Air Transport Association and interstate or local shipping regulations for diagnostic samples.

DIRECT DETECTION METHODS

Microscopic Methods

Laboratory methods may be used to detect histopathological changes and viral antigen and to observe virion morphology. Direct examination methods may utilize routine stains (e.g., hemotoxylin and eosin) to examine abnormal histopathology consistent with rabies encephalomyelitis and specialized stains (e.g., Seller's) to observe typical viral inclusions, such as classical Negri bodies (26, 43). Although of historical importance, these methods lack both the specificity and sensitivity of modern methods to detect specific rabies virus antigen. Properly performed, the Seller's staining technique may identify Negri bodies in 50 to 80% of rabid animals (33). Observation of virions by electron microscopy provides supportive evidence of a rhabdovirus infection, but this method is less sensitive and too costly for routine diagnosis (18).

ANTIGEN DETECTION

DFA Test

The standard test for rabies virus antigen detection in CNS tissues is the direct fluorescent-antibody (DFA) test. This test is easy to perform and highly specific, approximates 100% sensitivity, and can be completed within 3 to 4 h. A copy of the standard DFA may be obtained online at http://www.cdc.gov/ncidod/dvrd/rabies/Professional/publications/DFA_diagnosis/DFA_protocol-b.htm. The rabies virus diagnostic conjugates consist of antibodies to the whole virion or to the RNP, labeled with fluorescein isothiocyanate. The conjugates may be hyperimmune polyclonal or monoclonal antibodies directed against highly conserved rabies virus epitopes. The rabies virus infects neurons and replicates in the cytoplasm. Within infected cells there are abundant collections of rabies virus proteins (inclusions), especially RNP. When these diagnostic conjugates are added to rabies virus-infected, acetone-fixed brain impressions, the antibodies bind with the rabies virus proteins (antigens) in the cytoplasm of cells and the antigen-antibody complexes fluoresce an intense apple green color. Morphologically, rabies virus antigen may be fluorescent large or small inclusions, dust-like particles, or strands (Fig. 3). The standard protocol for rabies diagnosis includes guidelines for thorough sampling of tissues (complete cross-section of brain stem and cerebellum), optimizing antigen detection with the use of two antirabies conjugates, avoidance of cross contamination, the reduction of nonspecific staining, preservation of fluorescence with 20% glycerol Tris-buffered coverslip mounting medium (pH 9.0), interpretation of results, and repeat or confirmatory testing (35).

DRIT

The direct rapid immunohistochemical test (DRIT) uses a cocktail of purified biotinylated anti-rabies virus nucleocapsid monoclonal antibodies. The test has demonstrated preliminary sensitivity and specificity equal to that of the DFA test in detecting rabies virus antigen. Suggested uses include application to confirmatory DFA testing and enhanced field surveillance in suspect animal cases without human exposure. The advantages of this procedure include rapidity of test

TABLE 1 Routine tests for rabies diagnosis[a]

Analysis	Test method	Detection	Sample	Source	Requirements	Provider	Results
Microscopic examination	Specialized stains, e.g., H&E and Seller's	Eosinophilic intracytoplasmic inclusions	Postmortem CNS	Human or animal	Brain stem, cerebellum, hippocampus	Pathology laboratories (hospital, veterinary)	Detects eosinophilic intracytoplasmic inclusions, low sensitivity (60–80%)
Antigen detection by immunofluorescence	DFA	Rabies virus antigen	Postmortem CNS	Human or animal	Full cross-section of brain stem and cerebellum	State P.H., veterinary laboratories, CDC	Detects rabies virus-specific antigen, very high sensitivity (100%)
	DFA (cryostat)	Rabies virus antigen	Antemortem nuchal biopsy specimen	Human	Full thickness of skin (5–6-μm cryosections)	CDC (some regional or national labs)	Detects rabies virus-specific antigen
	FF DFA	Rabies virus antigen	FF postmortem CNS	Human or animal	Full cross-section of brain stem and cerebellum	CDC (some regional or national labs)	Detects rabies virus-specific antigen, very high sensitivity (~100%)
Antigen detection by IHC	IHC	Rabies virus antigen	FF postmortem CNS	Human or animal	Full cross-section of brain stem and cerebellum	CDC (some regional or national labs)	Detects rabies virus-specific antigen, very high sensitivity (~100%)
	DRIT	Rabies virus antigen	Postmortem CNS	Human or animal	Full cross-section of brain stem and cerebellum	CDC, USDA (some regional or national labs)	Detects rabies virus-specific antigen, very high sensitivity (~100%)
Nucleic acid	RT-PCR	Rabies virus RNA	Postmortem CNS	Human or animal	50 mg of brain stem and cerebellum	CDC (some regional or national labs)	Produces specific RT-PCR amplicons. Very sensitive, detects ~1 infectious unit, but related to primer specificity.
	Nested RT-PCR	Rabies virus RNA	Antemortem saliva, throat, skin, other	Human	100 μl of fluid samples or ≤50 mg of tissue	CDC (some regional or national labs)	Produces specific RT-PCR amplicons. Very high sensitivity, detects less than 1 infectious unit, but related to primer specificity.
Virus isolation	Cell culture (e.g., MNA) and animal inoculation (e.g., MI)	Infectious rabies virus	Postmortem CNS	Human or animal	20% brain homogenate (brain stem and cerebellum)	State P.H., veterinary laboratories, CDC	Detects infectious rabies virus
Serology—virus neutralization	RFFIT, FAVN, MNT	Rabies virus neutralizing antibodies	Antemortem or postmortem serum or CSF	Human or animal	Serum or CSF	CDC (some commercial, regional, or national labs)	Detects neutralizing antibodies. NT are useful in determining immunization status.
Serology—IFA	IFA	Rabies virus IgG or IgM antibodies	Antemortem or postmortem serum or CSF	Human or animal	Serum or CSF	CDC (some regional or national labs)	Detects specific rabies virus IgM and IgG antibodies from serum or CSF. Very sensitive as a diagnostic technique.

[a]Abbreviations: H&E, hematoxylin and eosin; MNA, mouse neuroblastoma; MI, mouse inoculation; MNT, mouse neutralization test; FF, formalin fixed; P.H., Public Health.

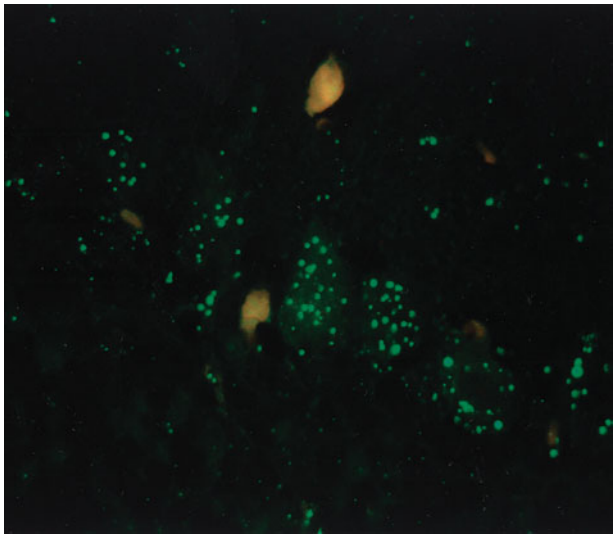


FIGURE 3 Rabies virus antigen detected by FF DFA test. Typical fluorescent (apple green) rabies virus antigen is detected in the cerebellum of a rabid brain by the FF DFA test. Large and small inclusions are seen within the cytoplasm of rabies virus-infected Purkenji cells by using a fluorescence microscope with ×200 magnification.

protocol; fixation of brain touch impression slides in formalin, which inactivates rabies virus; and use of a light microscope to observe test slides (25).

Formalin-Fixed Tissues

Routine formalin-fixed CNS tissue samples cannot be tested by the standard DFA test. The fixation process causes chemical cross-linking of proteins. Formalin-fixed tissue samples which have been processed, embedded in paraffin, and sectioned may be tested by a formalin-fixed DFA (FF DFA). The FF DFA includes modifications of the standard DFA, such as proteinase K digestion to disassociate chemical bonds and to expose rabies virus epitopes (50). An immunohistochemical test (IHC) for rabies virus antigen detection is an alternative protocol (15). All tests for rabies virus antigen require the same thorough tissue sampling (complete cross-section of brain stem and cerebellum) as the standard DFA test.

DFA Test of Nuchal (Neck) Biopsy Specimens

Antemortem DFA tests for rabies virus antigen are performed on serial 5- to 6-μm-thick tissue cryo-sections of neck skin biopsies and provide a rapid method for viral antigen detection in nerves at the base of hair follicles. The sample may be obtained by cosmetically removing a 5- to 6-mm-diameter biopsy specimen from a shaved area just within the hairline. The biopsy sample should contain a minimum of 10 hair follicles and be of sufficient depth (i.e., full skin thickness) to contain the nerves at the end of the follicles. This is an alternate method to brain biopsies for diagnosis of rabies in antemortem samples and is less invasive.

NUCLEIC ACID DETECTION

Reverse transcription (RT)-PCR methods are the most sensitive tests for rabies diagnosis. However, the reliability of RT-PCR depends in part on the sample (type and condition), the particular method of RNA extraction and RT-PCR, the primers selected for amplification, the quality of reagents, individual technical expertise, interpretation of the results, and confirmatory methods. The usefulness of RT-PCR as a routine diagnostic test on postmortem (fresh and fixed) CNS tissues is limited. Highly sensitive, broadly reactive, less expensive, and less time-consuming procedures for antigen detection by DFA are more efficient routine tests for rabies diagnosis. RT-PCR may be a useful tool for molecular typing and in some cases to confirm a positive diagnosis in CNS tissues, but the maximum utility is the detection of nucleic acid in non-CNS samples (e.g., antemortem saliva, skin, cornea impressions, tears, eye swabs, and throat swabs or postmortem vitreous fluid) when fresh CNS tissues are unavailable (31). When all conditions are optimal, RT-PCR can detect RNA in one infectious unit of rabies virus and nested RT-PCR increases the sensitivity (10- to 100-fold) to below one infectious unit of virus (32). RNA may be limited or degraded when examining non-CNS samples, and nested RT-PCR is usually required. The diversity among rabies virus variants and lack of specific nondegenerate universal primers (Table 2) make real-time PCR less useful for detection of rabies virus RNA in non-CNS samples when the rabies virus variant is unknown.

The use of N-gene primers 21g (RT) and 21g/304 (PCR) takes advantage of the conserved sequence at the initial methionine of the N and P proteins to produce an amplicon of 1,459 bp, which includes the entire N gene and hypervariable nontranslated region between N and P (Table 2). From experience, it can be stated that primers at this location usually amplify the variants of rabies virus found in the United States. However, no single rabies virus N primer set amplifies all lyssaviruses equally well. In some cases RNA may be degraded, and amplification of the 1,459-bp amplicon may be unsuccessful. In these cases, primers which amplify shorter regions, 398 bp (1066 deg/304) and 369 bp (1087 deg/304),

TABLE 2 Examples of synthetic oligonucleotides used in sample analysis[a]

Primer ID	Sense	Primer sequence	Gene target	SAD NT position
21g	Forward	5′ ATGTAACACCTCTACAATG	N	55–73
10g	Forward	5′ CTACAATGGATGCCGAC 3′	N	66–82
1066 deg	Forward	5′ GA(AG)AGAAGATTCTTCAG(AG)GA3′	N	1136–1155
1087i deg*	Forward	5′ GAGAA(AG)GAACTTCA(AG)GAiTA3′	N	1157–1176
1087s deg	Forward	5′ GAGAA(AG)GAACTTCA(AG)GA3′	N	1157–1173
504s	Forward	5′ TCATGATGAATGGAGGT 3′	N	1296–1312
304	Reverse	5′ TTGACGAAGATCTTGCTCAT 3′	N	1514–1533

[a] The N gene primers listed are used to amplify RNAs from the rabies viruses found in the United States. Multiple primer sets or degenerate primers are necessary when RT-PCR is used as a diagnostic tool. For example, the published primer 1087i deg* is a degenerate primer which will amplify all of the lyssaviruses with the exception of RNAs of skunk rabies viruses found in California. These RNAs possess two base changes at the 3′ end primer. An alternate primer (e.g., 1087s deg or 1066 deg) is necessary to amplify this region of the rabies virus N gene.

should be used. Degenerate primers are needed to amplify less conserved regions.

Measures to avoid cross contamination include preparation of sterile tips, tubes, and reagents in a clean area which has not been used for rabies testing; careful opening and closing of tubes; centrifugation of microcentrifuge sample tubes before uncapping; the frequent changing of gloves (immediately if contaminated); and use of separate laboratory areas where RNA is extracted and RT reactions occur from cDNA (PCR products). When selecting tissue for RT-PCR, try to cut small internal pieces of tissue, as these are less likely to be contaminated on the surface.

All positive diagnostic tests need to be confirmed by sequence analysis or restriction digest analysis to avoid misinterpretation of non-rabies virus amplicons, and negative diagnostic tests need to be confirmed with additional primer sets if there is a high suspicion of rabies.

ISOLATION OF RABIES VIRUS

Isolation methods are useful in detecting infectious virus in samples and may be applied as an alternate confirmatory test to the standard DFA. The classical methods include in vivo isolation in animals (usually intracerebral inoculation of young mice) and in vitro virus isolation in cell cultures. For most routine diagnostic needs, the inoculation of cell cultures, such as mouse neuroblastoma (obtained from Biowhittaker, Walkersville, Md.) or an alternative similar cell culture line, CCL 131 (ATCC, Rockville, Md.), provides the same sensitivity as animal inoculation, but with quicker results and without the maintenance required for the use of laboratory animals. Ideally, in vivo use should be reserved for purposes of efficacy and safety studies for biologics or virulence and pathogenesis studies. Cell cultures may be useful in the propagation, amplification, and quantification of virus and antibodies, to produce vaccines, determine the safety of vaccine lots, and study the pathogenesis of rabies virus in particular cells (45, 48, 49).

TYPING SYSTEMS

Rabies viruses are separated into terrestrial carnivore and bat variants by use of antigenic and molecular methods. In the United States, there are at least five major reservoirs detectable among wild terrestrial mammals, as identified by antigenic typing (Fig. 2). Genetic typing adds resolution and identifies seven distinct virus lineages among the current terrestrial variants. Antigenic typing is less useful in certain cases involving rabid bats. Nucleotide sequence analysis adds resolution when studying these samples. Typing methods are useful in a variety of circumstances, such as determining the rabies virus variants in human cases with unclear or unknown virus exposure histories; discovering the emergence of new viruses; monitoring the epidemiologic spread or reemergence of virus in defined geographical areas; detecting host switching or spillover of variants from the predominant host species to another species; and monitoring the success of rabies vaccination programs through typing of surveillance samples.

Antigenic Typing

Antigenic typing with monoclonal antibodies (MAbs) can be performed on acetone-fixed direct brain impression slides and rabies virus-infected cell culture slides. The best results are obtained from brain impressions if 75 to 100% of the microscope fields contain viral antigen. If there is insufficient antigen present, it is best to amplify virus by inoculating cell cultures or mice. The antigenic typing test is an indirect fluorescent-antibody (IFA) test. Murine anti-rabies-N MAbs (a panel of seven is commercially available) are used to distinguish rabies virus variants by the different reaction patterns. Antigenic typing is inexpensive, rapid, and easily performed, as a relevant method to determine rabies virus variants. Limitations include the necessity for amplification when antigen amounts are inadequate and a lack of resolution for certain terrestrial and bat rabies virus variants. Additional testing of these samples can be performed at reference laboratories, such as the CDC, which have additional MAbs and resources for sequence analysis (36–38).

Nucleotide Sequence Analysis

The use of genetic typing as a method for molecular epidemiologic studies has increased because more laboratories have the ability to extract RNA, perform RT-PCR tests, and sequence viruses. Methods of sequencing cDNA products have now become routine. Of the rabies virus genes of interest, the N protein gene has been one of the most frequently utilized in molecular epidemiology, and earlier studies usually analyzed short sequences, less than 400 nucleotides (40, 41, 46). Current analyses have included complete N gene sequences (23, 28). At present, there are thousands of N gene sequences (complete and partial) in GenBank. Lyssavirus researchers are now focusing on the G, P, and L genes (3, 22, 29). These data may assist in a better understanding of specific gene functions in viral pathogenesis and replication and virion formation.

SEROLOGIC TESTS

The serologic tests for rabies antibody include neutralization and IFA tests. Each varies in sensitivity, specificity, type of antibody detected, and the viral antigen (protein) recognized (8, 9, 39, 42).

Neutralization Tests

The neutralization test is the standard test for antibodies to the rabies G protein. The rapid fluorescent focus inhibition test (RFFIT), fluorescent-antibody virus neutralization test (FAVN), and mouse neutralization are highly specific tests which measure the ability of rabies virus antibodies in serum or cerebrospinal fluid (CSF) samples to neutralize a known standard challenge virus dose. The RFFIT and FAVN tests are comparable and are most frequently used today to determine the immunization status of vaccinated humans and animals, respectively. The FAVN, a modification of the RFFIT microneutralization test, is performed in microtiter plates instead of chamber slides. The results of RFFIT and FAVN are equivalent when converted to international units per milliliter, and the two tests exhibit the same sensitivity and specificity in determining rabies virus neutralizing antibodies (4, 8, 42).

IFA Test

IFA tests are sensitive methods for detection of specific rabies virus immunoglobulin M (IgM) and IgG antibodies in human serum and CSF for antemortem diagnosis. Unlike the NTs, the IFA detects antibodies to rabies proteins other than G, and predominately to the RNP. Serum or CSF samples are titrated and added to acetone-fixed cell culture slides infected with rabies virus (CVS-11). The endpoint antibody titer is the last dilution demonstrating specific fluorescence. The IFA titers cannot be interpreted as neutralizing antibody levels since the test is not G protein specific.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Since the development of the first rabies vaccine, accurate and timely diagnosis of rabies infections in animals has been essential for decisions regarding postexposure prophylaxis of humans. Of all the tests for rabies diagnosis, the DFA test performed on postmortem brain tissues is the standard by which all other rabies diagnostic procedures are evaluated. Misinterpretation of laboratory results may have a fatal outcome or cause unneeded and costly postexposure prophylaxis. Establishment of a standard DFA protocol addresses the common problems related to diagnosis by establishing requirements for sample tissues, sample integrity, evaluation of reagents, control samples, use of at least two rabies conjugates, equipment standards, evaluation of tests by two individuals, reporting results, confirmatory testing, specificity control reagents, and reference laboratory submissions. All rabies laboratory diagnosticians should provide reliable testing, be familiar with methods to avoid problem tests, and be aware of the resources available, such as the assistance of reference laboratories. Specific details for the interpretation and reporting of DFA test results, the availability of alternative tests, and related diagnostic methods are described in the standard protocol.

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Hendra and Nipah Viruses

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In September 1994, a respiratory disease was responsible for the sickness and death of several thoroughbred horses in Hendra, near Brisbane (Queensland, Australia) (30, 31). Two persons involved in the care and training of the horses developed a severe flu-like sickness within days after exposure. Both were hospitalized and one died. The causal virus, previously undescribed, was initially named equine morbillivirus but was subsequently renamed Hendra virus. In October 1995, a second outbreak near Mackay (Central Queensland, Australia) was retrospectively recognized when a horse owner died of a relapsing encephalitic disease. His probable infection occurred in August 1994 when two horses died after exhibiting severe respiratory distress and neurological symptoms. Since no known link could be traced between the two outbreaks, extensive surveys in domestic and wildlife animals were initiated around the two foci. In April 1996, anti-Hendra antibodies were detected in flying foxes (frugivorous bats, genus *Pteropus*) in Queensland. In September 1996, a virus identical to that found in the infected horses and humans was isolated from the reproductive tract of a gray-headed flying fox (*Pteropus poliocephalus*). Extensive serologic surveillance among flying foxes in Queensland as well as other areas within the geographic range of these bats demonstrated a very high prevalence of antibodies against Hendra virus. In January 1999, a fatal case of Hendra virus infection was reported in a horse near Cairns in northern Queensland. No secondary cases occurred in companion horses or in humans.

In September 1998, a highly contagious respiratory disease in pigs, with a low mortality, emerged in the northern part of Peninsular Malaysia. In the early months of 1999, an outbreak of severe febrile encephalitis in humans with a high mortality rate was recognized and Nipah virus, a previously unrecognized virus, was identified as the causal agent (7). The majority of affected humans had a history of contact with pigs, most of them being pig farmers. The outbreak subsequently spread to various states of the country and Singapore due to the movement of infected pigs. A total of 265 people were infected, of whom 105 died. The associated outbreak among pig abattoir workers in Singapore during March 1999 led to 22 cases, with one death. These workers had been handling pigs that had been imported from the outbreak areas in Malaysia. Antibodies were found in flying foxes in Malaysia (25). Nipah and Hendra viruses share ultrastructural, antigenic, and molecular characteristics.

Bangladesh was the site of successive outbreaks of Nipah encephalitis in 2001, 2003, and 2004 (1, 23, 50). Human encephalitis cases in Siliguri, India, in 2001 were retrospectively diagnosed as Nipah virus infections (4). No studies were conducted to identify potential reservoirs of infection in India; however, *Pteropus* fruit bats were found with Nipah virus antibodies in the outbreak areas of Bangladesh in 2004.

TAXONOMY AND DESCRIPTION OF THE AGENTS

Members of the family *Paramyxoviridae* are nonsegmented, negative-stranded RNA viruses composed of helical nucleocapsids enclosed within an envelope to form roughly spherical, pleomorphic virus particles (28). Within the family *Paramyxoviridae*, there are two subfamilies, the *Paramyxovirinae* and the *Pneumovirinae*. The subfamily *Paramyxovirinae* has been divided into five genera: *Rubulavirus* (prototype, mumps virus), *Respirovirus* (prototype, human parainfluenza virus 1), *Avulavirus* (prototype, Newcastle Disease Virus), *Morbillivirus* (prototype, measles virus), and the fifth genus, *Henipavirus*, which has been added recently, with Hendra virus as the prototype virus and Nipah virus as the second member. The complete sequences of Hendra and Nipah viruses are available from GenBank. The structure of the genome is consistent with those of the other members of the subfamily. The six transcription units encode six structural proteins: nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), glycoprotein (G), and large protein (L) or RNA polymerase, in the order 3′-N-P-M-F-G-L-5′ (31). The Nipah virus genome is 18,246 nucleotides in length, whereas that of Hendra virus is 18,234 nucleotides.

EPIDEMIOLOGY AND TRANSMISSION

The geographic distribution of Hendra and Nipah viruses should overlap with the distribution of the bat reservoirs, the flying foxes (genus *Pteropus*). About 60 species of bats are described in that genus, which inhabit an area extending from Madagascar and Mauritius to Australia. Many species are restricted to islands, but others are widespread in Australia and Southeast Asia. At this time, antibodies have been found in only a few species. Hendra antibodies were identified in black flying foxes (*P. alecto*), gray-headed flying foxes (*P. poliocephalus*), little red flying foxes (*P. scapulatus*), and spectacled

flying foxes *(P. conspicillatus)*. Hendra virus has been isolated from gray-headed flying foxes *(P. poliocephalus)* and from one black flying fox *(P. alecto)* (20). Nipah serological evidence has been found in *P. hypomelanus* and *P. vampyrus* in Malaysia (25); *P. lylei* in Cambodia (32); *P. hypomelanus, P. lylei, P. vampyrus*, and *Hipposideros larvatus* in Thailand (43); and *P. giganteus* in Bangladesh. Nipah virus has been isolated from a *P. lylei* urine specimen in Cambodia (37).

Several aspects of the epidemiological features of both viruses are not fully understood. The densities of bat populations and the prevalence of Hendra antibody positives among them can be very high in certain areas, but the frequency of transmission to human or susceptible intermediate animal hosts (horse or other mammals) seems to be rare. The virus has been circulating among bats since at least 1982 (14). Only a few bat isolates of Hendra virus are available despite numerous attempts at isolation. In bats, urine is the most likely route of excretion, and it is the source of infection of susceptible animals such as pigs or horses. Under laboratory conditions, the transmissibility of Hendra virus was very low. There was no transmission from bats to horses, horses to horses, or horses to cats, and only one case of transmission from infected cats to one horse was observed (46). Transmission and maintenance of Nipah virus among pigs, mostly via the respiratory route, were very efficient in Malaysia, as demonstrated by the epidemic spread within farms; farmers were mostly responsible for the transmission between farms by moving infected animals within states in Malaysia and to Singapore. Only a very severe culling strategy was effective in stopping the epidemic (15, 27). No nosocomial cases were described during the large Nipah outbreak in Malaysia. As for the Hendra virus, it was present earlier in Malaysia (retrospective diagnosis on pig disease in 1996). For both viruses, the amplification through a secondary host (horse or pig) was necessary for the infection of humans. Ecological and economical factors are certainly important in this sequential process.

CLINICAL SIGNIFICANCE

Only three persons are known to have been infected by Hendra virus, and two of them died (34, 35, 38). In all three cases, the disease started with a nonspecific flu-like syndrome including fever, myalgia, headache, lethargy, sore throat, nausea, and vomiting. Despite supportive treatment, the first patient developed respiratory and renal failure and died. The second patient recovered without sequela. The third patient recovered fully from this initial stage but a year later developed neurological signs with seizure and loss of consciousness and died 25 days after admission. The pathogenesis of this last patient's illness could be similar to that of a persistent measles virus infection, subacute sclerosing panencephalitis.

During the Malaysian epidemic, Nipah virus was identified as the cause of the encephalitis outbreak among pig farmers in Malaysia (5, 7, 16, 39). The incubation period ranged from 2 days to 1 month, but in most of the cases it lasted between 1 and 2 weeks. Prodromal signs and symptoms are nonspecific, with fever, headache, myalgia, and sore throat followed by drowsiness, confusion, and reduced level of consciousness after a few days (Table 1). Neurological symptoms usually appeared within a week after onset. The most common neurological signs were coma, hyporeflexia or areflexia, segmental myoclonus, gaze palsy, and limb weakness. Notable laboratory findings include thrombocytopenia, elevated alanine aminotransferase and aspartate aminotransferase, and abnormal cerebrospinal fluid (elevated protein and white-cell count). Systolic hypertension, tachycardia, high fever, and severe brain stem involvement were associated with poor outcome. Mortality occurred in 30 to 40% of the patients and was more frequent in patients with rapidly developing severe neurological signs (39). Residual neurological signs were common among the survivors.

Treatment is supportive including mechanical ventilatory support for patients in a deep coma and unable to maintain airways. Ribavirin has been used in humans during the Nipah outbreak in Malaysia with equivocal results: one study suggests that ribavirin was able to reduce the mortality of acute Nipah encephalitis (6); another saw no significant difference in outcome (16). Ribavirin showed no efficacy in in vitro tests and in a guinea pig model of the disease (P. E. Rollin, unpublished data).

There are no vaccines currently available for either Hendra virus or Nipah virus.

In horses, Hendra virus is responsible for a severe pulmonary syndrome with encephalitis in some animals (22, 44, 46).

TABLE 1 Signs and symptoms of patients with confirmed Nipah or Hendra infection

Signs and symptoms	No. of patients (%) exhibiting indicated symptom				
	Nipah virus infection			Hendra virus infection	
	Goh et al., 2000 (n = 94)	Sim et al., 2002 (n = 18)	Chong et al., 2000 (n = 103)	O'Sullivan et al., 1997 (n = 1)	Selvey et al., 1995 (n = 2)
Fever	91 (97%)	17 (94%)	100 (97%)	1	2
Headache	61 (65%)	12 (67%)	90 (88%)	1	2
Dizziness	34 (3%)	8 (44%)	40 (39%)	1	2
Vomiting	25 (27%)	7 (39%)	37 (36%)	1	1
Reduced (or loss of) consciousness	20 (21%)			1	
Sore throat			21 (21%)	1	
Diarrhea		1 (5%)	21 (21%)		
Nonproductive cough	13 (14%)	3 (17%)			
Myalgia	11 (12%)	4 (22%)	48 (47%)		
Neurological signs	10 (11%)	16 (89%)	5 (5%)	1	
Mortality	30 (32%)	11 (61%)	42 (41%)	1	1
Residual neurologic deficits	14 (15%)	6 (33%)	19 (19%)		0

Experimentally infected cats develop an acute febrile disease with respiratory signs similar to those observed in horses (45). A guinea pig model of infection has been described (47). In experimental infections, gray-headed fruit bats *(Pteropus poliocephalus)* seroconverted without clinical disease (46, 47).

Nipah virus is pathogenic for several animals. In pigs, as shown during the Malaysian outbreak and in experimental infections, the virus induces a respiratory and, less commonly, a neurological syndrome. Some animals remain asymptomatic, but in these animals, as in the one showing clinical signs, virus can be found in the respiratory secretions. Nipah virus is easily transmitted to contact animals (29). Respiratory and neurological disease was also produced in cats. Nipah virus can be recovered from urine and oropharyngeal secretions (29). During the Malaysian outbreak, fatal infections were described in dogs and a Nipah serological survey found that several dogs in an area adjacent to infected pig farms had antibody (2). Models of Nipah disease have been described in hamsters (48) and guinea pigs (Rollin, unpublished).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Hendra and Nipah viruses are classified as biosafety level 4 (BSL-4) agents in the United States and Australia, as there is a risk of infection of laboratory personnel and appropriate precautions must be taken (3). They are also in the United States classified as Select Agents by both the U.S. Department of Agriculture and the U.S. Department of Health and Human Services and should be handled as such (see chapter 9; http://www.selectagents.gov/forms Overview.htm).

For virus isolation, serum, heparinized plasma, whole blood, or cerebrospinal fluid (CSF) should be collected during the acute, febrile stages of illness and frozen on dry ice or in liquid nitrogen. Nasal or oral swabs and bronchial wash and urine specimens could also be collected and mixed with an equal volume of buffered diluents containing serum proteins to stabilize viral infectivity prior to freezing. In fatal forms, Nipah virus could easily be isolated from different parts of the central nervous system (CNS). Specimens collected for viral isolation are also suitable for testing by reverse transcriptase PCR (RT-PCR) and TaqMan; formalin-fixed tissues and paraffin-embedded blocks are also suitable for immunohistochemical identification of viral antigens and should be conserved and shipped at room temperature. Maintenance of samples at −20°C or below is sufficient to preserve antibody, but lower temperatures are required to preserve infectivity.

Manipulation of these specimens and tissues, including sera obtained from convalescent patients, may pose a serious biohazard and should be minimized. Although no nosocomial transmissions were described in Malaysia, proper barrier nursing procedures should be implemented and personnel caring for the patient and handling diagnostic specimens should wear disposable caps, gowns, shoe covers, surgical gloves, and face masks (N99 or N100 [HEPA] filters). Procedures generating aerosols (e.g., centrifugation) should be minimized and performed only if additional protective equipment, such as a flexible plastic film isolator capable of maintaining negative pressure and HEPA-filtered exhaust, is available. For specialized procedures, the infectivity of samples may be greatly reduced, if not totally inactivated, by the addition of Triton X-100 and heating, and the samples may be safely tested by serologic or antigen capture assays in the field. Heating to 60°C for 1 h renders diagnostic specimens noninfectious and is acceptable for measurement of heat-stable substances such as electrolytes, blood urea nitrogen, and creatinine. When the equipment is available, sterilization by ^{60}Co γ-irradiation is the preferred method. Extraction of RNA from infectious samples by using the guanidinium acid-phenol method should be conducted in a laminar-flow safety cabinet, but the extracted RNA is no longer infectious and can be manipulated at BSL-2.

For all testing of infectious material, samples should be packaged in accordance with current recommendations (International Air Transport Association [IATA]; http://www.iata.org/index.htm) and forwarded, after consultation, to one of the following laboratories, which maintain BSL-4 facilities and a diagnostic capability for these agents:

1. Centers for Disease Control and Prevention, Special Pathogens Branch, Division of Viral and Rickettsial Diseases, Center for Infectious Diseases, Atlanta, GA 30333. Phone: (404) 639-1115. Fax: (404) 639-1118.
2. AAHL Geelong, Chief, Diagnostic Sciences, 5 Portarlington Road, East Geelong, Private Bag 24 Geelong, Victoria 3220, Australia. Phone: +61 (3) 5227 5115. Fax: 61 (3) 5227 5555.

DIRECT DETECTION

Electron Microscopy

Electron microscopy (EM) examination (thin-section and negative-stain preparations) shows extracellular virions appearing as tangled collections of filamentous, helical nucleocapsids surrounded by the viral envelope. Particles were pleomorphic and varied greatly in size, averaging about 500 nm in diameter (range, 180 to 1,900 nm). Negative-stain EM revealed nucleocapsids with the typical herringbone appearance that is characteristic for paramyxoviruses. These measured an average of 21 nm in diameter, with a 5-nm periodicity, and up to 1.67 μm in length. In thin-section preparations, nucleocapsids averaged 18 nm in diameter, and spikes along the viral envelope, when seen, measured 12 nm in length (17) (Fig. 1).

EM examination of CNS specimens, collected from fatal cases of Nipah infection, showed typical *Paramyxoviridae* inclusions consisting of smooth, filamentous nucleocapsids associated with dense amorphous material. These inclusions were seen mostly in neurons and occasionally within endothelial cells. In addition, unusual cytoplasmic inclusions composed of aggregates of smooth curvilinear membranes were found in neurons (49).

Pathology and Immunohistopathology

During the Malaysian Nipah outbreak, tissues were collected in several human fatal cases. Histopathologic findings included a systemic vasculitis with extensive thrombosis and adjacent parenchymal necrosis, particularly in the CNS. Endothelial cell damage, necrosis, and syncytial giant cell formation were observed in affected vessels. Intracytoplasmic eosinophilic inclusions can be seen on hematoxylin and eosin staining in neurons and other cell types (Fig. 2). The widespread presence of Nipah virus antigens was seen by immunohistochemistry (IHC) in endothelial and smooth-muscle cells of blood vessels and in various parenchymal cells, particularly in neurons (Fig. 3). Plaques with various degrees of necrosis were found in both the gray and white matter. In non-CNS organs, multinucleated giant cells with intranuclear inclusions were occasionally noted in lung, spleen, lymph nodes, and kidneys. Infection of endothelial cells and neurons as

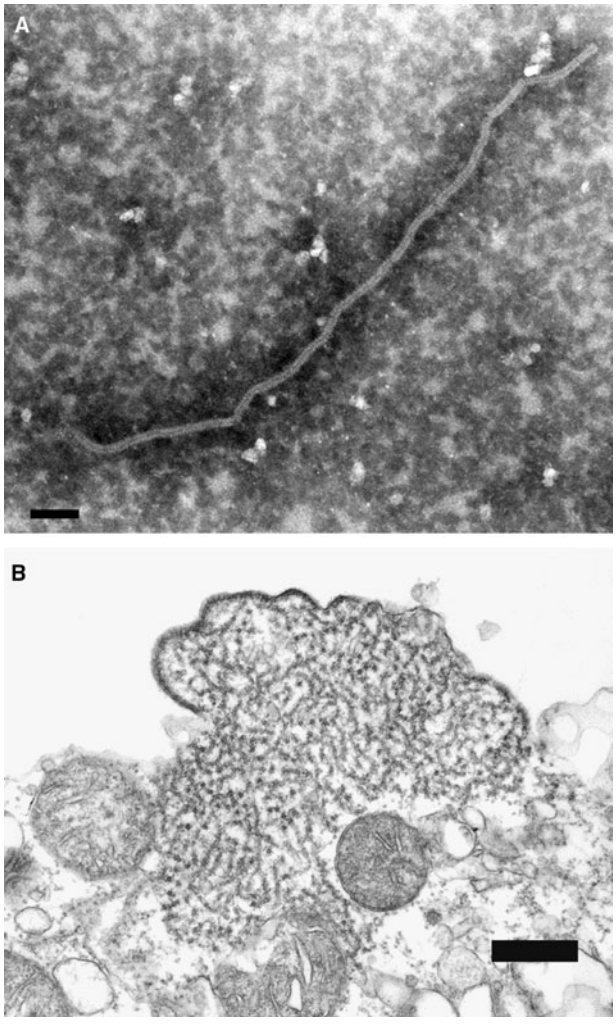


FIGURE 1 Ultrastructural characteristics of Nipah virus isolate in cell culture as seen by negative-stain (A) and thin-section (B) EM (courtesy of C. S. Goldsmith). (A) A single nucleocapsid shows the typical herringbone appearance characteristic of the family *Paramyxoviridae;* (B) Nipah virus nucleocapsids become tightly aligned along the plasma membrane as a particle prepares to bud. Scale bar, 100 nm.

well as vasculitis and thrombosis appears to be critical to the pathogenesis of this disease (24, 49).

The IHC technique is adapted from previously published hantavirus or filovirus detection (51). Briefly, 4-μm sections were predigested by proteinase K (Boehringer-Mannheim Corporation, Indianapolis, Ind.) for 15 min at room temperature and blocked with normal serum. Primary antibodies were applied for 1 h at room temperature. For anti-Hendra and anti-Nipah antibodies, the dilutions of the hyperimmune mouse ascitic fluids were 1:4,000 and 1:2,000, respectively. This step was followed by sequential application of biotinylated link antibody, alkaline phosphatase-conjugated streptavidin, and naphthol fast red according to the manufacturer's protocol (LSAB2 Universal Alkaline Phosphatase Kit; Dako Corporation, Carpinteria, Calif.). Sections were then counterstained in Meyer's hematoxylin (Fisher Scientific, Pittsburgh, Pa.).

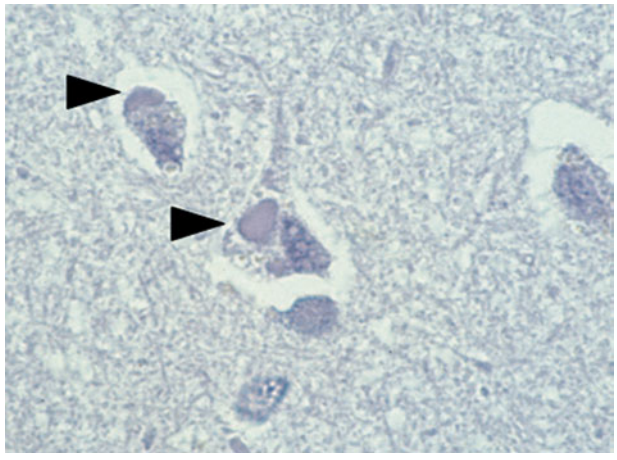


FIGURE 2 Typical eosinophilic viral inclusions (black arrows) in the cytoplasm of neurons (hematoxylin and eosin stain; original magnification, ×250).

Molecular Detection

During outbreaks, RT-PCR assays have been used successfully to detect RNA from Nipah and Hendra viruses from a variety of clinical samples including CSF, throat swabs, and urine samples (4, 7, 21). RNA can be extracted from samples by using the guanidium acid-phenol method or by using a standard commercial RNA extraction kit. RT-PCR is performed using standard conditions, with one-step RT-PCRs being preferred. PCR products are detected by agarose gel electrophoresis. The primers targeting 159- to 320-nucleotide regions of the N and M genes have given the best results with diagnostic specimens thus far (4, 21). Primer sequences can be designed to detect both Hendra and Nipah viruses or to detect each virus separately. For recent outbreak investigations, two primer pairs consisting of NVNF-4 (5′GGAGTTATCAATCTAAGTTAG′3) and NVNBR4 (5′CATAGAGATGAGTGTAAAAGC3′), which amplify a 159-nucleotide region of the N gene, and NVBMFC1 (5′CAATGGAGCCAGACATCAAGAG3′) and NVBMFR2 (5′CGGAGAGTAGGAGTTCTAGAAG3′), which amplify a 320-nucleotide region of the M gene, were used. Sequence analysis of the PCR products should be used to confirm the positive reactions, and sequence analysis allows differentiation between Hendra and Nipah viruses. In addition, phylogenetic analysis of the sequences can be used to study the evolution and transmission pathways of the viruses (4, 21).

Compared to standard RT-PCR techniques, real-time RT-PCR assays have greater sensitivity and higher throughput and are less labor-intensive and faster to perform. In addition, the TAQMAN format provides increased specificity because the identity of the amplified product is confirmed by hybridization to a target-specific, fluorogenic probe. Real-time RT-PCR assays have been developed to detect RNA from Hendra and Nipah viruses (19, 40), targeting sequences in the nucleoprotein and phosphoprotein genes. The lower limit of detection of Nipah RNA ranged from 10 (K. Hummel and P. Rota, unpublished data) to 1,000 copies of RNA (19) with excellent specificity. In addition to their critical role in diagnosis, real-time RT-PCR assays will facilitate research into the pathogenesis of Hendra and Nipah

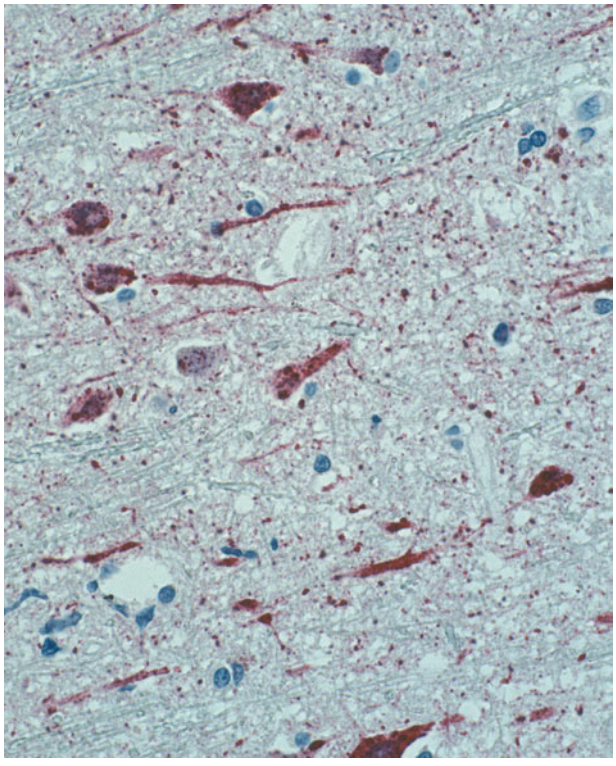


FIGURE 3 Nipah virus encephalitis: viral antigen present among numerous neurons. Note the abundance of extracellular antigen (immunoalkaline phosphatase with naphthol fast red substrate and light hematoxylin counterstain; original magnification, ×158).

viruses. For example, a real-time assay was used to determine viral loads in serum samples from experimentally infected hamsters (18).

ISOLATION PROCEDURES

Hendra and Nipah viruses are classified as BSL-4 agents in the United States and Australia, as there is a risk of infection of laboratory personnel and appropriate precautions must be taken (3). Virus isolation should be attempted only when the required laboratory containment is available.

African green monkey kidney (Vero) cells inoculated with high-titer samples contain antigen within a few days of inoculation. Cytopathic effect (CPE) is usually seen within 3 to 5 days. The CPE is noticeable by the formation of syncytia containing several nuclei. If no CPE is detected by 5 to 7 days, the sample is considered negative, but supernatant fluids should be blind passaged to confirm the absence of virus. CPE observed with Nipah virus is usually observed faster than with Hendra virus. To confirm the presence and identity of the virus, fixed cells are tested by immunofluorescence assay (see below) or supernatant fluids can be tested for evidence of viral replication by RT-PCR techniques (see above). Hendra or Nipah viruses can be reliably distinguished from each other only by neutralization tests. Other cells such as rabbit kidney (RK-13) are susceptible to Hendra and Nipah viruses. Suckling mice were susceptible by intracutaneous or intraperitoneal inoculations and died, but adult mice survived (44).

SEROLOGIC TESTS

Immunofluorescent Antibody Assay

Detection of antibodies by immunofluorescence is possible but is not routinely used by any of the reference laboratories. The preparation of spot slides with infected Vero cells would be identical to the procedure described for other viruses (26) and requires a BSL-4 facility for production.

ELISA

Enzyme-linked immunosorbent assay (ELISA) procedures for Hendra and Nipah virus-specific immunoglobulin G (IgG) and IgM have been developed and successfully used. The viruses are closely related antigenically, so Hendra or Nipah antigens could be used interchangeably in the ELISAs. Antigens are irradiated with 50 kGy prior to use under BSL-2 conditions.

IgM Detection

A Nipah or Hendra-Vero E6 cell slurry antigen was prepared in the BSL-4 laboratory and gamma-irradiated with 5×10^6 rads (50,000 Gy). A mock-infected Vero E6 control antigen was similarly prepared.

Microtiter plates were coated with goat anti-Mu chain antibody (Biosource International, Camarillo, Calif.) in coating buffer at a dilution of 1:500, held overnight at 4°C, and then washed. Fourfold serial dilutions of test sera (1:100 to 1:6,400) or CSF (1:20 to 1:1,280) in diluent and of a sample of negative and positive controls (to validate the cutoff values for the assays) were added, and the plates were incubated and washed. The Hendra or Nipah-Vero E6 cell slurry and mock-infected control were added to the top and bottom halves of the plate, respectively, at a dilution of 1:20. After incubation and washing, Nipah or Hendra hyperimmune mouse ascitic fluid was added at a dilution of 1:2,000 and the plates were again incubated and washed. Horseradish peroxidase-labeled anti-mouse IgG antibody (Biosource International) was added at a dilution of 1:2,000 before once more incubating and washing. The addition of substrate and the final incubation and wash steps were identical to those described above. A specimen is considered positive for the IgM assay if the sum of the adjusted optical densities from all of the dilutions (infected antigen less the mock-infected antigen) is >0.75 through the entire dilution series and the titer is ≥1:400 (≥1:80 for CSF).

IgG Detection

A Hendra or Nipah-Vero E6 cell lysate antigen and a mock-infected Vero E6 control antigen cell lysate were prepared under BSL-4 conditions and then gamma-irradiated as described above. Microtiter plates were again divided into positive and negative halves and coated with the respective antigens, both at a dilution of 1:1,000 as determined by checkerboard titration. Plates were kept overnight at 4°C and washed to remove unbound antigen, and fourfold serial dilutions of test sera (1:100 to 1:6,400) or CSF (1:20 to 1:1,280) were added. A sample of negative and positive controls was used to validate the cutoff values for the assays. After incubation and washing, horseradish peroxidase-labeled mouse gamma-chain-specific anti-human IgG antibody (Accurate Chemical, Grand Island, N.Y.) was added at a dilution of 1:10,000. The addition of substrate, final incubation, and wash steps were performed as described above. A specimen was considered positive in the IgG assay if the sum for the adjusted optical densities from all of the dilutions (infected antigen less the mock-infected antigen) was >0.90 through

the entire dilution series and if the titer was ≥1:400 (≥1:80 for CSF).

Nipah virus antigens have been expressed by recombinant DNA technology. These antigens, which are produced at BSL-2, have the potential to be used to configure serodiagnostic tests. So far, the extracellular domain of the G protein and the N protein virus have been expressed from recombinant baculoviruses and *Escherichia coli* (11, 12, 13, 33, 42) and have produced promising results in initial tests.

Virus Neutralization

Virus neutralization assays are the reference standard for confirmation of results obtained from ELISAs. In addition, because of antigenic differences in the G and F proteins of the viruses, neutralization is the only serologic test that can distinguish between Hendra and Nipah virus infections. The procedure involves handling infectious Nipah or Hendra viruses in a BSL-4 laboratory. Several techniques are available.

In the 50% tissue culture infective dose ($TCID_{50}$) assay, a constant predetermined dose (between 30 and 300 $TCID_{50}$ of Nipah or Hendra viruses) is allowed to react (60 min at 37°C) with varying dilutions of heat-inactivated serum to be tested (and negative and positive controls). This is then mixed with susceptible Vero cells and incubated at 37°C 5% CO_2 for 6 days. The cells are then observed for signs of CPE. The CPE in the wells can be scored by visual inspection after microscopic observation or by staining the monolayer with crystal violet. At 6 days postinfection, 10% NB formalin is added to the well and then crystal violet stain is added, allowed to absorb, and rinsed with distilled water. If the staining is to be done outside a BSL-4 facility, the plates are fixed with formalin, double bagged for removal from BSL-4, and irradiated with 2×10^6 rads before staining.

The virus control (back titration) of the test dose should give confirmation that the input dose of virus was between 30 and 300 $TCID_{50}$.

Neutralization titers of test samples are reported as the highest dilution in which cells are protected from CPE (the last well in which at least one-half of the monolayer was protected and stains purple).

A rapid immune plaque assay is also available. The technique allows the test to be performed in less time (9).

Western Blotting and Immunoprecipitation

In some cases, Western blot assays and immunoprecipitation assays have been used to confirm serologic results from ELISAs. Both Western blotting and immunoprecipitation can confirm the presence of IgG antibodies to individual structural proteins of Nipah or Hendra virus. Western blot assays are conducted by using standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%) and blotting techniques. No infectious Nipah or Hendra virus is required for the assays because baculovirus-expressed Nipah or Hendra virus nucleoprotein or inactivated native proteins are available for use as the test antigen and lysates of uninfected insect cells are used for the control antigen. The nucleoprotein antigens can be detected with a monoclonal antibody in the positive control reactions (P. A. Rota and B. H. Harcourt, unpublished data). To prepare radiolabeled antigen preparation for immunoprecipitation experiments, cells are infected with recombinant vaccinia viruses expressing the G, F, and N of Nipah or Hendra virus. Radiolabeling and immunoprecipitation are performed using standard conditions as previously described (41). Hyperimmune mouse ascites fluid, which recognizes all of the structural proteins of Nipah or Hendra viruses, is used as the positive control. Though this immunoprecipitation assay has been used to detect antibodies to Nipah virus in human serum samples, some samples may contain a high background of reactivity with vaccinia antigens if the patient has received smallpox vaccination.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

As for any infectious disease, the diagnosis should be based on a combination of clinical, epidemiological, and laboratory data. Several reviews have been published on the laboratory diagnosis of Nipah and Henda virus infections in humans and animals (10, 15). The proper timing and the type of specimens collected are essential. Virus can be isolated from throat or nasal swabs, tracheal secretions, CSF, and urine at the early stage of the disease, and the excretion rate decreases in the late phase when anti-Nipah antibodies are present (8, 16). Isolation attempts could also be attempted from brain tissues collected at autopsy. Ideally any virus isolation attempt should be performed in a BSL-4 facility. In the absence of BSL-4 conditions, primary virus isolation from suspect samples could be conducted under strict BSL-3 conditions. Cell culture and the manipulation of potentially infectious specimens should be stopped with the appearance of a CPE with syncytia. Fixed cells should be stained with proper reagents to confirm the diagnosis; in case of positive results, the specimen should be transferred to an appropriate laboratory.

Molecular diagnostic methods using standard or real-time RT-PCR techniques can be used on oropharyngeal swabs or secretions, CSF, CNS or other tissues, or viral isolates in cell culture. The specimens are inactivated by adding the lysis buffer used for the first step of the RNA extraction (usually containing 4 M guanidinium isothiocyanate), and the remainder of the RNA extraction and PCRs can be performed in any BSL-2 laboratory that is equipped for these techniques. Molecular techniques can be used to confirm Nipah or Hendra virus infection in a patient or to confirm isolation in cell culture. Positive PCRs should always be confirmed by amplification of more than one target sequence and by sequencing the PCR products. This sequence information clearly identifies the virus as being either Hendra or Nipah virus and may allow use of molecular epidemiologic techniques to study viral transmission pathways between and during outbreaks. However, the sensitivity of RT-PCR for identifying cases of Hendra or Nipah will, most likely, be lower than the sensitivity of serologic testing; the timing and the type of specimen obtained and the condition and handling of the specimen will all have an effect on the quantity and quality of detectable RNA in the sample.

The ELISA IgM test has been the most important technique for serological confirmation in acute-phase patients. The rate of IgM positivity in the serum increases from 50% on the first day of illness to nearly 100% 1 week after onset. For Nipah patients, 88% of the first samples tested were positive for IgM (36). Fewer patients have IgM in the CSF at onset, but nearly all are positive by days 10 to 15. Very few patients have IgG detectable by ELISA either in the serum or in the CSF at onset. All of them have IgG in the serum by day 17 or 18. There was no difference in the prevalence of antibody between encephalitic and nonencephalitic Nipah infection for IgM and IgG.

As mentioned before, neutralization tests should be used to determine the specificity (between Nipah and Hendra) of the antibodies detected by ELISA, particularly if no virus, or virus RNA, is available to facilitate molecular typing.

In some patients, the neurological deterioration may occur fast, and the only specimens available for testing are tissues collected at necropsy; or patients may die before the appearance of detectable antibodies. In such cases, the histopathological findings and specific IHC staining are very important for confirming the diagnosis and also help to understand the pathogenesis of the disease.

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the funding agency.

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Arboviruses*

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TAXONOMY

The term "arbovirus," a shortened form of "arthropod-borne virus," is used to describe a taxonomically diverse group of viruses that share the common feature of being biologically transmitted to vertebrate hosts by the bite of arthropod vectors (mosquitoes, sand flies, ticks, midges, etc.). This ecological grouping includes more than 500 viruses belonging to at least seven families, including the *Togaviridae, Flaviviridae, Bunyaviridae, Reoviridae, Rhabdoviridae, Orthomyxoviridae,* and *Asfarviridae* (16, 28). Over 100 of these agents have been associated with naturally acquired disease in humans and/or domestic animals. The *International Catalogue of Arboviruses* (28) also includes a number of other zoonotic viruses (i.e., hantaviruses, arenaviruses, and filoviruses) that are transmitted from animals to humans without the agency of a vector; these agents are not true arboviruses and are not included in this chapter.

DESCRIPTION OF THE AGENTS

All of the arboviruses affecting humans are RNA viruses (Table 1). Complete or partial genome nucleotide sequences have been determined for many of the medically important arboviruses, facilitating the use of nucleic acid detection techniques and the production of synthetic antigens for diagnosis and as candidate vaccines. Important antigenic determinants have been elucidated for the most important arboviruses, and virus- and group-specific monoclonal antibodies are available for taxonomic and diagnostic purposes.

Most arboviruses elaborate hemagglutinins that aggregate goose, chick, and/or human group O erythrocytes. Hemagglutination (HA) occurs optimally within a narrow and specific pH range, a property that may aid in viral identification. In general, serologic cross-relationships are most evident in hemagglutination inhibition (HI) and binding assays, e.g., enzyme-linked immunosorbent assays (ELISAs) and immunofluorescent antibody (IFA) tests, and occur to a lesser extent in complement fixation (CF) tests. These broad antigenic relationships can be refined in virus neutralization tests that differentiate individual viruses and even more specific subtype relationships through ratios of homologous and heterologous neutralization antibody titers. Genomic sequence divergence among strains of a single virus in separate geographic locations differentiates viral topotypes, which apparently result from the isolation and evolutionary divergence of these strains in distinct ecologic niches. Topotypic differences among populations of Japanese encephalitis (JE), tick-borne encephalitis (TBE), yellow fever (YF), dengue (DEN), West Nile (WN), eastern equine encephalitis (EEE), Venezuelan equine encephalitis (VEE), snowshoe hare, and LaCrosse (LAC, a serotype of California encephalitis virus) viruses, among others, have been described. Such genetic changes have been linked to differences in clinical expression of EEE, Sindbis (SIN), VEE, and St. Louis encephalitis (SLE) virus infections.

EPIDEMIOLOGY AND TRANSMISSION

The epidemiologic and clinical characteristics of the medically important arboviruses are summarized in Table 2. More comprehensive descriptions can be found elsewhere (17, 42, 52, 69). Current information on arboviral outbreaks and transmission patterns can be obtained from state health departments, from the World Health Organization (WHO) at http://www.who.org, and from the Centers for Disease Control and Prevention (CDC) at http://www.cdc.gov.

CLINICAL SIGNIFICANCE

The majority of arboviral infections in humans result in simple febrile illnesses that usually cannot be distinguished clinically from other common viral or bacterial infections. Clinical features that characterize acute arboviral fevers usually include a sudden onset of debilitating symptoms, such as malaise, extreme headache, myalgia, lumbar pain, and sometimes nausea, vomiting, and dizziness. In general, these illnesses are of relatively short duration (1 to 6 days) and resolve without sequelae, except for weakness and lethargy, which can persist for a week or more. In a few arboviral illnesses (e.g., Colorado tick fever [CTF] and Oropouche fever), a recrudescent course of fever and systemic symptoms can occur 10 to 14 days after the initial symptoms occur. In cases of Kyasanur Forest disease and Rift Valley fever (RVF), delayed syndromes of encephalitis or retinitis may follow weeks after the initial illness. Some important arboviruses, such as DEN, Ross River (RR), and WN viruses, also may produce a rash, but these

* This chapter contains information presented in chapter 103 by Theodore F. Tsai and Laura J. Chandler in the eighth edition of this Manual.

TABLE 1 Charactertistics of arboviruses

Family	Representative arboviruses affecting humans	Genome (no. of segments)	Vector(s)
Togaviridae	EEE, WEE, and VEE viruses	Positive, single-stranded RNA (1)	Mosquitoes, ticks
Flaviviridae	WN, SLE, DEN, YF, POW, and JE viruses	Positive, single-stranded RNA (1)	Mosquitoes, ticks
Bunyaviridae	LAC, Congo-Crimean hemorrhagic fever, and sandfly fever viruses	Negative- and ambisense single-stranded RNA (3)	Mosquitoes, ticks
Reoviridae	Colorado tick fever virus	Double-stranded RNA (10)	Mosquitoes, ticks
Rhabdoviridae	Vesicular stomatitis virus	Negative, single-stranded RNA (1)	Sand flies
Orthomyxoviridae	Thogoto virus	Negative, single-stranded RNA (6 or 7)	Ticks
Asfarviridae	None	Double-stranded DNA	Ticks

exanthems are not distinctive; in fact, even when outbreaks occur in epidemic proportions, diseases such as dengue fever are sometimes incorrectly diagnosed as rubella or measles. Likewise, diseases like influenza, leptospirosis, and some rickettsial infections are sometimes misdiagnosed as dengue. Several alphaviruses, such as the chikungunya, O'Nyong-Nyong, Mayaro (MAY), RR, SIN, and Barmah Forest viruses, produce an acute febrile illness with polyarthritis and exanthem that must be differentiated clinically from rubella, cytomegalovirus, parvovirus, or mycoplasma infections (63). Arboviruses can also cause congenital infections, especially when introduced into immunologically naïve populations (68).

Neurotrophic arboviruses such as JE, WN, SLE, TBE, Murray Valley, and LAC viruses cause aseptic meningitis, encephalitis, or even flaccid paralysis in only a small percentage of infected persons; most infections are asymptomatic or lead to a mild illness, sometimes with headache. Pathologically, global involvement of cortical, subcortical, and brain stem structures and spinal cord myelitis are typical in more severe forms; consequently, various neurologic presentations are possible, with combinations of coma, weakness, cerebellar and extrapyramidal movement disorders, cranial palsies, and other manifestations of bulbar dysfunction and myelitis.

Viscerotropic disease can occur with YF, DEN, RVF, and certain other arboviral infections, resulting in hepatitis and jaundice. Generalized hemorrhage and multisystem organ failure are sometimes the end results of fulminant YF, RVF, and dengue hemorrhagic fever, mimicking malaria, leptospirosis, and the viral hemorrhagic fevers produced by Lassa virus and other arenaviruses, the Ebola and Marburg filoviruses, and Crimean-Congo hemorrhagic fever virus. Although YF, RVF, and DEN viruses pose a low risk for person-to-person nosocomial transmission (e.g., through needlestick exposures), the other viruses are proven hazards to hospital and laboratory workers (40, 70).

Although more than 100 of the recognized arboviruses can cause illness in humans, the laboratory diagnosis of these infections can be simplified by first considering the patient's history of travel or exposure. Some arboviruses, such as the DEN viruses, are transmitted virtually everywhere in the tropics and subtropics, but others are much more limited in their geographic distribution. By considering the known incubation periods of the principal arboviral infections in the context of the itinerary of places and circumstances under which infection may have been acquired, the differential diagnosis can be narrowed considerably. However, microbiologists and clinicians should be mindful that arboviruses can occasionally appear unexpectedly in new geographic regions, as illustrated by the 1999 introduction of WN virus into North America and cases of chikungunya arthritis imported in 2006 to Europe from an Indian Ocean island outbreak.

Currently, there is no specific therapy available for arboviral infections; supportive care (e.g., fluid replacement, analgesics, control of convulsions, or blood products in the case of severe hemorrhage) is all that is available. The rationale for promptly establishing the laboratory diagnosis of an arboviral infection is to hasten the confirmation of other treatable conditions, to avoid unnecessary treatment, and to assist public health officials in identifying arboviral infections with epidemic potential so that appropriate control measures (e.g., vector control or vaccination) can be implemented.

COLLECTION AND STORAGE OF SPECIMENS

Other than perhaps serologic testing for WN virus infection, few primary diagnostic laboratories can justify routine arboviral diagnosis as a service; instead, most laboratories should focus on the collection of appropriate specimens for submission to a reference laboratory. Assistance with diagnosis of arboviral infections can be obtained from some commercial reference laboratories, selected university research groups, the Division of Vector-Borne Infectious Diseases of the CDC [phone: (970) 221-6400], some state public health laboratories in the United States, and the WHO Collaborating Centers for Arbovirus Reference and Research. A listing of the WHO Collaborating Centers may be found on the WHO website at http://www.bireme.br/whocc.

Most arboviruses produce a brief, low level of viremia in humans, and it is only by chance that a viral isolate can be recovered from blood in these instances. However, other arboviruses, notably DEN and YF viruses, produce higher levels of viremia ($>10^{3.5}$ PFU/ml) of sufficient duration (ca. 5 to 6 days after the onset of fever) that humans serve as hosts for epidemic transmission. From the perspective of the clinical laboratory, these infections can be diagnosed by isolating virus from blood obtained during the acute phase of illness or by detecting genomic sequences by use of nucleic acid amplification techniques (NAAT), such as reverse transcriptase PCR (RT-PCR). Viruses causing encephalitis usually can be recovered from blood only during the preneuroinvasive phase of illness, and then usually by chance. When donors are in the viremic phase of infection, both WN virus and DEN virus can be transmitted via blood transfusion or organ transplantation (8, 27, 45, 61). Retrospective testing of the donors' blood in such instances has confirmed viremia (60). CTF virus is exceptional among human arboviruses in that it infects bone marrow erythrocytic precursors, leading to infection of the erythrocyte for its lifetime; consequently, virus can be recovered from blood obtained for several weeks after clinical recovery and can be transmitted through transfusion (37, 75). TBE virus has also been transmitted by transfusion (37).

TABLE 2 Arboviruses associated with human illness by geographic region, mode of transmission, and clinical syndrome[a]

Location, mode of transmission, and virus	Febrile illness			Meningoencephalitis	Hemorrhagic fever	Other
	Nondescript	With rash	With arthritis			
North America						
Mosquito borne						
Cache Valley	○			○		
California encephalitis				○		
DEN 1-4	●	●			●	Hepatitis[b]
EEE				●		
Everglades (VEE type II)				○		
Jamestown Canyon				○		Respiratory symptoms
Keystone				○		
LAC				●		
SLE				●		
Snowshoe hare				○		
Tensaw				○		
Trivittatus				○		Respiratory symptoms
VEE (sylvatic subtypes 1D and 1E)	○			○		
WEE[b]				●		Pneumonitis
WN[b]	●	●	●	●		Flaccid paralysis, hepatitis, pancreatitis, myocarditis
Sand fly borne						
Vesicular stomatitis (New Jersey and Indiana)	○			○		
Tick borne						
Colorado tick fever	●			○	○	
POW				○		
Salmon River	○					
Central and South America						
Mosquito borne						
Bussuquara	○					
Cache Valley	○					
Catu	○					
Cotia	○					
DEN 1-4	●	●		○	●	Hepatitis[b]
EEE				○		
Fort Sherman	○					
Group C viruses (Apeu, Caraparu, Itaqui, Madrid, Marituba, Murutucu, Nepuyo, Oriboca, Ossa, Restan)	○					
Guama	○					
Guaroa	○			?		Hepatitis
Ilheus	○			○		
Maguari	○					
MAY	●	●	●			
Mucambo (VEE type IIIA)	○			○		
ORO	●			○		
Piry	○					
Rocio				●		
SLE	○			○		
Tacaiuma	○					Two cases with concurrent malaria were fatal
Tonate (VEE type IIIB)	○			○		

(Continued on next page)

TABLE 2 *(Continued)*

Location, mode of transmission, and virus	Febrile illness			Meningoencephalitis	Hemorrhagic fever	Other
	Nondescript	With rash	With arthritis			
Tucunduba				○		
VEE (epizootic subtypes IABC)	●			●		Abortion, CNS malformation after first-trimester infection
VEE (sylvatic subtypes 1D, 1E, and 1F)	○			○		Pneumonitis
WEE				●		
WN[b]	●	●	●	●		Flaccid paralysis, hepatitis, pancreatitis, myocarditis
Wyeomyia	○					
Xingu	○					Hepatitis?
YF	●				●	Hepatitis
Sand fly and/or midge borne						
Alenquer	○					
Candiru	○					
Chagres	○					
Changuinola	○					
Morumbi	○					
ORO	●	○		○		
Punta Toro	○					
Serra Norte	○					
Vesicular stomatitis (New Jersey, Indiana, and Alagoas)	○			○		
Europe						
Mosquito borne						
Batai	○			○		
Inkoo	○			○		Respiratory illness
SIN (Ockelbo)	●	●	●			
Snowshoe hare				○		
Tahyna	●			○		Respiratory illness
WN[b]	○	○	○	●		Flaccid paralysis, hepatitis, pancreatitis, myocarditis
Sand fly borne						
Sandfly fever (Naples)	●					
Sandfly fever (Sicilian)	●					
Toscana	●			●		Hepatosplenomegaly, DIC
Tick borne						
Bhanja	○			○		
Central European encephalitis[c,d]	●			●		Hepatitis, thrombocytopenia
Crimean-Congo hemorrhagic fever[d]					●	
Dhori	○			○[e]		
Kemerovo				○		
Lipovnik				○		
Louping ill[d]				○		
Thogoto	○			○		Hepatitis, optic neuritis

(Continued on next page)

TABLE 2 Arboviruses associated with human illness by geographic region, mode of transmission, and clinical syndrome[a] *(Continued)*

Location, mode of transmission, and virus	Febrile illness			Meningoencephalitis	Hemorrhagic fever	Other
	Nondescript	With rash	With arthritis			
Tribec				○		
Asia						
Mosquito borne						
Batai	○			○		
Chandipura	●			●		
Chikungunya		●	●	○	○	
DEN 1-4	●	●		○	●	Hepatitis[b]
JE				●		Abortion after congenital first- and second-trimester infection
Kunjin (WN)		○	○	○		
Semliki Forest (Metri)				○		
SIN	●	●	●	○	○	
Snowshoe hare				○		
Tahyna	●			○		Respiratory illness
WN[b]	●	●	●	○		Flaccid paralysis, hepatitis, pancreatitis, myocarditis
Yunnan	○					
Zika		●				
Sand fly borne						
Chandipura	●			●		
Sandfly fever (Naples)	●					
Sandfly fever (Sicilian)	●					
Tick borne						
Alma-Arasan	○					
Banna				●		
Crimean-Congo hemorrhagic fever[d]					●	
Dhori				○[e]		
Ganjam	○					
Issyk-kul	○					
Karshi	○					
Kemerovo				○		
Kyasanur Forest (Alkhurma)[d]				●	●	Pneumonia, retinitis
Negishi				○		
Omsk hemorrhagic fever				●	●	Pneumonia
POW				○		
Russian spring-summer encephalitis[c]				●		
Syr-Darya Valley		○				
Tamdy	○					
Wanowrie				○	○	
Africa						
Mosquito borne						
Babanki	●	●	●			
Bangui		○				
Banzi	○					
Bhanja	○			○		
Bunyamwera		●		○		
Bwamba		●		○		
Chikungunya		●	●	○	○	
DEN 1-4		●		○	●	Hepatitis[b]

(Continued on next page)

TABLE 2 *(Continued)*

Location, mode of transmission, and virus	Febrile illness			Meningoencephalitis	Hemorrhagic fever	Other
	Nondescript	With rash	With arthritis			
Garissa (Ngari)		○		○	●	Reassortant of Bunyamwera virus
Germiston		○		○		
Ilesha		●		○	○	
Koutango		○				
Lebombo	○					
Ngari				○		
Nyando	○					
O'Nyong-Nyong		●	●			
Orungo	●					
Pongola			○			
RVF[d]	●			●	●	Hepatitis, retinitis
Semliki Forest	○			○		
Shokwe	○					
Shuni	○					
SIN		●	●		○	
Spondweni		○				
Tahyna	●			○		Respiratory illness
Tataguine		●				
Usutu		○				
Wesselsbron	○					Hepatitis
WN[b]	●	●	●	●		Flaccid paralysis, hepatitis, pancreatitis, myocarditis
YF	●				●	Hepatitis
Zika		○				
Sand fly borne						
Chandipura	●			●		
Sandfly fever (Naples)	●					
Sandfly fever (Sicilian)	●					
Tick borne						
Abadina	○					
Bhanja	○			○		
Crimean-Congo hemorrhagic fever[d]					●	
Dugbe	○			○		
Nairobi sheep disease	○					
Quaranfil	○			○		
Thogoto	○			○		Hepatitis, optic neuritis
Australia and Oceania						
Mosquito borne						
Barmah Forest			●	○		Glomerulonephritis
DEN 1-4	●	●				Hepatitis[b]
Edge Hill			○			
GanGan			○			
JE				○		
Kokobera			○			
Kunjin (WN)	○		○	○		
Murray Valley				●		
RR		●	●	○		Glomerulonephritis
Sepik	○					
SIN	○	○	○			
Trubanaman	○					

[a] Key: ○, rare or sporadic; ●, frequent, epidemic. Only arboviruses causing illness after natural infection are listed; viruses causing illness after laboratory exposure only are excluded.
[b] Perinatal illness following third-trimester infection has also been described.
[c] Transmissible by ingestion of infected milk products.
[d] Transmissible through meat of infected animals.
[e] Syndrome reported only after laboratory-acquired infection.

Viral isolates can sometimes be recovered by biopsy or at autopsy from the viscera of patients with acute YF, dengue hemorrhagic fever, or other viscerotropic arboviral infections and from brain tissue or cerebrospinal fluid (CSF) of patients with central nervous system (CNS) infections. Brain samples should be taken from several areas, including the cortex, brain nuclei, cerebellum, and brain stem. Neurotropic arboviruses can occasionally be isolated from CSF obtained by lumbar puncture during the acute stages of encephalitis or aseptic meningitis. RT-PCR has often proved to be more sensitive in detecting specific arboviral genomic sequences in brain tissue or CSF. Alphaviruses have rarely been isolated from vesicular skin lesions, the joint fluids of patients with acute polyarthritis, or the upper respiratory tracts of patients with acute febrile illnesses. Under certain circumstances, arboviruses or their antigens have been detected in urine, milk, semen, and vitreous fluid (5, 66). Clinical laboratories sometimes also receive arthropods for viral isolation. These samples can be referred in the same manner as tissues to a reference laboratory, where they can be processed for virus isolation or nucleic acid or antigen detection procedures.

A plan for dividing available tissues or fluids for viral isolation, electron microscopy, and direct detection assays of antigen and genomic sequences should be devised. Tissues should be collected aseptically and rapidly transported to the laboratory in a viral transport medium or on a moist sponge. If the aliquot for viral isolation cannot be processed within 24 h, it can be maintained at 4°C; otherwise, the sample should be frozen immediately at −70°C or stored on dry ice. Samples for virus isolation and nucleic acid detection should be kept frozen continuously, avoiding freeze-thaw cycles, which may inactivate virus or disrupt nucleic acids. The aliquot for electron microscopy should be minced and placed directly in glutaraldehyde or another fixative. Autolytic changes occur rapidly, and tissues should be fixed as quickly as possible. To prepare sections for immunohistochemical examination, a portion of the sample should be either fixed in 10% buffered formalin or, preferably, added to freezing medium and frozen. Touch preparations are less reliable. Aliquots for antigen detection by other means and for PCR should be frozen at –70°C. Samples should be shipped by express mail in double, sealed containers according to local transport regulations. Frozen samples should be shipped separately on dry ice and labeled appropriately. International shipments usually require import and/or customs permits and clearances.

Serologic studies are best done by comparing antibody titers in paired serum samples drawn during the first week of illness and 2 to 3 weeks later. A single serum specimen may be sufficient for diagnosis of certain arboviral infections for which immunoglobulin M (IgM) assays are available. Detection of virus-specific IgM in CSF is a sensitive and highly specific approach to diagnosing CNS infection, but both CSF and serum samples should be tested. IgM capture ELISAs for arboviral CNS infections transmitted in the United States are offered by several reference and some state public health laboratories. Serum and CSF samples should be collected aseptically, stored either refrigerated or frozen, and transported according to current shipping regulations. Freeze-thaw cycles may reduce antibody titers and should be avoided. Blood or serum blotted and dried on filter paper may be suitable for some serologic assays.

No specific procedures for patient preparation are required. The isolation of certain arboviruses in host systems could be inhibited in patients treated with antiviral agents, such as ribavirin, or immunomodulators, such as alpha or gamma interferon. Some preparations of gamma globulin, including preparations for intravenous use, may contain arboviral antibodies and could interfere with the isolation of arboviruses from patient specimens or with arboviral serologic tests.

DIRECT EXAMINATION

Clinical application and experience with direct arbovirus detection methods generally have been limited, and specimens should be evaluated in parallel with conventional viral isolation, molecular testing, and serologic procedures.

Electron Microscopy

When tissue is available, direct examination by electron microscopy can sometimes provide rapid evidence of an arboviral infection. Experienced observers can identify virions morphologically, often to the level of a viral family, in thin tissue sections. Visualization of togaviruses in brain tissue has helped establish a diagnosis of EEE in some reported cases. Newer techniques using confocal microscopy combined with transmission electron microscopy have been used to diagnose human flavivirus infections (11). Virions may be absent, however, from autopsy tissues of patients who died after a prolonged course of illness and from whom infection had previously been cleared.

Detection of Viral Antigens by Use of IHC or Immunofluorescence

Immunohistochemical (IHC) staining of peripheral blood mononuclear cells or tissue sections has been successful in detecting arboviral antigens in CSF and brain specimens from encephalitis patients; in the viscera of patients with YF, DEN, and other viscerotropic infections; and in joint fluids of patients with acute arthritis (14, 21). CTF virus-infected erythrocytes occasionally can be identified directly by staining a simple smear of peripheral or clotted blood. Postmortem diagnosis of arboviral infections can be made from formalin-fixed sections by using IHC staining. Formalin-fixed tissue or paraffin-embedded tissue block samples can be used. The advantage of IHC is that samples can be examined months after collection and storage. For best results, formalin-fixed specimens should be transferred to 70% ethanol after 48 h for storage. Monoclonal antibodies specific to many medically important arboviruses are available; for more obscure arboviruses, hyperimmune mouse antibodies can be employed. Antibodies and information on their working dilutions can be obtained from the CDC and other reference laboratories. Although trypsinized, formalin-fixed tissues are suitable for IFA examination, frozen sections are generally preferred because trypsinization procedures have not been standardized for all arboviruses and because success varies with the duration of formalin fixation.

Formalin-fixed tissue or paraffin-embedded tissue block samples can also be sent to the CDC or to some academic pathology groups (e.g., University of Texas Medical Branch) for detection of specific arboviruses, such as WN virus. Arrangements can be made with the CDC pathology staff [phone: (409) 639-3133] before shipping any specimen.

Antigen Capture ELISA

Antigen capture ELISAs have been developed for several arboviruses; however, the formation of immune antibody-virus complexes in DEN and YF may interfere with the detection of viral antigens in viremic blood. Although complexes can be chemically dissociated, the assays have not been shown to be clinically useful. The detection threshold

of antigen capture ELISAs for SLE, WN, and JE viruses (ca. $10^{3.5}$ PFU/ml) is sufficiently sensitive for their application in the epidemiologic surveillance of infected arthropod vectors (26, 67). Several commercial antigen assay kits are available for detection of WN, SLE, and EEE viral antigens in mosquitoes (VecTest, Medical Analysis Systems).

Nucleic Acid Detection Techniques

A number of nucleic acid amplification strategies have been developed for the detection and identification of medically important arboviruses, including standard RT-PCR (with agarose gel analysis), real-time RT-PCR using fluorescent probes, and nucleic acid sequence-based amplification (NASBA). A review of the current literature reveals that in nearly all cases, the analytical sensitivity of any of the amplification assays is equal to or exceeds that of the most sensitive viral isolation procedures performed either with cultured cells or with neonatal mice. In addition, amplification assays can provide results in a much shorter time than isolation methods while allowing for the processing of greater numbers of samples. However, the clinical sensitivity of amplification assays is highly dependent upon the dynamics of in vivo viral replication and the tissue tropism of the virus. For example, WN virus infection produces a relatively short viremia which is often low or absent at the time of clinical presentation of CNS symptoms; in one study, WN viral RNA was detected in the CSF of serologically confirmed cases only 57% of the time with a real-time RT-PCR assay with an analytical sensitivity of 0.1 PFU (32, 33). In contrast, the DEN viruses typically achieve a much higher level of viremia with a longer duration, and virus can be detected readily in serum by virus isolation or amplification methods (77). In general, amplification assays have been successful in detecting arboviruses in tissues obtained from fatal human cases when the appropriate tissue target is known and assayed (i.e., brain tissue in WN, LAC, or EEE cases, liver tissue in YF cases, etc.). A commercially available WN viral RNA detection system is approved by the U.S. Food and Drug Administration (FDA) for screening donor blood, organs, cells, and tissues (Procleix WNV assay; Gen-Probe Inc.).

The use of amplification assays for the detection of arboviruses in surveillance protocols, particularly in mosquitoes collected in the field, has become the method of choice since large numbers of samples can be tested with minimum labor and the sensitivity often exceeds that of virus isolation or antigen detection assays. With the addition of automation, amplification approaches have been used to test hundreds of mosquito specimens in a single day (57). Amplification assays have also been utilized successfully in environmental surveillance by detecting arboviruses in tissues obtained from vertebrate hosts collected in the field. WN virus detection in dead birds collected in the field and EEE virus detection in fatal equine cases are typical applications of this technology.

RNA Extraction and Purification

The majority of arboviruses of medical importance are enveloped single-stranded RNA viruses, and therefore the sensitivity and specificity of any amplification strategy are dependent on the efficient extraction and purification of the target RNA template. Highly efficient RNA extraction and purification protocols are now commercially available from a number of sources. Most of these utilize a chaotropic lysis buffer which allows for both efficient solubilization of the sample and stabilization of the RNA, followed by binding to silica, subsequent washing, and then elution in a low-salt solution. Some purification protocols have also been automated, and hundreds of samples can be processed within a single day. The use of a chaotropic lysis buffer has also enabled the efficient extraction of intact RNA from sources that were problematic in the past due to ribonuclease activity, including serum/plasma, whole blood, CSF, tissues, and homogenized ticks or mosquitoes. Ticks and mosquitoes are particularly useful for environmental surveillance of arboviruses.

RT-PCR

Standard RT-PCR-based assays (compared to real-time assays [described below]) to detect arbovirus genomic sequences have been developed for a number of the medically important arboviruses (29). Most of the assays use virus-specific primers designed to amplify a single virus species or, in a few cases, serologically related viruses (i.e., the DEN viruses). The assays typically involve RT-PCR amplification followed by agarose gel electrophoresis, with DNA visualization by staining with ethidium bromide to characterize the amplified DNA by molecular weight. Visualization of a DNA fragment of the predicted size is considered diagnostic in some cases. Because nontarget amplification in some instances can generate DNA products with similar mobilities in agarose gels to that of the predicted fragment, generating false-positive results, additional characterization of the amplified DNA is often utilized. A variety of sequence-specific technologies for detecting and confirming the identity of the amplified DNA include hybridization with virus-specific probes (e.g., Southern blot, dot blot, or microtiter plate hybridization and simultaneous detection of multiple target sequences by capture oligonucleotide macroarrays), PCR amplification with primers internal to the original primers (nested or seminested PCR), restriction endonuclease digestion of the DNA product, or nucleic acid sequence analysis (18). Virus-specific assays are available to detect the DEN, YF, JE, western equine encephalitis (WEE), EEE, SLE, VEE, MVE, Powassan (POW), TBE, and WN viruses; the California serogroup viruses; and the RR, Ockelbo, and CTF viruses, among others. In contrast to virus-specific RT-PCR assays, there are also RT-PCR amplification strategies which utilize primers designed to amplify most or all viruses within a genus. Consensus RT-PCR assays have been described for alphaviruses, flaviviruses, and the California and Bunyamwera serogroup orthobunyaviruses (30, 47, 55). When consensus primers are utilized, a sequence-specific detection method (as described above) must be employed to specifically identify the resulting DNA, since by the design of the assay, all related viruses would generate DNA amplicons of identical size.

Real-Time 5′-Exonuclease Fluorogenic Assays

Real-time fluorogenic RT-PCR assays (commonly referred to as TaqMan assays) combine RT-PCR amplification with concurrent sequence-based detection of amplified DNA, using fluorescently labeled, virus-specific DNA probes. The assays offer many advantages over standard RT-PCR, namely, increased sensitivity, increased specificity, quantification, high throughput, a decreased likelihood of cross-contamination, and rapid results. These advantages are due to the use of the fluorescently labeled internal probe, which rapidly and specifically detects amplified DNA via hybridization. Subsequent *Taq* polymerase digestion of the probe releases the 5′-labeled nucleotide and results in a measurable increase in fluorescence. This sequence-specific detection obviates the need for any postamplification characterization of the amplified DNA, resulting in a much more rapid result. In addition, the likelihood of contamination is reduced because amplified DNA is not manipulated in the laboratory, as occurs with gel

electrophoresis and standard RT-PCR. Real-time fluorogenic assays also offer the added advantage of multiplexing, the ability to detect multiple targets at the same time in the same amplification reaction mix by including additional primers and probes containing unique fluorescent dyes. Several TaqMan assays for the detection of arboviruses have been described, including assays for WN, SLE, TBE, DEN, EEE, WEE, LAC, and chikungunya viruses (31, 33, 36, 44, 74).

NASBA

Another nucleic acid amplification technology that has been used for the detection and identification of arboviruses is NASBA. The initial stage of NASBA is similar to RT-PCR in that single-stranded target RNA is copied into double-stranded DNA via virus-specific primers in an RT reaction. However, NASBA subsequently differs significantly from RT-PCR in a number of ways: the amplification reaction is isothermic (41°C), different enzymes are used (reverse transcriptase, RNase H, and T7 RNA polymerase), and the final amplification product is single-stranded RNA. In NASBA, the oligonucleotide used to prime cDNA synthesis contains the T7 promoter sequence. The resultant reverse-transcribed DNA serves as a template for transcription by T7 RNA polymerase. Amplification is generally more rapid because it is not dependent on thermal cycling. Amplified RNA can be detected by hybridization to a virus-specific oligonucleotide probe either by utilizing an electrochemiluminescence detection format or in real time by using a fluorescently labeled molecular beacon probe. Both detection approaches have been employed successfully to detect a number of arboviruses, including WN, SLE, EEE, WEE, LAC, and DEN viruses (29, 30, 70).

VIRUS ISOLATION

Many of the medically important arboviruses have caused infections in laboratory workers. The pathogenesis of infections acquired from aerosols created by pipetting tissue, homogenization, centrifugation, and other procedures may differ from that of natural infection, with the possibility of direct CNS invasion through the olfactory epithelium (46). On the basis of this experience, the joint CDC-National Institutes of Health guidelines for biosafety in microbiological and biomedical laboratories recommend biosafety level 2 to 4 containment facilities for laboratory work with arboviruses (70). The essential elements of the various biosafety levels for activities with arboviruses and laboratory animals infected with them are detailed in these guidelines (70). Some arboviruses are also designated select agents and require additional levels of security. Laboratory staff should be immunized with available arboviral vaccines. Laboratories that process CNS specimens from patients with encephalitis should consider immunizing staff with rabies vaccine as well.

Most arboviruses can be cultivated in a variety of primary and continuous cell lines, including primary duck and chicken embryo cells, primary monkey and hamster kidney cells, C6/36 *Aedes albopictus* and AP-61 *Aedes pseudoscutellaris* mosquito cell lines, and Vero, LLC-MK_2, BHK-21, CER, SW13, and PK cell lines. The time to develop cytopathic effects (CPE) as well as the pattern of CPE varies in each virus-cell system, e.g., primary duck and BHK-21 cells are rapidly destroyed by most arboviruses, while the development of CPE in monkey kidney cell lines is generally slower. AP61 and C6/36 cells exhibit nearly universal susceptibility to the mosquito-associated arboviruses, including the DEN viruses, which grow poorly in continuous monkey kidney cell lines. The mosquito cell lines, which are generally incubated at 28°C, exhibit little evidence of viral CPE, and replication must be identified by an IFA test or other means. Many alphaviruses and some flavivirus strains produce CPE or form cell syncytia in these lines, but most arboviruses do not. The majority of arboviruses are lethal to suckling (2- to 3-day-old) mice, which exhibit signs of illness, paralysis, and death within days to 2 weeks after intracerebral inoculation. Some field isolates (e.g., DEN and sandfly fever group viruses) exhibit virulence for mice only after adaptation through blind passages. Infected suckling mouse brain is still the preferred antigen for many serologic tests and for the preparation of clean, high-titer immune reagents.

Since no single isolation system detects all arboviruses, tissues, fluids, and serum collected in the acute (febrile) phase of illness should be inoculated into cultures of several cell lines, including C6/36 or AP61 mosquito cells, Vero, LLC-MK_2, or BHK-21 cells, or primary embryonic cells, and into 2- to 4-day-old suckling mice (35, 62, 78). Direct inoculation of live colony-reared mosquitoes can also be used (54). Ideally, serum specimens should be inoculated undiluted and in 10^{-1} and 10^{-2} dilutions to avoid prozone effects due to viral autointerference and antiviral antibodies present in the specimen. Tissues should be homogenized and diluted similarly on a weight-to-volume basis (10 to 20%) with a protein- and antibiotic-containing medium, e.g., phosphate-buffered saline with 0.75% bovine serum albumin and 1% gentamicin. After adsorption to the drained cell monolayer, cell cultures are fed with medium and observed daily or more often for signs of CPE. Small flasks (12.5 or 25 cm^2), tubes, or coverslips in shell vials may be used. Viral growth in C6/36 and AP61 cells does not reliably produce CPE, and these cells must be examined for viral antigen and replication by an IFA test or other means (62). When medium from infected cultures is harvested for passage or storage, fetal calf serum should be added to a final concentration of 20 to 40% before the medium is frozen at –70°C.

Intracerebrally inoculated mice (one litter of 6 to 10 mice for each dilution) should be observed twice daily for up to 2 weeks for signs of illness and death (35). Ill and dead mice should be frozen at –70°C until infected organs can be harvested and passaged in cell culture. When a bunyaviral or orthomyxoviral etiology is suspected, it is prudent to harvest livers in addition to brains since certain orthobunyaviruses and thogotoviruses replicate to higher titers in the mouse liver than in the brain.

Although NAAT have the advantages of speed and sensitivity, virus isolation remains the "gold standard" against which other methods are compared. Virus isolation can detect new or unsuspected agents, as well as those agents for which NAAT have not yet been developed. Furthermore, genetic variants and mutations can lead to falsely negative NAAT results, yet the virus may be detected in culture.

VIRAL IDENTIFICATION

While identification of arboviral isolates previously depended upon their antigenic characterization, PCR and other molecular tests are now available for many of the medically important arboviruses. Provided that sufficient sequence information is available, analysis of the PCR product can provide more specific identification of the isolates than ELISA, IFA, or other rapid serologic assays. PCR-based assays that amplify sequences conserved among medically important alphaviruses, bunyaviruses, or flaviviruses permit the assignment of an unknown virus within a broad taxon, and sequence analysis of the amplified products can give

TABLE 3 Serological assays commercially available in the United States for diagnosis of arbovirus infections

Assay type	Virus(es)	Manufacturer	FDA status[a]
IFA for IgG or IgM	WEE, EEE, LAC, SLE	Focus Diagnostics	IVD
	WEE, EEE, LAC, SLE, WN, POW	PanBio	ASR
	WN	PanBio	ASR
ELISA for IgG and IgM	WN	Focus Diagnostics	IVD
	WN	PanBio	IVD
	DEN 1-4	Focus Diagnostics	RUO
	DEN 1-4	PanBio	ASR
ELISA for IgM	WN	InBios	IVD
ELISA for IgG	WN	InBios	RUO

[a] IVD, for in vitro diagnostics; ASR, analyte-specific reagent; RUO, for research use only. Additional products available for sale outside the United States include IFAs for WN, VEE, JE, YF, and DEN viruses; rapid lateral-flow and strip immunochromatographic tests for DEN virus, and IgM and IgG ELISAs for JE virus.

their precise identification. Such omnibus primers have been reported for alphaviruses and flaviviruses and for certain groups of bunyaviruses and reoviruses (10, 15, 30). Sequence information can also be used for molecular epidemiology, including identification of source strains during outbreaks and investigation of strain relationships that may have epidemiologic or clinical importance (34).

The identity of a suspected arbovirus can often be narrowed down from information on the circumstance, source, location, or other aspects of collection (Table 2). If no particular agent is suspected, polyclonal or monoclonal antibodies against selected prototype virus strains can be used to provide a provisional identification of the agent. Antibody panels prepared against representative serogroups of viruses, such as those prepared against the suspected etiologic agents of a specific clinical syndrome (i.e., encephalitis) or against viruses present in the area where the sample was collected or the patient was infected, are particularly useful. Once the virus is provisionally identified, PCR or specific antibodies can be used to type the virus.

Any of several standard serologic tests (ELISA, IFA, HI, and CF) can be used in this preliminary phase of identification. Complete identification requires partial sequencing or serologic comparison between reactivities of antisera prepared against the new isolate and a prototype strain. The neutralization test is the preferred and accepted method for such antigenic comparisons.

When screening with broadly reactive antibodies fails to place an unknown virus within a group of arboviruses, morphologic examination of the unknown by negative-stain electron microscopy can categorize the virus within a taxon (11). Virions generally can be found in cell culture medium or directly in clinical specimens that have infectivity titers of $\geq 10^6$ PFU/ml. Although most pathology laboratories do not routinely prepare negatively stained grids, examination of thin sections of infected cell cultures can often be arranged.

SEROLOGY

In most laboratories, diagnosis of arboviral infections relies mainly on serology. The classical serologic tests, HI and CF, have been abandoned by many diagnostic laboratories in favor of IgM capture ELISA or IFA. The HI and CF tests are now mainly used in research laboratories. A number of serologic assay kits are now available commercially for specific pathogens (Table 3). In the United States, indirect IFA and ELISA reagents and kits are confined to those for arboviruses most likely to be encountered by U.S. residents. The FDA status varies for each product. In some cases, a kit may be approved for diagnostic use for serum samples but not for CSF (49). Additional products, including rapid lateral-flow and strip immunochromatogenic tests, are available for a number of arboviruses important outside the United States.

ELISA

The antibody capture format for detection of IgM in serum or CSF is more sensitive and specific than indirect methods of IgM detection (2, 7, 71). Virus-specific IgM can be detected in the sera of 90 to 95% of patients with primary DEN virus infections by the sixth day after the onset of illness (71). IgM to WN virus is usually detected in CSF 3 to 5 days into clinical illness, although up to 8 days may be required (22, 49, 65). Serum IgM appears 3 days after CSF IgM. WN virus can be detected earlier in infection by PCR or culture, but once clinical illness develops, PCR is generally less sensitive than antibody-based methods. The presence of virus-specific IgM in CSF reflects intrathecal antibody production and is considered diagnostic of recent CNS infection. In serum, a declining level of virus-specific IgM can usually be detected for several months after infection and for up to a year after infection in some cases. In WN virus infection, specific IgM antibodies have been reported for some patients for up to 2 years or more (53); therefore, a positive IgM result for a single serum sample is considered only presumptive evidence of recent infection. An analogous IgA capture ELISA for RR polyarthritis was shown to be better suited for diagnosis of recent infection because the level of virus-specific IgA declined more rapidly than that of IgM (9). In addition, IgG avidity tests on acute-phase sera have been used to discriminate between primary and secondary infections (38, 41). Low-avidity antibodies are characteristic of recent infections, whereas high-avidity IgG antibodies are seen 6 months or more after the onset of symptoms. Arboviral IgG antibodies are usually detected by an indirect sandwich method.

Heterologous reactions among antibodies to antigenically related viruses are a vexing problem in areas where several related viruses cocirculate. Cross reactions between DEN viruses and JE virus, between YF virus and the numerous flaviviruses that circulate in West Africa, and between SLE, WN, and POW viruses in the United States frequently produce overlapping values that may be uninterpretable. Serologic cross reactions of WN virus with other flaviviruses, and less frequently with bunyaviruses, enteroviruses, and cytomegalovirus, can occur, especially with serum IgG (49). Because of cross-reactivity, a positive IgM assay should be followed by a neutralization test to determine the etiology of illness.

Numerous schemes have been devised to define the threshold of a positive or significant ELISA absorbance value. Some laboratories define a positive reaction in multiples (units) of the absorbance of a weak positive specimen, an approach that controls plate-to-plate variations in specimen reactivity. An alternative approach is to define the threshold absorbance as a multiple (e.g., 5 standard deviations) above the mean absorbance of samples from a population of uninfected persons. A simple multiple of two times the absorbance of a negative control generally produces a conservative interpretation, exceeding 5 standard deviations above the mean value for negative samples.

Indirect IFA

Some laboratories use IFA for differentiation of IgG and IgM responses. Infected cells fixed onto microscope slides with multiple wells are the usual antigen substrate. Kits for detection of antibodies to the most common arboviruses in the United States are commercially available (Table 3). The kits provide multivalent slides with four (EEE, WEE, LAC, and SLE viruses) or six (WN and POW viruses in addition to the other four) infected cell spots and uninfected control cells. WN viral antigen is also available as a single analyte (40). Positive and negative sera are supplied with the kits, and all reagents are ready to use and do not have to be titrated. As with other assays, sera from persons previously infected with WN, DEN, JE, POW, or other flaviviruses may cross react with the SLE antigen in these tests and vice versa; thus, confirmation of the diagnosis should be obtained with a neutralization test.

Anti-human IgG and IgM fluorescein isothiocyanate conjugates are commercially available, but they should be titrated to ascertain their optimal working dilutions. To conserve antigen, serum samples (1:16 dilution) and CSF can be screened and then tested in further twofold dilutions to the endpoint only if positive. Positive IgM reactions should be verified as specific by ruling out the presence of rheumatoid factor (serum IgM antibodies directed against IgG).

IgM antibodies detected by immunofluorescence become detectable within a few days after the onset of illness, and IgG antibodies appear shortly thereafter. IgG antibodies detected by IFA are long-lived, paralleling the longevity of HI and neutralizing antibodies. Fourfold changes are diagnostic of recent infection, and in some circumstances, single or stable elevated titers (>1:128) may indicate recent infection. The sensitivities and specificities of the IFA and HI tests are similar. Thus, cross reactions among related flaviviruses, e.g., among SLE, WN, and DEN viruses, sometimes present a problem in interpretation.

HI Test

For the arboviruses that hemagglutinate erythrocytes, i.e., members of the families *Flaviviridae, Bunyaviridae,* and *Togaviridae* and certain members of the *Rhabdoviridae* and *Orthomyxoviridae,* the HI test is a convenient screening serologic procedure (2, 24). HI antibodies rise rapidly within the first week after the onset of illness and are long-lived, persisting at low levels for decades in some instances. These kinetics are consistent with the mixed IgM and IgG isotype distribution of HI antibodies. Gander erythrocytes are preferred, although chick and trypsinized human O erythrocytes have also been used. Inactivated sucrose-acetone extracts of infected mouse brains provide a high-titer source of antigen; for viruses such as Oropouche (ORO) and group C viruses that do not propagate to high titers in mouse brain, livers, infected-cell-culture antigens, or twice-acetone-extracted sera from infected mice or hamsters can be used. For each virus, HA is optimal within a narrow pH range of less than one-half a pH unit. Before the HI test is performed, a preliminary HA test is done to determine the specific pH optimum and the antigen titer.

Serum samples are conventionally tested at a 1:10 dilution and at further twofold dilutions to the endpoint. A fourfold change between acute- and convalescent-phase samples is diagnostic of recent infection, a titer of >1:80 may be interpreted as presumptive evidence of recent infection, and a titer of >1:2,560 is evidence of a recent secondary antibody response to flaviviral infection.

The HI test is highly sensitive in confirming an infection when adequately timed serum pairs are available, but it is considerably less useful when applied to a single serum sample. Furthermore, HI antibodies tend to be broadly reactive, recognizing common epitopes within antigenic groups or complexes. For this reason, the HI test is useful for screening unknown samples and for demonstrating group relationships among arboviruses. But, as noted before, while a primary infection may lead to a specific antibody response, repeated infections with related viruses produce increasing levels of heterologous antibodies to the related viruses, often rendering the HI test results uninterpretable.

The HI test is useful in epidemiologic surveys because of its broad reactivity and the longevity of HI antibodies and because the procedure is robust. Blood samples collected on filter paper and dried can be stored for short periods without refrigeration, mailed, and tested by HI after reconstitution. Another advantage of the HI test is that the procedure does not require species-specific reagents and can be used for wildlife and animal surveys involving sera from a variety of birds, mammals, and reptiles.

CF Test

The CF test is moderately specific and is often used to narrow the definition of a heterologous HI antibody response (59). The half-lives of CF antibodies vary, but the antibodies may persist for several years. CF antibodies to some flaviviruses (DEN, WN, JE, MAY, and YF viruses) persist for many years. Thus, some laboratories use the HI test to screen samples and the CF test to define the specificities of reactive samples. Some arboviruses, such as CTF virus, certain rhabdoviruses, and the orbiviruses, do not hemagglutinate erythrocytes, so the HI test cannot be used and the CF test is then used for primary diagnosis. Because the test is relatively insensitive, it should be used in combination with some other procedure. A fourfold change is diagnostic of recent infection, and in some circumstances, a single or stable elevated titer of 1:32 may be accepted as presumptive evidence of recent infection.

CF antibodies generally rise slowly after infection, often peaking as late as 6 weeks after the onset of illness. Convalescent-phase serum samples should be obtained 2 to 3 months after onset to ensure against missing a late-rising CF antibody response. Some individuals fail to produce detectable CF antibodies, and advanced age may be associated with a blunted, delayed, or undetectable response.

Neutralization

The neutralization test is the most specific of the common serologic procedures and is used principally to sort out heterologous reactions observed in other assays (2, 25). The presence of virus-neutralizing antibodies is also considered the best indication of protective immunity, and the response to immunization is usually monitored by following neutralizing antibody levels. Neutralizing antibodies generally become detectable within the first or second week after the onset of

illness, peak in the following 2 weeks, and decay slowly, persisting for years and often over a lifetime. Reexposures and infections with related viruses (i.e., sequential DEN virus infections) may stimulate an accelerated secondary response. Although the neutralizing antibody response is relatively specific, repeated infections with related viruses produce a progressively broader heterologous immune response, with the possible extension of cross-protection in some instances. Sometimes, as in the case of repeated flavivirus infections, these extensive heterologous reactions cannot be resolved with cross-neutralization tests.

Infectious virus is used in the neutralization test; consequently, the procedure should be performed only in laboratories capable of working safely at the appropriate biosafety level. Neutralizing antibodies are best quantitated with a plaque reduction assay, in which dilutions of heat-inactivated serum are mixed with a fixed dose of virus and incubated for 1 h at 37°C or overnight at 5°C. The longer incubation time usually gives slightly higher antibody titers (greater sensitivity). Sometimes exogenous complement is also added to the mixture. The highest serum dilution inhibiting 80 to 90% of the infectious virus dose observed in virus control wells is the endpoint. In a typical test using 100 PFU as the viral input dose, the 90% endpoint is the last well showing 10 or fewer plaques. Serum samples are generally diluted 1:5, and with the addition of an equal volume of virus, the initial test dilution is 1:10. A fourfold change in titer is considered diagnostic of recent infection.

The neutralizing capacity of a serum sample can also be measured by testing a single dilution of the serum against a series of virus dilutions. The result is expressed as the serum's log neutralization index. For viruses that do not form readable plaques, neutralization tests can also be done by inoculation of laboratory animals (i.e., newborn or weanling mice) or by a fluorescent focus technique.

Other Procedures

Rapid procedures such as dipstick, dot blot, microsphere immunoassay, and immunochromatographic tests are commercially available outside the United States for several arboviruses (Table 3). These tests have shown good sensitivity and specificity for some arboviruses, including DEN and JE viruses (3, 12, 13, 20, 72, 76), although their diagnostic accuracy may be well below manufacturers' claims (3). The assay format is usually simple and robust enough for use in primary care hospitals or outpatient facilities and in field situations where the capacity for more complex testing, such as ELISA, is not available. Further clinical evaluations are needed, but such kits may bring the capacity for serologic diagnosis within the reach of a wider range of laboratories and medical care facilities in developed and developing countries.

As stated above, heterologous serologic reactions in patients who have had repeated arboviral infections are difficult to sort out, even after all available conventional tests have been exhausted. In an attempt to improve specificity, the narrow reactivity provided by viral monoclonal antibodies has been utilized in the development of serologic tests that combine a serum sample with a virus-specific monoclonal antibody in a competitive ELISA (4, 6). Virus-specific antibodies present in the test sample block binding of the monoclonal conjugate to viral antigen, thus reducing the absorbance. Synthetic antigens, expressed as polyproteins including areas of the envelope glycoprotein or produced by proteolytic cleavage, have allowed some differentiation of flaviviral antibody responses by ELISAs, by IHC staining of expressed antigens on cell surfaces, or by Western blotting (immunoblotting). Although these techniques may be applicable to population surveys, they are still largely research techniques, and their discriminatory power for routine clinical diagnostic purposes requires further confirmation.

Recently, discrimination between primary and secondary infections was performed by using IgG avidity tests on acute-phase serum samples (38, 41). This type of determination is especially helpful for hospitalized patients with dengue fever (41).

EVALUATION AND INTERPRETATION OF TEST RESULTS

Unlike herpesviruses, adenoviruses, and other viruses that produce persistent or latent infections, nearly all human arboviral infections are acute and are terminated by viral clearance. Thus, the isolation of an arbovirus from an ill patient or detection of viral nucleic acid in blood or tissues is virtually diagnostic. However, rare subacute, progressive, and recrudescent infections and experimental models of persistent infection have been reported for TBE, WN, SLE, JE, and ORO viruses (19, 48, 50, 52, 56, 64).

Surprisingly little is known about the effect of human immunodeficiency virus (HIV) infection on the clinical course of arboviral infections or vice versa. In one case of acute dengue fever due to type 1 DEN virus in an HIV-infected adult, the HIV viral load was reduced transiently by the intercurrent infection, and the clinical course of dengue fever was unremarkable (73). In contrast, recent reports of WN virus infections from the United States have documented that immunosuppressive therapy can predispose transplant recipients and patients on chemotherapy to severe neurological disease (23, 51).

Careful consideration and planning must be undertaken in the formulation of a molecular testing algorithm for arboviruses due to the potential for cross-contamination inherent with all amplification assays. In general, a good testing algorithm for an amplification assay would include independent confirmation of all positive results by another virus detection assay, such as an alternate amplification strategy, virus isolation, antigen capture ELISA, or direct IFA. In addition, the inclusion of an internal positive control (see below) is desired to ensure that all reagents and amplification parameters are optimal for target amplification. At a minimum, all positive amplification results should be confirmed by retesting using the same technology (e.g., RT-PCR) with a different primer set. Testing algorithms must also incorporate appropriate controls to ensure against false-positive or false-negative results. Negative controls should include reaction mixes with no RNA added (reagent controls) to ensure that amplification reagents are not contaminated with target RNA or DNA, as well as RNA extracted from negative specimens (extraction controls) to monitor contamination during sample processing. Positive controls included in each assay should contain various levels of target RNA (high, medium, low, etc.) to ensure that the sensitivity of the current assay is similar to those of previous tests. The use of an exogenously added RNA target along with a corresponding amplification primer set allows for the monitoring of every amplification reaction and can detect false-negative results; this strategy is particularly useful for testing specimens that may contain inhibitors of RT-PCR (e.g., blood, mosquito lysates, etc.). In all amplification-based technologies, it is essential that proper laboratory procedures be followed to prevent cross-contamination of clinical samples

with amplified DNA (amplicon) or RNA, which would result in the generation of false-positive results. In general, there should be complete physical separation of pre- and postamplification laboratory space and equipment. Finally, clinical samples should be tested in duplicate wells, at minimum, and ideally two primer sets should be utilized in independent reactions.

Due to the possibility of cross-reactivity, serologic results should always be interpreted in view of the patient's prior residence history, exposure, and immunization record. For example, recent immigrants may have resided in regions where DEN, YF, or JE is endemic and may have antibodies to the agents of these diseases. Vaccines for YF, JE, and TBE are commercially available in the United States and abroad and are widely used in some countries or for high-risk groups (e.g., military personnel). A vaccination history should be sought from the patient because evidence of prior immunization might mitigate against certain diagnoses and because vaccine-induced antibodies can interfere with the interpretation of serologic test results. The influence of immunosuppression on antibody responses to arbovirus infections is not well characterized, but results should be interpreted with caution, and in this instance, nonserologic methods should be employed. It is known, for example, that the antibody responses of HIV-infected children to live YF virus vaccine, essentially an attenuated infection, are poorer than those of healthy control children (58).

Finally, laboratory detection of a recent arboviral infection does not necessarily secure the clinical diagnosis because concurrent infections are possible. Arboviral infections may occur coincidentally with or may predispose patients to more serious secondary infections. In a review of ~2,800 pediatric dengue fever cases in Thailand, concurrent bacteremia and other infections complicated the illness in 14 cases (43). Furthermore, acute dengue fever has also been associated with reactivation of latent human herpesvirus 6 infection (1). Dual infection with an arbovirus and a protozoan or bacterium may also occur because certain vector species participate simultaneously in several transmission cycles. *Ixodes ricinus* ticks in Europe, for example, are vectors for TBE virus, anaplasmosis, and *Borrelia* sp. and *Ixodes scapularis* ticks in the United States can transmit POW virus, *Babesia microti,* and *Borrelia burgdorferi*. Dual human infections have been reported, possibly occurring from a single, dually infected tick or after simultaneous exposure to more than one tick, each infected with a different agent (39).

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Hantaviruses

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TAXONOMY

Hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS, also referred to as hantavirus cardiopulmonary syndrome) are rodent-borne zoonoses caused by certain members of the virus family *Bunyaviridae,* genus *Hantavirus*. This genus presently comprises 22 species (68). Ten of these species have been causally associated with human disease. (The terms "species," "strain," and "hantavirus" have been used synonymously in the literature.)

Specific rodents (i.e., one or two closely related members of the order Rodentia, family Muridae) are the principal hosts of the hantaviral species for which natural host relationships have been well characterized. Hantaviruses are divided into three groups based upon the taxonomic assignment of their principal host(s) into one of three subfamilies within the Muridae: Murinae (Old World rats and mice), Arvicolinae (voles and lemmings), and Sigmodontinae (New World rats and mice). Murine rodents are the principal hosts of hantaviral species that are associated with severe HFRS (Table 1). Voles are the principal hosts of *Puumala virus,* which is the cause of a relatively mild form of HFRS called nephropathia epidemica. Sigmodontine rodents are the principal hosts of the hantaviral species known to cause HPS (Table 1). Many hantaviruses, particularly those associated with rodents of the subfamily Arvicolinae, have not been associated with human disease.

The degree of genetic and antigenic relatedness among hantaviral species typically correlates with the degree of (phylo-) genetic relatedness among their respective rodent hosts. This observation has been attributed to virus-host coevolution or codivergence (66, 67, 81).

DESCRIPTION OF THE AGENT(S)

Hantavirions are pleomorphic, average 100 nm in diameter, and possess a lipid-bilayer envelope (87). The envelope displays a grid-like pattern apparent when negatively stained virions are viewed by electron microscopy (Fig. 1) (64). Protruding from the lipid envelope are spikes, approximately 6 nm in length, which are formed from the virus glycoproteins, G1 and G2 (87). The virion interior contains ribonucleocapsids—segments of single-stranded genomic RNA complexed with nucleocapsid protein and L protein (an RNA-dependent RNA polymerase). The genomes of hantaviruses consist of three unique negative-sense, single-stranded RNA molecules, designated L (large, approximately 6.5 kb long), M (medium, 3.6 to 3.7 kb), and S (small, 1.6 to 2.0 kb) (83). These RNA molecules encode the L protein, the glycoprotein precursor (which is cotranslationally cleaved to yield the envelope glycoproteins, G1 and G2), and the nucleocapsid protein, respectively (2). The ribonucleocapsids display helical symmetry and form circular structures as a result of base-pairing by conserved, inverse complementary sequences at the termini of each genomic RNA (87).

EPIDEMIOLOGY AND TRANSMISSION

Hantaan virus, the prototypical member of the genus *Hantavirus,* is the cause of a severe form of HFRS that is endemic in Korea, China, and eastern Russia. *Dobrava-Belgrade virus* is an agent of severe HFRS in the Balkans and Greece. Saaremaa virus, a variant of *Dobrava-Belgrade virus,* is an agent of HFRS in Europe (100). *Puumala virus* is the agent of nephropathia epidemica, which is endemic in Finland, Norway, France, and other western European countries. *Seoul virus* is a cause of HFRS in the Old World and in the New World (53). The case-fatality rate of HFRS ranges from 0.2% *(Puumala virus)* to 15% *(Hantaan virus)*. Approximately 150,000 HFRS cases occur each year worldwide (18). The majority (100,000) of these cases are caused by *Hantaan virus* or *Seoul virus* and occur in China.

The viral species causally associated with HPS include *Sin Nombre virus, Bayou virus, Black Creek Canal virus, New York virus,* and Choclo virus in North America, and *Andes virus* and *Laguna Negra virus* in South America (Table 1). From May 1993 through February 2006, 496 HPS cases were documented in North America (Canada, the contiguous United States, and Panama; http://www.cdc.gov/ncidod/diseases/hanta/hps/noframes/epislides/episls.htm) and 1,427 HPS cases were reported from South America (Argentina, Bolivia, Brazil, Chile, Paraguay, and Uruguay; http://www.paho.org/common/Display.asp?Lang=E&RecID=1971). Undoubtedly, *Sin Nombre virus* and *Andes virus* are the major causes of HPS in North America and South America, respectively. Although there are far fewer HPS cases than HFRS cases each year, outbreaks of HPS usually are highly lethal, with case-fatality rates of 40 to 60%.

Humans usually become infected with hantaviruses by inhalation of aerosolized droplets of infectious urine, saliva, or respiratory secretions from infected rodents, or by inhalation

TABLE 1 Hantaviral species known to cause human disease[a,b]

Virus species	Natural rodent host(s)	Known geographical distribution	Human disease
Principally associated with Arvicolinae[c]			
Puumala virus	*Clethrionomys glareolus* (red bank vole) and other *Clethrionomys* spp.	Scandinavia, western Europe	HFRS
Principally associated with Murinae[c]			
Dobrava-Belgrade virus	*Apodemus flavicollis* (yellow-necked field mouse)	Balkans	HFRS
Hantaan virus	*Apodemus agrarius* (striped field mouse)	China, Korea, Russia	HFRS
Seoul virus	*Rattus norvegicus* (Norway rat) and *Rattus rattus* (black rat)	Worldwide	HFRS
Principally associated with Sigmodontinae[c]			
Andes virus	*Oligoryzomys longicaudatus* (long-tailed pygmy rice rat)	Argentina, Chile	HPS
Bayou virus	*Oryzomys palustris* (marsh rice rat)	United States	HPS
Black Creek Canal virus	*Sigmodon hispidus* (hispid cotton rat)	United States	HPS
Laguna Negra virus	*Calomys laucha* (vesper mouse)	Bolivia, Paraguay	HPS
New York virus	*Peromyscus leucopus* (white-footed mouse)	United States	HPS
Sin Nombre virus	*Peromyscus maniculatus* (deer mouse)	United States, Canada	HPS

[a] Other hantaviruses that have been associated with human disease include Saaremaa virus in Europe; Monongahela virus in the United States; Choclo virus in Panama; Bermejo, Lechiguanas, and Oran viruses in Argentina; and Araraquara, Castelo dos Sonhos, and Juquitiba viruses in Brazil. Sareemaa virus is a variant (strain) of *Dobrava-Belgrade virus,* Monongahela virus is a strain of *Sin Nombre virus,* and Bermejo, Lechiguanas, and Oran viruses are strains of *Andes virus.* The taxonomic status of Choclo, Araraquara, Castelo dos Sonhos, and Juquitiba viruses within the genus *Hantavirus* has not been determined; however, these four viruses have been associated with HPS. Sigmodontine rodents are the natural hosts of Choclo, Bermejo, Lechiguanas, and Oran viruses. The natural hosts of Araraquara, Castelo dos Sonhos, and Juquitiba viruses are not known.

[b] Adapted from reference 68.

[c] Arvicolinae, voles and lemmings; Murinae, Old World rats and mice; Sigmodontinae, New World rats and mice.

of aerosolized particles of feces, dust, or other organic matter contaminated with infectious virus. The aerosol transmission of hantaviruses from rodents to humans has been well documented (46, 97). Other means of infection include contamination of cutaneous injuries with infectious virus, contact of infectious materials with mucous membranes, ingestion of food contaminated with infectious rodent secretions or excretions, laboratory-acquired infections (9, 46, 52, 57, 102), and—only in the case of *Andes virus*—contact with an HPS case during the acute phase of illness (75). Note that person-to-person hantavirus transmission has never been documented in Europe, Asia, or North America.

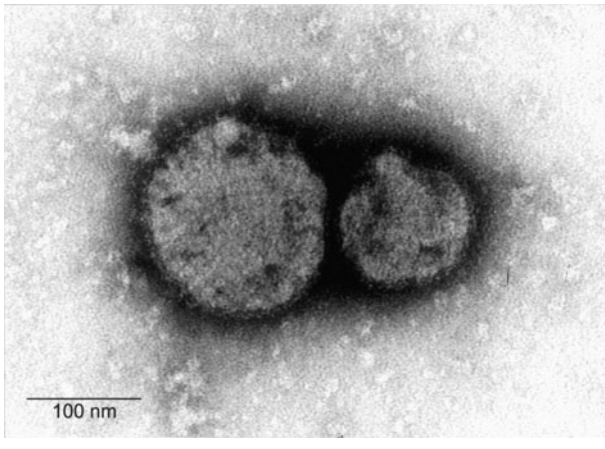


FIGURE 1 Electron micrograph of negatively stained Sin Nombre virus (2% phosphotungstic acid stain, pH 6.5). Courtesy of Charles Humphrey, Centers for Disease Control and Prevention.

In nature, the risk of infection in humans depends upon occupational or recreational activities, ecological factors which affect the abundance of infectious rodents, and other variables that affect the frequency and intensity of human exposure to infected rodents and their excreta. The cleaning of closed quarters occupied by infected rodents has been repeatedly associated with an increased risk of infection (3, 20, 65, 98).

CLINICAL CHARACTERISTICS OF THE HANTAVIRAL DISEASES OF HUMANS

The clinical features of HFRS, including nephropathia epidemica, and the clinical features of HPS were reviewed recently (24, 56). Both syndromes are associated with acute thrombocytopenia and a reversible increase in vascular (capillary) permeability. A major difference between the two syndromes is that the retroperitoneum is the major site of the vascular leak in HFRS, whereas the lungs are the major site of the vascular leak in HPS.

The length of the incubation period in HFRS and in HPS usually is 2 to 4 weeks but can range from a few days to 2 months. The clinical course of HFRS can be divided into five phases: prodrome, hypotensive, oliguric, diuretic, and convalescent. Similarly, the clinical course of HPS can be divided into four phases: prodrome, pulmonary edema and shock, diuretic, and convalescent. Death in HFRS usually is due to shock in the hypotensive or diuretic phase. Death in HPS usually is attributed to hypoxia or shock in the cardiopulmonary phase.

Nephropathia epidemica and the other forms of HFRS usually include renal and hemostatic disturbances. In severe HFRS caused by *Hantaan virus* or *Dobrava-Belgrade virus,* the prodrome usually lasts 3 to 7 days; begins with an abrupt onset of high fever, chills, headache, blurred vision, malaise,

and anorexia; and then includes severe abdominal or lumbar pain, gastrointestinal symptoms, facial flushing, petechiae, and an erythematous rash or conjunctival hemorrhage. The hypotensive phase lasts from several hours to 3 days, begins with a characteristic drop in platelet number, and is followed by defervescence and abrupt onset of hypotension, which may progress to shock and more apparent hemorrhagic manifestations. In the oliguric phase, which usually lasts 3 to 7 days, blood pressure returns to normal or becomes high, urinary output falls dramatically, concentrations of serum creatinine and blood urea nitrogen increase, and severe hemorrhage may occur. Spontaneous diuresis heralds the onset of recovery, with polyuria greater than 3 liters per day. Distinct clinical phases are less obvious in nephropathia epidemica and in HFRS caused by *Seoul virus,* and visible superficial hemorrhages usually do not occur in nephropathia epidemica (53). Pathological findings in HFRS at autopsy include enlarged, congested, hemorrhagic kidneys; retroperitoneal edema; and effusions in body cavities (59).

HPS was first recognized in 1993 as a highly fatal disease in the southwestern United States (69). The original description of HPS (21) subsequently was modified to include mild infections that do not result in radiographic evidence of pulmonary disease (47). It now is recognized that HPS sometimes may include renal impairment and, at least in South America, bleeding manifestations (8, 85). The prodrome in HPS typically lasts 3 to 5 days (range, 1 to 12 days) and is characterized by fever, myalgia, and malaise. Symptoms that may occur during the prodrome include headache, dizziness, anorexia, abdominal pain, nausea, vomiting, and diarrhea. Nonproductive cough and tachypnea usually mark the onset of pulmonary edema. Fully developed HPS is characterized by rapidly progressive (time span, 4 to 24 h) noncardiogenic pulmonary edema, hypoxemia, large volumes of pleural effusion, and cardiogenic shock. Hypotension and oliguria are the result of shock; myocardial depression may occur and contribute to shock. The diuretic phase is characterized by a rapid clearance of pulmonary edema and resolution of fever and shock. Hematologic abnormalities in HPS at hospitalization include thrombocytopenia, hemoconcentration, and the presence of large, reactive (immunoblastic) lymphocytes (48, 72). Other clinical laboratory abnormalities may include elevated levels of hepatic enzymes, hypoalbuminemia, metabolic acidosis, and, in severe cases, lactic acidosis. The gross features of HPS at autopsy have been reported previously (107) and include heavy, edematous lungs and large volumes of pleural fluid.

THERAPY

Successful management of HFRS and HPS begins with prompt recognition of the disease and hospitalization of the patient. Shock in HFRS usually can be effectively managed by judicious administration of vasopressors and intravenous fluids. Intravenous ribavirin given within the first few days of the onset of clinical disease has been shown to significantly decrease morbidity and mortality in HFRS (38). The oxygen status of an HPS patient should be closely monitored so that oxygen supplementation and mechanical ventilation can be provided if required. Because life-threatening pulmonary disease and cardiogenic shock can develop rapidly in HPS, transfer to a state-of the-art critical care facility with the capacity for extracorporeal membrane oxygenation should be an early consideration. The effect of ribavirin on the course and outcome of HPS has not yet been rigorously investigated (see "Antivirals" below).

VACCINES

Inactivated vaccines have been developed in Asia for the protection of humans against HFRS. These vaccines were prepared from the brains of suckling rats or mice or from cell cultures infected with *Hantaan virus* or *Seoul virus* (14, 58, 96, 105). Hantavax (GreenCross Vaccine Corp., Seoul, Korea) and other inactivated vaccines that have been tested in humans yielded only low levels of neutralizing antibodies at 1 year after the last vaccine dose, raising concern about the duration of protection afforded by these vaccines (12, 13, 78, 95). Optimization of vaccination schedules and advances in adjuvant technology may increase the duration of immunity elicited by inactivated vaccines. Strategies for the development of new hantavirus vaccines include recombinant viruses, alphavirus replicons, and naked DNA (35, 50).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Hantavirus-specific RNA has been detected in and infectious hantavirus has been isolated from blood, urine, and cerebrospinal fluid samples collected just after onset of clinical disease (9). Thus, blood, serum, urine, respiratory secretions, and other biological materials from HFRS and HPS patients, especially specimens collected during the acute phase of illness, should be considered potentially infectious to humans. Laboratorians should note that laboratory-acquired infections with cell culture-adapted *Hantaan virus* have occurred in individuals who have performed centrifugation of concentrated virus (84) and that dried, cell culture-grown virus maintained at room temperature has been infectious for up to 2 days (9). The U.S. Department of Health and Human Services has recommended the following precautions for the handling of biological specimens from HFRS or HPS patients: (i) potentially infectious specimens should be handled at biosafety level 2 (BSL-2) using BSL-3 practices, (ii) all procedures that could result in splatter or aerosolization of infectious materials should be done inside a certified biological safety cabinet, and (iii) isolation of infectious hantavirus from biological specimens should be carried out in a BSL-3 facility using BSL-3 practices and procedures (84). Additionally, large-scale growth and the preparation of concentrates of hantaviruses that are pathogenic for humans should be done in BSL-4 containment facilities (84). Hantaviruses are thermolabile (86) and can be inactivated by acids, alcohols, paraformaldehyde, detergents, disinfectants that disrupt lipid membranes, and UV irradiation (9, 49, 100).

Blood, serum, or plasma samples for serology may be stored at 4°C and shipped to the diagnostic laboratory on cold-packs if there is no significant delay between collection and testing. Otherwise, these specimens should be stored at −20°C or colder and shipped on dry ice. Blood, blood clots, solid tissues, and other samples intended for RNA extraction and subsequent testing by reverse transcription-PCR (RT-PCR) or virus isolation should be stored continuously at −70°C or colder and shipped on dry ice in order to preserve the integrity of the viral RNA and infectivity of the virus. Samples should be subjected to a minimum number of freeze-thaws. Samples shipped by air should be packaged, documented, and shipped in accordance with International Air Transport Association Dangerous Goods Regulations (http://www.iata.org). In the United States, ground shipments must comply with regulations issued by the U.S. Department of Transportation (49 CFR parts 171 to 178).

DIRECT DETECTION

Laboratory assays that have been used for diagnosis of hantaviral infections in humans have not been standardized, and to date, no hantavirus diagnostic assays have received approval from the U.S. Food and Drug Administration (32). Diagnostic test kits that are commercially produced in Europe are sold "for research use only" in the United States.

Microscopy

Direct electron microscopic examination of tissues is of limited diagnostic value but has been used to detect virions and viral replicative structures in postmortem samples. Electron microscopic examination of autopsy tissues from HFRS and HPS patients found that mature virions are infrequent in tissues and can be difficult to identify due to considerable polymorphism in size and shape (41, 72, 107). Structures determined to be hantaviral inclusion bodies were seen more often than intact virions (41, 107).

Antigen

Immunohistochemistry has been used to detect hantaviral antigens in formalin-fixed tissues from patients with fatal HPS (Fig. 2) (107, 108). Polyclonal antibodies (e.g., immune sera from humans, experimentally infected rabbits or rodents, or naturally infected rodents) and murine monoclonal antibodies have been used as primary antibodies in immunohistochemistry assays. Fatal HPS is associated with the widespread distribution of hantaviral antigen in lung, liver, spleen, kidney, and heart tissues, with antigen primarily localized within endothelial cells of capillaries and other small blood vessels (107). Immunohistochemistry assays for diagnosis of hantaviral infections in humans are limited to the few institutions that have access to the appropriate primary antibodies and control (comparison) tissues.

Nucleic Acid

Since the 1993 outbreak of HPS in the southwestern United States (69), RT-PCR assays have been employed extensively to detect hantaviral RNA in clinical samples from HPS and HFRS patients. Sets of oligonucleotide primers have been designed that anneal to regions of the S and M genomic segments that are highly conserved among the hantaviruses (43, 69, 77, 103). RNA can be isolated from tissues, blood, and serum and protected from ribonuclease degradation by using methods employing chaotropic salts such as guanidinium isothiocyanate (15). RT of hantaviral RNA and subsequent PCR amplification of the resulting cDNA usually can be accomplished by using a "single-tube" format in which a single set of oligonucleotide primers is used for both enzymatic reactions. Clinical specimens rarely yield an RT-PCR product that can be visualized by UV translumination of ethidium bromide-stained agarose gels; consequently, a second "nested" PCR amplification must be performed to obtain amplicons that can be detected on gels and purified for sequencing (69). The lower limit of detection for nested RT-PCR assays for hantaviruses has been reported to be less than one viral fluorescent focus unit, or approximately 316 median cell culture infectious doses of virus per 1.0 ml (26, 37). RT-PCR assays can be used to detect hantavirus RNA in fixed, paraffin-embedded tissues (89). Real-time RT-PCR assays have been developed for some hantaviruses and are in the process of being adopted for routine diagnosis (1, 26, 101). These assays match or exceed the sensitivity of nested RT-PCR assays, with less manipulation of reaction components and shorter times to detection, and can provide precise estimates of viral load in clinical samples (1, 26, 101).

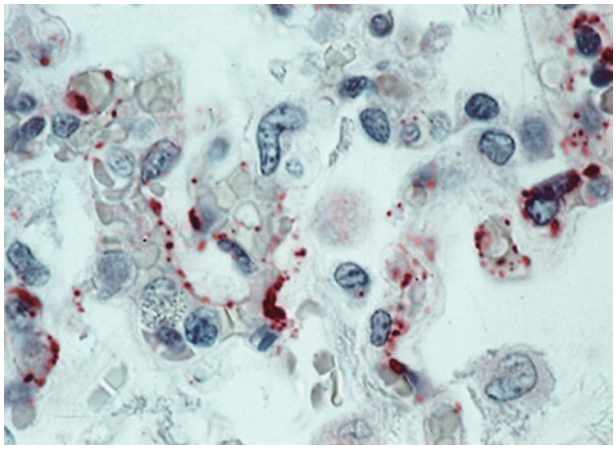


FIGURE 2 Photomicrograph of lung tissue showing hantaviral antigens in pulmonary microvasculature by using immunohistochemistry. Viral antigens are stained in red. Courtesy of Sherif Zaki, Centers for Disease Control and Prevention.

ASSAY FOR INFECTIOUS HANTAVIRUS

Compared with established RT-PCR assays for hantavirus-specific RNA, the isolation of infectious hantaviruses from blood, serum, urine, or tissues using cultured cells or live laboratory animals is substantively less sensitive and requires considerably more time. In addition, hantaviruses usually are extremely difficult to isolate from clinical materials. Thus, virus isolation is not commonly used for the diagnosis of hantaviral infections in humans.

The Vero E6 cell line (ATCC CRL-1586) has been used to isolate infectious hantaviruses from blood, solid tissue, and/or urine samples from HFRS patients and *Andes virus* from blood samples from an HPS patient. Typically, monolayer cultures of Vero E6 cells are inoculated with a crude or clarified tissue homogenate and then maintained under a fluid overlay for 10 to 14 days. Successful virus isolation may require repeated blind passages of inoculated cell culture material. Hantaviruses usually are neither cytopathic in cultured cells nor pathogenic in laboratory rodents. Consequently, detection of infection in cultured cells and in tissues of laboratory rodents often requires an indirect method, e.g., a fluorescent-antibody test for viral antigen or RT-PCR assay for hantavirus-specific RNA.

IDENTIFICATION

Historically, serological methods were used to define taxonomical relationships among the hantaviruses. Neutralization of infectivity in vitro, immunofluorescent-antibody assays (IFAs), enzyme-linked immunosorbent assays (ELISAs), and a host of other serological methods have been used to characterize hantaviruses isolated from rodents or clinical samples. The antibodies that have been used in these assays have included sera from HFRS or HPS patients (16, 54), sera from experimentally infected animals, sera from naturally infected rodents, and immune mouse ascitic fluids. Overall, serological cross-reactivity within the genus *Hantavirus* is greatest between hantaviral species principally associated with phylogenetically

closely related rodent species and usually is higher in antigen-binding assays such as IFA and ELISA than in neutralization assays. Neutralization of infectivity has been measured by plaque reduction in monolayer cultures of Vero E6 cells maintained under an overlay containing agarose and by focus reduction in monolayer cultures of Vero E6 cells maintained under an overlay containing agarose (99) or methylcellulose (4). The focus reduction neutralization test is used more widely than the plaque reduction neutralization test because some hantaviral strains do not consistently produce readily discernible plaques in monolayer cultures of cells stained with neutral red (70). Foci of infected cells (viral antigen) in the focus reduction neutralization test can be revealed by immunochemical staining (27, 91) or by chemiluminescence (30).

The use of genetic sequence data to define taxonomic relationships within the genus *Hantavirus* has become increasingly important, in part because some hantaviral species have never been adapted to growth in cultured cells and because our knowledge of the serological relationships among some of the hantaviral species is based on one strain per species. Since an RT-PCR or nested PCR product can be amplified directly from RNA isolated from clinical materials and then sequenced, analysis of sequence data (particularly from the G2 region of the glycoprotein precursor gene or the nucleocapsid protein gene) may provide the most rapid and specific means for determination of the identity of a hantavirus. Sequence data can also be used for subtyping of viruses (see next section).

TYPING SYSTEMS

Analysis of hantaviral sequence data provides the fastest and most discriminating method for hantavirus typing. An advantage of sequence data is that it is not affected by variation between different sources or lots of antibodies and antigens used in serological typing of hantaviruses (79). Additionally, sequence analysis may enable the detection of hantaviral strains that have arisen by genetic reassortment of genomic RNA segments or homologous recombination, two processes for which there is evidence in hantavirus evolution (31, 55, 92, 93). One of the criteria used to differentiate hantaviral species is at least a 7% difference in amino acid sequence identity in comparisons of complete glycoprotein precursor sequences and in comparisons of complete nucleocapsid protein sequences (68); however, there are some exceptions to this criterion (79). Analysis of sequence data also permits subtyping of hantaviruses, which often can identify geographic variants and variants associated with particular rodent-host subspecies or populations (7, 62, 80–82, 92, 101). Phylogenetic analysis of sequence data permits reconstruction of virus evolutionary trees, which have been invaluable in the establishment of virus-host relationships, virus evolutionary processes, and molecular epidemiology (34, 69, 99, 104). Microarray techniques show potential for genetic characterization of hantavirus RT-PCR products without sequencing (73).

SEROLOGY

Virtually all HFRS and HPS patients have high levels of hantavirus-specific immunoglobulin M (IgM) in serum or plasma at or soon after the onset of clinical disease (19, 42, 51, 71, 74). Measurable levels of hantavirus-specific IgM may persist as long as 6 months after the end of the acute phase of illness in nephropathia epidemica (23) and longer than 2 months after the end of the acute phase of illness in HPS (6).

Many HFRS and HPS patients have measurable levels of hantavirus-specific IgG during the acute phase of their illnesses (6, 22, 23, 42, 76). Levels of hantavirus-specific IgG increase through the end of the acute phase of illness, remain high for months to years, and then decline gradually (60). Hantavirus-specific IgG may persist as long as 10 years in HFRS cases (60) and longer than 3 years in HPS cases (106).

Neutralizing antibodies may appear during the acute phase of HFRS or HPS. A strong neutralizing antibody response early in the course of infection has been positively associated with successful recovery from HPS caused by *Sin Nombre virus* (4). High titers of neutralizing antibodies have been found in nephropathia epidemica cases 10 to 20 years after infection (36) and in HPS patients more than 3 years after infection (106).

The IgM response and IgG response in HFRS and in HPS are directed against the N protein and glycoproteins (25, 29, 33, 42, 60). The early IgM response and early IgG response are primarily directed against the N protein. The neutralizing response is directed against the glycoproteins, G1 and G2.

Antibodies (IgM and IgG) from patients infected with one hantavirus may cross-react with the nucleocapsid proteins and glycoproteins of other hantaviruses (22, 25, 40, 94). IgM and IgG in acute-phase samples can be highly reactive against the N proteins of heterologous hantaviruses (22). In contrast, IgG against G1 is more specific than IgG against the N protein (17, 42). Neutralizing antibody, even in acute- or early-convalescent-phase sera, can efficiently neutralize strains of several different hantaviral species and thus may not yield a species-specific diagnosis (61).

A variety of methods have been used for the detection of antibodies against hantaviruses in serum or plasma. These methods include high-density particle agglutination, indirect IFA, immunoprecipitation, radioimmunoassay, hemagglutination inhibition, plaque and focus reduction neutralization, Western (immuno-) blotting, μ capture (IgM capture) ELISA, and IgG ELISA. The most widely accessible serological methods for the diagnosis of hantaviral disease in humans are μ capture ELISA and IgG ELISA, which are done by many national, regional, and state public health laboratories and by some commercial reference laboratories.

The antigens used for serological assays for hantavirus-specific antibodies traditionally were prepared from cultures of virus-infected cells, concentrated virus, or tissues of naturally or experimentally infected rodents. Antigens must be rendered noninfectious prior to use, and this is usually accomplished by gamma irradiation (Co^{60} source). Heat treatment is an alternative method for making hantavirus antigens noninfectious prior to use in serological assays (86).

Recombinant nucleocapsid proteins or glycoproteins expressed in bacteria, yeasts, insect cells, or mammalian cells, as well as synthetic peptides, have been used as antigens in IFAs and ELISAs for the detection of antihantavirus antibodies (25, 28, 44, 45, 63, 88). The recombinant protein and synthetic peptide antigens can be more specific than native viral proteins in distinguishing between possible etiological agents of HFRS or HPS.

ANTIVIRALS

Presently, there are no antiviral compounds approved by the U.S. Food and Drug Administration for the prevention or treatment of HFRS or HPS. However, ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) has been shown to have activity against *Hantaan virus* (39, 90). In a double-blind, placebo-controlled study done in the People's

Republic of China, intravenous ribavirin therapy significantly reduced the morbidity and lethality of HFRS (38). In contrast, an open-label study done in the United States failed to demonstrate an appreciable effect of intravenous ribavirin therapy on the lethality of HPS (11).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Early diagnosis is critical to the successful management of HFRS and HPS and, at least in the case of HPS caused by *Andes virus,* implementation of appropriate isolation procedures to prevent virus transmission to health care providers and other humans. Diagnosis early in the course of disease is difficult because the hantaviral prodrome is similar to the prodrome of many other diseases. Fever and severe myalgia are prominent during the prodrome phase. These symptoms, especially in patients who develop thrombocytopenia, should lead health care providers in regions of endemicity to suspect hantaviral disease.

The laboratory criteria for diagnosis of HPS established by the Centers for Disease Control and Prevention (http://www.cdc.gov/ncidod/diseases/hanta/hps/noframes/phys/technicalinfoindex.htm) are as follows:

- Presence of hantavirus-specific IgM in persons who meet the case definition for HPS (see reference 10) or a fourfold or greater increase in titers of hantavirus-specific IgG in paired acute- and convalescent-phase serum samples
- Positive RT-PCR assay for hantavirus-specific RNA
- Positive assay for hantaviral antigens in lung, spleen, kidney, or other tissues

These criteria could be used for laboratory diagnosis of HFRS as well. Note that the extreme sensitivity of RT-PCR assays, especially those employing nested PCR or real-time PCR, predisposes these tests to generating false-positive results as a consequence of template contamination. Nucleic acid detection results should be run with the proper negative controls and confirmed by the results of tests for hantavirus-specific antibodies or antigen in clinical or autopsy specimens. Also note that proper controls for μ capture and IgG ELISAs (e.g., sera from laboratory-confirmed HPS cases) and immunohistochemical assays are essential for correct interpretation of the results of these assays. Most local laboratories do not have direct access to these materials. Thus, diagnostic testing is often limited to federal laboratories and to research institutions that have a specific interest in HFRS or HPS.

As noted previously (32), testing procedures for detection of hantaviral infections in humans have not been standardized. Thus, confidence in the results reported from individual laboratories varies substantially according to the tests used and the rigor of the standards for diagnosis. In an external quality control study for serological diagnosis of hantavirus infections involving 18 laboratories in Europe and Canada, only 53 and 76% of IgM- and IgG-positive samples, respectively, were diagnosed correctly (5).

The requirement for reporting HFRS and HPS varies from country to country and among local health agencies. In the United States, reporting of HPS cases is mandated at the state level. HPS is listed as a notifiable disease in the National Notifiable Diseases Surveillance System maintained by the Centers for Disease Control and Prevention, although reporting is not compulsory.

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Arenaviruses and Filoviruses*

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This chapter focuses on the viral hemorrhagic fever (VHF) viruses from two taxa, the families *Arenaviridae* (8) and *Filoviridae* (66). Some, but not all, members of these viral families cause severe, frequently fatal VHF in localized areas of the world. Although exotic in North America, these viruses are important public health problems in Africa and South America and have the potential of being introduced into the United States by travelers returning from these areas. The arenavirus and filovirus pathogens have been associated with serious nosocomial outbreaks involving health care workers and laboratory personnel. A set of guidelines, *Interim Guidance for Managing Patients with Suspected Viral Hemorrhagic Fever in U.S. Hospitals,* has been recently published (http://www.cdc.gov/ncidod/dhqp/bp_vhf_interimguidance.html). Continuing isolation of new arenavirus and filovirus subtypes from new geographic locations categorizes these agents as the causes of emerging infections, whose potential importance to public health is only recently becoming recognized (44). The similarity of initial isolation, clinical management, and viral diagnostic procedures for patients with suspected arenavirus or filovirus infections is the rationale for grouping these taxonomically distinct viruses in this chapter. Viruses from other taxonomic groups, including *Bunyaviridae* and *Flaviviridae* (chapters 97 and 98), have been associated with VHF (60).

Patients with VHF frequently present with similar, nonspecific clinical signs resembling malaria, typhoid fever, and pharyngitis. A detailed travel history, coupled with a high index of suspicion and availability of definitive virologic tools, should facilitate rapid diagnosis and the timely implementation of appropriate patient isolation, clinical management procedures, and public health measures.

TAXONOMY AND DESCRIPTION OF THE AGENTS

Arenaviridae

The family *Arenaviridae* comprises 22 named viruses which have unique morphologic and physiochemical characteristics. Antigenic relationships are established mainly on the basis of broadly reactive antibody binding assays, historically the complement fixation (CF) test (9), the indirect fluorescent-antibody (IFA) test (82), and the enzyme-linked immunosorbent assay (ELISA). Both serologic and phylogenetic analyses of viral RNA divide the arenaviruses into two complexes. The lymphocytic choriomeningitis (LCM) or Old World complex contains LCM virus and the Lassa viruses, including a number of apparently benign Lassa-like viruses from Mozambique, Zimbabwe, and the Central African Republic. On the basis of RNA sequence identities, these are unique viruses rather than substrains of Lassa virus. All have been isolated from rodents of the family *Muridae.* The Tacaribe or New World complex includes Tacaribe, Junin, Machupo, Amapari, Cupixi (8), Parana, Latino, Pichinde, Tamiami, Flexal, Guanarito, Sabia, Oliveros, Whitewater Arroyo, Pirital, and Bear Canyon (20) viruses. Chapare virus is a new arenavirus isolated from a human case in Bolivia; the reservoir is still unknown (S. Olson and our unpublished data). All New World complex viruses have been isolated from rodents of the family *Muridae* subfamily *Sigmodontinae* or, for Tacaribe virus, from bats. The Old and New World arenavirus complexes are distantly related; only when very-high-titer antisera are used can cross-reactions be observed. Monoclonal antibodies with specificities for structural proteins of arenaviruses suggest that the N protein is the group-reactive determinant whereas the envelope glycoproteins (G1 and G2) are responsible for type specificity (6, 7).

The morphology of arenaviruses is distinctive in thin-section electron microscopy (55) and was the basis for first associating LCM virus with Machupo virus and ultimately associating these viruses with all the viruses in the present family. Three major virion structural proteins are usually found (7). Two are glycosylated, G1 (50,000 to 72,000 Da) and G2 (31,000 to 41,000 Da), which constitute the virion envelope and spikes and which both serve as highly type-specific neutralization targets. The third, the N protein (63,000 to 72,000 Da), is clearly associated with the virion RNA and is considered to be the nucleocapsid protein.

Four RNA species with distinct oligonucleotide fingerprints can be isolated from intact arenavirus virions. Two species are virus specific and are ambisense in coding strategy: the small (S) RNA (22S), which encodes the N and GPC, the precursor of G1 and G2; and the large (L) RNA (31S), which codes for the viral polymerase and the Z protein, a regulatory element (8). The 28S and 18S species,

*This chapter contains information presented in chapter 104 by Peter B. Jahrling, Stuart T. Nichol, Pierre E. Rollin, and Thomas G. Ksiazek in the eighth edition of this Manual.

isolated in different proportions depending on external conditions, are ribosomal.

Arenaviruses mature by budding at the cytoplasmic membrane, and host proteins are incorporated into the virion envelope. Vero cells infected with each of the viruses contain distinctive intracytoplasmic inclusion bodies, immunoreactive with anti-N but not anti-G1 or anti-G2 antibody. All arenaviruses are readily inactivated by ethyl ether, chloroform, sodium deoxycholate, and acidic media (pH less than 5). β-Propiolactone (74) and gamma irradiation (19) are both reported to inactivate arenavirus infectivity while preserving reactivity in standard serologic tests.

Filoviridae

The filoviruses, Marburg and Ebola viruses, have a common morphology and similar genomic organization and complement of structural proteins (39). Marburg and Ebola virion RNAs are nonsegmented, negative, and single-stranded RNAs, 19.1 kb long, and 4.0×10^6 to 4.5×10^6 Da. The viral genomes are linearly arranged in a manner consistent with other nonsegmented, negative, and single-stranded RNA viruses. A substantial degree of similarity to the paramyxoviruses, especially in the nucleocapsid and polymerase genes, was noted. However, comparison with other filovirus protein sequences confirms that filoviruses are distinct. Furthermore, filoviruses are sufficiently distinct by ultrastructural and serologic criteria to warrant separate taxonomic status as members of the family *Filoviridae* (40, 66).

Marburg and Ebola viruses have at least seven virus-specific structural proteins, expressed from seven genes (66). For Ebola virus, the ribonucleoprotein complex contains L (180 kDa), N (104 kDa), VP30 (30 kDa), and VP35 (35 kDa) in loose association. L is an RNA-dependent RNA polymerase, and VP35 may play a role similar to that of the P protein of paramyxoviruses and rhabdoviruses. GP (125 kDa) is the major spike protein; VP40 (a matrix protein) and VP24 make up the remaining protein content of the multilayered envelope (40, 67). The GP of Ebola virus and that of Marburg virus can be differentiated by the presence or absence of N- and O-linked glycans and by the lack in Marburg virus of the second open reading frame coding for the small/soluble glycoprotein expressed during Ebola infection in vitro and in vivo. When grown in Vero (or Vero E6) or MA-104 cells, the GP of Marburg virus is totally devoid of terminal sialic acid whereas that of Ebola virus has abundant (2–3)-linked sialic acid. Phylogenetic analysis of GP genes from filoviruses clearly separates Marburg and Ebola viruses into two genera, *Ebolavirus* and *Marburgvirus,* and, furthermore, defines one species for Marburg virus (70) and four for Ebola virus: Zaire Ebolavirus, Sudan Ebolavirus, Reston Ebolavirus, and Tai Forest Ebolavirus (formerly known as Ivory Coast Ebolavirus).

Despite their unusual morphologic properties, Marburg and Ebola viruses resemble the other lipid-enveloped viruses, including the arenaviruses, in being susceptible to heat, lipid solvents, β-propiolactone (74), formaldehyde, UV light, and gamma radiation (19). These viruses are stable at room temperature for several hours but are inactivated by incubation at 60°C for 1 h.

EPIDEMIOLOGY AND TRANSMISSION

Arenaviridae

Arenaviruses are maintained in nature by association with specific rodent hosts (Table 1), in which they produce chronic viremia and/or viruria; they are routinely isolated from blood and urine samples. Naturally occurring human disease can usually be traced to direct or indirect contact with infected rodents. Aerosol infectivity is thought to be an important natural route of infection as well. Attempts to implicate arthropod vectors have been unsuccessful, but ectoparasites taken from viremic mammalian hosts have occasionally yielded arenavirus isolates. Of the 22 named members of the family *Arenaviridae* (8, 20), 7 are known to be human pathogens.

Filoviridae

Marburg and Ebola hemorrhagic fevers are caused by taxonomically distinct viruses which form two genera, *Ebolavirus* and *Marburgvirus,* within the *Filoviridae* (66). Marburg virus was first recognized in 1967, when 31 persons in Germany and Yugoslavia became infected following contact with monkey kidneys, primary tissue cultures derived from monkeys imported from Uganda, or sick patients (51). Ebola virus first emerged in two major disease outbreaks, which occurred almost simultaneously in Zaire and Sudan in 1976. Over 500 cases were reported, with case-fatality rates of 88% in Zaire and 53% in Sudan. Despite the simultaneous emergence of Ebola virus species from Zaire (Ebolavirus Zaire EBO-Z) and Sudan (Ebolavirus Sudan EBO-S) in 1976, these isolates are distinct species by serologic and sequence analysis criteria (71). More recently, two additional, distinct, and previously unrecognized Ebola virus species have been isolated. In 1989, a new strain of Ebola virus was isolated from cynomolgus monkeys being held in quarantine in Reston, Va., following importation from the Philippines (33). This species (Ebolavirus Reston EBO-R) may be less virulent for humans than are the African filovirus strains, since four workers at the quarantine facility seroconverted to EBO-R without experiencing disease. EBO-R reappeared in monkeys exported from the Philippines to Siena, Italy, in 1992 and to Alice, Tex., in 1996 (62). In 1994, another genetically distinct species of Ebola virus (Ebolavirus Ivory Coast) was associated with disease in a human and deaths among chimpanzees in the Ivory Coast (45). In 1995, EBO-Z reappeared in Zaire and disappeared without a trace, although subsequently (1996, 2001 to 2005) a number of outbreaks have occurred in Gabon and adjacent areas of the Republic of Congo among apes and humans, some human cases being associated with exposure to infected nonhuman primates (25, 63, 76). In 2000, EBO-S was responsible for a large outbreak in Gulu, in the northwest of Uganda. Approximatively 425 persons were infected, and 53% of them died (13, 58). In Yambio (south Sudan) in 2004, 17 persons were infected by EBO-S, and 7 of them died (77). An outbreak due to Marburg virus started in 1999 and continued into 2000 in eastern Democratic Republic of Congo (76). In the spring of 2005, a large outbreak of Marburg virus occurred in Uige, northern Angola. A total of 252 cases and 227 deaths from Marburg hemorrhagic fever were reported as of 24 August 2005 (78, 79). Reservoirs of filoviruses have remained elusive despite extensive searches during and between outbreaks. Recently, fruit bats have been reported as a potential reservoir for Ebola virus (47).

CLINICAL SIGNIFICANCE

Arenaviridae

Among the known arenavirus pathogens, LCM virus produces the least severe infection (46). A modest proportion of LCM infections are subclinical. A "typical" LCM case is

TABLE 1 Currently recognized arenaviruses and filoviruses and associated human diseases

Virus, date isolated	Natural host	Geographic distribution	Naturally occurring human disease	Human laboratory infections
Old World arenaviruses				
LCM, 1933	*Mus musculus* (house mouse)	Americas, Europe	Undifferentiated febrile illness, aseptic meningitis; rarely serious	Common; usually mild, but 5 were fatal
Lassa, 1969	*Mastomys* sp. (multimammate rat)	West Africa	Lassa fever; mild to severe and fatal disease	Common; often severe
Mopeia, 1977	*Mastomys natalensis*	Mozambique, Zimbabwe	Unknown	None; little experience
Mobala, 1983	*Praomys* sp.	Central African Republic	Unknown	None; little experience
Ippy, 1984	*Arvicanthus* sp.	Central African Republic	Unknown	None
New World arenaviruses				
Tacaribe, 1956	*Artibeus* sp. bats	Trinidad, West Indies	None detected	One suspected; moderately symptomatic
Junin, 1958	*Calomys musculinus*	Argentina	AHF	Common; often severe
Machupo, 1963	*Calomys callosus*	Bolivia	BHF	Common; often severe
Cupixi, 1965	*Oryzomys gaeldi*	Brazil	None detected	None detected
Amapari,1965	*Neacomys guianae*	Brazil	None detected	None detected
Parana, 1970	*Oryzomys buccinatus*	Paraguay	None detected	None detected
Tamiami, 1970	*Sigmodon hispidus* (cotton rat)	Florida	Antibodies detected	None detected
Pichinde, 1971	*Oryzomys albigularis*	Colombia	None detected	Occasional; mild to asymptomatic
Latino, 1973	*Calomys callosus*	Bolivia	None detected	None detected
Flexal, 1977	*Oryzomys* spp.	Brazil	None detected	One recognized (severe)
Guanarito, 1989	*Zygodontomys brevicauda*	Venezuela	Venezuelan hemorrhagic fever	None detected
Sabia, 1993	Unknown	Brazil	Viral hemorrhagic fever	Two recognized (both severe)
Oliveros, 1996	*Bolomys obscuris*	Argentina	None detected	None detected
Whitewater Arroyo, 1997	*Neotoma* spp.	New Mexico, Oklahoma, Utah, California	None detected	None detected
Pirital, 1997	*Sigmodon alstoni*	Venezuela	None detected	None detected
Bear Canyon, 2002	*Peromyscus californicus*	California	None detected	None detected
Filoviruses				
Marburg, 1967	Unknown	Imported in Germany and Yugoslavia; Kenya, Zimbabwe, Democratic Republic of Congo, Angola	Fatal cases	Yes
Zaire Ebola, 1976	Unknown, bats?	Democratic Republic of Congo, Gabon, Republic of Congo	Fatal cases	Yes
Sudan Ebola, 1976	Unknown	Sudan, Uganda	Fatal cases	None detected
Reston Ebola, 1989	Unknown	Philippines, imported in United States and Italy	Fatal asymptomatic cases	Seroconversion in animal handlers
Tai Forest Ebola, 1994	Unknown	Ivory Coast	Febrile illness, survived	None detected

usually heralded by fever, myalgia, retro-orbital headache, weakness, and anorexia. Especially during the first week, prominent symptoms include sore throat, chills, vomiting, cough, retrosternal pain, and arthralgia. Rash occurs but is infrequent. In about one-third of the patients, fever recurs, coinciding with the onset of frank neurologic involvement, usually aseptic meningitis but less frequently meningoencephalitis. Complete recovery is almost always the rule. Thus, LCM infections are temporarily debilitating but rarely fatal, even when neurologic complications arise. Although recognized only recently, in utero infections with LCM virus are a cause of significant birth defects of the central nervous and ocular systems (53, 80). Fatal forms of LCM infections were reported recently following transplantation of organs from two distinct LCM virus-infected donors (14, 59).

Lassa, Junin, and Machupo virus infections are much more severe. Lassa fever patients usually present at the hospital within 5 to 7 days of onset and complain of sore throat,

severe lower back pain, and conjunctivitis. Despite the usual antimalaria treatment, these symptoms usually increase in severity during the following week and are accompanied by nausea, vomiting, diarrhea, chest and abdominal pain, headache, cough, dizziness, and tinnitus. Later, pneumonitis and pleural and pericardial effusions with friction rub frequently occur. A maculopapular rash may develop, but frank hemorrhage is seen in only a proportion of the more severe cases. Oozing from puncture sites and mucous membranes and melena are more common. Approximately 15 to 20% of hospitalized patients die. Death occurs as a result of sudden cardiovascular collapse resulting from hepatic, pulmonary, and myocardial damage. Few Lassa fever patients develop central nervous system signs, although tinnitus or deafness may develop as recovery begins. Lassa fever is a particularly severe disease among pregnant women, for whom mortality rates are somewhat higher. The disease course in children is similar to that in adults, but in infants a condition described as swollen-baby syndrome, characterized by anasarca, abdominal distension, and bleeding, is typical. Clinical laboratory studies are not usually helpful for Lassa fever; specific virologic testing is required, especially in a setting where Lassa fever is less common or where mild, atypical cases are occurring.

The clinical pictures for Argentinian hemorrhagic fever (AHF) due to Junin virus and Bolivian hemorrhagic fever (BHF) due to Machupo virus are well characterized; for Venezuelan hemorrhagic fever due to Guanarito virus, Sabia virus in Brazil, or, more recently, Chapare virus in Bolivia, less information is available (60). However, all these infections are sufficiently similar to each other to be discussed as a single entity, i.e., South American or New World Arenavirus hemorrhagic fevers. Incubation periods range from 7 to 14 days, and very few subclinical cases are thought to occur. Following gradual onset of fever, anorexia, and malaise over several days, constitutional signs involving the gastrointestinal, cardiovascular, and central nervous systems become apparent by the time patients present to the hospital. On initial examination, AHF and BHF patients are febrile, acutely ill, and mildly hypotensive. They frequently complain of back pain, epigastric pain, headache, retroorbital pain, photophobia, dizziness, constipation or diarrhea, and coughing. Flushing of the face, neck, and chest and bleeding from the gums are common. Enanthem is almost invariably present; petechiae or tiny vesicles spread over the erythematous palate and fauces. Neurologic involvement, ranging from mild irritability and lethargy to abnormalities in gait, tremors of the upper extremities, and, in severely ill patients, coma, delirium, and convulsions, occurs in more than one-half of the patients. During the second week of illness, clinical improvement may begin or complications may develop. Complications include extensive petechial hemorrhages, oozing from puncture wounds, melena, and hematemesis. These manifestations of capillary damage and thrombocytopenia do not result in life-threatening blood loss. However, hypotension and shock may develop, often in combination with serious neurologic signs, among the 15% of patients who eventually die. Survivors begin to show improvement by the third week. Recovery is slow; weakness, fatigue, and mental difficulties may last for weeks, and a significant proportion of patients relapse with a "late neurologic syndrome," which includes headache, cerebellar tremor, and cranial nerve palsies. In contrast to Lassa fever, clinical laboratory studies are frequently useful. Total leukocyte counts usually fall to 1,000 to 2,000 cells/mm^3, although the differential remains normal. Platelet counts fall precipitously, usually to between 25,000 and 100,000/mm^3. Routine clotting parameters are usually normal or slightly abnormal; however, patients with severe cases may show evidence of disseminated intravascular coagulation.

Filoviridae

Marburg and Ebola virus infections are clinically similar, although the frequencies of reported signs and symptoms vary among individuals. Following incubation periods of 4 to 16 days, onset is sudden and is marked by fever, chills, headache, anorexia, and myalgia. These signs are soon followed by nausea, vomiting, sore throat, abdominal pain, and diarrhea. When first examined, patients are usually overtly ill, dehydrated, apathetic, and disoriented. Pharyngeal and conjunctival injection is usual. Within several days, a characteristic maculopapular rash over the trunk, petechiae, and mucous membrane hemorrhages appear. Gastrointestinal bleeding, accompanied by intense epigastric pain, is common, as are petechiae and bleeding from puncture wounds and mucous membranes. Shock develops shortly before death, often 6 to 16 days after the onset of illness. Abnormalities in coagulation parameters include fibrin split products and prolonged prothrombin and partial thromboplastin times, suggesting that disseminated intravascular coagulation is a terminal event and is usually associated with multiorgan failure. Clinical laboratory studies usually reveal profound leukopenia early, sometimes moderately elevated at a later stage. Platelet counts decline to 50,000 to 100,000/mm^3 during the hemorrhagic phase.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Safety and Security

Arenaviruses and filoviruses are classified as biosafety level 4 (BSL-4) agents, as there is a high risk of infection of laboratory personnel and appropriate precautions must be taken (12). They are also in the United States classified as Select Agents by the U.S. Department of Health and Human Services and should be handled as such (see chapter 9 and http://selectagents.gov/formsoverview.htm). At a minimum, barrier nursing procedures should be implemented and personnel caring for the patient and handling diagnostic specimens should wear disposable caps, gowns, shoe covers, surgical gloves, and face masks (preferably full-face respirators equipped with high-efficiency particulate air [HEPA] filters) (10, 11; http://www.cdc.gov/ncidod/dhqp/bp_vhf_interimguidance.html). Gloves should be disinfected immediately if they come in direct contact with infected blood or secretions. Manipulation of these specimens and tissues, including sera obtained from convalescent patients, may pose a serious biohazard and should be minimized outside a BSL-4 laboratory (10–12). Use of Vacutainer tubes is considered safer than use of syringes and needles, which must be disassembled before their contents are transferred to another tube. Procedures which generate aerosols (e.g., centrifugation) should be minimized and performed only if additional protective measures, such as keeping the equipment in a class I or II laminar flow hood, are taken. For specialized procedures, the infectivity of samples may be greatly reduced, if not totally inactivated, by the addition of Triton X-100 and heating. Heating to 60°C for 1 h renders diagnostic specimens noninfectious and is acceptable for measurement of heat-stable substances such as electrolytes,

blood urea nitrogen, and creatinine. When the equipment is available, sterilization by ^{60}Co γ-irradiation is the preferred method. Extraction of RNA from infectious samples by using guanidinium thiocyanate (with a proper ratio, at least 1 to 5 or 1 to 10 of lysis reagents) should be conducted in a laminar-flow hood, but after extraction, the extracted RNA is no longer infectious.

Specimen Collection

For virus isolation, antigen detection, and reverse transcription (RT)-PCR, serum, heparinized plasma, or whole blood should be collected during the acute, febrile stages of illness and frozen on dry ice or in liquid nitrogen vapor. Storage at higher temperatures (above −40°C) leads to rapid losses in infectivity. Blood obtained in early convalescence for serodiagnosis may be infectious despite the presence of antibodies, and it should be handled accordingly (10–12). In addition to blood samples, throat wash specimens could also be used for virus isolation during infections with arenaviruses (Lassa, Junin, and Machupo). LCM virus may be recovered from acute-phase serum samples obtained during the first week after onset or from cerebrospinal fluid during the period of meningeal involvement and from the brain at autopsy but is rarely, if ever, recovered from throat washings or urine specimens. Marburg and Ebola viruses are usually recovered from acute-phase serum samples; various specimens including throat washings, saliva, urine, soft tissue effusions, semen, and anterior eye fluid have yielded these viruses, even when the specimens were obtained late in convalescence.

Postmortem Specimens

Lassa, Machupo, Junin, Marburg, and Ebola viruses are all readily isolated from specimens of spleen, lymph nodes, liver, and kidney obtained at autopsy but rarely, if ever, from brain or other central nervous system tissues. Notably, Lassa virus is usually isolated from the placentas of infected pregnant women. In the recent severe and fatal clinical forms observed after organ transplants, LCM virus was isolated from several organs at autopsy. Formaldehyde-fixed tissues and paraffin-embedded blocks are also suitable for histopathology and immunohistochemical identification of viral antigens and should be conserved and shipped at room temperature.

Shipping

For all testing of infectious material, samples should be packaged in accordance with current recommendations (International Air Transport Association; http://www.iata.org/index.htm) and forwarded, after consultation, to one of the following laboratories that maintain BSL-4 facilities and a diagnostic capability for these agents:

1. Centers for Disease Control and Prevention, Special Pathogens Branch, Division of Viral and Rickettsial Diseases, Center for Infectious Diseases, Atlanta, GA 30333. Phone: (404) 639-1115. Fax: (404) 639-1118.
2. U.S. Army Medical Research Institute of Infectious Diseases, Headquarters, Fort Detrick, Frederick, MD 21702-5011. Phone: (301) 619-2833. Fax: (301) 610-4625.
3. Center for Applied Microbiology and Research, Special Pathogens Unit, Salisbury, Porton Down, Wiltshire SP4 0JG England. Phone: 0980-612224. Fax: 0980-611310.
4. National Institute for Communicable Diseases, Private Bag X4, Sandringham, Johannesburg, South Africa. Phone: 27 (11) 386 6336. Fax: 27 (11) 882 3741.

DIRECT EXAMINATION

Electron Microscopy

Individual arenavirus virions are pleomorphic and range in size from 60 to 280 nm (mean, 110 to 130 nm) (Fig. 1A). A unit membrane envelops the structure and is covered with club-shaped, 10-nm projections. No symmetry has been discerned. The most prominent and distinctive feature of these virions is the presence of different numbers of electron-dense particles (usually 2 to 10), which may be connected by fine filaments. These particles, 20 to 25 nm in diameter, are identical to host cell ribosomes by biochemical and oligonucleotide analysis. Immunoelectron microscopy techniques also work well for diagnosis of arenavirus infections, although the morphology of the virions is less striking for arenaviruses than filoviruses.

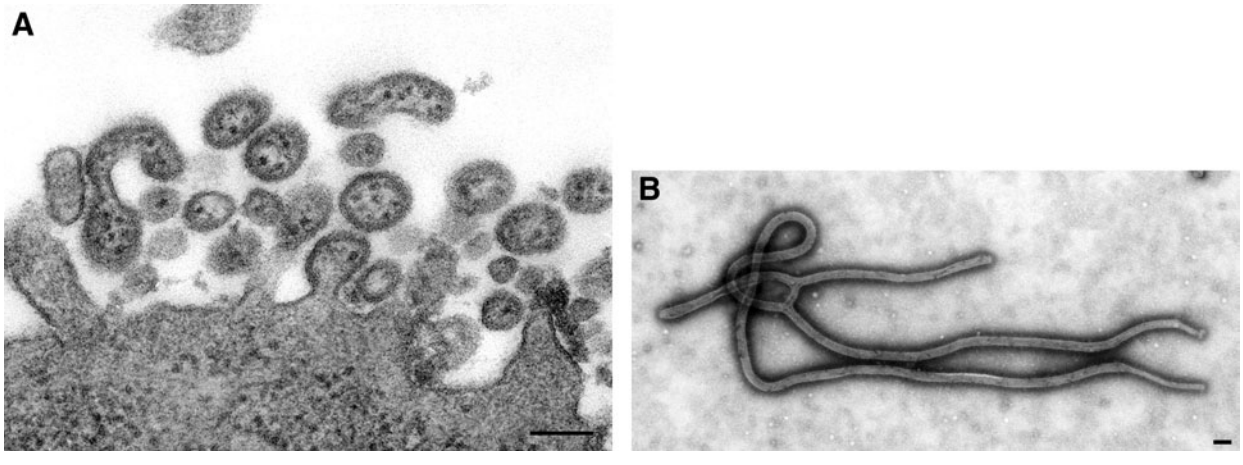


FIGURE 1 Ultrastructural characteristics of LCM and Ebola viruses as seen in tissue culture cells. (A) Lymphocytic choriomeningitis virus, an arenavirus, showing pleomorphic enveloped particles with internal ribosome-like granules; scale bar, 100 nm. (Courtesy of C. S. Goldsmith.) (B) Ebola virus isolate, a filovirus, showing enveloped filamentous particles around 80 nm wide. Some filaments can occasionally measure up to 15,000 nm in length; scale bar, 100 nm. (Courtesy of C. S. Goldsmith.)

Marburg and Ebola viruses have been successfully visualized directly by electron microscopy both of heparinized blood and urine obtained during the febrile period and of tissue culture supernatant fluids. The combination of the size and shape of the virions is sufficiently characteristic to allow a morphologic diagnosis of filovirus (21, 33, 54). The viral particles are very large, typically 790 to 970 nm long and consistently 80 nm in diameter. Bizarre structures of widely different lengths, sometimes exceeding 14,000 nm, as well as branching, circular, or "6" shapes, probably resulting from coenvelopment of multiple nucleocapsids during budding, are frequently found in negatively stained preparations (Fig. 1B) (21, 54).

Several Ebola viral inclusions are seen in thin-section electron micrographs of liver. The inclusions consist of viral nucleocapsids seen mostly in longitudinal section. Numerous viral particles are also seen in sinusoidal spaces (Fig. 2B). These virions can be differentiated and identified as Marburg or Ebola virus by immunoelectron microscopy techniques (22–24).

Antigen Detection

Immunohistochemistry

IFA staining of impression smears or air-dried suspensions of the liver, spleen, or kidney has been used successfully to detect cytoplasmic inclusion bodies associated with Marburg virus infection; clumps of Marburg virus antigen have also been observed by IFA examination of infected, dried, citrated blood smears. This approach was successfully adapted to the diagnosis of EBO-R in impression smears from blood, tissues, and nasal turbinates, as it was to the detection of Junin virus-infected cells in peripheral blood and urinary sediment. Hematoxylin and eosin staining of the liver reveals hepatocellular necrosis and numerous intracytoplasmic, eosinophilic Ebola viral inclusions, as well as sinusoidal dilatation and congestion (Fig. 2A). The development of immunohistochemical techniques for detection of filovirus and arenavirus antigens in formalin-fixed tissues has recently advanced to the point that their results are far more satisfactory than those of IFA examination of frozen, acetone-fixed sections (15, 16, 32, 83). Obtaining frozen sections for diagnosis is rarely worth the biohazard incurred, especially since the threshold sensitivity for detection exceeds 6 $\log_{10}$ PFU per g. For filoviruses, paraffin blocks of tissues are sectioned. Sections are deparaffinized, hydrated, digested with protease, and stained for the presence of viral antigens with immune rabbit serum or cocktails of murine monoclonal antibodies (32, 33, 83). Biotinylated antiserum is then allowed to react, and the product is developed with a streptavidin-alkaline phosphatase system. Substitution of other chromogens can further increase sensitivity while reducing background (15). Remarkable success was reported in the application of immunohistochemistry to the demonstration of Ebola virus

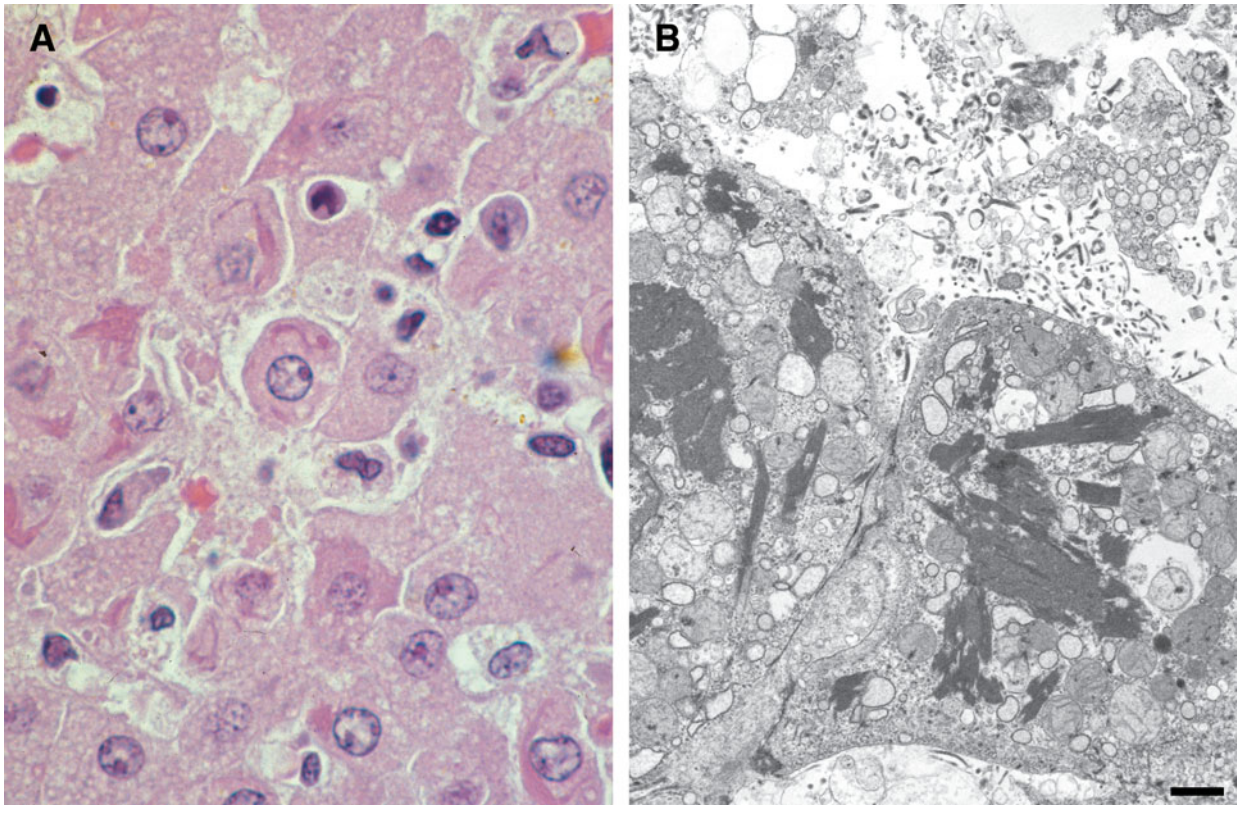


FIGURE 2 Ebola virus hemorrhagic fever. (A) Section of liver showing hepatocellular necrosis and numerous intracytoplasmic, eosinophilic Ebola viral inclusions, as well as sinusoidal dilatation and congestion. Hematoxylin and eosin stain; original magnification, ×250. (B) Several Ebola viral inclusions are seen in a thin-section electron micrograph of liver. The inclusions consist of viral nucleocapsids seen mostly in longitudinal section. Numerous viral particles are also seen in sinusoidal spaces; scale bar, 100 nm.

antigens in formalin-fixed skin biopsy specimens obtained from deceased Ebola virus patients in Zaire in 1995 (61, 83) (Fig. 3B). Immunohistochemistry was recently used to confirm the diagnosis of a fatal Lassa fever in a New Jersey patient returning from West Africa (Fig. 3A).

Antigen Capture ELISA

The recent development of an antigen capture ELISA for quantitative detection of arenavirus and filovirus antigens in viremic sera and tissue culture supernatants has facilitated the early detection and identification of these agents (2, 41, 42, 57). These tests reliably detect antigens in samples inactivated by either β-propiolactone or irradiation; therefore, they can be conducted safely without elaborate containment facilities. The threshold sensitivities for these assays are approximately 2.1 to 2.5 $\log_{10}$ PFU per ml, and so they are sufficiently sensitive to detect antigen in most acute-phase VHF viremias and to detect viruses at the concentrations present in throat wash and urine samples. Substitution of one or several monoclonal antibodies of high avidity and appropriate specificities for polyclonal sera (mostly against nucleoprotein and VP40) generally increases the sensitivities and specificities of these antigen capture ELISAs (30, 50, 56, 57, 65).

Based on the same capture principles, a rapid, colorimetric, column immunofiltration assay for the direct detection of Ebola antigen has been used in the field for the first time during an Ebola outbreak in the Republic of Congo (26).

Nucleic Acid Detection

RT-PCR followed by genome analysis is rapidly replacing identification methods based on serologic criteria and has the advantage of complete inactivation of the samples in the first extraction step. However, as RT-PCR is more prone to cross-contamination problems, we would still encourage its use in combination with traditional methods (virus isolation and/or serology). Real-time PCR combined with classical cell culture could also be used for testing antivirals against Lassa and Ebola viruses (27).

Arenaviridae

RT-PCR has also been evaluated in a small series of serum samples from Lassa fever patients in West Africa (17, 73). The sensitivity of PCR relative to conventional viral isolation

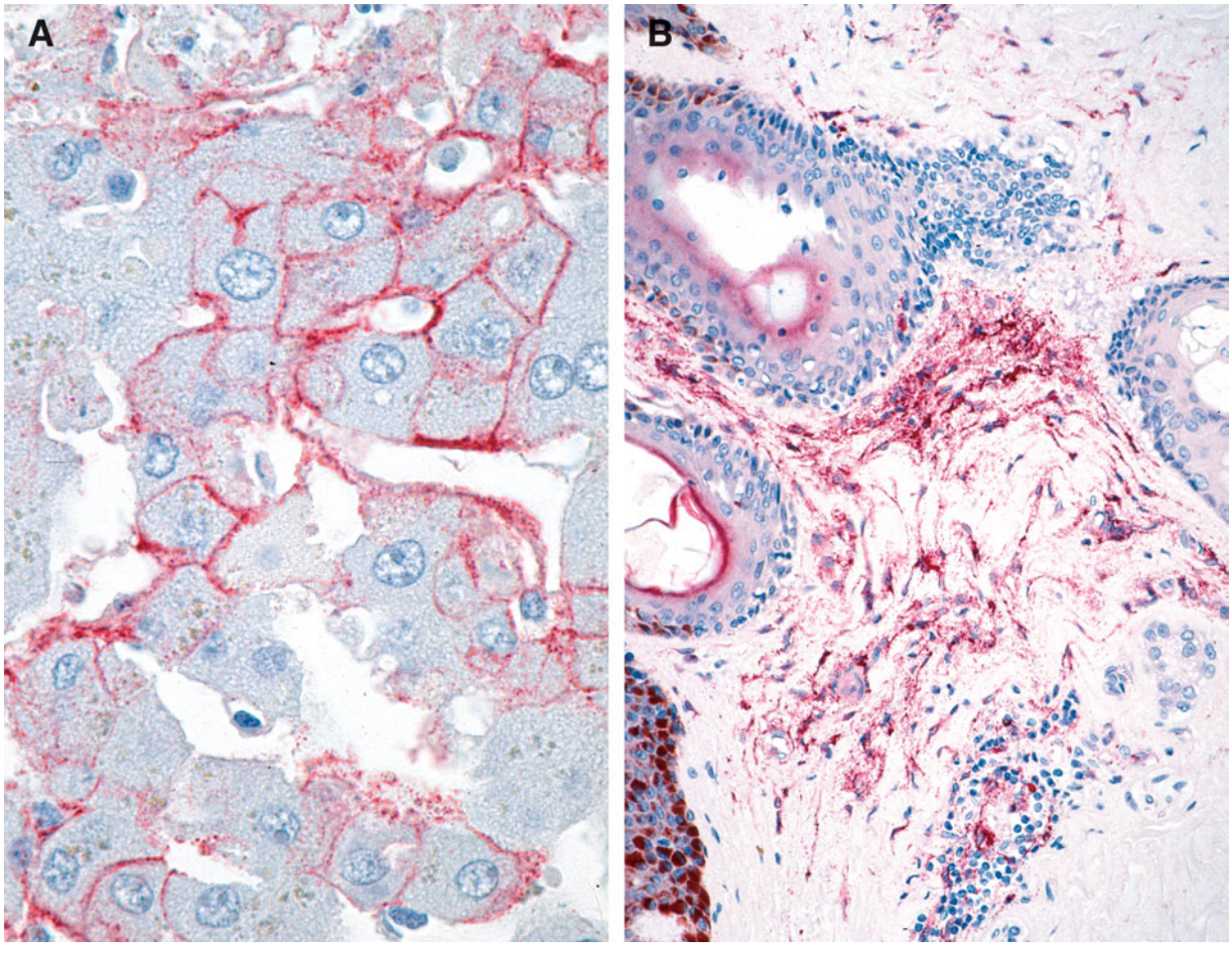


FIGURE 3 (A) By use of immunohistochemistry, abundant Lassa virus antigens are seen within the cytoplasm of hepatocytes and sinusoidal lining cells. Original magnification, ×158. (B) Skin showing massive viral burden as seen in this section immunostained for Ebola antigens; original magnification, ×50. (Immunoalkaline phosphatase staining, Naphthol fast red substrate with light hematoxylin counterstain.)

was 0.82, and the specificity was 0.68. PCR was positive throughout more of the disease course than was isolation, perhaps because of its greater sensitivity in the presence of the early antibodies associated with acute Lassa fever. Although systematic comparison with other techniques has not been made, improvement in the technique has occurred and suggests that considerable genetic variation among Lassa viruses requires the need for caution in primer design if diagnosis of infections in more than one geographic area is being considered (3–5). Real-time PCR assays are available for Lassa fever diagnostic but still lack field testing. Likewise, various strategies for RT-PCR have been devised to detect Junin virus RNA in clinical materials (3, 4, 48, 49). Some of these are reputed to be far more sensitive than conventional isolation procedures, especially in the presence of antibody. Any method which promises to be a reliable substitute for procedures that entail the manipulation of infectious BSL-4 virus deserves attention.

Filoviridae

Protocols which maximize detection are usually based on conserved sequences within the N, VP35, or L genes, while fine discrimination and phylogeny are based on the more variable GP region (68, 75). The sensitivity of RT-PCR for Ebola virus strains is similar to that of conventional isolation. During the EBO-S outbreak in Gulu, the RT-PCR-based assay was very valuable because of its ability to identify some patients prior to identification by any other available test and after clearing detectable antigen. Using NP-based real-time quantitative RT-PCR, the viral load was found to correlate with disease outcome (72). During the recent outbreak in Angola, Marburgvirus real-time PCR assays were developed and effectively applied in the laboratory in Luanda and in the field in Uige (26).

ISOLATION PROCEDURES

Cell Culture

Clinical specimens and clarified tissue homogenates (usually 10% [wt/vol]) are diluted in a suitable maintenance medium and adsorbed in small volumes to cell monolayers grown in suitable vessels, such as T-25 tissue culture flasks. If no antigen is detected by IFA by 14 days, the sample is considered negative, but supernatant fluids should be blind-passaged to confirm the absence of virus. To confirm the presence and identity of the virus, scrapped cells are tested by immunofluorescence assay and supernatant fluids could be tested by antigen capture ELISA or RT-PCR techniques.

Arenaviridae

Cocultivation of Hypaque-Ficoll-separated peripheral blood leukocytes with susceptible cells has increased the isolation frequency of Junin virus. Cocultivation of lymphocytes from spleens of experimentally infected animals has yielded Lassa virus late in convalescence, even after neutralizing antibody has appeared. The technique merits systematic development for the remaining arenavirus and filovirus pathogens.

Although historically Machupo and Junin viruses were isolated by inoculation of newborn hamsters and mice, respectively, Vero cells are approximately as sensitive and are far less cumbersome to manage in BSL-4 containment. Furthermore, Vero cells usually permit isolation and identification within 1 to 5 days, a significant advantage over the use of animals, since 7 to 20 days of incubation is required for illness to develop in the animals.

Filoviridae

The best general method currently available for isolation of filoviruses is the inoculation of appropriate cell cultures (usually Vero cells) followed by IFA or other immunologically specific testing of the inoculated cells for the presence of viral antigens at intervals. Other cell lines, including human diploid lung (MRC-5) and BHK-21 cells, also work; MA-104 cells (a fetal rhesus monkey kidney cell line) may be more sensitive than Vero cells for some strains.

Animal Inoculation

Arenaviridae

Intracranial (i.c.) inoculation of weanling mice is still regarded by some as the most sensitive established indicator of LCM virus (28), although adequate cell culture systems for the isolation of LCM virus exist. Care must be taken to use mice from a colony known to be free of LCM virus. Many LCM virus isolates produce a characteristic convulsive disease within 5 to 7 days, which is nearly pathognomonic. Brains from dead mice may be used to prepare ELISA antigens or may be subjected to IFA or immunohistochemistry staining to obtain presumptive identification. Clarified mouse brain may also be used as the antigen for confirmatory testing by neutralization or RT-PCR.

Most LCM virus strains are also lethal for guinea pigs. The pathogenicity of virulent Lassa virus strains for outbred Swiss albino mice inoculated i.c. seems to vary with different sources; mice should not be seriously considered for Lassa virus isolations.

For the New World arenaviruses, particularly Junin virus, young adult guinea pigs inoculated either i.c. or peripherally have been used. Guinea pigs die 7 to 18 days after Junin virus inoculation. Strain 13 guinea pigs are exquisitely sensitive to most Lassa virus strains and uniformly die 12 to 18 days after inoculation; outbred Hartley strain guinea pigs are somewhat less susceptible. Newborn mice (1 to 3 days old) are highly susceptible to Junin virus inoculated i.c.; newborn hamsters are believed to be more sensitive to Machupo virus.

Filoviridae

Marburg virus and the EBO-Z and EBO-S strains of Ebola virus produce febrile responses in guinea pigs 4 to 10 days after inoculation; however, none of these viruses kills guinea pigs consistently on primary inoculation, and only EBO-Z and Marburg virus have been adapted to uniform lethality by sequential guinea pig passages. EBO-Z is usually pathogenic for newborn mice inoculated i.c., but EBO-S, EBO-R, and Marburg virus are not.

IDENTIFICATION OF VIRUS

Typing Antisera

Detection of viral antigens in infected tissue culture cells (usually Vero or MA-104) permits a presumptive diagnosis, provided that the serologic reagents have been tested against all the reference arenaviruses and filoviruses expected in a given laboratory, thus permitting an interpretation of virus cross-reactions. Virus isolates in cell culture supernatant fluids or tissue homogenates are presumptively or specifically identified by their reactivity with diagnostic antisera in various serologic tests (see below). Specific polyclonal antisera are prepared in adult guinea pigs, hamsters, rabbits, rats, or mice inoculated intraperitoneally with infectious virus. Rhesus and cynomolgus monkeys that are convalescent from

experimental infections are also reasonable sources for larger quantities of immune sera. Polyvalent polyclonal "typing" sera or ascitic fluids made by immunization with a number of viruses within the families have also been useful in early identification of virus isolates and in immunohistochemistry on unknown patient materials. Diagnostic antisera produced by a single injection of infectious virus are less cross-reactive and usually have higher titers than those produced by multiple injections of inactivated antigens. To further reduce the induction of extraneous antibodies, input virus should be derived from tissues or cells homologous to the species being immunized; likewise, the virus suspension should be stabilized with homologous serum or serum proteins. Sera produced for use in the IFA tests and ELISAs should be collected 30 to 60 days after inoculation; sera for neutralization tests should be collected later. All sera must be inactivated and rigorously tested for the presence of live virus before being removed from a BSL-4 environment.

Production and use of specific murine monoclonal antibodies with fine specificities for N and GP epitopes of LCM virus, Junin virus (69), and other arenaviruses have been reported, as well as monoclonal antibodies for Lassa, Ebola, and Marburg viruses. Reference reagents for all these viruses are not generally available outside the appropriately equipped specialized laboratories.

Immunofluorescence

To process infected cells for IFA examination and presumptive identification, inoculated cell monolayers are dispersed by using glass beads or a rubber policeman or trypsinization, washed, and placed onto circular areas of specially prepared Teflon-coated slides. These "spot slides" are air dried, fixed in acetone at room temperature for 10 min, and either stained quickly or stored frozen at −70°C. Although acetone fixation greatly reduces the number of infectious intracellular viruses, spot slides prepared in this manner should still be considered infectious and handled accordingly. Gamma irradiation has been used to render spot slides noninfectious (19), with no diminution in fluorescent-antigen intensity. Alternatively, infected cells may be biologically inactivated with β-propiolactone (74). Gamma irradiation is recommended if the appropriate equipment is available.

For direct FA tests, specific immune globulins conjugated to fluorescein are used with Evans blue counterstain. Specific viral fluorescence is characterized as intense, punctate to granular aggregates confined to the cytoplasm of infected cells. Specific Marburg and Ebola virus fluorescence may include large, bizarrely shaped aggregates up to 10 μm across. Nonspecific fluorescence is rarely a problem in IFA procedures for these viruses. Detection of Marburg, Ebola, Lassa, and LCM virus antigens by the IFA test is usually considered sufficient for a definitive diagnosis, although Lassa and LCM viruses cross-react at low levels in this test. Detection of Junin or Machupo virus antigens by the IFA test constitutes a presumptive diagnosis, since these viruses can be reliably distinguished from each other only by neutralization tests. IFA formats for viral detection are more cumbersome and cross-reactive but can be used if direct conjugates are unavailable. Immunohistochemical techniques for detecting arenaviruses (15) and filoviruses (16, 32) with a variety of chromogens can also be applied.

CF Test

The CF test is rarely used now, since reliable, simplified, and more sensitive immunofluorescence, ELISA, and RT-PCR procedures have been developed. Detailed instructions for performing the CF test for arenaviruses are available (9).

SEROLOGIC DIAGNOSIS

IFA Test

Until recently, the IFA test was widely regarded as the most practical single method for documenting recent infections with Marburg and Ebola viruses or large prevalence studies (1). Preparation of spot slides with infected Vero cells is identical to the procedure described above. Some refinements to enhance reproducibility and quality between spot slides lots have been suggested (18). Although monovalent spot slides are usually desired and are prepared with cells optimally infected with a single virus, polyvalent spot slides can also be prepared by mixing cells infected with different viruses selected from these or other taxonomic groups which have similar geographic distributions (37). The BSL-4 facility requirement to produce the spot slides could be avoided by using recombinant expressing cells (29). Discrepancies in titers determined by different laboratories, or even different investigators, are common. In addition, in most of the severe and fatal forms of these diseases, the patients never develop a humoral response and die without antibodies. This technique is not recommended for acute diagnosis of hemorrhagic fevers.

ELISA for Detection of IgG and IgM Antibodies

For all intents and purposes, the immunoglobulin G (IgG) and IgM ELISAs have replaced the more subjective IFA tests as the serologic tests of choice (41, 81). ELISA procedures for Lassa virus-specific IgG and IgM have been developed and successfully used on field-collected human sera (2, 57). When this ELISA is used in combination with the Lassa antigen capture ELISA described above, virtually all Lassa fever patients can be specifically diagnosed within hours of hospital admission. A simplification of this procedure, which entails the use of infected Vero cell detergent lysate as the antigen, diluted in SerDil and adsorbed directly to the microtiter plate wells, has been developed for Ebola virus (41, 43). Test sera are serially diluted, initially 1:100, and incubated with antigen in a format analogous to the antigen capture ELISA described above. Following incubation, washing, and addition of horseradish peroxidase-conjugated anti-human serum, ABTS [(2, 2′-azinobis(3-ethylbenzthiazolinesulfonic acid)] substrate is added for color development. Species-specific conjugates allow testing of other animal species during epidemiological studies. To avoid the use of several conjugates during epidemiological studies, proteinA-proteinG conjugate can be used. More recently a competition assay using an antibody-phage indicator has been proposed but has never been used in the field (52). Samples are considered positive if the optical density at 410 nm exceeds the mean plus 3 standard deviations for the normal-serum controls. This procedure can be further modified to detect virus-specific IgM by coating the plates with anti-human IgM followed by test serum dilutions and cell slurry antigens and then using the antigen capture protocol. These IgG and IgM ELISA procedures have worked well with specimens obtained from humans during natural infection and with animals experimentally infected with Lassa, Machupo, Junin, Marburg, and Ebola viruses. A simplification of the ELISA plate procedure, substituting filter paper disks, has been reported for EBO-R (38); it appears to sacrifice some sensitivity and precision in comparison with the more

established procedures but may find application in a field setting. All of these developmental assays are sufficiently robust to warrant field testing. Recombinant full or truncated proteins have also been proposed and used as antigen for ELISAs (31, 64).

Neutralization Tests

For the arenaviruses, plaque reduction tests with Vero cells are generally used. For measuring the levels of neutralizing antibody to Lassa and LCM viruses, which are both difficult to neutralize and are poor inducers of this antibody, test sera are diluted, usually 1:10, in medium containing 10% guinea pig serum as a complement source and mixed with serial dilutions of challenge virus. Titers are expressed as a $\log_{10}$ neutralization index, defined as ($\log_{10}$ PFU in control) − ($\log_{10}$ PFU in test serum). For Junin and Machupo viruses, the more conventional serum dilution-constant virus format is normally used, although the constant serum-virus dilution format is equally useful for distinguishing among virus strains. Neutralizing-antibody responses require weeks to months to evolve but persist for years (36). Performance of these tests is restricted to laboratories equipped to handle the infectious viruses.

In survivors, neutralizing antibodies to Lassa virus first appear very late in convalescence (6 weeks or later), long after the viremia has disappeared. This pattern of early IFA and delayed neutralizing-antibody response is similar for LCM virus infections. Neutralizing antibodies against Junin and Machupo viruses become detectable 3 to 4 weeks after onset, soon after the termination of viremia. While these antibodies are thought to be important in protection against reinfection, their role in resolution of acute infections is less firmly established (34, 35).

As described above, reliable tests for measuring the levels of neutralizing antibody to Marburg and Ebola viruses are not currently available.

Western Blotting

Western blotting is feasible for demonstrating antibodies to arenaviruses and filoviruses. However, it has never been applied systematically or routinely to diagnosis, although it was proposed as a confirmatory test to supplement the IFA test for filovirus antibodies (18). Detection of the nucleocapsid (N) band plus either VP30 or VP24 was taken as diagnostic. The Western blotting procedure was further refined by miniaturization, using the Phast system sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transblot apparatus (Phast Western blot system). This test is not used anymore for antibody detection.

Other Serologic Tests

Other serologic tests have been applied to diagnosis but are abandoned. They include gel diffusion for arenaviruses, CF tests, reversed passive-hemagglutination (and inhibition) test involving Lassa virus antibody-coated erythrocytes, and a radioimmunoassay using ^{125}I-labeled staphylococcal protein A for Ebola. Western blotting and radioimmunoassay procedures are used in specialized laboratories to determine the fine specificities of monoclonal antibodies and occasionally to confirm the results of serosurveys based on ELISA.

EVALUATION AND INTERPRETATION OF RESULTS

Early diagnosis of arenavirus and filovirus infection is desirable since specific immune plasma and appropriately selected antiviral drugs are in certain cases effective when treatment is initiated soon after onset. Early recognition of these infections should also trigger strict isolation procedures to prevent the spread of disease to patient contacts. In areas where specific viruses are endemic, the index of suspicion is often high and experienced clinicians may be remarkably accurate in rendering an accurate diagnosis of fully developed cases on clinical grounds alone. However, even in these areas, specific virologic and serologic tests are required to confirm clinical impressions, since many other diseases, including malaria, typhoid, rickettsial infections, idiopathic thrombocytopenia, and viral hepatitis, may masquerade as an arenavirus or filovirus infection.

Since patients with most of the severe and fatal forms of these diseases can die without developing detectable levels of antibody, timely diagnosis requires a means of detecting infectious virus or antigen in the field. The antigen capture ELISA holds promise for use in detecting clinically relevant concentrations of virus in infectious or gamma irradiation-inactivated or β-propiolactone-inactivated sera, body fluids, and tissues, and in blood and tissues of humans and nonhuman primates infected with Machupo, Lassa, Ebola, and Marburg viruses (2, 33, 41, 42, 62).

RT-PCR assays may eventually eliminate almost all need to isolate infectious virus to establish a definitive diagnosis, but virus isolation remains important for subsequent genetic and pathogenesis studies. The ability to amplify viral genomes from infected tissues, and even from formalin-fixed tissues, and to sequence the reaction products does eclipse serologic methods of identification and classification of arenaviruses (3–5) and filoviruses (70, 71). With the exception of LCM virus, which may be handled at a lower containment level, isolation attempts of these viruses should not be done outside a BSL-4 laboratory (12). A combination of several laboratory techniques should be used to confirm any clinical suspicion of hemorrhagic fevers.

Among arenavirus infections, Lassa virus is usually recovered (by isolation, antigen detection, or RT-PCR) from acute-phase sera of hospitalized patients soon after admission, frequently in the presence of specific IgM antibody, and a detectable antibody response does not necessarily signal imminent recovery; viremia frequently persists, and some patients die despite an antibody response. Junin, Machupo, and LCM viruses are recovered less frequently, and diagnosis is usually based on seroconversion. The presence of specific IgM ELISA titers in the cerebrospinal fluid of LCM patients constitutes a definitive diagnosis, and for all arenavirus infections, the presence of specific IgM antibodies is indicative of recent infection. The extent to which heterologous arenavirus infection and/or reinfection broadens antibody specificity has not been systematically evaluated for any of the available serologic tests. Neutralizing antibodies against arenaviruses persist for long periods, perhaps for life, and thus provide the most reliable basis for determining the minimum resistance of a population to reinfection. The role of neutralizing antibody in acute recovery is less clear. The protective efficacy of passively administered immune plasma is believed to be a function of neutralizing-antibody titers, and plasma should be selected on this basis, especially for Junin and Lassa fever (34).

For filoviruses, the combination of antigen detection, RT-PCR, and IgM is the most valuable for acute-case diagnosis. Because of the time required for culture and the biohazard, virus isolation data for these viruses are usually available only retrospectively. Marburg virus and Ebola viruses are usually isolated from acute-phase serum samples. A rising IgM or IgG ELISA titer constitutes a strong presumptive

diagnosis. Since IgM titers do not persist for long, a decreasing titer suggests a recent infection which occurred perhaps several months previously. The IFA test has yielded misleading results when applied to diagnostic and population-based serosurveys and should be abandoned. For unknown reasons, the filoviruses are notoriously poor inducers of neutralizing antibody. The role of neutralizing antibodies for Ebola virus protective immunity is unclear, although passively administered IgG with very high neutralizing-antibody titers conferred only partial protection to experimentally infected primates (34, 35).

The highest priority for future development is refinement of the available diagnostic tools to permit definitive virus identification in the field. PCR-based assays add another dimension to the capability of field laboratories to diagnose acute disease almost in real time. Proper tailoring of primers should permit the design of tests with the proper degree of specificity. The emergence of three new arenavirus pathogens, Guanarito, Sabia, and Chapare viruses, as well as EBO-R, Whitewater Arroyo, Pirital, and Bear Canyon viruses in the past decade, serves as a reminder that broadly reactive grouping reagents are still required to augment the newly evolving tools of PCR and capture ELISAs based on extremely specific monoclonal antibodies and gene sequences.

An investment in rapid diagnosis should result in more timely intervention with effective treatment regimens and, through implementation of appropriate public health measures, contribute to reduce the dissemination of these highly virulent viral pathogens.

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Herpes Simplex Viruses and Herpes B Virus

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TAXONOMY

Herpes simplex virus (HSV) types 1 and 2, formally designated *Human herpesvirus 1* and *Human herpesvirus 2,* respectively, are members of the family *Herpesviridae.* Along with varicella-zoster virus (VZV) *(Human herpesvirus 3)* and a number of viruses affecting primarily nonhuman hosts, they constitute the subfamily *Alphaherpesviridae.*

DESCRIPTION OF THE AGENT

Historical Aspects

The herpesviruses are thought to have coevolved with their hosts (57, 58), and thus, the earliest documentation of presumptive herpes simplex infection appears shortly after the development of writing and clinical observation. The infectious nature of HSV was demonstrated in 1919 (90). The concepts of seropositivity and recurrence developed in the 1930s (15, 20). In the 1960s antigenically distinct strains (HSV-1 and HSV-2) were identified. HSV-1 and HSV-2 are two of eight known human herpesviruses (118). In addition, herpes B virus *(Herpesvirus simiae),* a cercopithecine herpesvirus, is an important zoonotic pathogen in humans (Table 1).

Genetic Structure

The linear, double-stranded genome is 152 kbp for HSV-1 and 155 kbp for HSV-2. HSV-1 and HSV-2 share 83% nucleotide identity within their protein-coding regions. The genome is organized into a unique long (UL) and unique short (US) region, each of which is flanked by inverted-repeat regions. Although significant stretches of sequence are conserved across unrelated clinical isolates, isolates can be epidemiologically linked by restriction endonuclease digestion (116, 117), PCR (92), or sequencing (103).

Virion Structure

The HSV virion is 120 to 300 nm in diameter, with a central electron-dense core containing the DNA, an icosahedral capsid consisting of 162 capsomeres surrounding the core, and a tegument layer surrounded by a spiked envelope containing viral glycoproteins that aid in attachment, penetration, and immune evasion (119). The envelope is a trilaminar, lipid-rich layer derived largely from the nuclear membrane of the infected cell. Because the viral envelope is lipid rich, the virus is readily inactivated. Lipid solvents, exposure to pH of <4, or temperatures of $>56°C$ for 30 min all eliminate infectivity (39).

Life Cycle

Viral replication begins with attachment to the target cell via cell type-dependent host receptors (129). After attachment the viral envelope and cell membrane fuse, releasing the capsid into the cell. The capsid is translocated to the nuclear pores (11, 135), and DNA is released into the nucleus. The tegument protein VP16 induces expression of the immediate early, or alpha, proteins, which in turn transactivate the expression of the early, or beta, genes. Another immediate early gene, ICP47, acts as an important mediator of immune evasion by inhibiting the loading of peptide onto major histocompatibility complex class I molecules by the transporter associated with antigen processing, or TAP (55, 62). The early genes are maximally expressed 5 to 7 h after infection and are involved in the synthesis of progeny viral DNA. DNA replication is required for optimal synthesis of the late, or gamma, genes, which are mainly structural proteins. Progeny DNA is processed and assembled into preformed capsids within the cell nucleus. The DNA-containing capsids attach to patches of the nuclear membrane that contain viral tegument proteins and viral glycoproteins. In neurons, nucleocapsids are transported anterograde to the axon terminus, where the final assembly of enveloped virus occurs (66). The replication cycle is complete within about 20 h in epithelial cells.

Immunobiology

Innate immune mechanisms such as activation of macrophages, NK cells, and interferon production play a predominant role in the control of primary infection, while both innate and adaptive immunity are important in the control of recurrences. Although both cellular and humoral immunity are thought to be important, persons with defective cellular immunity often show severe HSV infections, while those with agammaglobulinemia do not (4). $CD4^+$ T cells infiltrate mucocutaneous lesions early in lesion development (41), followed by cytotoxic T lymphocytes (81). T cells may also play an important role in controlling viral reactivation at the dorsal root ganglia (76), perhaps through the action of gamma interferon.

Functional antibody responses include complement-independent and complement-dependent viral neutralization

TABLE 1 Herpesvirus family members pathogenic to humans

Designation	Common name	Subfamily
Human herpesvirus		
1	Herpes simplex virus type 1	Alpha
2	Herpes simplex virus type 2	Alpha
3	Varicella-zoster virus	Alpha
4	Epstein-Barr virus	Gamma
5	Cytomegalovirus	Beta
6a	Human herpesvirus 6a	Beta
6b	Human herpesvirus 6b	Beta
7	Human herpesvirus 7	Beta
8	Human herpesvirus 8	Gamma
Cercopithecine herpesvirus 1	Herpes B virus or herpesvirus simiae	Alpha

(46). Immunoglobulin G (IgG), IgM, and IgA responses to individual viral proteins arise within the first weeks after infection (4). IgM wanes after 2 to 3 months, appearing sporadically thereafter, and in about one-third of patients after recurrent genital herpes episodes. Antibody titer rises may or may not occur after recurrences (113). However, IgG titers are maintained for years after primary infection.

Most epitopes are shared between HSV-1 and HSV-2. As a result, it is difficult to distinguish antibodies to HSV-1 from those to HSV-2 (3, 6). In particular, in HSV-1-seropositive patients, seroconversion to HSV-2 is accompanied by brisk anamnestic responses to HSV-1, resulting in predominant antibody titers to HSV-1 rather than to HSV-2 for prolonged periods (3). While type-specific epitopes have been demonstrated on the major viral proteins, only glycoprotein G (gG) elicits predominantly type-specific responses.

EPIDEMIOLOGY AND TRANSMISSION

HSV infections occur worldwide with no seasonal distribution. The virus is spread by direct contact with virus in secretions. Incubation periods range from 1 to 26 days. The prevalence of HSV-1 infection increases gradually from childhood, reaching 80% or more in later years (101). In contrast, the seroprevalence of HSV-2 remains low until adolescence and the onset of sexual activity. The incidence of antibodies to HSV-2 in the United States is increasing; currently about 22% of the general U.S. population is seropositive (52). The rate of seropositivity varies widely, reaching more than 50% in some demographic groups (52, 71). In general, the seroprevalence of HSV-2 is higher in the United States than in other developed countries (93, 128). Importantly, a large percentage of individuals seropositive for HSV-1 and/or HSV-2 are unaware of the infection (52). Such persons comprise an important reservoir of infection.

CLINICAL SIGNIFICANCE

Primary Infection

Most HSV-1 infections are acquired early in childhood as subclinical or unrecognized infections (131). Young children may present with classic primary HSV-1 infection characterized by gingivostomatitis, fever, and marked submandibular lymphadenopathy (131). Oral lesions progress to ulceration and heal without scarring over 2 to 3 weeks. Adolescents may present with pharyngitis and mononucleosis.

Primary infection with HSV-2 classically presents as herpes genitalis, with extensive, bilateral vesicles, fever, inguinal lymphadenopathy, and dysuria (33). Lesions ulcerate and heal without scarring within 3 weeks. Secondary lesions may develop in the second to third week. Subclinical or unrecognized primary infection with HSV-2 is common.

The proportion of primary genital infections due to HSV-1 is increasing (19), from about 10% in 1983 to 32% in 1995 (33, 137), reaching 50% or more in certain populations (115). This trend is thought to result from changing sexual practices, including increased oral-genital exposure (115). The clinical presentation of primary genital HSV-1 cannot be distinguished from that of HSV-2. However, recurrent disease (see below) is less common with genital HSV-1 than HSV-2 (33, 49), and thus, determining the infecting virus type is useful for prognostic purposes.

Latency and Recurrent Disease

Primary infection with HSV-1 or HSV-2 is followed by the establishment of latency in the dorsal root ganglia, typically the trigeminal ganglia for orolabial disease and the lumbosacral ganglia for genital disease. Periodically the virus reactivates and travels via the nerve axon to oral or genital sites, resulting in release of infectious virus and, in some cases, lesion formation. Recurrent disease has milder symptoms and a shorter time to lesion healing than primary episodes (33, 131). The frequency of HSV-2 genital recurrences varies widely among individuals, ranging from none to 12 or more per year (12) with a mean rate of 0.33 recurrences/month. Orolabial HSV-1 recurrence rates are lower, with a mean of 0.12 episodes/month, while genital HSV-1 infection occurs even less frequently (mean 0.02 episodes/month) (84). While HSV-2 may be isolated from the pharynx during primary genital herpes episodes, orolabial HSV-2 recurrences are extremely infrequent (84).

Asymptomatic or Subclinical Infection

Approximately 70 to 90% of individuals with HSV-2 antibodies have not been diagnosed with genital herpes (52). Over one-half of such persons recognize and present with symptoms after education regarding manifestations of HSV disease (54, 88). In addition, about 20% of patients presenting with first episodes of genital herpes have serologic evidence of having been infected for some time (45). These episodes most closely resemble recurrent disease, with mild symptoms. The risk of recurrence is similar for patients presenting with true primary infection and for those with first recognized recurrent episodes (45). Most patients with genital herpes have episodes of subclinical virus excretion from anogenital sites; these constitute an important source of transmission. The copy number of HSV DNA can sometimes be as high during subclinical episodes as when symptoms occur (140). Oral shedding of HSV-2 is rare, particularly outside the setting of newly acquired genital HSV-2 (84, 141).

Neonatal Herpes

The most serious consequence of genital HSV infection is neonatal herpes. Infection usually occurs during vaginal delivery when the infant is exposed to HSV in maternal secretions. The risk of maternal-to-infant transmission is 10-fold higher in mothers experiencing unrecognized primary infection during the time of labor than in those shedding HSV as a result of recurrent, subclinical reactivation. This conclusion was drawn in part from the finding that the neonatal infection rate among HSV-seronegative mothers is 54 per 100,000, 26 among mothers with only HSV-1 antibody, and 22 among all mothers with HSV-2 antibody (18, 111). Transmission of

HSV-1 occurs at a significantly higher rate than that of HSV-2 (18). Nevertheless, the relative preponderance of recurrent genital HSV-2 disease means that most cases of transmission occur during HSV-2 recurrences.

Infected neonates can present with HSV disease localized to the skin, eyes, and mucosa or more serious central nervous system (CNS) or disseminated disease but often have nonspecific presentation (82). The mortality rate for untreated neonates with disseminated disease exceeds 70%. Early diagnosis and antiviral therapy while disease is localized to the skin can substantially reduce the morbidity and mortality associated with neonatal infections (78, 146, 147). PCR should be used to test cerebrospinal fluid (CSF), plasma, and peripheral blood mononuclear cells (44).

Herpesvirus CNS Disease in the Immunocompetent Host

HSV is the most common cause of fatal sporadic encephalitis in the United States (105). HSV encephalitis presents as fever, behavioral changes, and altered consciousness, resulting from localized temporal lobe involvement. Without treatment, mortality exceeds 70%, and few survivors recover normal neurologic function. Early treatment with acyclovir reduces morbidity and mortality; however, residual neurologic impairment is common (59). HSV PCR of CSF is the test of choice, being far more sensitive than culture (27, 47).

Genital HSV may be followed by sporadic meningitis or recurrent meningitis (Mollaret's syndrome) characterized by headache, fever, photophobia, and lymphocytic pleocytosis (123). The condition is self-limiting and typically resolves within 1 week (149a). HSV also can be associated with myelitis, radiculitis, ascending paralysis, autonomic nerve dysfunction, and possibly Bell's palsy.

Ocular Herpesvirus Infections

HSV is the most common viral cause of corneal infection in the United States, affecting 400,000 to 500,000 people (42, 108). Most corneal HSV infections are limited to the epithelial layer, causing characteristic branching ulcerations, pain, photophobia, and blurred vision. HSV epithelial keratitis responds well to oral or topical therapy (see below). Such superficial infections heal without loss of vision. HSV infection may extend to the corneal stroma leading to scarring and opacification of the cornea. Stromal HSV infections respond favorably to a combination of corticosteroids and topical or oral antivirals (75).

Herpes in the Immunocompromised Host

Immunosuppressed individuals with defective cell-mediated immunity frequently develop symptomatic HSV disease. HSV infections in such individuals can be severe, with extensive mucocutaneous necrosis and involvement of contiguous tissues leading to esophagitis or proctitis. HSV occasionally disseminates to multiple organs, leading to meningoencephalitis, pneumonitis, hepatitis, and coagulopathy. Disseminated infections require intravenous antiviral therapy and monitoring for development of antiviral resistance.

Antiviral Therapy

Several antiviral drugs including acyclovir, valacyclovir, penciclovir, and famciclovir are now widely used to treat mucocutaneous and genital herpes (24) and for long-term suppression of recurrent episodes. These drugs are selectively activated by the viral thymidine kinase and have minimal side effects. Suppressive therapy reduces the risk of viral transmission between HSV-discordant partners (36). *N*-Docosanol, a nonprescription topical medication, and topical acyclovir are used for the treatment of herpes labialis. HSV conjunctivitis, blepharitis, and dendritic keratitis are treated either topically with trifluridine, idoxuridine, or vidarabine or orally with acyclovir, valacyclovir, or famciclovir (75). In cases with stromal involvement the addition of topical corticosteroids reduces the persistence of inflammation (75). Neonatal herpes, herpes encephalitis, and severe infections in immunocompromised patients require prompt intravenous antiviral therapy (43). Foscarnet, a viral DNA polymerase inhibitor, is reserved for acyclovir-resistant herpes infections (77).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Lesions or Mucocutaneous Sites

Specimens require special viral transport medium (VTM) and controlled temperature to retain optimal infectivity for culture (7). VTM is made of balanced salt solutions such as Hanks' balanced salt solution, Stuart's medium, Leibovitz-Emory medium, veal infusion broth, or tryptose phosphate broth buffered to maintain a neutral pH. Protein-stabilizing agents such as gelatin or bovine serum albumin are added as well as antibiotics to prevent bacterial overgrowth (130). Most VTM are inappropriate for bacterial or chlamydial transport. However, Multi-Microbe Medium (M4-RT) from MicroTest (Lilburn, Ga.) can be used for bacterial, chlamydial, or viral specimens. In one comparison study, Bartel's VTM (ViraTrans; Trinity Biotech, Bray, Ireland), cell culture medium (Dulbecco's with bovine serum albumin and gentamicin), and tryptose phosphate broth did not preserve HSV infectivity as well as Richards' VTM or Virocult Medium (Medical Wire and Equipment, Cleveland, Ohio) (69).

Samples should be shipped cold (on ample ice packs) but not frozen. Prolonged (>48 h) storage should be at –70°C. HSV stability is reduced 2- to 10-fold at 22°C compared with 4°C; in one study, median half-lives of four strains held in Richard's VTM was 72 days at 4°C but only 8 days at 22°C. With other transport media, half-lives ranged from 0.5 to 4 days at 22°C and from 1 to 180 days at 4°C (69).

Necrotic debris or exudate should be removed from mucosal sites (endocervix, anogenital areas, conjunctiva, and throat) or lesions with a cotton swab prior to sampling. The area must be vigorously rubbed with Dacron, rayon, or cotton swabs on aluminum shafts to ensure that infected cells at the base of the lesion are collected. Calcium alginate swabs and swabs with wooden shafts inhibit infectivity (38, 130). Swabs are then broken off into VTM for transport.

A single transport vial may provide specimen for both viral culture and direct antigen detection tests. Alternatively, slides for direct fluorescent-antibody (FA) detection can be prepared immediately after collection by gently spreading the material on a swab in a thin layer over a clean microscope slide. The slide is air dried and fixed in cold acetone before transport.

Collection for DNA Amplification (PCR)

Special collection and handling methods are essential to avoid contamination of the specimen with exogenous viral DNA (31). For lesions or mucocutaneous sites, a dedicated specimen should be collected with a Dacron swab in VTM or digestion buffer (120). CSF does not require special handling. Blood from neonates is collected in lavender-topped tubes (EDTA) for PCR of peripheral blood mononuclear cells and plasma (44). Specimens for PCR can be maintained

at 4°C for up to 72 h, but longer storage should be at –20°C (31, 70, 142).

Other Samples

Fluids such as tracheal aspirates should be collected aseptically and transported without VTM. Urine should be collected by clean catch and refrigerated before transport. Once in the laboratory, urine is diluted 1:1 with culture medium. Corneal samples are obtained with a no. 15 scalpel blade, and cells are suspended in VTM. Tissue is placed in VTM in a sterile container. Prolonged storage of tissue requires immersion in sterile 50% neutral glycerol in saline or, alternatively, in culture medium with 5% fetal bovine serum. Fresh tissue samples can also be frozen, sectioned, applied to slides, and fixed in acetone.

DIRECT EXAMINATION

Detection of virus in patient samples without an intervening culture amplification step provides the most rapid diagnosis. Immunostaining methods to detect antigen require less expertise than cytopathic effect (CPE)-based culture methods and are usually less expensive than culture.

Tzanck Test

Changes that are characteristic for HSV are sometimes visible in fixed, stained cells from lesions (Tzanck preparations) or cervical scrapings (Papanicoalaou stains) (100) or in hemotoxylin and eosin stains of fixed tissue. Enlarged or degenerating cells, syncytium formation, chromatin margination, a "ground glass" appearance of the cytoplasm, and nuclear inclusions are typical of HSV-infected cells. These methods are widely available but lack sensitivity and specificity (34). Virus-specific methods are preferred.

Fluorescent-Antibody Detection (DFA or IFA)

For FA detection, slides are prepared with cells from the patient's specimen and fixed with acetone (7). Cells are coated with antibody preparations against HSV-1 and HSV-2 or against type common epitopes. If the antibodies are linked to a fluorophore such as fluorescein isothiocyanate, the test is a direct fluorescent-antibody assay (DFA). If bound anti-HSV antibodies are detected with secondary antibodies conjugated to fluorophore, the test is an indirect fluorescent-antibody assay (IFA). Same-well testing can be performed using antibody conjugates with different fluorophores for HSV-1 and HSV-2 (see Table 3) (25). DFA is 10 to 87% as sensitive as culture, with higher sensitivity from vesicular lesions and poor sensitivity from healing lesions (85). DFA is far less sensitive than PCR (37).

Antibodies give their own distinct patterns (see Table 3). For example, HSV-1-infected cells have a halo of apple-green fluorescence with monoclonal antibodies from MicroTrak (see Fig. 2B). Staining patterns require skill to interpret; nonspecific staining can be distinguished from staining of viral antigen by a trained reader.

Cytospin DFA

An effective modification of DFA employs a cytocentrifuging step to apply cells from the sample to slides for subsequent DFA testing. The cytospin step results in better adherence of cells to the slides and fewer inadequate specimens than what is obtained with drip application methods (87) while allowing a <2-h turnaround time. The sensitivity of cytospin DFA has been reported to be higher than that of culture for detecting HSV from swab specimens (87), while other researchers, using different DFA antibody and cell culture systems, reported sensitivity of 31% versus culture (122).

EIA

Enzyme immunoassay (EIA) is rapid and amenable to automation. Sensitivity can be as good as culture for diagnosis from lesions (32) or as low as 35% in asymptomatic patients (16, 136). Dako USA (Carpinteria, Calif.) currently markets an amplified enzyme-linked immunosorbent assay (ELISA) based on monoclonal antibodies to HSV-1 and HSV-2 (IDEIA).

Direct DNA Detection

In situ hybridization or solution hybridization methods are not as widely used as IFA or DFA. These tests use DNA or RNA probes, some of which are type specific. The hybrid capture method (Digene Corp.) used a cocktail of RNA probes targeting type-common or type-specific regions of the HSV genome. DNA/RNA hybrids were then detected by ELISA (40). The sensitivity of direct hybridization methods is limited (approximately 1×10^5 copies/ml for the Digene assay), and thus, these approaches have been largely replaced by PCR (see below).

PCR-Based Detection Methods

The most sensitive method for direct detection of HSV is the PCR (127). Precautions to prevent contamination of the sample and the inclusion of frequent negative controls within the assay are critical (83, 86). Some laboratories include isopsoralens or uracil-*N*-glycosylase in their PCR amplifications, preventing the amplification of PCR products in subsequent reactions.

The likelihood of laboratory contamination by amplicon is reduced with the new real-time PCR detection systems (see below) that eliminate the need for postamplification manipulation of the PCR product. Laboratories performing PCR testing should participate in a regular proficiency testing program, such as the Nucleic Acid Amplification ID survey from the College of American Pathologists (http://www.cap.org). It is important to consider performance data, including test turnaround times, before selecting a provider of DNA amplification testing.

Primer Selection and HSV Typing

Depending on the primers and detection methods, PCR can be set up to detect both HSV-1 and HSV-2 (120) or to allow distinction of HSV-1 from HSV-2 (79, 120). PCR primers have been described amplifying portions of many HSV genes, including those encoding thymidine kinase (U_L23), DNA polymerase (U_L30), DNA binding protein (U_L42), and glycoproteins B, C, D, and G (U_L27, U_L44, U_s6, and U_s4, respectively) (133). Distinction of HSV-1 and HSV-2 can be achieved using type-specific primers or probes, melting curve analysis, restriction enzyme analysis, or direct sequencing. At our institution, we detect both HSV-1 and HSV-2 by PCR using a real-time detection system (see below) with the type-common gB forward primer CCG TCA GCA CCT TCA TCG A, reverse primer CGC TGG ACC TCC GTG TAG TC, and probe CCA CGA GAT CAA GGA CAG CGG CC (70).

Detecting PCR Products

Methods to detect PCR products include intercalating agents such as ethidium bromide, Southern blot hybridization, and liquid hybridization (29, 65, 120). Of these, liquid

hybridization gives the greatest sensitivity (64). The herpes simplex 1/2 consensus assay and the herpes consensus generic (which can detect HSV-1, HSV-2, VZV, Epstein-Barr virus, cytomegalovirus, and HHV-6) (Argene) detect amplification product by hybridization with biotinylated probes (14). The Light Diagnostic HSV OligoDetect assay uses a biotinylated primer set to generate the amplification product, which is then denatured and captured on microtiter plates via hybridization with an immobilized oligonucleotide probe. A recent improvement has been the development of HSV assays using real-time detection of PCR products (51, 70, 74, 102). These systems allow constant monitoring of the amount of PCR product by detection of a fluorescent signal generated as PCR product accumulates. The Cepheid HSV Non-typing ASR utilizes primers directed to the HSV type 1 and type 2 polymerase gene to generate a 92-bp amplicon, which is then detected using a specific fluorophore (6-carboxyfluorescein [FAM])-labeled probe. The Cepheid HSV Typing ASR uses primers targeting the glycoprotein D gene of HSV-1 and the glycoprotein G gene of HSV-2, which are then detected with FAM- and Alexa 532-labeled probes, respectively. The LightCycler-HSV 1/2 Detection Kit (Roche) uses a pair of detection probes designed such that hybridization to PCR product brings a donor fluorophore into proximity with an acceptor fluorophore, allowing fluorescence of the acceptor (94). In addition to the commercial assays, a large number of laboratories offer in-house-validated assays using various real-time detection approaches. Real-time systems decrease the likelihood of laboratory contamination and allow more accurate quantification than is possible with older quantitative-competitive assays (120). The ability to quantitate virus may be useful in monitoring response to antiviral therapy, particularly in HSV encephalitis or neonatal herpes infections (1).

ISOLATION PROCEDURES

Culture

Virus detection methods including culture and modified culture are used to diagnose mucocutaneous, genital, and ocular lesions (Table 2). Conventional culture uses cells that are permissive for HSV-1 and HSV-2 as well as other viruses that may be of diagnostic importance. Inoculated cells are examined frequently for CPE (see below). In our laboratory, 95% of HSV isolates produce CPE by day 5; only 5% require 5 to 14 days. Mink lung cells, rhabdomyosarcoma cells, human diploid fibroblasts such as MRC-5 and WI-38, and human epidermoid carcinoma lines such as HEp-2 and A549 are commercially available (68, 72).

Direct comparisons suggest that test sensitivity can be markedly affected by the cells used, particularly with a low-titer HSV inoculum. Mink lung cells are reportedly more sensitive than MRC-5 cells, while Vero cells have had low sensitivity reported even with high-titer isolates (150).

CPE

Infected cells develop cytoplasmic granulation and then become large, round, and refractile. Clusters or foci of infected cells appear early after inoculation in diploid fibroblast or epidermoid carcinoma lines (Fig. 1). CPE in A549 cells is characterized by syncytium formation, particularly with HSV-2 (68). Cells then lyse and detach from the substrate with eventual destruction of the monolayer.

IDENTIFICATION AND TYPING

Other viruses, as well as toxic factors in specimens, can mimic the CPE of HSV (Fig. 2A). Confirmation of putative HSV CPE is accomplished using commercially available polyclonal

TABLE 2 Selected diagnostic tests for HSV

Syndrome	Sample	Laboratory tests	Comments
Oral or genital lesion	Swab in VTM	Culture, shell vial FA, cytospin FA PCR	Viability must be preserved. Cell architecture must be maintained.
Recurrent genital symptoms; culture negative	Serum Serum Serum Capillary blood	WB HerpeSelect ELISA HerpeSelect Immunoblot Captia ELISA HSV-2 *biokit* Rapid HSV-2	Limited availability. Good confirmatory test. FDA approved; Focus (Cypress, Calif.) FDA approved; Focus (Cypress, Calif.) Trinity Biotech (Bray, Ireland) Biokit USA (Lexington, Mass.); point-of-care, FDA approved; HSV-2 only
Neonatal herpes	CSF; blood in EDTA Eye, mouth, nasal, and rectal swabs	PCR Culture, FA, PCR	Collect separate CSF for PCR. Blood in EDTA (purple top)
Ocular herpes	Swab in VTM	Culture, FA, PCR	Clinical presentation may be sufficient for diagnosis.
Conjunctivitis; dendritic corneal ulcers	Corneal scraping	Culture, FA, PCR	
Encephalitis	CSF	PCR	Culture far less sensitive than PCR.
Recurrent lymphocytic meningitis	CSF	PCR	

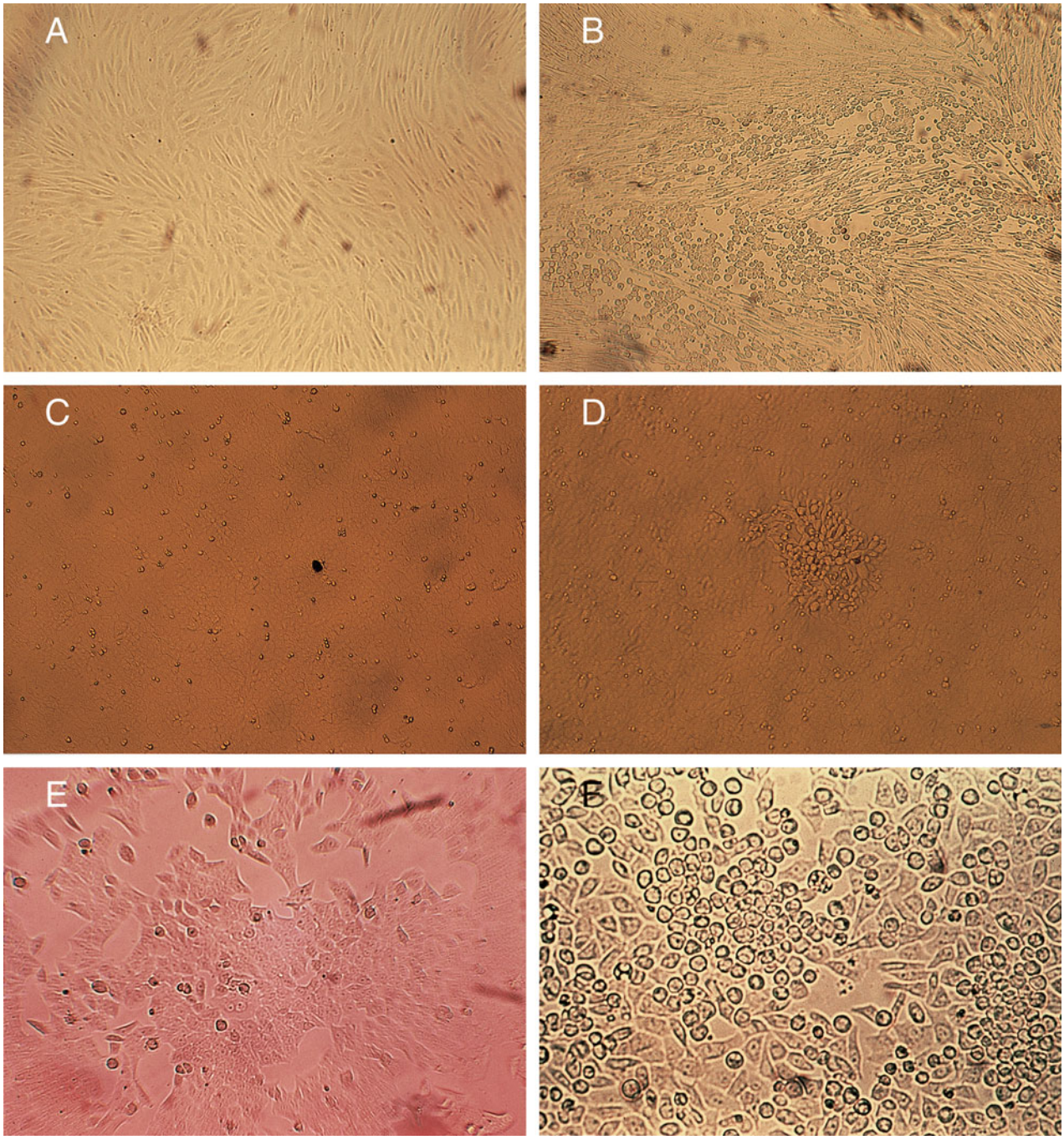


FIGURE 1 (A and B) Normal and HSV-1-infected human diploid fibroblasts, respectively (magnification, ×400). (C and D) Normal and HSV-2-infected mink lung cells, respectively (magnification, ×100). (E and F) Normal and HSV-2-infected HEp-2 cells, respectively (magnification, ×400).

or monoclonal antibodies against type-common or type-specific epitopes. The most widely used reagents are fluorescein conjugated for use in fluorescent-antibody tests (Table 3 and Fig. 2B). Trinity Biotech and Diagnostic Hybrids, Inc. (Athens, Ohio) both supply these reagents. SimulFluor HSV/VZV (Chemicon, Temecula, Calif.) can detect and type HSV as well as VZV from a single sample. Peroxidase-conjugated antibodies against HSV-1 and HSV-2 are also used in in situ EIA formats (150).

Spin-Amplified Culture

Spin amplification methods reduce the time required to detect HSV in culture by detecting viral proteins before CPE develops (150). The most widely used technique involves centrifuging the sample onto monolayers of cells on coverslips that are placed in the bottom of small vials ("shell vials") or in the wells of flat-bottomed plates. After 16 to 48 h of incubation, the coverslips or plates are subjected to antigen detection tests for HSV (25). When compared to standard

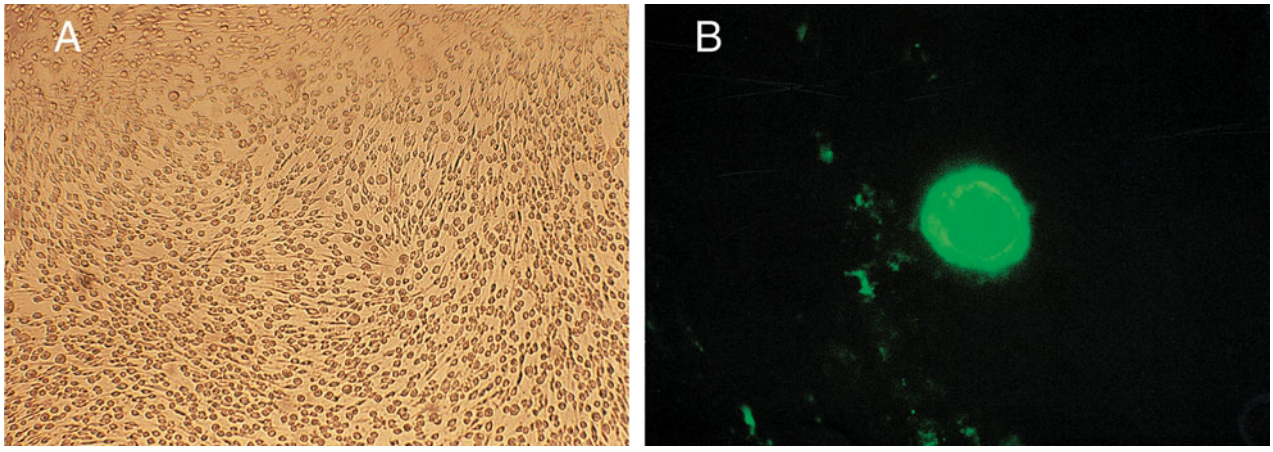


FIGURE 2 (A) Toxicity in human diploid fibroblasts (magnification, ×100). (B) HSV-2 DFA confirmatory test.

culture, reported sensitivity ranges from 71 to 97% (50, 110, 149). Sensitivity is affected by time of incubation before staining, with 48 to 72 h yielding more positives than 16 to 24 h. The cell line also affects sensitivity, with mink lung or A549 cells having greater sensitivity than MRC-5 cells (110).

Mixed FreshCell (Diagnostic Hybrids, Inc.) is a combination of CV-1 and MRC-5 cells in a shell vial format to detect a large number of human viruses including HSV-1 and HSV-2. Combined with immunodetection tests and spin amplification, this approach is intended to provide a rapid definitive diagnosis of HSV-1, HSV-2, or VZV from lesions.

Genetically Altered Cell Lines

The enzyme-linked virus inducible system (ELVIS) is based on BHK cells with a reporter gene for β-galactosidase that is driven by the promoter from the HSV-1 UL39 gene. Other viruses do not transactivate this promoter (132). Infected cells express the reporter gene, resulting in a color change visualized by light microscopy (132). The ELVIS-Id (Diagnostics Hybrids, Inc.) system allows HSV-positive cells to be tested for the presence of HSV-1 or HSV-2 (107). ELVIS-based systems can have sensitivity comparable to that of standard culture (107) and spin-amplified cell culture (89).

SEROLOGIC TESTS

Because nearly all HSV structural proteins have extensive antigenic cross-reactivity, only IgG tests based on the type-specific HSV gG accurately distinguish HSV-1 and HSV-2 antibodies (3, 9, 95). Tests based on crude antigen mixtures are still on the market from Wampole Laboratories (Cranbury, N.J.), Zeus Scientific (Raritan, N.J.), and DiaSorin (Minneapolis, Minn.) and have unacceptably low sensitivity and specificity, especially for detecting new HSV-2 infections in patients with prior HSV-1 infections (3, 95). Type-specific IgM tests are not available. FDA-approved IgM tests decrease the time to detecting seroconversion in new infections but cannot accurately distinguish new from established infections (99). Tests for HSV-2 antibody avidity can discriminate accurately between first episodes (low avidity) and recurrent episodes (high avidity) in most cases. However, overlapping avidity values occur in the mid-range (97).

WB

The HSV Western blot (WB) used by the University of Washington laboratory uses nitrocellulose blots prepared with human diploid fibroblast-infected cell proteins. WB detects antibodies to multiple viral proteins, including those to the type-specific glycoprotein gG-2 (2). About 20% of sera must be preabsorbed against Sepharose beads coated with HSV-1 or HSV-2 proteins and retested to give definitive results (5). This combination of tests gives a very accurate determination of HSV-1 versus HSV-2 antibody status (2).

Commercial Type-Specific G-Based Assays

The following tests use gG as the target antigen: HerpeSelect ELISA and HerpeSelect immunoblot tests from Focus Diagnostics (Cypress, Calif.; formerly MRL). These

TABLE 3 Monoclonal antibodies for HSV-1 and HSV-2 detection, confirmation, and typing by immunofluorescence

Reagent	Source	Comments
SimulFluor	Chemicon International, Inc., Temecula, Calif.	HSV-1: whole-cell stain (apple green); HSV-2: cytoplasmic or membrane stain (yellow); simultaneous testing for both HSV-1 and HSV-2
PathoDx	Diagnostics Products Corp./Viromed, Minneapolis, Minn.	HSV-1: cytoplasmic or whole-cell stain; HSV-2: nuclear stain
MicroTrak	Trinity Biotech, Jamestown, N.Y.	HSV-1: nuclear staining; HSV-2: strong nuclear stain, with speckled cytoplasmic staining

tests have been approved by the FDA for detection of HSV-1 or HSV-2 antibodies in adults and in pregnant women. The Captia ELISA for either HSV-1 or HSV-2 by Trinity Biotech was recently FDA cleared. A point-of-care test, *biokit* Rapid HSV-2 assay (Biokit USA, Lexington, Mass.) or SureVac-HSV-2 (Fisher HealthCare), was FDA approved under the original brand name, POCkit-HSV-2, for use with capillary blood.

The Focus and Biokit tests perform as well as or better than other gG-based tests in clinically defined populations and can be used for serum or plasma (9, 26). When compared with the University of Washington Western blot, HerpeSelect ELISA for HSV-1 had 91 to 96% sensitivity with 92 to 95% specificity. HerpeSelect Immunoblot had 99 to 100% sensitivity with 93 to 96% specificity. For HSV-2, HerpeSelect ELISA had 96 to 100% sensitivity with 96 to 97% specificity while HerpeSelect Immunoblot for HSV-2 had 97 to 100% sensitivity and 94 to 98% specificity. The POCkit-HSV-2 test from Diagnology (now Biokit USA) had 93 to 96% sensitivity with 95 to 98% specificity in comparison tests (9).

Median time to seroconversion is approximately 3 weeks for HerpeSelect-HSV ELISA (10) and about 2 weeks for the biokit-HSV-2 assay (8).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Laboratory results are best interpreted in the context of the patient's presentation and history and with the knowledge of the natural history of HSV infection.

Interpretation of Virus Detection Tests

Interpretation of positive test results also depends upon the specificity of the test. Specificity of culture is virtually 100% if confirmatory tests are performed. In situ techniques for antigen detection (FA or immunoperoxidase staining of infected cells) have better specificity than other antigen detection methods because the cell's staining pattern allows the technologist to confirm that the signal is specific (Table 3). Finally, PCR for HSV is most reliable when closed system methods are used and laboratory practices are strictly met to avoid contamination of specimens with exogenous HSV DNA. Given these caveats, in practice positive cultures are highly diagnostic in patients with lesions due to gingivostomatitis, genital herpes, or ocular infections. Positive FA tests from lesions or ocular swabs are considered reliable. HSV found in the central nervous system, in tissues, in blood, or in the eye by culture or PCR techniques is diagnostic. Positive cultures or positive PCR tests in symptomatic infants at 1 to 3 weeks of life are highly diagnostic (78).

For other patients, interpretation of a positive viral detection test requires caution. For example, respiratory secretions from immunocompromised patients may contain HSV in addition to the causative agent such as respiratory syncytial virus, parainfluenza virus, or adenovirus. The laboratory should be engaged in working up this type of specimen in appropriate viral isolation systems and appropriate antigen detection tests to identify the relevant pathogen. HSV may also be shed concurrently with other pathogens from oral or anogenital sites or may not be the cause of the episodic syndrome that triggered testing. Syphilis and chancroid are the most likely alternative causes of genital ulcers in the United States. In a series of patients with genital ulcers other than those typical of herpes (vesiculopustular lesions), 65% had only herpes detected while 20% had both syphilis and herpes detected (96). HSV results from such patients need to be interpreted in the context of laboratory test results for other pathogens in the differential diagnostic list (61). Symptoms of HSV gingivostomatitis or genital infection can be mimicked by noninfectious syndromes, such as Stevens Johnson syndrome, as well as by infectious agents. Because of the sporadic nature of asymptomatic HSV shedding, swabs of oral or anogenital sites may yield positive HSV culture, PCR, or antigen test results when the underlying cause of disease is not herpes.

Positive cultures or PCR results from skin, conjunctival, mouth, or nasal swabs from neonates less than 24 h old may reflect maternal virus, not neonatal infection. Neonates born to mothers found to be shedding HSV from the genital tract at labor and delivery may be tested 24 to 72 h after birth; a likely time frame for receiving maternal culture results. Most babies with neonatal herpes present between 9 and 14 days of life (147), well beyond the time frame during which maternal viral contamination would be a problem.

False-negative tests may occur because of poor technique in sampling (not obtaining infected cells), because of prolonged transport time or exposure of samples to high ambient temperature (reducing viability for culture) or because the specimen has low levels of virus. Recurrent episodes yield lower titers of virus for shorter periods (33). In many cases, a combination of tests or repeated testing is the most sensitive approach. Performing both culture and FA tests can increase sensitivity by 20% or more from genital lesions; repeating testing at a later recurrence nearly doubles the sensitivity (85). PCR is the most sensitive test available and is the test of choice for herpes encephalitis, disseminated neonatal herpes (see below), and, increasingly, diagnosis of anogenital herpes (140). However, in areas without local PCR testing, direct or indirect FA methods and culture may yield more timely results. Antiviral therapy should be started empirically if disseminated herpes, neonatal herpes, or encephalitis is suspected.

Interpretation of PCR Results

PCR has adequate sensitivity to detect shedding of HSV in individuals with asymptomatic infection or negative cultures (139) and also to detect HSV in CSF, a specimen with a low yield of HSV by culture. The sensitivity of HSV PCR for HSV encephalitis is about 96%, while specificity is 99% (13). Similarly, PCR has about three times the sensitivity for genital HSV-2 detection both from lesions and during periods of subclinical shedding (140). PCR can remain positive for several days after HSV lesions become negative by culture (29). PCR positivity in this case probably represents infectious virus below the limit of detection by culture, and PCR positivity is quickly lost in the absence of active shedding (138). False negatives can occur in PCR, due to reaction failure or the presence of inhibitors in clinical specimens (21, 114). It is therefore critical that only negative reactions accompanied by positive internal controls be accepted as true negatives (30). Importantly, a negative PCR result for HSV does not rule out HSV disease, since specimens taken very early or late in the course of disease may not have viral DNA (112). This is especially true in pediatric populations; for example, in one series nearly 25% of infants with CNS HSV infections had negative CSF PCR results (78). In patients with CNS HSV infections, HSV PCR may become negative after about 7 days of antiviral therapy (13). The persistence or reemergence of virus after antiviral therapy has been associated with a poor clinical outcome (80).

Quantitative PCR may be useful in monitoring the response to antiviral therapy. Successful antiviral therapy is associated with a decline in viral load in the CSF (1, 48, 114,

148). Quantitation of HSV may also have prognostic value; patients with >100 copies/μl (100,000 copies/ml) of CSF were reported to have worse outcome than patients with lower levels (48). However, other groups have reported no association between CSF viral load and clinical outcome (114, 148).

Interpretation of Type-Specific Serology

Type-specific serology is useful when culture or other virus detection methods are not available or when specimen collection or transport is inadequate. Serology based on gG-1 and gG-2 is vital for diagnosing subclinical and unrecognized HSV infections. In fact, many experts believe that better recognition of genital HSV-2 infections by increased use of accurate gG-based serologic tests could help slow the spread of genital herpes (17, 35, 60).

As with other herpes laboratory tests, serology tests based on gG must be ordered and interpreted with care. First, due to the low specificity of non-gG-based commercial tests, it is important to determine that a gG-based test was used. If so, a positive test for HSV-2 antibodies for a patient with genital lesions is highly likely to be a true positive. A positive HSV-2 antibody test for a patient without a history compatible with genital herpes may be false positive and should be confirmed by a different type-specific test (98). Negative HSV-2 test results for a patient with symptomatic genital disease may indicate genital HSV-1; no test can distinguish between antibodies elicited by oral versus genital HSV-1. False-negative tests may also occur because the patient is seroconverting; sera drawn 4 to 6 weeks later should be tested. IgM tests based on gG-1 and gG-2 are not available commercially. The available IgM tests cannot distinguish new from established symptomatic episodes with sufficient accuracy and are not recommended (99). However, avidity tests based on the Focus ELISA may be useful to identify recent infection (97).

The role of type-specific serology in pregnancy is controversial. Determining that a woman is HSV-2 seropositive early in pregnancy has been recommended so that treatment options can be considered and careful checks for lesions can be performed at delivery (17). Further, because the risk of herpes transmission to the neonate is 10-fold higher when maternal infection is acquired late in pregnancy, serologic screening of pregnant women and their partners has been recommended to identify seronegative women at risk of acquiring herpes (17). If a herpes culture from the anogenital region is positive at labor or delivery, a negative maternal type-specific serology indicates high risk (30 to 50%) for neonatal herpes. A positive maternal serology by WB indicates a lower risk of transmission (1 to 3%).

HERPES B VIRUS

Description of the Agent

Herpes B virus is similar to HSV in terms of genome size and structure and in terms of virion morphology. Herpes B virus DNA has 161 ± 12 kbp (91) and is extremely (approximately 75%) G+C rich.

The replication cycle of herpes B virus is very similar to that of HSV. Herpes B virus is detectable as soon as 6 h postinfection, and titers of virus stabilize by 24 to 36 h (63). At least nine herpes B virus glycoproteins have been identified, and at least two of these share antigenic determinants with glycoproteins B and D of HSV (104). As an enveloped virus, herpes B virus can be inactivated by lipid solvents, UV light, or heat, and thus, cell-free virus is inactivated rapidly in the environment.

Clinical Significance

Herpes B virus is the simian counterpart to HSV in Old World monkeys of the genus Macaca, including the rhesus, cynomolgus, Japanese, Taiwan, and stumptail macaques. The virus can affect the oropharynx or genital areas and is spread between animals via biting, sexual activity, or other close contact (144, 145). The seroprevalence of herpes B virus among macaques in the wild is unknown, but among animals housed in outdoor breeding corrals the seroprevalence is approximately 22% before 2.5 years of age, rising to more than 97% among animals 2.5 years or older (143). Primary infection in the macaque is often asymptomatic, but the virus establishes latency and can reactivate. Reactivation can be triggered by stress, such as the transition of animals from freedom to captivity, or crowded conditions. Recurrent disease in macaques is typically characterized by vesicular lesions of the tongue and buccal mucosa, progressing to ulceration. Encephalitis is rare but can occur. Asymptomatic shedding of virus can occur.

In 1932 a researcher was bitten by a macaque and developed ascending myelitis and encephalitis culminating in death. Herpes B virus was subsequently described (56, 121). About 40 cases of herpes B virus infection in humans have been described in the literature. Humans can be infected via animal bites, mucosal or eye exposure (23), inoculation of broken skin, needle sticks, or, potentially, via aerosols. The possibility of herpes B virus transmission makes macaques unsuitable as pets (106). Safety precautions for workers with close contact with macaques have been summarized recently (67).

Herpes B virus disease is severe in humans, with a mortality rate of 70% or higher without treatment. The first symptoms usually appear 3 to 5 days after exposure (although they can appear up to several weeks later) and include a localized vesicular lesion at the site of inoculation, erythema, and edema. Lymphangitis and lymphadenopathy follow, with fever, myalgia, vomiting, and cramping. Neurologic signs develop quickly, starting with meningeal irritation, diplopia, and altered sensation, progressing to paralysis, altered mentation, respiratory depression, seizures, and death within 10 days to 6 weeks. Human-to-human transmission appears to be extremely rare (22). Asymptomatic infection in humans also appears to be rare. In one study of over 300 primate handlers (of whom more than 150 had a history of exposures), none had antibody to herpes B virus (53).

Acyclovir is effective against herpes B virus, although the 50% effective dose is 10-fold higher than for HSV. Ganciclovir is somewhat more effective than acyclovir (151), although clinical experience with ganciclovir is limited. Foscarnet has also been used in some cases (23). Postexposure therapy can often prevent the development of acute disease (67). The preferred drug for postexposure prophylaxis is oral valacyclovir, due to its improved bioavailability (28). Clinical disease necessitates intravenous antiviral therapy. Intravenous therapy should be continued until symptoms resolve and two or more viral cultures are negative after having been held for 10 to 14 days, at which time therapy can be switched to oral antivirals (28). Reactivation and relapse have occurred in some patients upon cessation of antiviral therapy. Many clinicians therefore recommend continuing oral antiviral therapy indefinitely.

Collection, Transport, and Storage of Specimens

Because herpes B virus should be propagated only under biosafety level 4 conditions, diagnostic specimens are best handled by specialized reference laboratories (see below). Special precautions should be taken by other laboratories so

that herpes B virus specimens are not confused with HSV specimens and thus cultured under lower biosafety conditions, since amplification of virus in culture increases the risk to laboratory personnel.

Reference Laboratories for Herpes B Virus

Lesion swabs should be taken from each collection site by using a separate, sterile Dacron or cotton swab with wooden or plastic shafts. Swabs or biopsy tissue should be placed into separate tubes containing 1 to 2 ml of VTM. Specimens should be stored on dry ice or at <–60°C until shipped. Advice regarding proper specimen collection and submission of specimens for herpes B diagnosis can be obtained from the National Institutes of Health B Virus Resource Laboratory, Atlanta, Ga., phone (404) 651-0808, or from their website http://www.gsu.edu/bvirus.

Identification of Virus and Serodiagnosis

Herpes B virus can be cultured in monkey kidney and chick embryo cells. A characteristic CPE similar to that of HSV is seen, with polykaryon formation and intranuclear Cowdry type A inclusion bodies. Confirmation of herpes B virus in culture can be done using monoclonal antibodies or molecular techniques. PCR for herpes B virus (109, 124, 125, 126) is generally preferred over culture for diagnostic purposes, since it is comparatively rapid and highly sensitive and specific and avoids the need to amplify infectious virus to high titers.

Serodiagnosis of herpes B virus infections has been complicated by extensive cross-reactivity with HSV-1 and HSV-2. However, a competitive ELISA that allows discrimination of antibodies to herpes B virus, HSV-1, and HSV-2 has been developed (73). Serologic testing can be useful for evaluation of potentially infected animals involved in human exposures and for screening research animals. Serial determinations of serostatus from potentially exposed individuals are also useful adjuncts for diagnosis (23).

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Varicella-Zoster Virus

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101

TAXONOMY

Varicella-zoster virus (VZV) is a member of the *Herpesviridae.* It is classified as an alphaherpesvirus along with the herpes simplex viruses (HSV). It has been designated human herpesvirus 3 by the International Committee on Taxonomy of Viruses.

DESCRIPTION OF THE AGENT

VZV has a morphology typical of the *Herpesviridae* (12). It has a linear double-stranded DNA genome of approximately 125,000 bp ([80 ± 3] × 10^6 Da) that exists as four isomers. The genome encodes at least 70 open reading frames (ORFs) and consists of a 67 × 10^6-Da long unique DNA segment, U_L, and a 3.4 × 10^6-Da short unique sequence, U_S. Each unique sequence is flanked by internal and terminal repeats (Fig. 1). The virion has a diameter of 150 to 200 nm and consists of an inner icosahedral capsid composed of 162 capsomeres surrounded by a tegument and of an envelope composed of two or more membranes. The envelope contains essential lipid; therefore, virus infectivity is inactivated by lipid solvents. The viral genome encodes nonstructural and structural proteins, including at least seven glycoproteins (gps), designated E, B, H, I, C, K, and L, which are at least partially analogous to the gps of HSV (20, 22). Unlike HSV, VZV has no glycoprotein D (gD). The gps are required for viral attachment and penetration of host cells. Immune recognition of gps and other virion components results in humoral and cellular responses to VZV. Immunity to nonstructural antigens undoubtedly also plays a role in host defense.

There are at least three distinct VZV genotypes based on nucleotide analysis (83). Antigenic variation, however, of VZV isolates has not been demonstrated. Restriction enzyme profiles of VZV isolates reveal that (i) there are no detectable differences between varicella and zoster isolates from the same individual (23), (ii) the agent is remarkably stable after passage in vitro and in vivo, and (iii) non-epidemiologically related strains may be differentiated with molecular assays (54). VZV shares major antigens with herpesviruses that produce varicella-like disease in various simian species (15), and it appears to share minor antigens with HSV (67).

EPIDEMIOLOGY AND TRANSMISSION

VZV is a highly cell-associated virus that is spread through the body by cell-to-cell contact. However, during acute infection, highly infectious cell-free VZV is produced in skin vesicles, accounting in large part for the high degree of contagion of varicella and zoster (9). VZV spreads by the airborne route and infects the respiratory mucosa; the source of most of the airborne virus is the vesicular skin lesions. Humans are the only natural host for VZV, so there is no animal reservoir for this virus.

CLINICAL SIGNIFICANCE

Varicella (chicken pox) and zoster represent different clinical manifestations of VZV infection. Varicella occurs most frequently in children and is characterized by a generalized vesicular exanthem often accompanied by fever. Zoster (shingles) usually occurs in adults or immunocompromised patients and consists of a painful, circumscribed eruption of vesicular lesions with accompanying inflammation of associated dorsal root or cranial nerve sensory ganglia. Varicella is the primary infection with VZV, whereas zoster is a secondary infection due to reactivation of latent VZV in sensory and cranial nerve ganglia. The following observations indicate that zoster results from reactivation of latent virus rather than reintroduction of virus into the host: (i) zoster does not exhibit the seasonal prevalence seen with varicella (late winter and spring in temperate climates); (ii) zoster does not occur commonly in parents of young children with chicken pox; (iii) latent virus is detectable in the sensory ganglia of autopsy specimens as indicated by the presence of VZV DNA (51), RNA (37, 49), and certain latency-associated proteins (50); and (iv) viruses causing the primary infection and subsequent zoster are the same as demonstrated by molecular studies of VZV isolates from patients with zoster after either natural chicken pox or varicella vaccination (73). Studies after natural varicella or immunization indicate that reinfection and reactivation of VZV may occur in the absence of clinical symptoms and that second attacks of clinical varicella are infrequent but may occur (24, 53). VZV infection may cause severe or fatal disease in individuals who are receiving immunosuppressive therapy or who have abnormalities in cell-mediated immune responses. Progressive, generalized varicella may occur in as many as 30% of children who acquire chicken pox while receiving chemotherapy and radiotherapy for cancer, with mortality of 7% (18).

VZV infections can be treated with acyclovir (ACV), and disease can be prevented by vaccination. Live attenuated

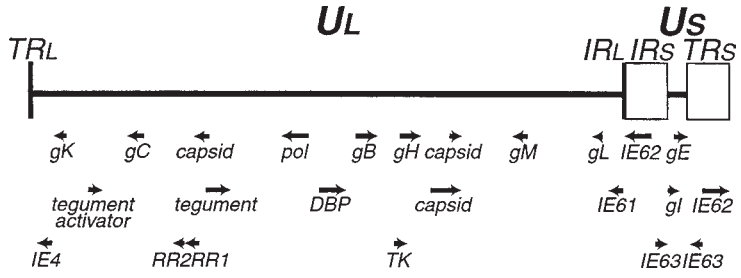


FIGURE 1 Diagram of the structure of the VZV genome. The linear double-stranded DNA consists of a long unique segment, U_L, of approximately 100 kbp, and a short unique sequence, U_S, of approximately 5.4 kbp, flanked by internal and terminal repeats of 6.8 kbp (TR_S and IR_S) and repeats flanking U_L (TR_L and IR_L). The ORF and direction of transcription (arrows) of some of the important genes are indicated below the map. RR, ribonucleotide reductase; DBP, DNA binding protein. Reprinted from reference 20.

varicella vaccine derived from the Japanese Oka strain was licensed in the United States for routine use in susceptible healthy persons in 1995. Since licensure, the incidence of varicella has decreased in sentinel counties in the United States by 71 to 84% (72). Approximately 15% of vaccinated individuals may develop "breakthrough" varicella caused by wild-type (WT) virus months to years after vaccination (23) because the vaccine is not 100% protective. However, it is 97% protective against severe disease (82). Vaccine-induced zoster is rare but has been reported in healthy and immunocompromised patients (45, 73). Infection of highly susceptible patients can be prevented through administration of varicella immunoglobulin.

Although the clinical presentations of varicella and zoster are likely to be easily recognizable to medical personnel, clinical symptoms such as rash may be diagnostically deceiving. Eruptions caused by VZV and HSV can be confused with each another. In one study, 13% of patients diagnosed as having zoster were found to have HSV infection on laboratory testing (36). The diagnosis of acute VZV disease is best made by testing vesicular skin lesion specimens rather than by determining relative antibody levels in acute- and convalescent-phase sera. Discrimination between VZV infection and other causes of vesicular eruptions such as enteroviruses, bacterial agents, and hypersensitivity reactions may also be necessary. Rapid diagnosis of suspected infection is important in a number of situations so that appropriate medical management such as antiviral therapy or passive immunization with varicella immunoglobulin prophylaxis for high-risk exposed persons can be initiated. These settings include (i) immunocompromised patients and their contacts, (ii) uncommon presentations such as pneumonia or encephalitis, (iii) some cases of breakthrough after vaccination, (iv) suspected reactions in the days to weeks following vaccination, and (v) zoster sine herpete, the presentation of cutaneous pain in a dermatomal distribution in the absence of a rash.

It is possible, although potentially inaccurate and slow, to diagnose acute VZV infection by measurement of immunoglobulin (IgM) titers to VZV; these determinations may have false-positive and false-negative results. Antibodies to VZV after varicella begin to be detected only after onset of rash, usually within 3 days. Titers of IgG rise for about 10 days and then fall, but not to susceptible levels in most cases. Zoster develops despite the presence of VZV antibodies, often in high titer. Serology thus is not the best way to diagnose acute VZV disease.

Determination of VZV serostatus may be helpful in several settings other than acute disease. Determining immune status is useful for guiding the management of exposed, high-risk immunocompromised individuals. It may also be useful for healthy adults with no history of varicella. Only approximately 25% of such individuals are susceptible (21); however, these susceptible adults have an increased risk of developing severe varicella. Adults raised in countries with tropical climates are also at increased risk of varicella compared to those from countries with temperate climates (18). Ideally, measurement of antibodies to VZV could be used to determine whether immunization has been successful in vaccinees, but available serologic tests are not sensitive enough for this purpose (see below).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

To make a laboratory diagnosis of acute VZV infection, the presence of the virus or one of its products should be demonstrated in tissues or fluids obtained from the patient. Specimens usually examined for VZV are smears of vesicular lesions, skin scrapings, vesicular fluid, and tissues obtained at autopsy. Detection of VZV in affected tissues or fluids is diagnostic because asymptomatic patients do not shed virus. Tests to detect VZV and acceptable specimens are shown in Table 1 (29). Depending upon the individual circumstances, it is optimal to obtain material for immunofluorescence (IF) staining, PCR, and/or culture. If insufficient material is available for all determinations, IF and/or PCR is preferred and virus isolation may be forgone. A few diagnostic laboratories no longer use culture for diagnosis because IF and PCR are faster, less expensive, and more accurate.

Smears of Cellular Material from Lesions

To obtain specimens from lesions, a sterile needle is used to lift off the top of a vesicle. A sterile swab moistened in sterile transport medium is then used to vigorously swab the base of the lesion, applying enough pressure to collect epithelial cells without causing bleeding. Swabs can be placed in viral transport medium and taken rapidly to the laboratory where the laboratory personnel will prepare the slides by using methods such as vortexing and cytospin to concentrate cellular material. Alternatively, for direct detection of antigens, lesion material can be applied directly to slides at the bedside (11). Slides can then be transported to the laboratory for fixation and staining. It is essential that lesion

TABLE 1 Diagnostic tests for acute VZV infection[a]

Test	Comments
PCR	Becoming the diagnostic standard. Useful with scabs and skin lesions several days old, when culture is negative. Most sensitive assay. Useful for CSF, other body fluids, and tissues. Used to differentiate between WT and vaccine VZV disease in vaccine recipients. Rapid results (1–2 days).
DFA	Commercial kits available to use with fluorescein-conjugated monoclonal antibody to VZVgE. Results potentially available within a few hours. Possible to distinguish rapidly between HSV, VZV, and poxviruses (which would yield negative results).
Culture	Traditionally the diagnostic gold standard. May take up to 1 week for CPE. Positive only in the first 1–2 days of rash.
Serology	Limited value; requires 7–10 days between acute- and convalescent-phase sera. IgM may yield false-positive or false-negative results.

[a] Adapted from Hambleton and Gershon (29).

specimens contain a large number of infected epithelial cells for successful detection of antigen.

The time between specimen collection and testing is particularly critical for successful VZV isolation because the virus is very labile and infectivity is transient. Lesions present on skin for more than 4 days rarely yield infectious virus, but scrapings may give positive results by IF and/or PCR (5, 11, 16, 38, 40, 47, 52, 58, 82). Crusts from lesions do not yield infectious virus and are not suitable for IF staining or other specific antigen detection methods. However, crusts are very useful for PCR and may be expected to yield positive results in VZV infection.

Vesicular Fluid

Vesicular fluid contains cell-free virus. This material is therefore suitable for a variety of diagnostic assays (Table 1) but not for IF staining (due to its lack of cellularity). Fluid can be collected into capillary pipettes or syringes or on swabs. For culture, collection on swabs is least preferable. Virus collected on swabs is less stable than in vesicle fluid. In addition, medium used for specimen transport or virus elution during swab processing may impede recovery due to dilution and loss of virus infectivity.

Tissue

Smears of autopsy tissue for IF staining are prepared by excising three or four pieces of autopsy tissue about 10 to 15 mm in size, holding the tissue with forceps, and gently pressing the cut surface to the clean area of a slide. A series of impressions is made over an area 30 to 40 mm in length. Three or four slides should be prepared from each specimen. The slides are dried at room temperature for 15 to 20 min, fixed in cold acetone for 10 min, and stained by IF as described below. Suspensions of tissue are prepared for virus isolation as described in chapter 80. Skin biopsy material, for example, from a vesicular lesion, can be fixed, paraffinized, sectioned, and then stained with anti-VZV antibodies for demonstration of VZV antigens. Tissue samples can also be used for PCR analysis, but this will require nucleic acid extraction prior to amplification.

Blood (Buffy Coat) and CSF

Virus has been isolated from buffy coat cells (3, 55) and extremely rarely from cerebrospinal fluid (CSF) (35) from immunocompetent and immunocompromised patients. Demonstration of VZV in CSF by PCR appears to be more sensitive and useful than culture (33, 38, 52, 58, 63, 77, 89, 92). Whole blood should be promptly submitted to the laboratory in sterile anticoagulant other than heparin, for example, EDTA. CSF should be sent in a sterile tube.

Storage and Transport of Specimens

Specimens for virus isolation attempts should be inoculated into suitable cell cultures as soon as possible after collection. If inoculation must be delayed (ideally for no longer than 12 h), specimens may be transported or held (preferably in transport medium) on wet ice or in a refrigerator at 2 to 8°C. For longer storage, freezing at −70°C (or on dry ice) is required to preserve virus for isolation. Refrigeration is preferred for smears to be examined for viral antigen, but it is not necessary for cytological examination or for PCR. Specimens from vesicular lesions may be infectious; thus, suitable precautions should be taken in packing and storage to protect postal and laboratory personnel.

DIRECT DETECTION

Microscopy

For cytological examination of smears from vesicular lesions (Tzanck smears), cellular material from the base of vesicular lesions is collected and processed as described above. Prepared slides are fixed with methanol and stained with Giemsa stain buffered to pH 7.0 to 7.2. Microscopic examination reveals the presence of multinucleated giant cells with altered chromatin patterns (Fig. 2). Multiple viral capsids in the nucleus of the infected cell appear as eosinophilic intranuclear inclusions. This appearance is characteristic of infection with either VZV or HSV. Due to the availability of PCR and IF, this nonspecific technique is rarely useful today and cannot be recommended.

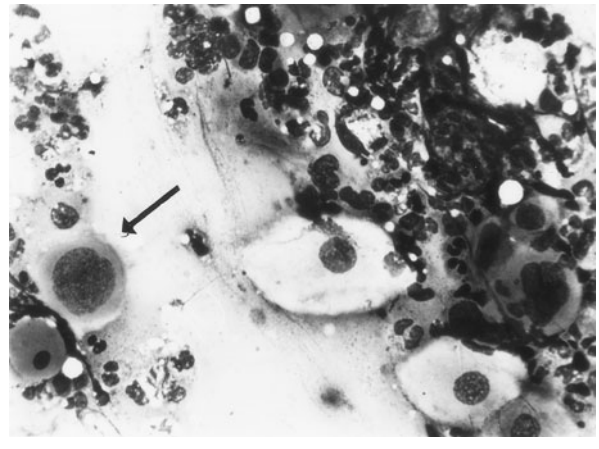


FIGURE 2 Giemsa-stained preparation of material from the base of a vesicular lesion. Magnification, ×102. The arrow indicates a giant cell with a folded nucleus characteristic of VZV or HSV.

Vesicular fluids can be examined by electron microscopy (EM), but it is not possible to differentiate between different herpesviruses by this method. The main diagnostic use of EM is to distinguish between a herpesvirus and agents such as poxviruses. To prepare slides for negative staining, a drop of the specimen is placed on a grid and blotted with filter paper. A drop of 3% phosphotungstic acid (prepared in distilled water buffered to neutrality with 1 N KOH) is then added and blotted. The specimen should be examined in an electron microscope as soon as possible. The demonstration of virus with typical herpesvirus morphology (Fig. 3) identifies the etiological agent as a member of this group and distinguishes it from the poxvirus group, but it does not provide a specific diagnosis of VZV infection. For confirmation of results, vesicular fluid can be fixed with glutaraldehyde and centrifuged. The pellet is then dehydrated and embedded in epoxy resin for examination by standard EM.

Antigen Detection by IF Staining

To prepare slides from swabbed lesions submitted in viral transport medium (VTM), swabs are vortexed in the original transport tube to release collected cells. The swab is discarded, and tubes are centrifuged to concentrate cells. Supernatant is decanted, and cells are resuspended in a small volume of phosphate-buffered saline (PBS). Cell suspensions are applied directly to slides or by using a cytospin. Most laboratories utilize Teflon-coated slides for this purpose. If individual direct IF staining for VZV and HSV is to be performed, at least three slides should be made. Slides are fixed in cold acetone for 15 min and air dried.

Slides of lesion specimens can be stained by the direct method using conjugated antibodies or with indirect methods (unlabeled anti-VZV antibodies followed by labeled secondary antibody). Monoclonal antibodies, which are preferred, have overcome problems previously encountered with immune VZV serum including the presence of unwanted antibodies to cellular proteins (19, 86). The use of human sera of uncertain antibody content is considered unreliable because these sera may contain antibodies to other human herpesviruses such as HSV and cytomegalovirus (CMV), from which it is important to distinguish VZV.

Direct fluorescent-antibody (DFA) staining is more specific than indirect staining, due to the potential for nonspecificity when more than one antibody is used in IF. Directly labeled antibodies to VZV gps are available from commercial sources such as Merifluor (Meridian Biosciences, Cincinnati, Ohio). There is extensive diagnostic experience with commercially produced monoclonal antibodies to VZV gE. In one study involving 133 patients, 79 (56%) specimens were positive for VZV: 40 (51%) by DFA alone, 2 (3%) by culture only, and 37 (47%) by both culture and DFA (11). The sensitivity of DFA was 97.5% (77 of 79) and 49.4% (39 of 79) for viral culture. Both procedures were highly specific. Rapid differentiation between HSV and VZV by simultaneously using antibody pools labeled with different fluorophores on a single cell spot such as SimulFluor reagents has been reported (8, 71). Slides with wells are commercially available (Trinity Biotech, Co., Jamestown, N.Y.) and highly convenient. Commercial monoclonal antibodies are usually supplied at working concentrations. Antibodies from noncommercial sources require titration against VZV-infected smears to determine appropriate concentrations for use in staining protocols. Working dilutions of these immune conjugates are usually prepared in PBS.

For staining, conjugated antibody is applied to the slide in a volume sufficient to cover the lesion smear (about 0.05 ml). Positive and negative control slides for VZV should also be stained with each conjugate as controls for conjugate reactivity and specificity. After addition of antibody, slides are incubated in a humidified atmosphere at 18 to 25°C for 30 min, rinsed twice in PBS (5 min for each rinsing), rinsed once in distilled water, and then air dried. Commercial mounting medium or buffered glycerol solution (9 parts glycerol and 1 part PBS) and a coverslip are then placed over the stained cells.

Examination with a fluorescence microscope at a magnification of ×100 to ×400 should reveal specific staining of 3+ to 4+ intensity, especially associated with the cytoplasm of the rounded epithelial cells. Positive specimens should contain two or more cells with typical apple green fluorescence (Fig. 4). Vesicular lesion specimens containing too few basal epithelial cells for definitive examination should be reported as unsatisfactory rather than negative.

Unconjugated mouse monoclonal antibodies can be used for indirect detection of VZV in clinical specimens. When using the indirect method, it is essential to use a conjugate against mouse immunoglobulins that is free from nonspecific reactivity with human tissue and serum components. This is determined by testing dilutions of the conjugate against infected and uninfected cell smears in the absence of intermediate mouse antibodies. The appropriate working dilutions of the VZV reagent and the anti-mouse globulin conjugate are determined by preliminary titration.

Nucleic Acid Amplification

Molecular methods are immensely practical for demonstrating VZV infection, and they are becoming the standard for diagnosis. In the past few years there has been a veritable explosion in publications concerning molecular techniques to identify VZV infections. Although the virus has been demonstrated using in situ hybridization (1, 74), this technique is

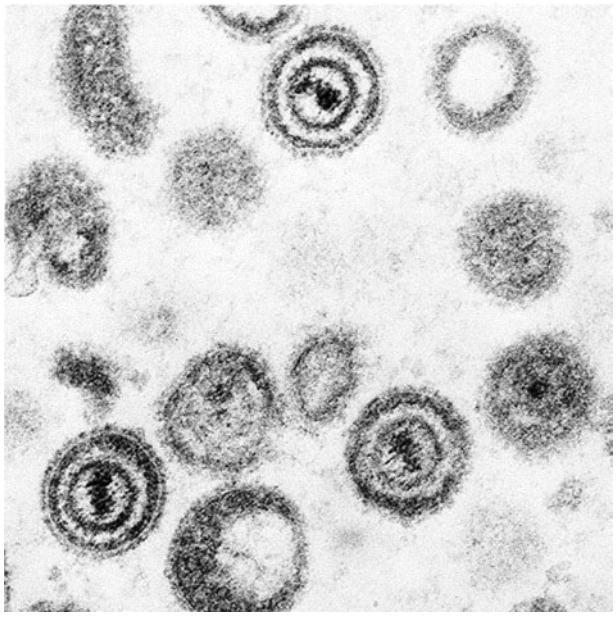


FIGURE 3 Electron micrograph of VZV. The specimen was obtained from a skin vesicle from an infant on day 1 of varicella. Magnification, ×100,000. Reprinted from *Cell* (9) with permission of Elsevier.

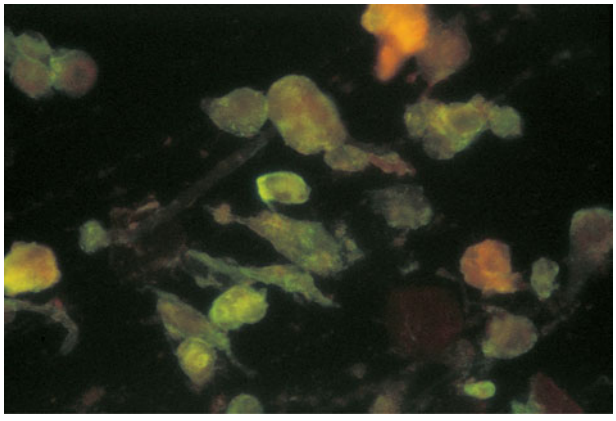


FIGURE 4 IF assay for detection of VZV antigens. A swab was obtained from an unroofed skin vesicle from a patient with a localized unilateral vesicular rash, from which a smear was made. The specimen was fixed and stained with monoclonal antibody to VZV gE. Numerous VZV positive cells are shown, establishing the diagnosis of zoster. Magnification, ×200.

not nearly as well developed nor as practical as nucleic acid amplification by PCR. Nucleic acid amplification is more sensitive than virus isolation partly because infectious virus is more labile than viral DNA (42, 70, 78, 81, 82).

PCR has been employed successfully for VZV detection from skin scrapings and vesicular fluid (4, 5, 16, 42, 44, 47, 70, 78, 81, 82, 85), respiratory and ocular secretions (10, 63, 78, 81, 84), blood (32, 76, 77, 84), and CSF (10, 13, 33, 38, 52, 58, 63, 77, 89, 92). Because PCR is often used to distinguish between vaccine and WT VZV infections, ORFs 54, 38, and 62 have been developed as popular amplification targets.

Many conventional PCR methods have been described (4, 7, 16, 33, 39, 40, 42, 47, 48, 52, 58, 89); however, homogeneous (real-time) PCR methods have quickly become the new standard due to faster processing time, decreased labor and expense, lower risk of laboratory contamination with amplicons, and comparable sensitivity to conventional PCR (6, 32, 70, 77, 78, 81, 84, 85). Table 2 describes information from published real-time PCR methods for VZV.

A significant advance associated with real-time PCR is the multiplex assay, used for simultaneous diagnosis of VZV and other viruses from a single specimen (10, 13, 52, 58, 76, 77). Real-time PCR assays have been employed to determine VZV viral loads in blood, a technique which may prove to be useful in the management of immunocompromised patients (32, 84).

Reagents for VZV DNA amplification by conventional (multiplex herpesvirus PCR; Argene, Inc., North Massapequa, N.Y.) and real-time PCR (Qiagen Diagnostics, Valencia, Calif.) are commercially available. Table 2 indicates published methods used for DNA extraction prior to amplification. Internal controls spiked into samples have been used to determine the efficacy of DNA extraction and if extraction has removed inhibitors of thermostable polymerases (6, 78). It is possible to purchase quantified VZV DNA for use as positive controls. Potential sources are Advanced Biotechnologies, Inc. (Columbia, Md.) and Zeptometrix Corp. (Buffalo, N.Y.).

Molecular methods for discriminating between WT and vaccine (Oka) strains directly in patient specimens have been developed (39, 42, 63, 73, 82). Such assays are useful in distinguishing the causes of rash in a number of settings including possible vaccine reactions (17, 73) and the rarely observed transmission of vaccine virus from healthy (41, 62, 73) or leukemic vaccinees (31) to varicella-susceptible contacts. Molecular typing has been reported for the rare identification of Oka-mediated herpes zoster in a vaccinated immunocompromised patient (45).

Conventional PCR assays with restriction fragment length polymorphism (RFLP) analysis of ORF6 and a combination of ORFs 38 and 54 (ORF38/54) (Table 3) have been described (39, 79). In a recent comparison of different typing methods it was found that BglI and PstI do not distinguish between parental Oka and Oka vaccine. In addition, inconsistent patterns of ORF62 BssHII and ORF6 AluI RFLP were found in vaccine preparations and vaccine-derived rashes. ORF62 SmaI and NaeI RFLPs, however,

TABLE 2 Real-time PCR for VZV

Target ORF (size in bp)	Sample(s)	DNA extraction method	Instrument	Probes	Positive control material	Reference
4 (266)	Swab in VTM	Manual spin column[a]	LightCycler[b]	SYBR green; T_m analysis	VZV genomic DNA standard[c]	70
28 (63)	CSF, swab, biopsy specimen, blood	Manual spin column[a]	LightCycler[b]	TaqMan	Cloned amplicon	86
29 (202)	CSF, serum, plasma	Manual spin column[d]	LightCycler[b]	FRET[e]	Quantified genomic DNA[f]	77
38 (82)	Swab in VTM	Automated extraction[g]	ABI Prism 7700[h]	TaqMan	Quantified genomic DNA[f]	78
38 (82)	Not specified	Manual spin column[a]	ABI Prism 7700[h]	TaqMan	Plasmids and virus	85
38 (89)	Various	Automated extraction[g]	ABI Prism 7700[h]	TaqMan	Phocine (seal) herpesvirus internal control	81

[a] QIAamp DNA Blood Mini Kit (Qiagen, Valencia, Calif.).
[b] Roche Diagnostics, Indianapolis, Ind.
[c] Swedish Institute for Infectious Disease Control.
[d] Roche High Pure Viral Nucleic Acid kit (Roche Applied Sciences, Indianapolis, Ind.).
[e] Fluorescent resonance energy transfer with two adjacent probes.
[f] Advanced Biotechnologies, Inc., Columbia, Md.
[g] MagNA Pure LC (Roche Diagnostics).
[h] Applied Biosystems (Foster City, Calif.).

TABLE 3 Genotyping of VZV vaccine and WT strains

ORF(s)	Amplicon size (bp) or real-time PCR	RFLP or SNP[a]	Virus strains[b]	Reference
38	350	PstI (250, 100)	WT +/POka[c] and vaccine −	39
54	222	BglI (137, 85)	WT −/POka and vaccine +	
62[d]	268	SmaI (+, 112, 79, 41, 36; −, 153, 79, 36)	−, numerous WT; +, 1 strain from U.S. vaccinee	48
62[d]	Real-time PCR	WT, T; Vaccine, C	Numerous WT; 1 vaccine strain (U.S. vaccinee)	47
6	418	AluI (+, 205, 112, 101; −, 317, 101)	+, WT and POka; −, 6 vaccines	79
62[e]	Real-time PCR	WT, T; Vaccine, C	Numerous WT; vaccine, 1 Canadian vaccinee	7
62[d]	244[f]	SmaI (113 + 131)	−, numerous WT and POka; +, vaccines	60
62	781	NaeI (517 + 264)	−, WT and POka; +, vaccines	60

[a] SNP, single nucleotide polymorphism. T, thymidine; C, cytosine.
[b] Wild-type (WT) or vaccine. +, RFLP is present; −, RFLP is absent.
[c] POka, WT parent Oka strain from which Oka vaccine was obtained. POka is still circulating in some geographic areas such as Japan.
[d] Nucleotide no. 106262 of VZV genome (GenBank accession number, X04370).
[e] Nucleotide no. 107252 of VZV genome.
[f] Size variation relative to reference 48 is due to difference in primer design.

were present in all vaccine preparations and vaccine-derived rashes, but not in all WT viruses tested. Therefore, SmaI and NaeI sites can be used successfully to identify rashes caused by varicella vaccine (60).

Real-time PCR assays based on sequence polymorphisms in ORF62 (7, 48) and ORF38 (80) have also been published. Such molecular assays reflect differences between circulating VZV strains; however, none have been identified as vaccine virus-attenuating mutations. The molecular basis for Oka vaccine strain attenuation is still under investigation. Because VZV varies in different geographical locations (60), analyses of ORFs 6 and 62 have been useful in Japan, while those involving ORF38/54, ORF62, and ORF38 are especially useful in western countries (59, 65) (Table 3).

ISOLATION

Host Systems

Diploid human cell lines or primary human cell cultures are the most sensitive host systems for isolation of VZV from clinical materials. Human fetal diploid kidney or human fetal diploid lung (HFDL) cells have been found to be highly satisfactory for primary isolation of VZV and are available from commercial sources (see chapter 81). Some laboratories have used human foreskin fibroblasts for isolation of VZV, but their sensitivity, compared to that of HFDL cells, is uncertain. Primary monkey kidney cells and embryonic guinea pig cells support growth of VZV but are less sensitive than HFDL cells. A549 cells, a human cell line of lung carcinoma cells, are sometimes used in the clinical laboratory in preference to HFDL (11, 64). MeWo cells, a human melanoma cell line, support growth of VZV (28), but plaques are more difficult to identify than those in fibroblast cultures. Cell cultures are initiated with Eagle minimal essential medium prepared in Hanks' or Earle's balanced salt solution (BSS) and supplemented with 10% heat-inactivated (56°C for 30 min) fetal bovine serum. Inoculated cell cultures are maintained in Eagle minimal essential medium prepared in Earle's BSS supplemented with 2% inactivated fetal bovine serum. Although this medium maintains the cultures for 14 days without a fluid change (a period usually sufficient for the development of a specific viral cytopathic effect [CPE]), a change of medium at least once a week is preferable. VZV cannot be isolated in mouse or embryonated egg host cell systems.

Virus Subpassage and Storage

Infectious VZV remains in close association with the host cell in cell cultures, and therefore it is necessary to use virus-infected cells rather than culture fluids as an inoculum for serial propagation of the virus. Trypsin-dispersed infected cells are most suitable for subpassage of VZV. The medium is removed from cell cultures in which CPE involves approximately 50% of the cell sheet and rinsed briefly with PBS, and about 1 ml of 0.25% trypsin solution is added to each tube culture. After the cells begin to round up (usually after 60 s at room temperature), most of the trypsin is removed, and the small amount of residual trypsin is distributed over the monolayer. As soon as the cells detach from the surface, they are dispersed in the original volume of growth medium and inoculated into fresh cell cultures.

Virus in infected cells can be stored in the frozen state if the viability of the cells is maintained through the use of a cryoprotective agent such as dimethyl sulfoxide (DMSO) in the freezing medium. Infected cells are dispersed with trypsin as described above and then suspended in 80% heat-inactivated fetal bovine serum and 20% DMSO. Infectious virus can be recovered after storage at −70°C for 18 months or more, but infectivity is preserved more effectively by storage of the infected cells in liquid nitrogen. High-titer cell-free virus prepared by sonic treatment of infected cells is stable at −70°C in Hanks' BSS for at least 5 years, although titers decrease with time (68).

IDENTIFICATION

Presumptive Identification

Presumptive identification of VZV may be made on the basis of cells in which virus grows and CPE. Unlike HSV, VZV fails to produce CPE in rabbit or hamster kidney cell cultures. Initially, VZV CPE consists of small, discrete crescent-shaped foci of rounded and swollen refractile cells (Fig. 5). CPE may appear from 2 to 14 days after inoculation of the cultures with clinical materials, but in most instances it is first apparent after 3 to 7 days. The foci of infected cells enlarge and may slowly involve much of the monolayer. The spread of infection can be accelerated by trypsin treatment and replating.

VZV CPE is more focal in nature and progresses more slowly than that of HSV. The specimen source and clinical presentation should usually prevent confusion between VZV and human CMV. However, it may be necessary to distinguish between these herpesviruses if isolations are made from tissue specimens. Production of CPE by CMV generally is slower and more focal in nature than that produced by VZV. Unlike CMV, VZV usually produces CPE in primary monkey kidney cells after initial isolation.

Specific Identification

Identification of VZV isolated in cell cultures is performed by direct IF staining when the inoculated cultures show distinct plaques. Trypsin-dispersed cells from an infected tube culture are suspended in 3 ml of PBS with 2% fetal bovine serum, and cells are sedimented by centrifugation at 1,000 × *g* for 5 min. Cells are resuspended in 0.2 ml of PBS and spotted onto slides. Alternatively, slides can be prepared using cells that have been scraped into a small volume of PBS after monolayers are washed twice with PBS. The slides are fixed and stained as described above for antigen detection by DFA. Positive-control slides should be included in each run. Positive results are based upon staining of 3+ to 4+ intensity with the VZV conjugate. Infected cells can be easily distinguished from uninfected cells by using the Evans blue counterstain reagent that is included in commercial kits.



FIGURE 5 CPE of VZV in human fetal diploid lung (HFDL), showing a characteristic viral plaque. Magnification, ×100.

Identification of VZV Isolates by Use of Shell Vials

The shell vial method is a combination technique involving virus culture and early specific identification of virus with monoclonal antibody prior to development of obvious CPE. It is more rapid and sensitive for VZV detection than conventional tube culture (26, 66, 87). The disadvantages of this method include the lack of a viable isolate after the virus has been identified and the inability to identify other viruses in clinical specimens. Therefore, some clinical laboratories may also inoculate standard tube cultures during specimen processing if a virus resistant to antivirals is suspected.

Shell vials containing coverslips with monolayers of human diploid fibroblast cells are commercially available from many sources. One useful product is H&V Mix (Diagnostic Hybrids, Athens, Ohio). This is a proprietary mix of CV-1 and MRC-5 cells sold in shell vial and plate format for the detection of HSV, VZV, and CMV (30). Swabs or vesicular fluids are diluted in approximately 1 ml of medium, and 0.2 ml is inoculated directly into shell vials from which medium has been removed. Vials are centrifuged for 40 min at 700 × *g* at room temperature. After centrifugation, 2 ml of maintenance medium is added to each vial. One set of positive and negative controls should be set up daily. Vials can be incubated at 35 to 37°C for 1 to 3 and 5 days. HSV can be detected after 24 h of incubation, but VZV usually takes an additional day, and CMV may not be obvious for 5 to 7 days. After incubation, the vials are washed twice with 1 ml of PBS and monolayers are fixed in 2 ml of cold acetone for 10 min. Coverslips are either stored at −70°C or used immediately. For staining, one vial is incubated with 1 or 2 drops of commercial fluorescein-conjugated monoclonal antibody to VZV (see "Antigen Detection by IF Staining" above for reagents) for 15 to 30 min at room temperature or 37°C. The vials are washed with PBS twice and quickly rinsed with distilled water once. The coverslips are removed from the vials, air dried, and placed cell side down on microscope slides in mounting medium. Positive results are based on staining of 3+ to 4+ intensity with the VZV conjugate.

Preparation of Control Slides for IF Staining

Control slides are required for IF staining for antigen detection in lesion smears, for identification of viral isolates, and as an antigen substrate for antibody assays. Negative control slides are prepared from uninfected monolayers. Positive control slides are prepared using stock virus that has been subpassaged two or three times to increase infectivity. Fresh monolayers are infected with subpassaged virus, and cells are harvested when cultures show about 50% CPE, usually 24 h after inoculation. Infected and uninfected cells are dispersed with trypsin, suspended in 0.1 M PBS (pH 7.2 to 7.5) with 2% fetal bovine serum, and sedimented by centrifugation by 1,000 × *g* for 5 min. The supernatant fluids are removed, and the packed cells are resuspended in PBS with 2% fetal bovine serum, using a volume of 0.01 ml per tube culture or 0.4 ml per 32-oz (ca. 0.95-liter) flask culture. Smears approximately 5 mm in diameter are made from this cell suspension by placing small drops (approximately 0.025 ml) on microscope slides or by using a cytospin apparatus. The smears are dried at room temperature, fixed with cold

acetone for 10 min, and then dried at room temperature. Smears can be stored at −70°C until they are used. Before staining, the smears are ringed, if necessary, with a liquid embroidery pen, wax pencil, or diamond pen to retain the conjugate.

SEROLOGICAL METHODS

Serological tests are used for laboratory diagnosis of varicella or zoster infections and for determining immunity status to varicella. Serologic testing is particularly useful for adults who have no history of varicella, since over 75% have detectable VZV antibody and therefore presumably experienced mild or subclinical varicella.

Today, viral diagnostic laboratories are using mainly enzyme immunoassays (EIAs) as their principal serological tool. Alternative methods for serodiagnosis of VZV infections are indirect IF with fluorescent-antibody staining of membrane antigens in VZV-infected cells (FAMA) (88), anticomplement immunofluorescence (ACIF) staining (57), and latex agglutination (LA) (21, 75). Neutralizing antibody to VZV can be assayed by plaque reduction techniques with cell-free virus obtained by sonic treatment of infected human diploid cells (69); however, this is primarily a research rather than a routine diagnostic tool.

The FAMA assay is generally considered the "gold standard" for VZV serological procedures. The sensitivity and specificity of other serologic assays have been compared to those of FAMA in a few studies. In one study of healthy adults, the sensitivity, specificity, positive predictive value, and negative predictive value of EIA compared to FAMA were 86.1, 98.6, 98.9, and 82.1% (14). FAMA and LA assays are also more sensitive than EIA for demonstration of antibodies after vaccination (21, 61, 75). In a study of vaccinated adults, the sensitivity and specificity of EIA (VIDAS Varicella-Zoster IgG; bioMerieux, Hazelwood, Mo.) were 74 and 89% compared to those of FAMA (61). It is noteworthy that 10% of adults were positive by EIA but negative by FAMA prior to vaccination in this study, suggesting that this EIA is less specific than FAMA.

FAMA and LA results have correlated well with resistance or susceptibility to infection in clinical settings (18). In a study comparing FAMA with LA, approximately 80% of 77 sera from healthy adults tested were seropositive up to 11 years after vaccination; there were no significant differences between the rates of positivity of LA and FAMA (21). A newly developed IF antibody assay using a cell line of lung carcinoma infected with VZV has been described and compares favorably with FAMA (64). While it is sensitive, the FAMA method is somewhat cumbersome. Other disadvantages include the requirement for live unfixed VZV-infected cells and the lack of automation.

Traditionally for serodiagnosis of infection, acute- and convalescent-phase sera must be analyzed in the same test run. A fourfold or greater increase in IgG antibody titer between acute- and convalescent-phase sera is considered diagnostically significant. For EIAs, however, fourfold increases in antibody are not measured. Values for significant increases in antibody titer must be determined on an individual basis for each particular assay. For commercial kits, the diagnostically significant increase is determined by the manufacturer and provided in the instruction manual. In varicella, antibodies are usually demonstrable within several days after onset of rash, and they reach a peak 2 to 3 weeks later. The antibody increase is usually more rapid in zoster, and antibodies are detectable at the time of rash onset.

EIAs

A number of EIAs are commercially manufactured and have been evaluated (14, 43, 61). A few examples of some commercial tests are VZV ELISA (Zeus Scientific, Raritan, N.J.), VZV IgG ELISA (Trinity Biotech, Bray, Ireland), VIDAS immunoassay system (BioMerieux, Durham, N.C.), VZV EIA (Panbio, Sinnamon Park, Queensland, Australia), and VZV ELISA (Walpoole Labs, Princeton, N.J.). The main advantages of these assays are their automated formats and availability. Assays are performed according to the manufacturer's package inserts. In general, these tests are fairly specific; however, because they are relatively insensitive, some adults with immunity to varicella may be misidentified as susceptible to chicken pox. False-positive tests have also been reported (61). EIAs are more effective for screening individuals (especially adults) who might have had previous natural varicella than for identifying persons who have responded to immunization. It is not uncommon for a vaccinee to have a negative EIA but have antibodies to VZV detectable with a more sensitive test.

FAMA Test

For the FAMA test, serial twofold dilutions of serum are made in PBS beginning at 1:2 in a 96-well round-bottom microtiter plate. Heat inactivation of serum is not required and should not be done since it may lead to nonspecific results. Cells infected with VZV are harvested when they exhibit 50 to 75% CPE. Medium is poured off the cultures, and the cells are scraped into any remaining medium and centrifuged at 700 × *g* for 2 min. The cells are taken up in PBS (about 1.5 ml/150 cm^2 flask) and dispersed in 0.025-ml aliquots into microtiter plates containing dilutions of serum. Cells are then incubated for 30 min at room temperature in a moist chamber. PBS is then added to fill the wells, and the plate is centrifuged at 1,000 × *g* at 4°C for 10 min. PBS is removed by rapidly flipping the plate upside down, and the washing step is repeated. A working dilution of fluorescein-labeled anti-human immunoglobulin (0.025 ml) is added to each well, and plates are incubated for 30 min at room temperature. This assay may also be used to measure specific VZV IgG, IgM, or IgA antibody by using corresponding fluorescein-labeled anti-human conjugates. After two washing steps, a drop of buffered glycerol (9 parts glycerol to 1 part PBS) is added to each well, and the cells are removed with an aspirator and placed on microscope slides. The cells are covered with a glass coverslip, which is sealed with nail polish. The slides are then examined by fluorescence microscopy. The antibody titer is the highest dilution of serum yielding membrane fluorescence.

The sensitivity of this assay is likely due to the preservation of VZV glycoprotein antigen conformation on the surface of infected cells. These structures are probably altered by fixation, leading to decreased sensitivity by other methods. A modified FAMA assay in which cells are briefly fixed in dilute glutaraldehyde (0.075% for 60 s at 0°C) and stored at −70°C has, however, been described (90). The advantage of this particular method is that virus-infected cells may be stored, and a continuous culture for VZV is not required in the laboratory. A positive FAMA test from a seropositive individual is shown in Fig. 6.

ACIF Test

The ACIF method has a sensitivity comparable to those of FAMA and LA, permitting examination of serum dilutions as low as 1:2 or 1:4, and avoids nonspecific reactivity indirect IF staining (57). The advantage of the ACIF test is its

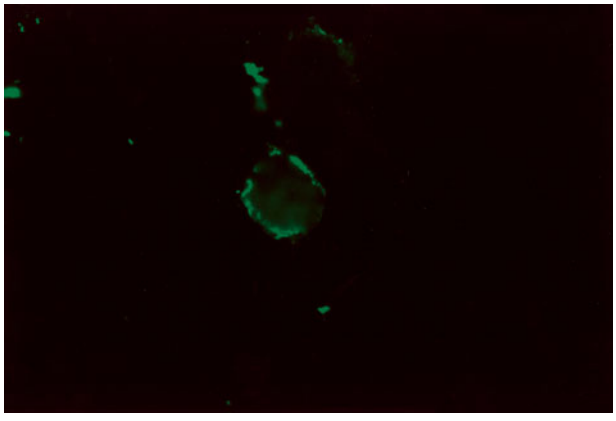


FIGURE 6 Positive FAMA test for VZV antibodies. Note surface staining of unfixed HFDL infected with VZV. Positive results of ≥1:4 are consistent with immunity to VZV. Magnification, ×525.

specificity; its disadvantages include the inability to automate the test, the high degree of training required to perform it, and the inability to distinguish between IgG and IgM against VZV.

LA Assay

The LA method is a commercial assay (VZVScan; Becton Dickinson, Sparks, Md.) that uses emulsified latex particles coated with VZV gps (21, 75). Prior to the test, all reagents stored at 4°C are warmed to room temperature. Serial twofold dilutions of sera are made in 1-cm-diameter flat circles on a wax-coated 10- by 12-cm card supplied by the manufacturer, and the serum is spread evenly over the circle. A 0.025-ml drop of latex emulsion is added to each circle directly from the supplied reagent bottle. The card is briefly rotated by hand and placed on a mechanical rotator for 10 min at 90 to 110 rpm in a moist chamber. Circles are then examined for agglutination (obvious clumping of the latex particles) using a high-intensity desktop lamp. To screen for immunity status, it is recommended that sera be tested at 1:4 and 1:40 dilutions, although the package insert specifies an initial dilution of 1:2. A titer of 1:4 is more consistent with immunity to varicella in our experience. A positive reaction in one or both wells is highly correlated with immunity to varicella. If possible, sera (particularly from adults) that are negative at screening dilutions should be retested at dilutions of 1:4 to 1:64 to avoid false-negative results due to prozone phenomena. In our experience, testing of serial dilutions is more likely to provide an accurate assessment of immunity to varicella. False-positive reactions are rare (75). To diagnose VZV infection, serial twofold dilutions of acute- and convalescent-phase sera are tested beginning at a dilution of 1:4. In convalescent-phase specimens, care must be taken to make certain that high antibody titers such as those greater than 1:4,096 are not missed due to a prozone effect. Thus, if a negative result is seen in a convalescent-phase serum specimen, the serum should be retested using higher serum dilutions. The chief advantages of the LA test are its simplicity, low cost, and the rapidity with which it may be performed (15 to 30 min). Its disadvantages include lack of automation, inability to demonstrate IgM, requirement for training to correctly interpret the appearance of agglutinated latex, and the occurrence of prozone reactions in 1 to 5% of sera.

ANTIVIRAL SUSCEPTIBILITIES

Methods to detect viruses resistant to ACV have not been standardized and are not widely performed. Most VZVs are sensitive to 1.0 to 2.0 μg/ml concentrations of ACV (reported range, 0.3 to 10.8 μg/ml) (2). VZV isolates that are resistant to ACV are unusual but have been described, particularly from patients with underlying human immunodeficiency virus infection who have received multiple courses of ACV for frequent recurrences of VZV infection (34, 46, 56). One ACV-resistant Oka strain virus has been described, from an immunocompromised patient with a chronic form of zoster following immunization (45).

EVALUATION AND INTERPRETATION OF RESULTS

Demonstration of virus growth, specific antigen, or other viral products such as DNA in skin lesion material or autopsy tissue is diagnostic of active VZV infection, since asymptomatic shedding, as occurs with some of the other herpesviruses such as HSV and CMV, is not known to occur. The presence of VZV IgM in serum is suggestive but not definitive of acute VZV infection because false positives occur.

The combination of IF staining and/or PCR is used in some laboratories as the first line for rapid diagnosis. Obtaining smears from vesicular lesions for IF staining should be attempted wherever feasible, because this method is more rapid and sensitive than culture (11, 82, 91). PCR is more sensitive than IF (82). Studies have shown virus isolation, even in optimal cell culture systems, to be less sensitive than IF staining or PCR on lesion materials for diagnosis of VZV infection (5, 27, 82). Infectious virus persists for a shorter length of time in vesicles and is more labile than viral particles and antigens. The lability of viral infectivity in vesicular lesions and the host immune response that inactivates virus in the skin with time probably both contribute to the difficulty of virus isolation. Shell vial methods are a practical alternative to tube culture since they are more sensitive and rapid, providing significant information within 24 to 48 h.

Currently the disadvantages of VZV PCR include false-positive reactions due to contamination of specimens with previously amplified VZV DNA (a problem commonly encountered with PCR in general) and the somewhat limited availability of commercial reagents. These drawbacks are minor and easily managed, so virus isolation and IF are being increasingly supplanted by PCR for diagnosis of VZV infection.

In the absence of a similar rise to HSV antigen, a ≥4-fold increase in IgG titer to VZV antigen in convalescent serum or a significantly increased titer by EIA is diagnostic of a current VZV infection. However, a high proportion (up to one-third) of individuals with primary HSV infections who have experienced a prior VZV infection show a heterotypic antibody response to VZV antigen, making the differentiation between VZV and HSV infection difficult in the absence of clear-cut clinical findings. Thus, not only is serology slower than IF and PCR for diagnosis of VZV infection, but also it is less reliable. The best method for diagnosis of acute VZV disease today, including possible rashes associated with varicella vaccination, continues to be by demonstration of the virus, its antigens, or its DNA.

Although negative results by FAMA, ACIF, LA, and EIA are reliable indicators of susceptibility to varicella, the occurrence of clinical varicella in a few individuals with low titers of VZV antibody demonstrated by FAMA and ACIF indicates that low levels of antibody demonstrable by these

sensitive methods are not always a reliable indication of protection against clinical illness. This is particularly true for immunocompromised patients. Patients who have received blood transfusions may have falsely positive FAMA tests for some months. Occasional second attacks of varicella can occur in VZV-seropositive healthy individuals. Detection of serum antibody to VZV by FAMA in healthy individuals has correlated with protection in up to 96% of persons in one study (25). More recently, similar accuracy levels of the FAMA and LA assays have been demonstrated with healthy subjects (21, 75). In general, testing sera from immunocompromised persons for antibodies to VZV may be less reliable for determining immunity status to varicella than simply asking the patient whether he/she had previous varicella. In contrast, testing sera from healthy individuals, particularly adults with no history of varicella, is a useful and reliable means to identify persons susceptible or immune to varicella.

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Human Cytomegalovirus

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TAXONOMY

Human cytomegalovirus (CMV), formally designated human herpesvirus 5 (HHV-5) by the International Committee on Taxonomy of Viruses, is a member of the family *Herpesviridae,* which includes herpes simplex virus types 1 (HHV-1) and 2 (HHV-2), varicella-zoster virus (HHV-3), Epstein-Barr virus (HHV-4), and human herpesviruses 6, 7, and 8. It is classified in the subfamily *Betaherpesvirinae* with cytomegaloviruses of other animal species based on its tropism for salivary glands, slow growth in cell culture, and strict species specificity. Human CMV is the type species of the genus *Cytomegalovirus,* and its name is derived from the enlargement of cells (cyto = cell, mega = large) infected by the virus. Regions of genome sequence homology between CMV and herpesviruses 6 and 7 have been identified, and HHV-6 and HHV-7 are now classified with CMV among the betaherpesviruses.

DESCRIPTION OF THE AGENT

Complete CMV particles have a diameter of 120 to 200 nm and consist of a core containing a 235-kb linear double-stranded DNA genome, an icosahedral capsid with 162 capsomeres, an amorphous tegument or matrix, and a surrounding phospholipid-rich envelope. Electron microscopic features of CMV include virions morphologically indistinguishable from those of other herpesviruses, a high ratio of defective viral particles, and the presence of spherical particles called dense bodies. Viral replication occurs in the nucleus of the host cell and involves the expression of immediate-early (α), early (β), and late (γ) classes of genes. The viral envelope is formed as assembled nucleocapsids bud from the inner surface of the nuclear membrane.

Molecular virologic techniques have been used to study variation among CMV strains. DNA-DNA reassociation kinetics analysis has shown that various CMV strains have considerable homology to AD-169, a standard laboratory strain; restriction endonuclease analysis has shown that DNAs from various strains have similar but distinctive fragment migration patterns. These studies suggest that CMV strains are closely related to each other, more so than are herpes simplex virus types 1 and 2. Antigenic heterogeneity among CMV strains has been detected in cross-neutralization and other serologic assays, but evidence for distinct serotypes is limited.

CMV is inactivated by a number of physical and chemical treatments, including heat (56°C for 30 min), low pH, lipid solvents, UV light, and cycles of freezing and thawing.

EPIDEMIOLOGY AND TRANSMISSION

CMV has a worldwide distribution and infects humans of all ages, with no seasonal or epidemic patterns of transmission. The seroprevalence of CMV increases with age in all populations and ranges from 40 to 100%; the virus is acquired earlier in life and the prevalence is highest among lower socioeconomic groups in crowded living conditions. CMV can be transmitted vertically and horizontally, and infections are classified as being acquired before birth (congenital), at the time of delivery (perinatal), or later in life (postnatal).

Most infections are acquired by direct close personal contact with individuals who are shedding virus. Since CMV has been detected in many body fluids, including saliva, urine, breast milk, tears, stool, vaginal and cervical secretions, blood, and semen, it is clear that transmission can occur in a variety of ways. Prolonged shedding of virus after congenital or acquired CMV infection contributes to the ease of virus spread; virus may be excreted for weeks, months, or even years following a primary infection.

Transplacental infection of the fetus can occur following primary or recurrent infection of a pregnant woman, but the risk of CMV transmission to the fetus and the rate of symptomatic fetal infection are much higher with primary maternal infection. Newborns can also acquire infection at the time of delivery by contact with virus in the birth canal. Nearly 10% of women shed CMV in the genital tract at or near the time of delivery, and virus is transmitted to approximately 50% of the newborns. Such infants begin to excrete virus at 3 to 12 weeks of age but usually remain asymptomatic. Mother-to-infant transmission of CMV through breast milk is very common; low-birth-weight premature infants are at greatest risk for developing disease. Of children who attend day care centers and enter as toddlers, 20 to 70% experience CMV infection over a 1- to 2-year period. Infection is usually asymptomatic, but the children may transmit CMV to their parents and other caregivers, posing a risk to an unborn fetus if a woman is pregnant at the time. In adolescents and adults, sexual transmission of CMV may occur and is an important route of CMV spread.

Similar to infections with other herpesviruses, primary infection with CMV results in the establishment of a persistent or latent infection. The sites of latent infection are thought to include various tissues, endothelial cells, and leukocytes. Therefore, CMV can be transmitted by blood transfusion and organ transplantation. Reactivation of the virus can occur in response to different stimuli, particularly immunosuppression.

CLINICAL SIGNIFICANCE

CMV infections are common and usually asymptomatic in otherwise healthy children and adults; however, the incidence and spectrum of disease in newborns and in immunocompromised hosts establish this virus as an important human pathogen.

CMV infection has been detected in 0.2 to 2.5% of newborn infants and is the most common identified cause of congenital infection. Fewer than 5% of congenitally infected infants develop symptoms during the newborn period; possible manifestations range from severe disease with any combination of intrauterine growth retardation, jaundice, hepatosplenomegaly, petechiae, thrombocytopenic purpura, myocarditis, pneumonitis, central nervous system abnormalities, and chorioretinitis to more limited involvement. Symptomatic infants may die of complications within the first months of life; more commonly, they survive but are neurologically damaged. It is now recognized that even congenitally infected infants who are asymptomatic at birth may develop hearing defects or psychomotor and/or intellectual disabilities later in life. Thus far, it appears that perinatally infected infants do not develop late neurologic sequelae of infection.

The vast majority of immunocompetent children and adults who acquire CMV infection postnatally remain asymptomatic. In high-risk premature newborns infected as a result of blood transfusions, morbidity and mortality can be significant and hepatosplenomegaly, thrombocytopenia, atypical lymphocytosis, and hemolytic anemia have been described. Symptoms in young adults can mimic the infectious mononucleosis syndrome caused by Epstein-Barr virus and include prolonged fever (persisting for 2 to 3 weeks), malaise, an atypical lymphocytosis, and mild hepatitis without the production of heterophile antibody. More recently, a link between CMV infection in the immunocompetent host and atherosclerosis has been suggested, and there is growing evidence that CMV is a contributing factor to vascular disease in certain individuals (for a review, see reference 74).

CMV infections are frequent and occasionally severe in children or adults with congenital or acquired defects of cellular immunity, such as patients with AIDS, cancer patients (particularly those with leukemia and lymphoma receiving chemotherapy), and recipients of solid-organ and bone marrow transplants. Infections in these patients may be due to reactivation of latent virus or primary infection or reinfection with exogenous virus, which may be introduced by blood transfusions or by the grafted organ. Symptoms tend to be most severe after primary infection; however, reactivation infection or reinfection in a severely immunocompromised host may also cause serious illness. Active infection usually occurs between 1 and 4 months after transplantation, when patients are at the height of their immunosuppression, or when $CD4^+$ lymphocyte counts drop below 50 cells/μl for individuals infected with the human immunodeficiency virus. The frequency and severity of CMV infection in organ transplant recipients are variable and depend on the type of transplant, the source of the donated organ, the immune status of the recipient, and the duration of the immunosuppressive therapy. The major symptoms in these patients usually include fever, malaise, lethargy, myalgia or arthralgia, leukopenia, thrombocytopenia, and hepatitis. Specific organ damage may lead to pneumonitis in recipients of lung or heart-lung transplants; the development of myocarditis, retinitis, or accelerated vascular damage and atherosclerosis after cardiac transplantation; hepatitis and pancreatitis in liver and pancreas transplant recipients, respectively; and gastrointestinal disease. CMV infection in transplant recipients has also been associated with delayed or failed bone marrow engraftment, an increased incidence or severity of graft-versus-host disease, and an increased risk of graft rejection in solid-organ transplants. Death may occur as a result of various complications, including bacterial and fungal superinfections. CMV infection, particularly when associated with pneumonitis, is an important cause of morbidity and mortality after bone marrow transplantation. In patients infected with human immunodeficiency virus, CMV is an important cause of fever, sight-threatening retinitis, encephalitis, polyradiculomyelopathy, and gastrointestinal infections including esophagitis, gastritis, and ulcerative colitis.

Antiviral drugs, including ganciclovir, valganciclovir, foscarnet, cidofovir, and fomivirsen, have been licensed by the U.S. Food and Drug Administration (FDA) for treatment of CMV retinitis in immunocompromised hosts, particularly in patients with AIDS. Ganciclovir, its oral prodrug, valganciclovir, valacyclovir, cidofovir, and foscarnet have been used with or without immunoglobulin products containing CMV-specific antibodies in various strategies as universal prophylaxis, preemptive therapy, and treatment for CMV disease in transplant recipients (for a review, see reference 84). Experience in using these antiviral drugs in the settings of congenital and perinatal CMV infection is limited. There are currently no licensed vaccines available for prevention of CMV disease. A number of candidate vaccines have been developed and are in various stages of preclinical and clinical evaluation (for a review, see reference 91).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

At present, a variety of methods are available for use in the diagnosis and management of patients infected with CMV. These include isolation of the virus in cell culture, histologic and cytologic techniques, assays for the direct detection and/or quantification of viral proteins or nucleic acids, serologic tests, and phenotypic and genotypic tests to screen for antiviral drug resistance. The selection of assays to perform and the choice of specimen(s) to be tested depend on the patient population and clinical situation and the intended use of the individual tests. In the immunocompromised host, virologic or serologic detection of CMV indicates infection but does not establish whether the infection is responsible for symptomatic illness. CMV is ubiquitous, and there is a high rate of asymptomatic excretion in patients who never progress to disease. Reactivation of latent virus is also common, and other pathogens are simultaneously present in patients with overt disease. To implicate CMV as the cause of an illness, laboratory confirmation of active infection in an appropriate clinical setting is required.

Specimens for Direct Detection

Tissue specimens, respiratory secretions, urine sediment, cerebrospinal fluid (CSF), amniotic fluid, and peripheral blood

leukocytes have been used for the direct detection of CMV antigens or nucleic acids. Whole blood, plasma obtained from anticoagulated whole blood, serum obtained from clotted blood, or purified peripheral blood leukocytes have all been used to quantitate CMV DNA in molecular amplification assays (20, 72, 83, 97). There is considerable debate about which blood compartment is best suited for the detection of CMV DNA. The most experience to date is with peripheral blood leukocytes and plasma. More recently, whole blood has been shown to yield the highest levels of CMV DNA of all blood compartments examined (83) and may represent the most practical specimen for laboratories to process for use in the diagnosis and monitoring of CMV disease since it requires little processing prior to extraction of the DNA. Also, delays in sample preparation can result in lysis of leukocytes after blood collection, which may result in inaccurate quantitation of CMV DNA if either whole blood, purified leukocytes, or plasma is used as specimen sources (88). A potential limitation for the use of whole blood is the possibility of variation in leukocyte counts from patient to patient; this may lead to erroneous quantitative measurements if fluctuations in cell numbers are not taken into consideration. Plasma or serum may be preferable in neutropenic patients who may have inadequate numbers of leukocytes for testing. For the CMV antigenemia assay, a total of 4 to 7 ml of whole blood is usually recommended for collection, but at least 10 ml of blood may be required for patients with severe neutropenia (e.g., absolute neutrophil counts of less than 200/mm^3). Any type of anticoagulated blood can be used, including blood collected in heparin, EDTA, sodium citrate, or acid citrate dextrose, although the most extensive experience has been with using heparin or EDTA. The blood should be kept at 4°C during storage and transport and should be processed within 6 to 8 h of collection for accurate and reliable quantitation of the viral load and within 24 h and no later than 48 h for qualitative testing (59). A decrease in quantitative antigenemia levels after storage of blood specimens for 24 to 48 h has been described, although most positive specimens remain positive after this time when held at 4°C (11, 59). Specimens for molecular amplification should be stored at 4°C immediately after collection and then promptly transported to the laboratory for processing. Single-use aliquots of processed specimens should be placed in multiple cryovials for testing and storage. By being processed in this manner, specimens are not frozen and thawed repeatedly and are never returned to the original specimen cryovial, thereby avoiding possible degradation of the DNA and cross-contamination of specimens, respectively. If not tested immediately, specimens should be promptly frozen and stored at –70°C. Impression smears, frozen sections, or formaldehyde-fixed and paraffin-embedded material can be used for in situ hybridization or histopathologic examination of tissue specimens.

Specimens for Virus Isolation

CMV can be isolated from a variety of body fluids; however, urine, respiratory secretions (e.g., saliva, throat washings, and bronchoalveolar lavage fluid), and anticoagulated whole blood (leukocytes) are most common for diagnostic purposes. Urine specimens should be clean-voided specimens. Because excretion of CMV in urine is intermittent, increased recovery of the virus is possible by processing more than one specimen. In the evaluation of immunocompromised patients, blood leukocyte cultures are particularly useful. Detection of CMV in leukocytes is often a better indicator of symptomatic CMV infection than is shedding of virus in urine or respiratory secretions. Bronchial washings and tissue biopsy and autopsy specimens, particularly of lungs, kidneys, spleen, liver, brain, and retinas, also can be processed for virus isolation. Recovery of CMV from tissues is strong evidence for organ involvement. All tissue specimens should be placed in a suitable viral transport medium immediately after collection. The details of specimen collection and processing are given in chapter 80. Since CMV loses infectivity when subjected to freezing and thawing, specimens for virus isolation should be kept at 4°C in an ice-water bath or refrigerator until they can be used to inoculate cultures, preferably within a few hours after collection. When prolonged transport times are unavoidable, infectivity is reasonably well preserved for at least 48 h at 4°C. In general, it is never a good idea to hold specimens for CMV isolation at room temperature and specimens should be transported to the laboratory as quickly as possible after collection to maintain viability of the virus. If freezing the specimen is necessary, an equal volume of 0.4 M sucrose-phosphate added to the specimen helps preserve viral infectivity. All frozen specimens should be stored at −60 to −80°C or in liquid nitrogen. Loss of virus infectivity occurs if specimens are stored at −20°C.

Specimens for Serologic Testing

Single serum specimens are useful in screening for evidence of past infection with CMV and for identifying individuals at risk for CMV infection. This approach is especially helpful in testing sera from organ transplant donors and from donors of blood products that are to be administered to premature infants or bone marrow transplant patients. For serologic diagnosis of recent CMV infection, paired sera should be obtained at least 2 weeks apart when testing for immunoglobulin G (IgG) antibody. The acute-phase serum sample should be collected as soon as possible after onset of illness and tested simultaneously with the convalescent-phase serum sample. If congenital infection is suspected, both maternal and infant sera should be submitted. Detection of IgM in a single serum specimen may be beneficial. Testing saliva or oral fluids for CMV-specific antibodies has been suggested as a noninvasive alternative to the collection of blood from children (1). In patients with CMV neurologic disease, CSF may be tested for viral antibody if paired with a serum specimen collected on or close to the same date.

DIRECT EXAMINATION

Histopathologic Testing

Histologic examination of Wright-Giemsa-, hematoxylin and eosin-, or Papanicolaou-stained lung or other biopsy specimens can be useful in the diagnosis of localized CMV organ disease. Characteristic large cells (cytomegalic cells) with basophilic intranuclear inclusions and, on occasion, eosinophilic cytoplasmic inclusions can be seen in routine sections of biopsy or autopsy material (Fig. 1). The nuclear inclusion has the appearance of an "owl's eye" because it has marginated chromatin that is typically surrounded by a clear halo that extends to the nuclear membrane. The presence of characteristic cytologic changes suggests CMV infection and correlates with active disease in most cases, although additional virologic or serologic confirmation is suggested. Overall, histopathologic diagnosis of CMV disease involves time and labor and is relatively insensitive, and since CMV can infect tissues without producing morphologic changes, failure to find typical cytomegalic cells does not exclude the

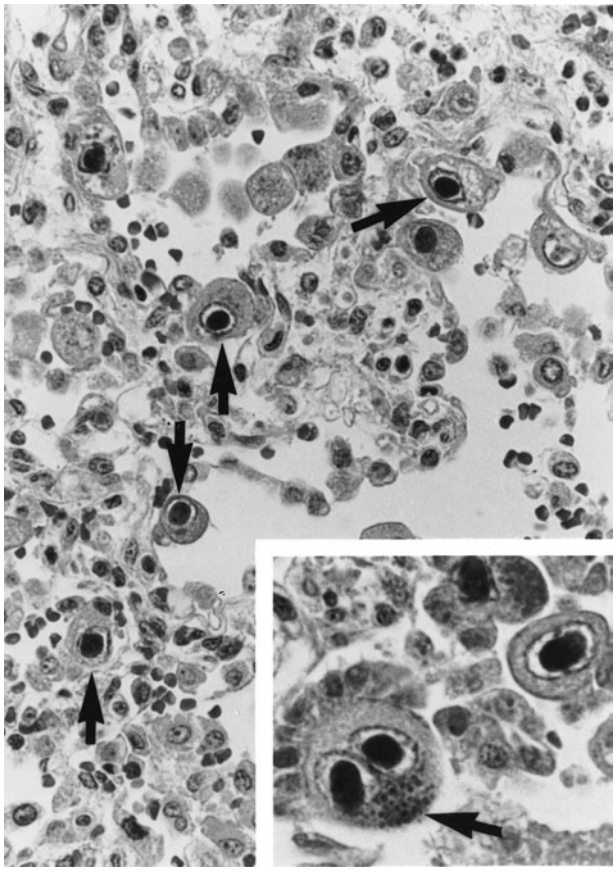


FIGURE 1 Fixed hematoxylin-eosin-stained lung tissue from a patient with interstitial pneumonia. Note the numerous giant cells that possess large intranuclear inclusions surrounded by characteristically clear halos (arrows). Less pronounced granular inclusions may also be present in the cytoplasm (inset). (Courtesy of Eduardo Ruchelli.)

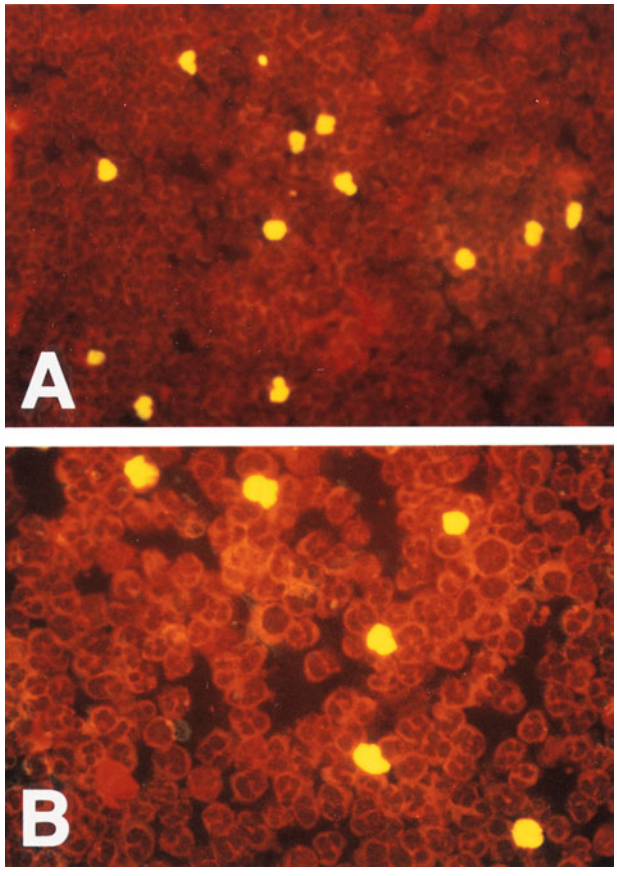


FIGURE 2 CMV antigen-positive polymorphonuclear leukocytes. Note the nuclear staining when a monoclonal antibody directed against pp65 is used. (A) Magnification, ×100; (B) magnification, ×400.

possibility of CMV infection. The sensitivity of histopathologic testing can be increased somewhat by using immunohistochemical staining to detect CMV antigens or in situ hybridization to detect CMV nucleic acids. A major obstacle to tissue diagnosis of CMV is the need to perform invasive procedures to obtain specimens for testing.

Antigenemia Assay

The CMV antigenemia assay is widely used for the direct detection and quantitation of CMV from blood leukocytes (102, 103, 106, 107) in recipients of solid-organ and bone marrow transplants and individuals infected with human immunodeficiency virus (HIV). There have been numerous studies comparing the performance of this assay to conventional and shell vial culture isolation of CMV from blood (for a review, see reference 10). The antigenemia assay is a sensitive, specific, and rapid method for the early diagnosis of CMV infection and can be used for routine monitoring of patients at high risk for severe CMV disease. The test is relatively simple to perform and is based on immunocytochemical detection of the 65-kDa lower-matrix phosphoprotein (pp65) in the nuclei of peripheral blood leukocytes (Fig. 2). By using this assay, CMV can be detected before the onset of symptoms and the viral load can be quantified to assist in predicting and differentiating CMV disease from asymptomatic infection (36, 58). The procedure has also been used to evaluate the efficacy of antiviral therapy (39, 105), to prompt the institution of preemptive therapy, and to detect CMV in leukocytes of CSF from AIDS patients with infections of the central nervous system (86). The pp65 protein has also been found in endothelial cells circulating in the blood of immunocompromised patients, and some investigators have suggested that infection of these cells is associated with organ involvement and more advanced disease (37, 87). The results of the antigenemia assay correlate well with the quantitative detection of CMV DNA in leukocytes or plasma in molecular amplification assays (13, 34).

In the antigenemia assay, leukocytes (mainly polymorphonuclear leukocytes) are enriched from freshly collected whole blood by sedimentation with dextran. More recently, a method for direct lysis of erythrocytes and subsequent isolation of leukocytes from whole blood has been described (48). This modification allows for a shorter total assay time and the capability of processing more specimens. Following sedimentation, the remaining erythrocytes are lysed with ammonium chloride, the granulocytes are counted in a hemocytometer or automated cell-counting instrument, and a known number of cells (usually 2×10^5 although variations of 5×10^4 to 10^6 cells have been used) are cytocentrifuged onto microscope slides. The cells are fixed with formaldehyde or

paraformaldehyde and then permeabilized with the nonionic detergent Nonidet P-40 and stained with suitable monoclonal antibodies directed against CMV pp65. These processes are followed by incubation of the cells with a fluorescein isothiocyanate-labeled secondary antibody diluted in a counterstain. Slides are read by microscopy at ×200 to ×400 magnification. Positive results are viewed as homogeneous apple green fluorescence within the nuclei of infected cells. Quantitative results are usually expressed as the number of antigen-positive cells per total number of leukocytes evaluated. No absolute CMV antigenemia values or breakpoints for symptomatic disease have been established for interpretation of quantitative testing; they appear to be different between patient populations. Therefore, it is important to monitor patients and observe relative rises and/or falls in the level of antigen-positive cells in multiple blood specimens collected over time. The presence of small numbers of antigen-positive cells generally indicates asymptomatic infection, whereas increasingly larger numbers are more strongly associated with clinically significant disease. In patients with severe immunosuppression (e.g., after allogeneic bone marrow transplantation), however, even very small numbers of antigen-positive cells may be significant. For transplant recipients, the frequency and extent of monitoring vary with the institution and from one transplant population to another and depend on the medical approach taken to manage CMV infection and disease (for a review, see reference 84).

The major advantages of the antigenemia assay are that it is more sensitive than either conventional tube or shell vial cultures for the detection of CMV from blood; it can be completed in 2 to 4 h, providing same-day turnaround of results; and the procedure can be readily modified for quantitative measure of the viral load. The assay has the disadvantages of being labor-intensive and time-consuming, particularly when large numbers of specimens are being processed; and it should be performed by personnel with experience in immunocytochemical techniques. Considerable time, effort, and expertise are needed in isolating and counting cells, adjusting the cell concentration to 10^6/ml before preparing the slides for staining, and then reading the stained slides. Also, interlaboratory variability exists with regard to the exact procedures used to perform the antigenemia assay and there is a definite need for assay standardization (35, 101, 108). Commercial kits containing monoclonal antibodies and other reagents needed to perform the antigenemia assay are available (Table 1). Several of these assays have been licensed by the FDA, and comparative studies have demonstrated equivalent performances (25, 57, 60, 99, 109).

Molecular Amplification

Molecular amplification assays are progressively replacing nonmolecular assays for the diagnosis and monitoring of CMV infections. PCR is currently the most widely used molecular method for the detection of CMV DNA and mRNAs. Many laboratory-developed and some commercial qualitative assays (64, 96) are available, and the sensitivity and specificity of PCR for diagnosis of active CMV infection have been evaluated (22, 51, 75, 92, 114). Amplification has been performed with a variety of primer pairs from the immediate-early antigen 1, the major immediate-early antigen, glycoproteins B and H, the EcoRI D fragment, the HindIII X fragment, pp65, pp67, and the major capsid protein gene fragments. In several studies, the sensitivity of the assay was increased by amplifying genomic regions from both the immediate-early and the late CMV genes or by using nested primers to a single gene fragment. The use of both gene fragments enabled the detection of a variety of clinical isolates, indicating that strain variability is not a limiting factor for PCR diagnosis of CMV. PCR has been used successfully to detect CMV DNA in a variety of clinical specimens from organ transplant recipients, patients with AIDS, and infants with congenital infection. It also has been used for the continued surveillance of

TABLE 1 Available CMV antigenemia assays

Manufacturer	Product name	Recommended blood collection and processing			Cell separation technique	No. of cells per slide	Monoclonal antibody used	Total assay time (h)	FDA license[a]
		Amount (ml)	Anticoagulant used	Storage conditions					
Biotest Diagnostics, Denville, N.J.	CMV Brite	5–10	Heparin or EDTA	20–25°C for 6–8 h	Dextran sedimentation	1.5×10^5	C10/C11	4–5	Yes
	CMV Brite Turbo	3–5	EDTA	20–25°C for 6–8 h	Direct erythrocyte lysis with NH_4Cl	2.0×10^5	C10/C11	2	Yes
Argene, North Massapequa, N.Y.	CINAkit Rapid Antigenemia	7	Heparin or EDTA	2–8°C for up to 24 h	Dextran sedimentation or direct erythrocyte lysis with NH_4Cl	2.0×10^5	1C3 + AYM-1	4–5 or 2	Yes
Chemicon International, Temecula, Calif.	Light Diagnostics CMV pp65 Antigenemia	5–10	Heparin or EDTA	20–25°C for up to 24 h	Dextran sedimentation or direct erythrocyte lysis with NH_4Cl	2.0×10^5	Proprietary	4–5 or 2	No

[a] FDA license for qualitative testing only.

immunocompromised patients and for evaluation of the therapeutic efficacy of antiviral drugs (38, 39, 103).

Of concern with PCR for CMV diagnosis is whether the test can distinguish between active disease and asymptomatic infection or latency. CMV viremia is considered to be the best predictor of CMV disease, and CMV DNA has been successfully detected in whole blood, purified peripheral blood leukocytes, plasma, and serum by PCR (38, 92, 97, 103). However, PCR is extremely sensitive and the qualitative detection of CMV DNA in these specimens has limited value in predicting symptomatic disease and in monitoring the success of antiviral therapy in immunocompromised patients. CMV DNA can be detected in blood by PCR in the absence of disease and can be found for weeks or months after successful therapy of symptomatic patients (5, 39, 98). At present, the clinical significance of results provided by qualitative PCR is unclear, but the method appears to be useful in certain settings. These include diagnosis of less common forms of CMV infection, such as detection of CMV in CSF of patients with encephalitis or polyradiculomyelitis, in urine, tissue, amniotic fluid, or fetal blood for diagnosis of congenital CMV infection; in the aqueous or vitreous humor in patients with CMV retinitis, and in the blood of patients at high risk of severe infection, such as CMV donor-positive, recipient-negative transplant patients, so that antiviral therapy can be started early in the course of infection. Also, a negative result from qualitative PCR of blood has a high negative predictive value for ruling out systemic CMV disease.

Similar to the antigenemia assay, measuring the level of CMV DNA in blood appears necessary to predict and diagnose CMV disease (for reviews, see references 10 and 50). Consequently, a number of quantitative and semiquantitative molecular assays have been developed and a variety of kits and analyte-specific reagents are now commercially available. These include target and signal amplification methods such as conventional PCR (12, 13, 16, 17, 94) and real-time quantitative PCR (20, 30, 42, 46, 47, 63, 66, 71, 89), branched-chain DNA technology (18, 28), and a solution hybridization antibody capture assay (17, 69, 90). The performances of individual assays have been compared to the antigenemia assay and culture (12, 16, 69, 90, 94) as well as to each other (4, 7, 78, 82, 104, 111). Overall, these molecular methods provide quantitative results comparable to those obtained by the antigenemia assay; they are also more sensitive than culture for detecting CMV infection and can detect CMV before the onset of clinical symptoms. It has been shown that transplant recipients and AIDS patients with active CMV disease have higher levels of CMV DNA and that a rapid rise in the CMV DNA copy number correlates with the presence of symptoms and drug failure during treatment (18, 23, 31, 34, 117). Also, Rasmussen et al. (81) have determined that quantitation of CMV DNA from peripheral blood leukocytes can be used to identify HIV-infected patients at risk for development of symptomatic CMV retinitis. It has also been determined that immunocompromised patients with CMV disease have more CMV DNA in either plasma or leukocyte fractions than do patients without disease but that the level of CMV DNA in leukocytes is higher than that in plasma (34, 118). Quantitation of CMV in plasma, therefore, may be less sensitive for monitoring CMV infection, although a positive result may correlate better with active disease. As in the antigenemia assay, absolute CMV DNA levels or breakpoints for symptomatic disease have not been determined, and it is currently more important to monitor the relative changes in DNA levels from serial blood specimens collected over time. Quantitative PCR methods for the estimation of CMV DNA levels in CSF, amniotic fluid, tissue, and urine of CMV-infected patients have been described (4, 28, 29, 85, 93), but more research is needed to assess the clinical relevance of quantitating CMV DNA from these specimens. It has been shown that the prognosis of congenital CMV infection may be directly related to the amount of CMV in the urine of an infected neonate and that quantitating CMV DNA in the CSF of AIDS patients may help determine if disorders of the central nervous system are attributed more to CMV than to the direct effects of HIV or other opportunistic pathogens.

As an alternative to using quantitative assays, reverse transcriptase PCR assays have been used to demonstrate that qualitative amplification of specific CMV mRNA transcripts that are expressed only during active infection may make it possible to identify the patients at greatest risk for developing symptomatic infection (6, 43, 54, 73). Commercial assays involving nucleic acid sequence-based amplification have been developed, and the diagnostic value of monitoring the expression of CMV immediate-early and late pp67 mRNA has been evaluated for blood leukocytes and CSF specimens (8, 9, 17, 32, 33, 45, 116). Assays used to detect CMV mRNA are usually less sensitive than other methods, but they have a high specificity for diagnosing CMV disease.

In general, molecular assays for the detection and quantification of CMV DNA are sensitive, specific, and reproducible and significantly reduce the time necessary to report results that may have an impact on the care and management of patients. They differ in the types of specimens used, the nucleic acid target selected, the choice and design of suitable oligonucleotide primers and probes, optimization of specimen extraction and PCR amplification conditions, the controls and quantitative standards used, the method chosen to detect and/or quantify the amplified product, and the number of DNA copies reported from one laboratory and method to another. Many of the assays require additional validation, and there is a definite need for standardization of both the qualitative and quantitative test methods. Most methods also have the distinct disadvantages of being technically demanding and expensive and requiring considerable technical expertise to perform. These concerns have diminished as many of the steps involved in molecular testing are now being automated. Ultimately, the clinical utility of these assays will depend on their accuracy, reproducibility, precision, ease of use, cost, and predictive value. Molecular methods, however, are proving to be valuable additions to the analytical tools already being used to provide a rapid diagnosis of established disease, identify patients at risk of developing disease, assess the progression of disease and the risk of relapse, direct the initiation of preemptive therapy, monitor the response to therapy, and predict viral resistance and treatment failure.

Other Direct Detection Methods

Electron microscopy, exfoliative cytology, and molecular dot blot and in situ hybridization techniques have also been used for the direct detection of CMV from tissues, bronchoalveolar lavage specimens, blood leukocytes, cervical secretions, and urine sediments. An enzyme-linked immunosorbent assay and a dot immunoperoxidase assay involving antisera to viral proteins have also been used for the direct detection of CMV antigens from clinical specimens. Overall, these methods lack sensitivity and are not used routinely in most clinical laboratories.

ISOLATION PROCEDURES

Processing of Specimens

Adjustment of urine specimens to pH 7.0 with 0.1 N NaOH or 0.1 N HCl is recommended to reduce toxicity to cell cultures. Centrifuging urine specimens to obtain sediment-enriched samples has been advocated but is usually unnecessary. Sediment-enriched urine samples from renal transplant recipients may produce toxicity more frequently than do uncentrifuged urine samples.

A number of procedures for obtaining peripheral blood leukocytes have been described. However, density gradient centrifugation with either Ficoll-Hypaque or a mixture of sodium metrizoate and dextran 500 (PMN; Robbins Scientific, Sunnyvale, Calif.) is most suitable for a clinical laboratory. Fresh blood collected in the presence of heparin, sodium citrate, acid citrate dextrose, or EDTA may be used. Both mononuclear cells and granulocytes are efficiently separated from erythrocytes in a single step. The procedure is rapid and easy to perform, and the reagents are commercially available. Compared with traditional sedimentation methods, the technique results in a greater number of virus isolates and an increased yield of infectious foci or plaques. Alternatively, the direct lysis procedure described above may be used, although a reduced ability to culture CMV following direct erythrocyte lysis has been reported (56).

Respiratory, biopsy, autopsy, and other specimens are processed as described in chapter 80. All specimens are treated with antibiotics before inoculation of cell cultures. Portions of specimens not used to inoculate cell cultures should be mixed with equal volumes of 0.4 M sucrose-phosphate and frozen at −70°C or in liquid nitrogen; some infectivity may be preserved if further isolation attempts are necessary.

Cell Cultures

Human fibroblasts best support the growth of CMV and therefore are used for diagnostic purposes. Acceptable fibroblast cultures include those prepared from human embryonic tissues or foreskins and serially passaged diploid human fetal lung strains such as WI-38, MRC-5, or IMR-90. Diploid fibroblast cells should be used at a low passage number, since they may become less susceptible to CMV infection with increasing cell generations. Several of these fibroblast cell lines are commercially available. CMV can infect and replicate in certain human epithelial cells, but its growth is limited. CMV infection of nonhuman cells can occur but leads to abortive replication with expression of only immediate-early genes and proteins.

Isolation of Virus in Conventional Tube Culture

Specimens to be tested are added in a volume of 0.2 ml to duplicate tubes of confluent fibroblasts maintained in Eagle minimal essential medium with 2% fetal bovine serum. Alternatively, the tubes are drained of medium, the inocula are absorbed for 1 h in a stationary position or by centrifugation at 700 × *g* for 45 min at 30 to 33°C, and then fresh medium is added. After inoculation, the tubes can be rolled or kept stationary at 37°C. At 24 h later, the medium is changed in tubes inoculated with urine or leukocyte specimens. Thereafter, and for other types of specimens, the medium is changed once a week or more frequently as the pH of the culture medium changes or if toxicity appears. When toxicity necessitates passage of the culture, cells rather than culture medium should be passaged, since CMV remains cell associated. Cells are removed by addition of 0.25% trypsin–0.1% EDTA to the monolayers and incubation at 37°C for approximately 1 min. When the cells detach, Eagle minimal essential medium with 2% fetal bovine serum is added, and the cells are used to inoculate fresh tubes. Tubes are examined for cytopathic effect (CPE) daily for the first 5 days and then twice a week for at least 4 weeks for most specimens (6 weeks for leukocyte specimens). Control, uninoculated cultures are handled in the same manner as those inoculated with clinical specimens.

The time of appearance and the extent of CPE depend on the amounts of virus present in specimens. In cultures inoculated with urine from a congenitally infected newborn, CPE may develop by 24 h and progress rapidly to involve most of the monolayer if the virus titer in the urine is extremely high. More commonly, foci of CPE, consisting of enlarged, rounded, refractile cells, appear during the first week, and progression of CPE to surrounding cells proceeds slowly (Fig. 3). In cultures inoculated with urine or respiratory specimens from older individuals, CPE usually appears within 2 weeks. Leukocyte cultures may not become positive until after 3 to 6 weeks. The usual slow progression of CPE in cultures inoculated with clinical specimens is due, at least in part, to limited release of virus into extracellular fluid. With strains of CMV that have been serially passaged, including laboratory-adapted strains, greater amounts of extracellular virus are released and CPE progresses more rapidly.

For storage of fresh isolates, monolayers exhibiting CPE are treated with trypsin-EDTA and the cells obtained are suspended in Eagle minimal essential medium with 10% fetal bovine serum and 10% dimethyl sulfoxide and then frozen at −70°C. Infectivity can be better maintained for long periods by storage in liquid nitrogen.

Identification of Isolates

In some laboratories, CMV isolates are identified solely on the basis of characteristic CPE and host cell range. However, viruses such as adenovirus and varicella-zoster virus occasionally produce a CPE indistinguishable from that of CMV. Thus, suspected CMV isolates are best confirmed by an immunofluorescence assay (IFA) using monoclonal or polyclonal antibodies that are available from a variety of commercial sources. The appearance of typical nuclear fluorescence of infected cells indicates the presence of CMV. PCR or other molecular amplification methods also can be used for confirmation of suspected isolates.

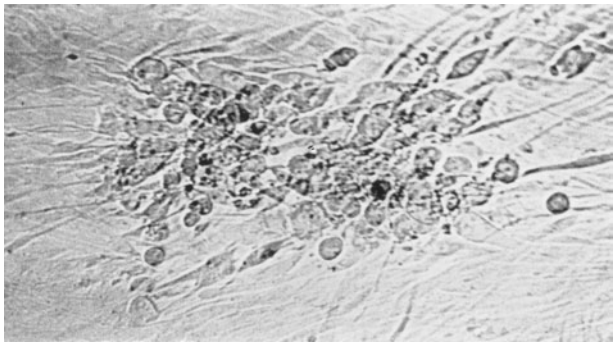


FIGURE 3 CPE produced by a CMV isolate in human skin fibroblasts 10 days postinoculation. Unstained preparation; magnification, ×100. (Courtesy of Sergio Stagno.)

Spin-Amplification Shell Vial Assay

The spin-amplification shell vial assay as described by Gleaves et al. (41) has been used extensively as a rapid culture method for the detection of CMV in clinical specimens. The technique is based on the amplification of virus in cell cultures after low-speed centrifugation and detects viral antigens produced early in the replication of CMV, before the development of CPE. Even low titers of virus present in specimens are easily amplified and rapidly detected, within 24 h. Monoclonal antibodies are commercially available and have been used for the detection of CMV early antigens. In situ hybridization with commercially available DNA probes to CMV has also been used.

MRC-5 fibroblast cells are grown to confluency on 12-mm round coverslips in 1-dram (3.7-ml) shell vials and inoculated with 0.2 ml of specimen. Shell vials of MRC-5 cells can be obtained commercially or prepared in the laboratory. Monolayers should be inoculated within 1 week after preparation, since older monolayers demonstrate decreased sensitivity to CMV and increased toxicity (27). Pretreatment of the monolayers with dexamethasone, dimethyl sulfoxide, or these agents plus calcium may increase the sensitivity of the cells to CMV infection (112, 113), although other investigators have been unable to confirm these results (26). Two vials should be inoculated for urine, tissue, and bronchoalveolar lavage fluid specimens, and three should be inoculated for blood specimens (77). Alternatively, disruption of purified leukocytes by sonication before their use in the shell vial assay may increase the sensitivity of CMV detection from blood (115). Increasing the frequency of blood collection and the volume of blood obtained also may enhance the diagnostic yield of the shell vial assay from this specimen (76). After inoculation, the vials are centrifuged at 700 × *g* for 40 min at 25°C and then 2.0 ml of Eagle minimum essential medium containing 2% fetal bovine serum and antibiotics is added. The cultures are incubated at 37°C for 16 to 24 h, fixed with acetone, and stained. A longer incubation time may be used, but the time should be determined by each laboratory on the basis of individual experience, reagents, staining technique used, and whether monolayers are purchased or prepared in the laboratory. Uninfected and CMV-infected monolayers are included as negative and positive controls, respectively. Mink lung (ML) cells, a nonhuman continuous cell line, are comparable to MRC-5 fibroblasts for the detection of CMV in clinical specimens by shell vial culture (40, 67). A distinct advantage in using ML cells is that this cell line can be propagated and passaged in the laboratory for a long time without a decrease in susceptibility to CMV. Significantly less toxicity and an increase in the number of CMV-positive nuclei were also observed with ML cells. Coverslips are scanned at ×200 to ×250 magnification, and specific staining is confirmed at ×400 to ×630. Positive cells contain apple green fluorescent nuclei against a red cytoplasmic background. Staining of immediate-early antigen appears as an even matte green fluorescence with specks of brighter green (Fig. 4A). Viral inclusions (owl's eyes) may be visible in the nuclei (Fig. 4B).

The spin-amplification shell vial assay has been a valuable adjunct to conventional virus isolation. It has the important features of being rapid, sensitive, and specific. However, skilled technical personnel and close attention to the quality of specimens, monolayers, and reagents are required for optimum performance.

SEROLOGIC DIAGNOSIS

A variety of tests with high sensitivities and specificities are available for the detection of antibodies to CMV (for an overview, see reference 49). In deciding which test to perform, one should consider such factors as the number of specimens to be tested, the patient population, cost, turnaround time, equipment needs, and ease of performance. The method that is chosen depends on the needs of individual laboratories. For small-volume laboratories, IFA or passive latex agglutination tests may be more cost-effective and practical, while enzyme immunoassays (EIA) may be more suitable for laboratories with higher volumes of specimens. Overall, detection of CMV-specific IgM or determination of a positive seroconversion can be useful for the diagnosis of primary CMV infection in certain clinical settings, and the screening of blood and organ donors and recipients plays an important role in preventing the transmission of latent CMV to patients at high risk for severe CMV disease.

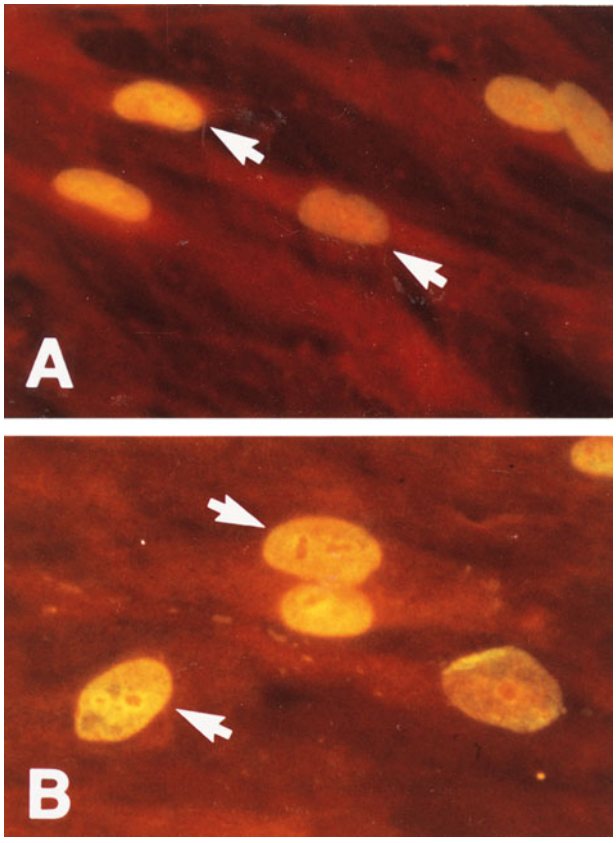


FIGURE 4 Demonstration of CMV early antigens in the nuclei (arrows) of infected MRC-5 cells following shell vial culture and IFA staining. Staining of immediate-early antigen appears as an even matte green fluorescence with specks of brighter green (A). Viral inclusions (owl's eyes) may be visible in the nuclei (B). Magnification, ×400.

EIA

Over the years, EIA have largely replaced other traditional methods for detecting antibodies to CMV. The main advantages of the EIA are that it is rapid, sensitive, and specific (68, 70, 79). In addition, multiple specimens can be handled daily at a relatively low cost. Kits that detect CMV IgG are available from a number of commercial sources. The kits are easy to use, and the manufacturers have provided detailed instructions. All the materials necessary to perform the assay are included, and the reagents are stable with time. Some

companies also provide a spectrophotometer and automated plate washer, which otherwise must be purchased separately at considerable expense. The development of robotics technology has led to the commercial availability of both fully automated and semiautomated EIA instruments, including sample dispensers, diluters, washers, and spectrophotometers with complete computer programming and generation of written reports.

IFA

Indirect and anticomplement IFA are commonly used methods for detecting CMV antibodies (53, 80). In the indirect IFA, dilutions of test serum are incubated with virus-infected cells that have been fixed to a glass microscope slide. Specific antibody-antigen complexes are detected using an anti-human antibody conjugated with fluorescein isothiocyanate and fluorescence microscopy. Anticomplement immunofluorescence is similar to the indirect IFA. It differs, however, in that the test serum is first heat inactivated to remove endogenous complement activity and then incubated with virus-infected cells on glass slides. Complement is added and bound by any specific antigen-antibody complexes that have formed. A fluorescein-labeled anticomplement antibody is then added; it binds to the C3 component of complement, and the slides are read using a fluorescence microscope. IFAs are useful and inexpensive methods that offer the advantages of speed and simplicity for the qualitative and quantitative detection of CMV antibodies. Commercial kits are readily available, or antigen-coated slides and labeled secondary antibodies can be purchased separately. The major disadvantages of IFA systems are that they require a fluorescence microscope and darkroom for examining slides and that extensive training is needed to read and interpret the test results.

Passive Latex Agglutination Test

The passive latex agglutination test provides a simple, rapid, and inexpensive means of detecting antibodies to CMV in human sera and plasma (3, 52, 68, 70). A suspension of latex particles coated with viral antigens is mixed with a test serum and allowed to incubate with rotation for a short time. The antigen-coated particles clump and produce agglutination in the presence of CMV-specific antibody; the agglutination is visible to the naked eye. The procedure detects both IgG and IgM antibodies but does not differentiate between the two classes of immunoglobulins. The assay can be completed in 10 to 15 min and does not require extensive washes, long incubation times, or expensive equipment. The main disadvantages are that agglutination patterns can be difficult to discern and readings are subjective. Commercial kits are readily available.

CMV IgM Antibody Measurements

Current methods for measuring CMV IgM antibodies include IFA and EIA. The procedures are essentially the same as those used to detect IgG antibodies, except that anti-human IgM antibodies labeled with suitable markers are used. A recognized pitfall of CMV IgM assays is the occurrence of false-positive and false-negative reactions. False-positive reactions occur when sera contain unusually high levels of rheumatoid factor in the presence of specific CMV IgG (21). Rheumatoid factor is an immunoglobulin, usually of the IgM class, that reacts with IgG. It is produced in some rheumatologic, vasculitic, and viral diseases, including CMV infection. IgM rheumatoid factor forms a complex with IgG that may contain CMV-specific IgG. The CMV IgG binds to CMV antigen, carrying nonviral IgM with it; in this setting, a test designed to detect IgM will produce a false-positive result. False-negative reactions occur if high levels of specific IgG antibodies competitively block the binding of IgM to CMV antigen. Therefore, it is highly recommended to separate IgM and IgG fractions before testing to decrease the incidence of both false-positive and false-negative IgM test results.

Rapid and simple methods for the removal of interfering rheumatoid factor and IgG molecules from serum have been developed. These include gel filtration, affinity chromatography, selective absorption of IgM to a solid phase, and removal of IgG by using hyperimmune anti-human IgG, staphylococcal protein A, or recombinant protein G from group G streptococci. Serum pretreatment methods are now incorporated in the procedures of commercially available IFA and EIA kits, which have resulted in more-reliable IgM tests. More recently, reverse capture solid-phase IgM assays have been used as an alternative approach to avoiding false-positive or false-negative results. This method uses a solid phase coated with an anti-human IgM antibody to capture the IgM from a serum specimen, after which competing IgG antibody and immune complexes are removed by washing. The bound IgM antibody is then exposed to specific CMV antigen, and an enzyme-conjugated second antibody and substrate are added. The development and use of recombinantly derived CMV proteins as a source of antigenic substrate also have greatly improved the performance of IgM assays (55, 110).

Although the detection of CMV-specific IgM may be useful in the determination of recent or active infection, the results should be interpreted with caution. Because IgM does not cross the placenta, a positive result from a single serum specimen from an infected newborn is diagnostic. However, there may be a lack of or delay in production of IgM in the newborn. Testing for the presence of CMV-specific IgM antibody beyond the newborn period is usually not recommended since IgM antibody can appear in both primary and reactivated CMV infections and can persist for extended periods after a primary infection. This complicates the interpretation of test results, particularly for pregnant women or immunocompromised patients, and may not be predictive of recent or active infection. Similar to newborns, immunocompromised individuals also may have a delay in IgM production or may be unable to mount a significant IgM antibody response. Lastly, patients with Epstein-Barr virus-induced infectious mononucleosis may produce heterotypic IgM responses, resulting in false-positive CMV IgM test results.

IgG Avidity Assay

The detection of CMV-specific IgM in maternal serum is not predictive of transmission to the fetus, since fewer than 10% of pregnant women who are positive for CMV IgM actually give birth to a congenitally infected infant. More recently, it has been suggested that finding low-avidity CMV-specific IgG antibody in CMV-IgM-positive pregnant women may improve the diagnosis of recent primary CMV infection and may have prognostic value with regard to fetal infection (44, 61). In solid organ transplant recipients, it has also been shown that a delay in the development of high-avidity CMV-specific IgG antibody correlates with prolonged antigenemia and a poor prognosis (62). Measurements of CMV-specific IgG avidity are accomplished by conventional EIA protocols, with one modification. Following the addition of patient serum to antigen-coated wells and prior to the addition of the conjugated secondary antibody, any CMV-specific IgG bound to the antigen source is treated with a

denaturing agent such as urea to determine the strength, or avidity, of the binding. Avidity levels are expressed as the percentage of IgG bound to the antigen when test results are generated and compared in the presence and absence of the urea pretreatment step. CMV-specific IgG of low avidity is produced during the first weeks to months following primary infection, whereas IgG antibody of increasingly higher avidity is produced with past or nonprimary infections.

Other Serological Tests

Complement fixation, immune adherence hemagglutination, indirect hemagglutination, and the neutralization test are other serologic tests used to measure CMV antibody. These methods are currently performed in only a limited number of diagnostic laboratories; some of the procedures have the disadvantages of being insensitive, poorly standardized, and relatively labor-intensive and time-consuming.

ANTIVIRAL SUSCEPTIBILITY TESTING

Resistance of CMV to ganciclovir has been reported in immunocompromised patients, particularly patients with AIDS receiving long-term ganciclovir treatment for CMV retinitis. Ganciclovir-resistant clinical isolates have also been recovered from bone marrow and solid organ transplant recipients and a patient with chronic lymphocytic leukemia. Although foscarnet is the alternative therapy for ganciclovir-resistant CMV infections, clinical CMV isolates resistant to foscarnet alone or in combination with ganciclovir resistance have been described. The mechanisms of resistance to ganciclovir in CMV include mutations in the catalytic domain of the UL97 phosphotransferase gene, leading to a deficiency in drug phosphorylation, and alterations in the UL54 viral DNA polymerase gene; the latter is also responsible for resistance to foscarnet (for a review, see reference 24). Ganciclovir-resistant CMV isolates with mutations in the UL54 gene are generally cross-resistant to cidofovir, and isolates resistant to ganciclovir, foscarnet, and cidofovir have also been reported.

The described emergence of antiviral drug resistance has led to a definite need for in vitro antiviral susceptibility testing. Laboratory confirmation of drug resistance is essential for defining the mechanisms of antiviral resistance, for determining the frequency with which drug-resistant CMV mutants emerge in clinical practice, for predicting treatment failure and identifying cross-resistance to other antiviral agents, for instituting the most appropriate alternative therapy, and when evaluating new antiviral agents. A number of phenotypic and genotypic assays have been described for testing the susceptibility of CMV to antiviral agents (for an overview, see references 65 and 100). Phenotypic assays measure the ability of CMV to grow in cell culture in the presence of various concentrations of antiviral drug, and they generally require isolation and passage of the virus in cell culture before testing begins. The activity of the antiviral agent against the replication of the virus is measured by one of many methods, including the inhibition of viral plaque formation or virus-induced CPE; a decrease in the production of viral antigens, enzyme activities, or total virus yield; and a reduction in viral nucleic acid synthesis. With each method, results are expressed as the drug concentration that causes a 50% inhibition in the growth of the virus. Genotypic assays have been used to screen CMV isolates for the identification of mutations in the CMV UL97 phosphotransferase gene and the UL54 DNA polymerase gene that confer resistance to ganciclovir and/or foscarnet (2, 19, 95). These assays normally involve using PCR for amplification of specific viral genes and direct sequencing of the amplified products; some assays use specific probes to detect selected mutations or use restriction endonuclease digestion of amplified products to identify alterations in the viral genome known to be associated with viral resistance to a given viral agent. Phenotypic assays offer the distinct advantage of being a direct measure of CMV susceptibility to any antiviral drug and can provide data on the concentration of drug needed to inhibit viral replication. However, phenotypic assays for CMV are cumbersome, labor-intensive, and expensive and have long turnaround times. Genotypic assays offer speed and efficiency in analyzing large numbers of CMV isolates and may allow an earlier detection of the emergence of drug resistance than phenotypic assays. Current genotypic assays, however, detect only known drug-resistant mutations, and the results may be confounded by the presence of mutations that have no bearing on drug resistance. Phenotypic assays are still required to identify drug-resistant viruses with novel mutations for antiviral resistance. The overall complexities of phenotypic and genotypic assays make either method less than routine for most clinical laboratories.

REPORTING, EVALUATION, AND INTERPRETATION OF RESULTS

There are a number of points to consider when choosing which tests to perform for the detection of CMV. Conventional tube cultures are highly specific and can detect unexpected viruses, but are generally too slow and insensitive to have an impact on clinical decision making when considering CMV as the primary pathogen. Shell vial cultures decrease the time required for detecting CMV in culture, but the sensitivity varies by specimen type and from laboratory to laboratory. Cultures also are often positive in the absence of true CMV disease; this is particularly true when screening urine or respiratory specimens for CMV, where shedding of CMV is common during asymptomatic infection and is usually not suggestive of more severe illness. Isolation of CMV in culture may be useful for postpartum and in utero diagnosis of congenital CMV infection and tissue invasive disease, and CMV isolates are also needed for phenotypic antiviral susceptibility assays.

In the immunocompromised host, the use of direct detection methods to diagnose CMV disease is most beneficial for patient care. In this regard, most clinical virology laboratories have largely replaced their conventional tube and shell vial culture systems with CMV antigenemia assays or molecular methods for accurate CMV detection and/or quantification from blood. Quantitative measures of CMV pp65 antigen or CMV DNA or qualitative detection of CMV mRNA can provide a rapid diagnosis of established disease, identify patients at risk of developing disease, assess the progression of disease and the risk of relapse, direct the initiation of preemptive therapy, monitor the response to antiviral therapy, and predict treatment failure and the emergence of drug-resistant virus. Histopathologic examination can be useful in immunocompromised hosts for the diagnosis of tissue invasive disease, although the procedures involve considerable time and labor and are of moderate sensitivity and specificity. Also, detection of CMV in tissue specimens must be interpreted with caution, since other pathogens may also be present; when dual infections are documented, the relative importance of each pathogen in producing clinical illness may be difficult to determine.

The isolation of CMV from urine, respiratory secretions, or other body fluids within the first 3 weeks of life is the traditional means of confirming the diagnosis of congenital infection in newborns. Urine is the preferred specimen because it contains greater amounts of virus, and the virus therefore grows quickly in culture. PCR has also been used to find CMV DNA in urine of congenitally infected infants, and viremia in infants with congenital infection has been detected by the antigenemia assay and PCR. Attempts to isolate or detect virus from maternal blood leukocytes and from amniotic fluid and fetal tissue by molecular- and/or antigen-based tests may provide useful information for prenatal diagnosis of congenital CMV infection (for a review, see reference 85). While such prenatal testing is a sensitive indicator of maternal or fetal CMV infection, positive results do not predict which infants will have disease. Recently, it has been demonstrated that elevated levels of CMV DNA in blood during early infancy is associated with hearing loss in newborns with symptomatic and asymptomatic congenital CMV infection (14, 15). Infants not previously tested but found to be excreting virus after 3 weeks of age may have either congenital or acquired infection. Standard serologic or virologic tests do not differentiate between these possibilities.

In general, serologic tests for CMV-specific IgG antibody have limited value for the diagnosis of acute infection since the results are obtained retrospectively and are not predictive of disease. Also, the transplacental passage of maternal IgG antibody confounds the diagnosis of CMV infection in the neonate, and immunocompromised hosts may not mount a normal immune response or they may have been given blood transfusions or intravenous immunoglobulins that contain detectable CMV IgG antibodies. Nonetheless, a history of seroconversion from a negative to a positive IgG antibody response to CMV is diagnostic of primary infection and may be beneficial in evaluating a pregnant woman with symptoms of viral disease. For pregnant women with preexisting CMV-specific IgG and/or IgM antibody, however, IgG avidity testing may be more helpful in distinguishing primary from nonprimary infections and for predicting CMV transmission to the fetus. Use of the IgG avidity assay in immunocompromised hosts may have value in detecting those at highest risk for CMV disease. Whenever possible, serologic diagnoses of CMV infection should be confirmed by other virologic methods. CMV-specific IgG serologic assays play a more important role in the determination of an individual's immune status to CMV. Detection of CMV-specific IgG in a single serum specimen indicates exposure to CMV at some time in the past. Negative serum antibody titers may exclude previous CMV infection. In the evaluation of an organ donor and recipient, a seronegative recipient who receives an organ or blood products from a seropositive donor is at increased risk for developing primary CMV infection and serious disease. Knowing the serostatus of the donor and recipient is therefore important in determining the treatment or prophylaxis to be used and in considering the type of donor and blood products to be given.

The application of virologic methods, including conventional and shell vial culture, rapid direct-detection assays, and serologic testing, should be combined with clinical assessment of the patient to provide an accurate, reliable diagnosis of CMV infection and disease and to allow subsequent prompt, appropriate patient management and timely intervention with specific antiviral therapy. There is a particular need to differentiate CMV infection from graft rejection in transplant patients, since the administration of potent immunosuppressive antirejection drugs during active CMV infection can result in life-threatening disease.

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Epstein-Barr Virus

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TAXONOMY

The Epstein-Barr virus (EBV), also known as human herpesvirus 4, belongs to the subfamily *Gammaherpesvirinae* in the *Herpesviridae* family.

DESCRIPTION OF THE AGENT

EBV has the morphology of a herpesvirus, with 162 capsomeres in icosahedral arrangement surrounded by a lipid-rich envelope. It has a double-stranded 172-kbp DNA, which is linear in the mature virion and circular and episomal in latently infected cells. The genome consists of a series of unique sequences alternating with internal repeats, all sandwiched between two terminal repeat elements. These are joined during circularization. Integration of viral DNA into host chromosomal DNA has been shown, mainly in lymphoblastoid cell lines (46).

EBV primarily infects B cells, which carry the receptor for EBV, the CD21 complement receptor. It has the ability to transform precursor and mature human B lymphocytes to lymphoblastoid cell lines. B cells are the site of latency, but epithelial cells, not expressing the receptor, are the main producers of progeny virus. Also monocytes may be productively infected by EBV, and the infection may affect the virus-host interaction (33, 71). In the B cells, the EBV nuclear antigens (EBNA), EBNA 1, EBNA 2, EBNA 3 (also referred to as EBNA 3a), EBNA 4 (EBNA 3b), EBNA 5 (EBNA LP), and EBNA 6 (EBNA 3c) and latent membrane proteins (LMP 1 and 2A and B) can be expressed. Three types of B-cell latency have been defined, based on their varying expression of the latency-associated proteins (58). EBNA 1 is always present. In addition, all latently infected B cells contain high copy numbers of EBV-encoded RNAs, known as EBER1 and EBER2. These are nonpolyadenylated RNAs with unknown functions. In vitro, EBV has been propagated only in B lymphocytes from human and subhuman primates (46).

EPIDEMIOLOGY AND TRANSMISSION

Transmission of EBV occurs by salivary or sexual contact (13, 19, 68). Virtually everyone becomes infected with the virus at some time. Primary infections occur during the first decade of life in areas with crowded living conditions and poor hygiene. In industrialized countries at least 50% of the population becomes EBV seropositive before puberty. Familial transmission of EBV infections is common (31). Infectious virus and viral genomes can be detected in materials from the oropharynx of the majority of seropositive individuals, including infectious mononucleosis (IM) patients (20). Detectable, infectious virus and viral DNA in saliva are more common in immunosuppressed than in immunocompetent persons (54). Viral DNA is detectable by nucleic acid amplification techniques (NAAT) in leukocytes, both from healthy carriers and from IM and immunosuppressed patients. However, except in immunocompromised patients, EBV transmission via blood products is not of clinical importance. EBV strains may be classified into types A and B (sometimes referred to as types 1 and 2) based on the polymorphism of their EBNA genes. Serology and PCR investigations indicate that both types have worldwide distribution, although type A appears to be more prevalent than type B and is the dominant type in IM (75). Dual infections with both types are not uncommon. In immunosuppressed individuals, coinfection by multiple strains of EBV has been demonstrated (30).

CLINICAL SIGNIFICANCE

IM

Primary EBV infections are mostly asymptomatic, especially in early childhood. Around 50% of primary infections in young adults are associated with classical IM. This is a self-limiting, lymphoproliferative illness, ranging from mild to severe (65). The clinical manifestations of primary EBV infections are mainly immunomediated (44). The various traits of IM are presented in Table 1. The incubation time is 30 to 50 days, and the disease generally lasts 1 to 4 weeks. However, protracted illness and tiredness for up to a year are not uncommon. Complications (Table 1) are frequent and can sometimes be the only sign of the primary infection. Very rare fatalities can occur due to splenic rupture, overwhelming infection, or hemophagocytic lymphohistiocytosis. In adults, the last is often associated with EBV reactivation and lymphoma (42). In immunocompromised patients, the primary infection often becomes severe and may be lethal. The severity of IM, the many atypical manifestations, and its similarity to malignant disease often warrant a laboratory confirmation of primary EBV infection. Mononucleosis can also be induced by other agents, such as cytomegalovirus

TABLE 1 Conditions and malignancies verified or suggested to be associated with EBV

Condition	Clinical traits
Nonmalignant	
Classic IM	Fever, malaise, pharyngitis, tonsillitis, lymphadenopathy, periorbital edema, hepatomegaly, splenomegaly, lymphocytosis with atypical lymphocytes and elevated liver enzymes
Complications of IM and other nonmalignant manifestations	
Hematological	Anemia, thrombocytopenia, granulocytopenia, hemophagocytic lymphohistiocytosis
Neurological	Meningoencephalitis, cranial nerve palsies, Guillain-Barré syndrome, seizures, mononeuritis, myelitis, psychosis
Visceral	Pericarditis, myocarditis, airway obstruction, laryngitis, epiglottitis, tracheitis, interstitial pneumonia, hepatitis, splenic rupture, splenic pseudotumor
Skin/epithelium	Exanthema, mosquito bite hypersensitivity, oral hairy leukoplakia, genital ulcers
Other	Chronic active EBV infection
Malignant/premalignant conditions	
High degree of association	Burkitt's lymphoma, posttransplant lymphoproliferative disorders, peripheral NK/T-cell lymphomas, peripheral NK/T-cell lymphomas including skin localizations, leiomyosarcoma in immunosuppression, lymphoepithelioma-like gastric carcinomas
Varying degree of association	Non-Hodgkin lymphoma, Hodgkin's disease, salivary gland and other head and neck tumors
Possible association	Gastric adenocarcinomas, breast cancer
Genetic	X-linked lymphoproliferative syndrome

(CMV), adenovirus, and *Toxoplasma gondii,* and etiologic differentiation may have important implications for treatment. To verify the diagnosis may also be cost saving since many other examinations may be avoided (5).

EBV and the Immunocompromised Host

In immunocompromised patients, EBV reactivations are associated with a number of lymphoproliferative disorders (LPD), ranging from benign, polyclonal hyperplasias to oligoclonal or monoclonal, malignant lymphomas (28, 53). The lymphomas are often localized to the brain or intestine. The risk of posttransplant lymphoproliferative disorders (PTLDs) is around 1% in hematopoietic stem cell transplant recipients and varies from 1 to 20% in solid organ recipients, depending on the type of organ. Risk factors for PTLD are EBV-seronegative recipients with seropositive donors; young age; long-standing, severe T-cell depletion of donor cells or recipient; transplantation of small intestine as well as heart, lung, and liver; CMV infection; and high degree of mismatch (11). In late-stage AIDS, the risk for LPD is also very high. EBV also may play a role in rare leiomyosarcomas, occurring in immunocompromised children (72). In oral hairy leukoplakia, replicating EBV is found in the oral epithelium. The lesions appear as white patches at the border of the tongue, mainly in human immunodeficiency virus-infected patients (88).

X-linked lymphoproliferative syndrome (XLP) is a rare, inherited immunodeficiency, involving primarily T and natural killer (NK) cells (25). Affected boys become symptomatic after exposure to EBV, developing acute fatal IM or lymphoproliferative disorders and/or dysgammaglobulinemia. The gene responsible for XLP is the SH2 domain containing 1A (SH2D1A). Multiple mutations of that gene have been identified in XLP (59, 83). Untreated, the disease is eventually lethal, but transplantation and gene therapy are possible therapeutic approaches, making the identification of affected persons important (25).

EBV-Associated Tumors

EBV is associated with a variety of tumors not related to obvious immunosuppression (62, 86). In >95% of endemic Burkitt's lymphoma and nasopharyngeal carcinoma (NPC), 40 to 50% of Hodgkin's disease, 2 to 16% of gastric adenocarcinoma, >90% of T/NK lymphomas, and 44 to 93% of lymphoepithelioma-like carcinomas, EBV genomes have been found in tumor cells. EBV-associated tumors are frequently preceded by detectable EBV genomes in serum or cerebrospinal fluid (CSF) and increased virus load in peripheral blood mononuclear cells. The reason for this is not clear. The EBV latency is different in different kinds of tumors. Burkitt's lymphoma has latency I, Hodgkin's disease and NPC have latency II, and PTLD, like lymphoblastoid cell lines, has latency III (58).

PROPHYLAXIS AND TREATMENT

There is no registered vaccine against EBV, but two approaches have been tried. One is to use glycoprotein (gp) 340 to induce neutralizing antibodies; the other is to use peptide vaccines based on major histocompatibility complex class 1-restricted epitopes of latent genes (56, 93). A gp340 vaccine is presently in clinical trials. Nucleoside analogues such as acyclovir, ganciclovir, and famciclovir, as well as the pyrophosphate analogue foscarnet, inhibit replication of EBV. Treatment of IM with nucleoside analogues results in termination of viral shedding but does not affect the symptoms, probably since these are mainly immunomediated (1, 89). Of immunomodulators, cortisone has been systematically tried against IM and may be beneficial for impending airway obstruction. Gamma interferon treatment is useful only in patients lacking this cytokine (65). The pathophysiology behind hemophagocytic lymphohistiocytosis is hypercytokinemia, and treatment with combinations of cyclosporin, methothrexate, anti-thymocyte globulin, and etoposide has been tried with some success (42).

For LPD treatment, anti-CD20 monoclonal antibodies and EBV-specific cytotoxic T lymphocytes are cornerstones (27). Adoptive transfer of cytotoxic T lymphocytes may also be beneficial in other EBV-associated malignancies (16). Despite lack of proof that antivirals have any beneficial effect, immunomodulation is almost always combined with nucleoside analogues in treatment of EBV-associated malignancies. Since the presence of EBV in tumors may have therapeutic consequences, the demonstration of the virus in tumors can be of vital importance (80).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Virus Isolation and Antigen Detection

For isolation of excreted virus, 5 to 10 ml of throat gargle fluid, collected in serum-free tissue culture medium or Hanks' balanced salt solution, is satisfactory. Five to 10 ml of heparinized blood is needed for culture to detect EBV-transformed B lymphocytes. The same material can be used for antigen detection in B cells by immunostaining. Blood specimens should be processed as soon as possible, although refrigeration for up to 24 h is acceptable for antigen detection. Storage at room temperature is preferable for lymphocyte transformation assays. Fresh biopsy specimens or thin (5-μm) cryosections are preferred to formalin-fixed material for antigen detection in other tissues.

Nucleic Acid Detection

Whole blood (collected in EDTA; heparin may inhibit the PCR), leukocytes, serum, and plasma are useful materials for monitoring of EBV presence and of viral load by NAAT. The choice of material is dependent on the clinical condition of the patient (Table 2). There is no agreement on whether leukocytes, whole blood, or serum or plasma should be used for monitoring EBV lymphoproliferative disease. Leukocytes and blood give higher sensitivity for EBV detection, while serum or plasma gives higher specificity for EBV-associated disease. The amount needed varies depending on the technique used at the individual laboratory, but it is advisable to send at least 1 ml of serum or plasma or 3 ml of whole blood. If the initial volume available is less than required, the material may be diluted, but this lowers test sensitivity. Blood specimens should be processed as soon as possible. If transport time is less than 24 h, whole blood can be kept at room temperature until separated. The separated materials can thereafter be stored at 4°C for a few days. If leukocytes or serum or plasma is to be used, the material should be separated before freezing. Whole blood can be frozen without further processing. For long-term storage, freezing at –70°C is recommended. For detection of EBV by DNA hybridization in peripheral blood lymphocytes, 5 to 10 ml of blood collected in heparin or EDTA is needed. CSF is useful for diagnosis of EBV-associated central nervous system disease, though the relation of EBV DNA to disease is not completely elucidated (15, 81). CSF is also useful for diagnosis of EBV-associated brain tumors in immunosuppressed patients. Sterile CSF without additives should be sent and can be stored in the same way as serum. The amount of CSF needed for NAAT varies depending on the amplification technique used at the laboratory. Though sometimes as little as 10 to 20 μl is sufficient for one examination, it is advisable to send at least 0.5 ml. Tissues to be examined for viral nucleic acids and antigens by NAAT should be collected aseptically and refrigerated in saline or balanced salt solutions. Suitable for this purpose are lymph nodes, spleen, liver, brain, and biopsy specimens of tumors. Fresh, frozen, but also paraffin-embedded specimens may be used.

Serodiagnosis

Only a single acute-phase serum sample is required for serodiagnosis in most cases. The amount needed varies depending on the method(s) used in the individual laboratory. Fifty microliters or even less may be enough, but it is advisable to send 1 to 5 ml. Convalescent-phase serum collected 1 to 2 months after onset is occasionally needed if ambiguous results were obtained for the first sample. Plasma can be used, but serum is preferable. Plasma and serum titers should not be compared to determine changes over time. Aseptically collected serum can be stored at 5°C for several months. For long-term storage, freezing at –20 or –70°C is recommended.

TABLE 2 Materials and methods to be used for diagnosis and monitoring of disease caused by EBV, for identification of EBV-associated malignancies, or for verification of EBV association in already diagnosed tumors

Suspected EBV-associated condition	First-choice method	Suitable material	Second-choice method	Suitable material
IM	Serology	Serum	PCR[b]	Serum or plasma
LPD outside CNS[a] immunosuppressed patients	Quantitative NAAT	Whole blood, serum or plasma, leukocytes	In situ hybridization and/or antigen detection	Tumor biopsy specimen
CNS lymphoma in immunosuppressed patients	Quantitative or qualitative NAAT	CSF	In situ hybridization and/or antigen detection	Tumor biopsy specimen
Nasopharyngeal carcinoma	Serology	Serum	Quantitative or qualitative NAAT	Plasma or serum
XLP	Genetic analysis	Cells	Serology	Serum
Other EBV-associated tumors	In situ hybridization and/or antigen detection in tumor cells	Biopsy specimen	Quantitative or qualitative NAAT[b]	Plasma or serum

[a] CNS, central nervous system.
[b] Still not fully evaluated, but promising in preliminary reports.

DIAGNOSTIC METHODS

Direct Detection

Electron Microscopy

Electron microscopy is not an appropriate diagnostic technique for EBV, since latency is the norm and virions rarely are present in sufficient amounts in infected tissues.

Antigen Detection

EBNA 1 is the only antigen expressed in all EBV-infected cells. Staining for EBNA 1 in tissues and cell lines by anticomplement immunofluorescence (ACIF) with carefully selected polyclonal sera or a monoclonal antibody (29, 61) has been the main method used for detection of EBV. For differentiation between latent and lytic infection and for characterization of type of latency associated with different EBV-associated tumors, immunohistochemistry is widely used (39, 61). A number of investigational-use antibodies against both latent and lytic antigens are available (29, 66, 90, 96). In practice, staining for EBNA 2 and LMP 1 is sufficient to define the form of latency (61). However, detection of nucleic acids is more sensitive if knowledge of the mere presence of EBV is desirable.

Nucleic Acid Techniques

Molecular techniques are important tools for detection of EBV RNA and DNA in clinical specimens. For the clinical laboratory, the choice of which EBV nucleic acid technique to use is dictated by the availability of methods and specimens, as well as the medical questions to be answered. A variety of methods, such as in situ hybridization on frozen or paraffin-embedded sections, cyto-hybridization on cell suspensions, dot blot hybridization, Southern blot hybridization, or NAAT, may be used (17, 32, 34, 91, 92). Today, NAAT methods are probably the most commonly used tools for detection of EBV and can be performed on many different clinical specimens (Table 2) after RNA or DNA extraction. A variety of primers, probes, and techniques have been presented (35). Two general-purpose probes are widely used for EBV detection. The conserved BamHI W region, coding for a long internal repeat sequence of EBNA 1, is present in multiple copies in EBV-infected cells. Probes targeting this region have maximal sensitivity in both DNA hybridization and genome amplification assays on clinical samples (10, 91). Also the LMP 1 gene is conserved and suitable for NAAT primers (19). In situ NAAT can detect one EBV genome in formalin-fixed and paraffin-embedded tissue or in whole blood (85). Quantitative real-time PCR is probably the most popular method today for monitoring EBV in patients at risk for PTLD (53). The Namalwa cell line, containing two integrated EBV copies per cell, is frequently used as a standard for determination of the number of EBV copies in a sample (19). Since increases in genome levels often signify a pathological process, the criteria for a significant increase must be determined by establishing the expected interassay variation by repeated examination of the same material (70, 79). Different statistical calculations can be used to determine the limit. A variation of 0.5 $\log_{10}$ (threefold) is often used as indication of a change that is above the expected interassay variation. Appropriate controls for contamination and inhibition should always be included. Participation in external quality control programs is important to ensure the sensitivity, specificity, and correct quantification (see evaluations at http://www.QCMD.org).

Like NAAT, in situ hybridization and cytohybridization require small amounts of sample. They can also be combined with immunohistochemical staining, and they are ideally suited for the detection of EBV in tumor tissues. Sequence-specific RNA or DNA in a single cell can be detected by in situ hybridization, also allowing for cellular localization of the EBV infection. Both RNA and DNA probes can be used for hybridization (55, 60). The EBER1 and EBER2 genes, each coding for a small RNA expressed in high (10^6 to 10^7) copy numbers, were found to be highly sensitive in detecting latently infected tissues and tumors (67). The blot techniques require larger samples but are useful when multiple analytic probes are needed.

Isolation Procedures

EBV isolation is not a routine method but may be important for research purposes. For EBV isolation, freshly fractionated human cord blood lymphocytes are inoculated with cell-free and filtered saliva or throat gargle specimens and monitored for 4 weeks (1). Individual viral isolates can be characterized by molecular techniques, on the basis of polymorphism of the EBNA 2 and 3 genes. In addition, the sizes of many of the EBNA proteins (EBNA 1 to 6) are strain dependent. Each isolate induces proteins with distinctive patterns by Western blotting, thus allowing the fingerprinting or "EBNo-typing" of each virus (31).

Serologic Tests

Heterophile Antibodies

Serologic testing is still the method of choice for the diagnosis of primary EBV infections (38, 40, 64, 68). For patients with classical IM, demonstration of Paul-Bunnell heterophile antibodies is diagnostic and no further testing is necessary (66). In-house Paul-Bunnell assays are rarely used at present. Instead, rapid qualitative agglutination or immunochromatographic assays for heterophile antibodies are widely available. They are all intended for laboratory and point-of-care use and are effective for 80 to 85% of the IM patients (6, 50). However, performing the agglutination assays may be unsuitable for persons without laboratory training, since the evaluation of a true positive agglutination result demands some experience. A strict adherence to the procedure described by the manufacturer concerning the time frame within which to read results is required, since spontaneous agglutination always appears over time. Also in comparative studies, immunochromatographic assays have performed better than agglutination assays (6).

EBV-Specific Antibodies

Around 15 to 20% of patients with EBV IM lack heterophile antibodies, and for these, EBV-specific serology is necessary for confirmation of the diagnosis. Traditionally, in the qualified virus laboratory, antibodies to four antigen complexes are measured, including the virus capsid antigen (VCA), a combined assay for the diffuse component of the early antigen (EA/D) and the early antigen-restricted component (EA/R) and the EBNAs. In addition, differentiation of immunoglobulin G (IgG), IgM, and sometimes IgA to VCA is made. The development of the antibody profile for evaluation of the EBV serological status of a patient with suspected mononucleosis is presented (Fig. 1). The immunofluorescence assay (IFA) methods are still the "gold standard" in EBV serology, but a large number of serologic assays using different methodologies are in use today. Commercially available kits are of three types: IFA, enzyme-linked immunosorbent assay (ELISA), and immunoblot assays. IFAs have fairly uniform performance characteristics as a group, principally because the cell substrates provided by various suppliers

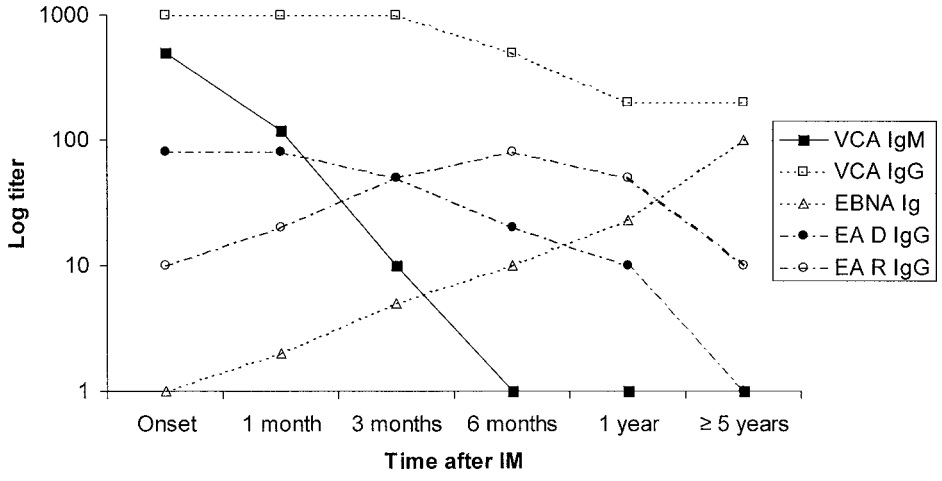


FIGURE 1 Development of titers to some EBV antigens over time after primary infection, as measured by IFAs using cell-bound antigens. Although marked interpatient and interlaboratory variation in titers occurs, the figure gives the characteristic development of titers in relation to each other.

are similar. The performance of ELISAs is much more variable, due to the plethora of antigen preparations used in the different kits. They range from cell extracts to recombinant or fusion proteins and synthetic peptides (40). Therefore, the reference criteria for interpretations established for the IFAs (Table 3) may not apply to all ELISAs, even when the antigens are referred to by the same name. For example, EBNA is a complex of several large, native proteins detected in Raji cells by ACIF, whereas the same designation may be given to a single oligopeptide deduced from EBNA 1 or an EBNA 1 recombinant protein in ELISAs. Recombinant proteins and peptides, however, are easier to standardize than cell-grown antigens, and possibly assays using synthetic antigens will be more widely used in the future.

There are many published evaluations of varying quality concerning the performance of commercial ELISAs (6, 21, 22, 26, 41, 84). Lack of standardization for the evaluations, batch-to-batch variation of some assays, and varying names and distributors of the assays in different countries and over time, as well as different demands, economy, and routines at individual laboratories, make it impossible to provide firm advice as to what brand to choose. In general, before selecting an assay, one must ascertain that it has been thoroughly investigated and related to reference IFA methodology. Examination of a series of consecutive clinical samples should be performed to determine the sensitivity and specificity in each laboratory setting, and a selection of problematic sera (mainly sera from patients with other herpesvirus infections and from patients with autoimmune diseases) should also be examined. Further, to ensure batch-to-batch stability, internal serum controls, apart from the controls provided by the manufacturers, and participation in external quality control programs, are necessary.

Antibodies against Individual EBV Antigens

VCA

The main VCA is a 150-kDa protein, but p18 and p23 proteins are also included in the VCA complex and used for serology (40). Thus, what is called VCA in reality is a mixture of antigens expressed by the P3HR1 cell line entering the lytic phase, to which human sera react (68). VCA-ELISAs containing antigens from infected cells have been available for a long time, and assays using affinity purified antigen still perform better than ELISAs with recombinant or synthetic antigens (22). Recently, ELISAs and line blot assays using recombinant VCA proteins (p23-p18) and a peptide (p18) have been reported to perform adequately in relation to IFAs (8, 41, 87).

TABLE 3 Typical EBV serological profiles under different conditions[a]

Condition	Presence of antibodies to antigen and Ig isotype[b]:						
	VCA, IgG	VCA, IgM	VCA, IgA	EA/D, IgG	EA/R, IgG	EBNA, Ig	EBNA 1, IgG
Seronegative	−	−	−	−	−	−	−
Ongoing primary infection	++	+++	+	+	±	−	−
Recent primary infection	++	+	+	+	+	±	−
Past infection	+	−	±	−	±	+	+
Chronic active EBV infection	+++	+	++	++	+	±	±
NPC	+++	−	+++	+++	+	+++	+++
XLP	±	−	−	−	−	−	−

[a] The most frequently employed antigens and Ig isotypes are included.

[b] −, antibodies completely absent; ±, antibodies absent or present in low titers; +, antibodies present; ++, antibodies present in elevated titers; +++, antibodies present in strongly elevated titers.

EBV Early Antigens

Anti-EA/D and EA/R antibodies are traditionally measured with Raji cells, activated by, e.g., phorbol ester and sodium butyrate, to enter the lytic phase. Ethanol fixation eliminates the EA/R complex, and cells fixed in ethanol are used for studies of EA/D antibodies, but separation of EA antibodies is seldom made for diagnostic purposes today. ELISAs with cell-derived and recombinant EA proteins are available, but especially the EA IgM assays are often lacking somewhat in both specificity and sensitivity (6, 84).

Anti-EBNA

The ACIF assay or assays with equally high sensitivity must be used to detect the low concentration of EBNA antibodies by IFAs. ELISAs can be made more sensitive than ACIF, but they have been adjusted to be of sensitivity similar to that of ACIF since the late seroconversion to EBNA 1, as measured by ACIF, is an important part of diagnosis. The complement-fixing antibodies detected by ACIF may be of almost any Ig isotype and should be referred to as EBNA Ig. The EBNA staining pattern is nuclear and should be present in all EBV-infected cells. The preparations should contain cells from an EBV-negative lymphoblastoid cell line, or EBV-negative cells could be incubated in parallel staining, to ensure that the nuclear staining is not due to antinuclear factors present in the patient's serum.

Commercial ELISAs containing cell-derived EBNA antigen or recombinant EBNA 1 antigen are available. The recombinant antigen assay containing p72 has ≥95% sensitivity and specificity compared to the ACIF assay for total EBNA Ig (23, 84). The same antigen is used in line blot assays. Antibody responses to individual EBNA components can be measured using ACIF with transfected cell cultures (18).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Diagnosis of EBV IM (Table 3)

Heterophile Antibodies

High levels of heterophile antibodies are seen during the first month of IM, normally followed by a rapid decrease. As a rule, in typical cases, a positive assay for heterophile antibodies is sufficient confirmation of a primary EBV infection. False-positive results are found mostly in individuals with low but persisting levels long after their primary illness. However, other viral infection agents such as CMV may also cause false-positive results, especially in the agglutination assays. False negatives (mean, 10 to 15%) are the rule among young children (82) but also occur in adolescents and adults. For these patients, EBV-specific serologic testing is needed.

Antibody Reactivity against Various EBV Antigens

Most IM patients have peak titers of both IgG and IgM antibodies to various antigens belonging to the EBV lytic phase (VCA and EA) by the time they consult their physicians but lack antibodies to the EBNA antigens, as measured by the standard ACIF assay using Raji cells (Fig. 1). IgM VCA starts to disappear after 4 weeks, and the levels of IgG VCA decline with time. However, IgG VCA persists for life, and its presence is the most reliable test for EBV seropositivity. The EBV status of the patient can generally be determined from a single serum sample by measuring antibodies to a panel of EBV-associated antigens. The characteristic antibody profile is sufficiently distinct in at least 90 to 95% of the cases to allow classification as to whether the patient (i) is still susceptible to EBV, (ii) has a current primary infection, (iii) has had a recent primary infection (within 2 to 3 months), (iv) has a past infection, or (v) may be having reactivated EBV infection (Table 3).

A cost-effective, stepwise analysis of sera for serologic diagnosis of heterophile-negative IM could start with analysis for antibodies to EBNA 1 (47). These antibodies normally do not appear until 6 weeks after onset of IM, and the assay should be negative in acute IM (37). They are maintained for life but have been reported to be of low titer and sometimes undetectable in EBV-seropositive children (82) even without recent primary infection. With severe immunosuppression, the anti-EBNA titers may gradually decrease but rarely disappear entirely. Only sera with no or very weak EBNA 1 reactivity need to be further examined and analyzed for IgG and IgM to VCA. If both are positive, a primary EBV infection is confirmed. Diagnosis of primary EBV infections should not rely only on the detection of IgM-VCA since both false-positive and false-negative results occur in IgM VCA testing. The former is mostly due to the presence of rheumatoid factor (36), other herpesvirus infections, and antinuclear factors (49). False negatives may result from late collection of serum samples or insufficiently sensitive methods. Some ELISAs use EA instead of VCA for confirmation, but the EA IgM ELISA especially has a low specificity (84). Inclusion of measurement of IgG to EA by IFA may increase the diagnostic discrimination concerning EBV IM, but the use of commercial assays with inclusion of the p18 protein or Western blots including an array of proteins may be more valuable (40). Further, avidity indices of VCA IgG antibodies can add information, especially if serum is drawn late after onset of disease and EBNA antibodies have already appeared (4, 74). In a laboratory using Western blot routinely for other agents, a commercial blot method could be used for primary screening for EBV IM, or as a second line, after the exclusion of EBNA-positive patients. It can also be combined with avidity testing, and may then be of sensitivity and specificity equal to those of the IFA reference methods (4, 22). EBNA 1 to EBNA 2 titer ratios were used for some time to verify chronic or complicated EBV infections (37, 47). However, no clear association can be made between the anti-EBNA 1 to EBNA 2 ratio and the duration or nature of illness, and the significance of antibodies to other EBNA components is still not firmly settled.

The interpretation of the specific antibody status must always be related to the condition of the patient, above all to the immunological status. In immunocompetent individuals, only three interpretations are relevant in routine diagnostics: primary infection, past infection, and EBV susceptibility (23). In Table 2, the methods of choice depending on the condition of the patient and the suspected EBV-related disease are presented.

The exact titers to each EBV antigen and the time needed to develop a full spectrum of antibodies vary widely between individuals. Significant changes in EBV antibody titers can be demonstrated only with the consecutive samples analyzed in the same assay. In general, the antibody titers to the various antigens are remarkably stable after adolescence in immunocompetent individuals. Significant changes in titers are seldom seen except during the early acute phase of the infection. Many apparently completely healthy individuals may maintain EBV antibodies at high levels throughout life (95). High titers alone are therefore not diagnostic of EBV-associated disease, but the combination of very low or absent EBNA 1 antibodies and high titers to other EBV antigens indicates a pathological virus-host relationship (63). Whether EBV is the cause of disease or immune aberrancies induced by the disease

cause an asymptomatic EBV reactivation, however, is always difficult to establish. Each laboratory not using well-characterized, commercial assays must establish its own range of "normal" titers in healthy, EBV-seropositive individuals. Titers need to be established using the laboratory's own microscope and other equipment and healthy donor sera. Due to the complexity of EBV serology, written interpretations of results should be sent to the clinicians.

EBV can be detected by NAAT in serum in most IM patients until around 2 weeks after onset of disease (3, 20), but the role of NAAT in the routine diagnosis of IM is not established. The main problem is that EBV genomes may be detected in healthy individuals. The level is normally low (20), but studies of blood from healthy donors indicate that temporary increases in levels may occur quite frequently (51, 57).

Chronic Active EBV Infections

During the 1980s, the diagnosis of chronic EBV (7) infection was given to patients with a variety of diffuse symptoms, often dominated by fatigue and fever. The EBV diagnosis was based mainly on the finding of antibodies to EA. There now seems to be a general agreement that symptomatic EBV reactivations are very uncommon. The term "chronic active EBV infections" could be given to rare, severe EBV-associated conditions, often ending in EBV-associated malignancies or other diseases with lethal outcome (63, 73). The serologic profile should be similar to what is found in primary EBV infection. However, most likely due to long-standing antigen stimulation during chronic reactivation, VCA and EA/D IgG titers are usually higher than during primary infection and may reach extremely high levels. VCA IgM titers may be very low or undetectable (Table 3).

EBV-Associated Malignancies and XLP

For patients with chronic conditions other than malignancies affecting the host-EBV balance, profiles of EBV antibodies compatible with EBV reactivation are already established by the time most patients seek clinical diagnosis. Their EBV serological profiles do not undergo observable change. Whether there is a relation between EBV and the symptoms or the EBV reactivation is an asymptomatic consequence of their immune disorder is seldom possible to discern. EBV serology for these patients is not clinically indicated. In nasopharyngeal carcinoma, elevated antibody levels of various isotypes to a variety of antigens, e.g., VCA and LMP 2 (48), LMP 1 (94), thymidine kinase (12), ZEBRA (14), and EBNA 1 (9), have been identified. The assays could be used for screening of high-risk populations. Positive findings should lead to close examination of the nasopharynx. Maintenance of high antibody levels after treatment correlates with a bad prognosis. The best-documented antibodies for monitoring are VCA IgA antibodies. The presence of EBV DNA in plasma has recently been suggested as a more reliable marker, both for diagnosis and monitoring of NPC (52, 76, 77). However, DNA detection is expensive, and serology may still be more suitable for the large-scale general screening that may be indicated in affected geographic areas.

Monitoring for EBV-associated lymphoproliferative disease in transplant recipients is indicated for those at high risk (see above). The EBV serostatus should always be clarified before transplantation since seronegativity is one risk factor. Thereafter, serology is of little value since the heavy immunosuppression of transplant recipients prevents adequate antibody formation. Monitoring of viral load by NAAT is the method of choice (69). NAATs detect EBV in some asymptomatic persons, and cutoff levels for significant viral loads must be defined for each patient group and for each material. Saliva and leukocytes have low predictive value for disease, since in healthy seropositive individuals, EBV genomes can be detected in saliva (20, 54), and there may be up to 1 to 50 EBV-infected B cells in 1 million B cells (45). Detectable EBV genomes in serum or plasma are a more reliable sign of a pathological virus-host relationship (2, 78). However, any immunocompromised patient may lack EBV-associated symptoms despite detectable EBV in these materials. Irrespective of the material, diagnostic cutoffs at low copy numbers give low specificity for tumor development, while a high cutoff gives low sensitivity (24). A significant increase in genomes is a relatively strong marker for an ongoing pathological process and is an indication for the need for a further search for EBV-associated malignancies in at-risk patients. Since the disease and also its treatment have severe consequences, additional easy-to-use but more precise markers for risk of PTLD would be valuable.

EBV-positive CSFs from HIV-infected individuals have been reported to be significantly associated with primary central nervous system lymphoma (10), but the findings have not been fully verified in recent studies (43). In nonimmunocompromised individuals it is found in EBV encephalitis, but the specificity for EBV-associated disease is not 100% (81). For unambiguous laboratory diagnosis of possible EBV-associated lymphoproliferation or other malignancies, detection of EBV genomes by amplification should be complemented by other laboratory data, such as in situ immunohistochemical detection of LMP 1 or EBERs in hyperplastic or neoplastic cells.

XLP is very rare, but the severity and the heredity of the disease make it important to identify. Typically, these patients do not develop EBNA antibodies, and usually they have low titers to other EBV-associated antigens in comparison to other patient groups with chronic lymphoproliferative diseases. The ultimate diagnosis rests on genetic analysis for mutations in the SH2D1A gene domain, and such analysis should certainly be performed in the few cases where the questions may arise.

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Human Herpesviruses 6, 7, and 8

PHILIP E. PELLETT AND GRAHAM TIPPLES

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The viruses discussed in this chapter are *Human herpesvirus* 6 variants A and B (HHV-6A and HHV-6B), *Human herpesvirus* 7 (HHV-7), and *Human herpesvirus* 8 (HHV-8; Kaposi's sarcoma-associated herpesvirus). These viruses cause diseases that are clinically significant primarily in small children and in immunocompromised patients (Table 1). The clinical challenges relate to differential diagnosis, identification of efficacious therapies, and discrimination between their adventitious and etiologic associations. Diagnostic challenges include a lack of licensed diagnostic kits and limited commercial availability of specific reagents. This leaves it up to the local laboratory to devise in-house assay validations and quality control and quality assurance programs that meet regulatory requirements. A limited array of laboratory assays for these viruses is available through commercial reference laboratories; the information provided here will help in deciding when such assays are needed, choosing the most appropriate test, and interpreting test results.

HHV-6

Taxonomy

HHV-6A and HHV-6B are the most closely related pair of known herpesviruses that warrant designation as individual species (75). Along with HHV-7, which is somewhat more distantly related, these viruses comprise the *Roseolovirus* genus of the betaherpesvirus subfamily. Roseoloviruses share many features of their genomic architecture and genetic content, the ability to replicate and establish latent infections in lymphocytes, associations with febrile rash illnesses in young children, and being opportunistic pathogens in immunocompromised patients. For convenience we will frequently refer to HHV-6A and HHV-6B collectively as HHV-6.

Description of the Agent

Like all herpesviruses, HHV-6 virions consist of four concentrically arranged major components: a double-stranded DNA genome (160 to 170 kb) that is contained in the core of an icosahedral capsid, which is surrounded by a proteinaceous tegument, all of which is surrounded by a lipid bilayer envelope that is studded with a variety of virally encoded proteins and a sprinkling of cellular proteins. The viral genomes encode approximately 100 unique genes.

Epidemiology and Transmission

Epidemiology

Collectively, HHV-6A and HHV-6B are highly prevalent, with seroprevalences in many populations exceeding 90% (30). Most individuals become infected by the age of 2 to 3 years (98). HHV-6B is the main source of the nearly universal early childhood HHV-6 infections, and HHV-6A prevalence may exceed 50% by adulthood.

Tissue Distribution

The cell surface receptor for HHV-6 is CD46, a widespread cell surface antigen. HHV-6B DNA has been detected in as many as 90% of peripheral blood mononuclear cell (PBMC) specimens, >80% of brains, >90% of tonsils (in epithelial cells of tonsilar crypts), and 40 to 90% of saliva or oral fluid specimens; it has been detected less frequently in skin, lungs, endomyocardial biopsy specimens, goblet cells and histiocytes in the large intestine, and cervical fluids. During primary infection in young children, HHV-6A was found by PCR in 2.5% of PBMCs and in 17% of cerebrospinal fluid (CSF) specimens, while HHV-6B was found in 99% of PBMCs and 86% of CSF specimens (some specimens were coinfected) (42). The median salivary viral level of HHV-6 increased from approximately 10^3 copies of HHV-6/ml at 1 week postinfection to 10^5 copies of HHV-6/ml by 8 weeks postinfection (98). HHV-6A DNA has been detected in over one-half of lung and skin specimens, mostly in conjunction with HHV-6B. In a study of adult brains, 28% were positive for HHV-6A and 75% were positive for HHV-6B (some were dually positive) (18). In bone marrow transplant recipients, HHV-6A and HHV-6B were both detected in plasma, while HHV-6B was the variant detected in PBMCs (68). A small percentage of individuals (0.5 to 1%) harbor germ line integrated HHV-6A or HHV-6B genomes, and thus all of their cells are positive for viral DNA, which may be a source for the occasionally detected persistence of high levels of HHV-6 DNA in serum (92).

Latency and Persistence

Other than during the acute phase of roseola, HHV-6 can seldom be detected by culture, even though its DNA can be intermittently detected in most children by PCR, and transcripts can be occasionally detected in asymptomatic children (~1% of specimens) (14). This suggests that a true

TABLE 1 Disease associations

Virus	Age at primary infection	Principal disease associations	
		Primary infection	Immunocompromised patients
HHV-6A	?	Occasional roseola	Disseminated infections, CMV-like and CMV-associated disease, pneumonitis, graft loss
HHV-6B	<2 yr	Most roseola, related febrile/rash illness	Disseminated infections, CMV-like and CMV-associated disease, pneumonitis, graft loss
HHV-7	>50% by 2 yr	Occasional roseola	CMV-like and CMV-associated disease
HHV-8	During childhood in Africa; postadolescence in the United States and Europe	?	KS, MCD, PEL, acute bone marrow failure in transplant recipients

latent infection is established in PBMCs. Latent HHV-6 has been detected in monocytes, their $CD34^+$ bone marrow progenitors, and in PBMC-derived dendritic cells. HHV-6 DNA is more frequently detected in salivary glands than in saliva, in contrast with HHV-7, which is frequently detected in both the gland and the fluid.

HHV-6A was reactivated in 53% of patients in an intensive care unit for medical or surgical indications or trauma (79). Extensive HHV-6 activity (variant not determined) was also seen in another study of intensive care unit patients who were experiencing multiple organ failure syndrome (33); there was no linkage of viral activity to disease severity or outcome in either study. Stress-induced HHV-6 activity thus represents a possible confounder of studies of the relationship between the virus and specific disease, as well as a possible subsequent contributor to the pathogenic process.

Transmission

HHV-6B primary infection typically occurs within a matter of months of the waning of maternal antibody, transmission likely being via saliva. Asymptomatic congenital transmission has been documented (at a rate of approximately 1%), one-third of these infections being with HHV-6A (41). Breast milk is not a significant vehicle for transmission. Little is known of HHV-6A transmission.

Clinical Significance

Infections with HHV-6 are generally mild or subclinical in immunocompetent individuals, but clinically important disease does occur and can be severe in immunocompromised persons.

Primary Infection

HHV-6 primary infection causes roseola (roseola infantum, sixth disease, exanthem subitum) in approximately one-quarter of children. While HHV-6B is the predominant etiologic agent of roseola, rare cases of HHV-6A-associated roseola or febrile illness have been reported. About 90% of children are symptomatic at the time of primary infection, with specifically associated symptoms including fever (58%), fussiness (70%), rhinorrhea (66%), diarrhea (26%), rash (31%), and roseola (24%) (98).

Classic roseola symptoms include an abrupt rise in temperature (39 to 40°C), which persists for 2 to 5 days. Fever abatement coincides with the onset of a maculopapular rash (only rarely vesicular) (Fig. 1), similar to measles and rubella, which resolves in 1 to 3 days. The initial appearance of the rash is on the neck, behind the ears, and on the back, followed by spread to the rest of the body, usually excluding the face and distal extremities. The illness lasts 2 to 7 days, usually with no sequelae. Other clinical symptoms may include palpebral edema, and suboccipital, postauricular, and cervical lymphadenopathy prior to rash onset.

Primary HHV-6 infection may also present as fever without rash or rash without fever. Less common but more severe forms of primary HHV-6 infection may include a body temperature of >40°C, respiratory tract distress, tympanic inflammation, diarrhea, and convulsions. Primary HHV-6 infection, with or without clinical roseola, accounts for a significant proportion of febrile seizures in young children who



FIGURE 1 Child with roseola during primary HHV-6B infection. Photo courtesy of Stephen Dewhurst, University of Rochester. Previously published in reference 36.

are taken to emergency departments (91). However, there were no seizure cases among 130 prospectively monitored children who had laboratory-confirmed primary HHV-6 infection (98), which suggests that relatively few primary infections result in seizures. Other serious but rare complications reported for HHV-6 primary infections include hepatosplenomegaly, Gianatti-Crosti syndrome (an acrodermatitis of childhood that has also been associated with hepatitis B virus and Epstein-Barr virus [EBV] infections), bulging fontanels, aseptic meningitis, encephalitis (perhaps 1% of meningitis and encephalitis cases) (91), seizures and poor neurologic outcome following congenital infection, or disseminated fatal infection.

Primary HHV-6 infection in adults is rare but can be severe. Clinical presentation in these cases may include mononucleosis-like illness, prolonged lymphadenopathy, and fulminant hepatitis.

Immunocompromised Patients

Under immunocompromised conditions, such as in the transplantation setting, or in AIDS patients, HHV-6 can reactivate, sometimes causing disease.

Transplant Recipients

HHV-6 reactivation occurs in approximately one-half of bone-marrow transplant recipients and one-third of solid organ transplant recipients (30). There appears to be a temporal pattern of HHV-6, HHV-7, and human cytomegalovirus (HCMV) reactivation for solid organ transplant recipients, with the median times posttransplantation being 20 days, 27 days, and 36 days, respectively (30).

HHV-6 is a consistent, low-to-moderate frequency contributor to posttransplant illness that can sometimes be severe, and its activity might be both a marker of and contributor to immune suppression (88). In bone marrow and stem cell transplant recipients, HHV-6 has been associated with encephalitis/encephalopathy, pneumonitis, delayed engraftment, erythematous papular rash, fever, CMV-like disease, graft-versus-host disease, seizures, hepatitis, diarrhea, and bone marrow suppression (reviewed in references 13, 30, and 95). In solid organ transplant patients, HHV-6 has been associated with encephalitis, pneumonitis, graft-versus-host disease, fungal disease, and bone marrow suppression. HHV-6B is detected more frequently than HHV-6A in transplant patients. Partitioning of HHV-6A into plasma and HHV-6B into both plasma and circulating lymphocytes (68) suggests that studies that focused on one or the other of these compartments may have missed part of the story.

Human Immunodeficiency Virus (HIV)-Infected Patients

Although HHV-6 infects and replicates in $CD4^+$ T cells, no specific role has been established for HHV-6 in AIDS; some interesting data suggest possible pathogenic interactions (26, 51, 75).

Infections in the Brain

HHV-6 can grow in neuronal cells, and neurological symptoms are clearly associated with primary infection. Numerous studies have shown that HHV-6 is a common inhabitant of the brain, and there have been many suggestions of a possible involvement of the virus in multiple sclerosis (MS) (25). While many studies have seen associations between the virus and disease in a subset of patients and others have found no difference between cases and controls, no study has shown an inverse association, which would be expected if the results were simply random. In addition, the more precisely linked the specimens have been to diseased tissue (e.g., microdissected lesions versus CSF versus serum), the stronger the associations have been. Thus, while the HHV-6–MS association is still a research question, it may soon be of greater relevance to diagnosticians, and some physicians and patients request such testing. Improved and standardized assays and validated controls are needed to allow proper comparison of research results, and potentially for monitoring patients.

In addition to the established etiologic role of JC polyoma virus in progressive multifocal leukoencephalopathy (PML), HHV-6 was more frequently detected in archival brain tissues from PML cases than in control tissues using in situ PCR and immunocytochemistry (64). In contrast, active HHV-6 infection as detected by HHV-6 PCR in CSF was not detected in a study of AIDS patients with progressive multifocal leukoencephalopathy (29).

Other Clinical Manifestations

Drug Hypersensitivity Syndrome

Exposure to various drugs occasionally results in a severe and sometimes fatal reaction variously known as drug hypersensitivity syndrome, drug rash with eosinophilia and systemic symptoms, and anticonvulsant hypersensitivity syndrome. The illness is characterized by rash, fever, and internal organ involvement. In a number of these patients, HHV-6 reactivations have been identified by very large increases in HHV-6 immunoglobulin G (IgG) antibody titers 3 to 4 weeks after onset (93). It remains to be seen whether HHV-6 contributes to either initiation or exacerbation of the events or whether either antiviral therapy or HHV-6 monitoring might be useful.

Rosai-Dorfman Disease

Rosai-Dorfman disease (histiocytosis with massive lymphadenopathy) patients have had increased HHV-6 antibody titers, HHV-6 DNA detected by in situ hybridization, and strong immunohistochemical staining for HHV-6 antigen in abnormal histiocytes.

Other Diseases

There is no conclusive evidence for an association between HHV-6 and malignancy or chronic fatigue syndrome, but the lingering possibility of an association with the latter leads to frequent requests for HHV-6 diagnosis. In our opinion, such information is of little clinical value.

Collection, Transport, and Storage of Specimens

For virus isolation, blood should be collected in an anticoagulant (such as heparin) and processed through a Ficoll gradient within 24 h; cells for virus isolation should not be frozen. After isolation, HHV-6-infected cells (both primary cells and cell lines) can be stored in standard cryopreservation media. Saliva specimens can be collected for virus detection (e.g., by PCR) by various methods, including filter paper strips (97), oral swabs, throat swabs, and expectorated saliva. Serum, plasma, and cells intended for PCR can be shipped frozen.

Direct Detection

Direct detection of HHV-6 can be done by in situ hybridization and in situ PCR (12, 71) and by immunohistochemistry (IHC) (55, 67). While those methods have their niches, the

most common method of direct detection is by PCR. The sensitivity of PCR is of particular value for the detection of HHV-6, given that even during periods of viral activity, viral DNA can be at a relatively low concentration relative to cellular DNA. A multitude of PCR strategies that utilize numerous HHV-6 genomic targets have been published. No genomic target or strategy has emerged as clearly superior; more needs to be done to compare and standardize methods. The list of methods includes single-target and multiplex PCR strategies, qualitative and quantitative assays, and methods that target either DNA (PCR) or RNA (reverse transcriptase [RT]-PCR). HHV-6A and HHV-6B can be readily distinguished by using variant-specific PCR primers, amplimer size differences, variant-specific hybridization probes, restriction enzyme analysis of PCR amplimers, or by melting curve analysis using real-time PCR.

Qualitative PCR for HHV-6 in the clinical laboratory can be set up as a component of a herpesvirus multiplex PCR assay that enables simultaneous analysis for multiple human herpesviruses (46, 49, 78). When multiplex assays are used, it is important to consider the clinical management implications and clinical significance (or lack thereof) of positive results for viruses that would not normally be included in the differential diagnosis.

Quantitative PCR (from whole blood or PBMCs) is particularly useful for the determination of baseline viral load levels in transplant patients, with significant increases above baseline indicating active infection. A LightCycler-based assay that quantitates and discriminates among HHV-6A, HHV-6B, and HHV-7 has been described (81).

We suggest the use of quantitative real-time assays that incorporate a coamplified positive control and allow the HHV-6 variants to be distinguished. Nested-PCR strategies are to be avoided because of the problem of contamination control. Important variables that need to be carefully standardized and then incorporated into the sensitivity calculation and interpretation are the amount of material initially lysed to prepare the template (e.g., volume of fluid or number of cells) and then the amount of this material that is included in each reaction (63). Sources for commercial PCR testing are listed in Table 2. Quantitated HHV-6A and HHV-6B DNA for use as PCR controls is available from Advanced Biotechnologies (Columbia, Md.).

Isolation Procedures

Although HHV-6 can be isolated from PBMCs by cocultivation of patient PBMCs with stimulated human umbilical cord blood lymphocytes (CBLs) (8, 31), primary isolation from clinical specimens is not routinely done in the clinical diagnostic laboratory. The primary cell culture medium consists of RPMI-1640, 10% fetal bovine serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 0.29 mg of L-glutamine per ml. Cells are cultured at starting densities of 10^6 cells per ml. Ficoll-purified primary CBL are first stimulated for 2 to 3 days at 37°C, 5% CO_2 in cell culture media containing 0.01 mg of hydrocortisone/ml and 2 μg of phytohemagglutinin/ml. Stimulated CBLs are then cocultured with an equal volume of Ficoll-purified patient PBMCs in the medium described above, except that the PHA is replaced with recombinant interleukin-2; interleukin-2 concentrations used in various laboratories range from 0.1 to 5 U/ml. Cultures are monitored daily for cytopathic effect (CPE). Typical HHV-6 CPE consists of loss of CBL clumping, the appearance of large balloon-like cells, and syncytium formation. After 7 to 10 days the culture is passaged into freshly stimulated CBLs. Cultures that remain healthy after another 7 to 10 days are considered negative. HHV-6 infection should be confirmed by immunofluorescence or PCR, as described below.

Isolates from primary CBL cultures can be adapted for propagation in continuous cell lines as follows: HHV-6A isolates in HSB-2 or J-JHAN cells, and HHV-6B isolates in Molt-3 cells. HHV-6B strain Z29 grows well in Molt-3 cells of the NIH lineage but not in Molt-3 cells from the ATCC lineage. Culture conditions are similar to those used for propagation in primary cells. Propagation of HHV-6 can be moderately tricky; more extensive procedural details are provided elsewhere (8, 9, 72). It is important to monitor cultures by an immunofluorescence assay (IFA) in addition to CPE. Nonoptimal culture conditions can lead to extensive CPE but little virus production.

Identification

Electron Microscopy

HHV-6 virions have typical herpesvirus morphology by electron microscopy: approximately 200-nm-diameter spherical enveloped virions, containing a 90- to 110-nm icosahedral capsid with an electron-dense core surrounded by an amorphous tegument.

MAbs

Numerous monoclonal antibodies (MAbs) are available (both commercially and from research laboratories) that react with both HHV-6A and/or HHV-6B. MAbs are useful

TABLE 2 Some commercial sources for HHV-6, HHV-7, and HHV-8 testing

Source (URL)	Serology[a]			PCR[b]		
	HHV-6	HHV-7	HHV-8	HHV-6	HHV-7	HHV-8
Labcorp (http://labcorp.com)	IgG			q, V		
Specialty Labs (http://specialtylabs.com)	IgG IgM	IgG IgM	IgG	q		q
Viracor (http://viracor.com)				Q	Q	Q
Focus Diagnostics (http://focusdx.com)	IgG IgM	IgG IgM	IgG	Q	Q	q

[a] Serologies are by IFA, reported as endpoint titers.
[b] Q, quantitative PCR; q, qualitative PCR; V, variant discriminating.

for confirming cell culture CPE for HHV-6 infection, for antigen detection assays, and for flow cytometry. Well-characterized commercially available antibodies include MAbs against p41 (DNA polymerase accessory factor) and gp116/54/64 (glycoprotein B) (both from Advanced Biotechnologies, Inc.), and a B variant-specific MAb against the tegument protein 101K/p100 (Chemicon International, Temecula, Calif.). MAbs are also used for IHC analysis, such as in brain sections from patients with MS or in lymph node biopsy specimens. Although not all MAbs react well with antigens that have been fixed and then embedded in paraffin, the MAbs described above against gp116/54/64 and 101K are useful for such specimens. A list of published antibodies, their targets, variant specificity, and commercial sources is provided in reference 3.

Typing Systems

HHV-6A and HHV-6B can be typed through the use of variant-specific MAbs and variant-specific PCR assays (described above).

Serologic Tests

Numerous enzyme-linked immunosorbent assay (ELISA) methods for detecting HHV-6 antibodies have been described. Most are based on the use of lysate from HHV-6-infected cells; purified viral antigens can also be used. The most sensitive HHV-6 antigen in immunoblot assays (IgG and IgM) is a virion protein of approximately 100 kDa, designated either p100 or 101K. IFA is commonly used for the detection and quantification of HHV-6-specific IgM and IgG antibodies. Recent versus past infections can be discriminated through the use of IFA-based avidity antibody tests. Maturation from low- to high-avidity HHV-6 antibody following primary HHV-6 infection takes approximately 5 months. Antibody avidity testing can also be used to differentiate between HHV-6 and HHV-7 primary infections (90). Neutralizing antibodies typically become detectable 3 to 8 days following the onset of fever during primary infection. Sources for commercial antibody testing are listed in Table 2. Research antibody test kits (IFA and ELISA) are available from Advanced Biotechnologies and Biotrin (Dublin, Ireland). Slides and control sera are available from Panbio Diagnostics (Columbia, Md.) and Bion Enterprises (Des Plaines, Ill.).

Sensitivity and Specificity

ELISA-based methods are generally thought to be more sensitive than IFA, but this is not always true in practice and may depend on whether the definition of sensitivity is endpoint titer or simply detection of infection.

Specificity issues for HHV-6 serological methods involve cross-reactivity between HHV-6A, HHV-6B, HHV-7, and CMV (13, 90). Current serological methods based on whole infected cells as antigen do not differentiate between HHV-6A and HHV-6B antibody responses. Although adsorption studies have demonstrated the presence of cross-reactive antibodies to HHV-6 and CMV, the cross-reactivity is generally not sufficient to confound methods based on HHV-6 whole-cell lysates. The simultaneous rise in antibodies to both HHV-6 and CMV seen in some patients is not fully understood; it may be due to varying combinations of simultaneous activity of both viruses and some individuals responding to cross-reactive antigens. Limited cross-reactivity has also been found between HHV-6 and HHV-7 antibody responses; adsorption methods and antibody avidity can be used for differentiation (90).

Antiviral Susceptibilities

Antiviral treatment of HHV-6 has been reviewed in depth (30). The clinical circumstances for which antiviral intervention for HHV-6 might be appropriate include HHV-6 reactivation in immunocompromised individuals. There are presently no antivirals licensed for specific treatment of HHV-6; however, an approach similar to that for HCMV has been used. The best in vitro antiviral sensitivities and selectivities have consistently been for foscarnet, cidofovir, and ganciclovir. Similar to the mechanism of ganciclovir resistance for HCMV, there is in vitro and in vivo evidence that mutations in the HHV-6 U69-encoded protein kinase or the HHV-6 DNA polymerase gene can cause HHV-6 ganciclovir resistance.

Evaluation, Interpretation, and Reporting of Results

Methods used in the clinical virology laboratory must be thoroughly evaluated in the relevant clinical context (e.g., febrile and/or rash illness in children under 3 years of age, and specific forms of disease in immunocompromised individuals such as transplantation patients) to assess sensitivity, specificity, and positive and negative predictive values. Whether using in-house or commercial assays, the panel of specimens used in the evaluation should be validated with respect to a "gold standard" test or marker, such as virus isolation or seroconversion.

Laboratory diagnosis of HHV-6 is challenging given the many biological and molecular similarities between HHV-6A, HHV-6B, and HHV-7; the association between CMV and HHV-6 reactivation; and the need to differentiate between primary and reactivation disease. Also, other than for roseola and related febrile rash illnesses in children, the profiles of viral markers and immunologic responses during the course of HHV-6 primary infection and reactivation are still not well defined (11, 22). With these limitations, the interpretations of laboratory results for the detection of primary HHV-6 infections and the detection of active HHV-6 infection (which includes both primary and reactivation) are presented in Table 3. We also refer the reader to the elegant treatment of this issue by Ward (90).

Primary Infection

The following definitions have been used to diagnose HHV-6 primary infection: (i) antibody seroconversion between acute- and convalescent-phase serum or plasma specimens (collected 2 to 4 weeks apart); (ii) detection of HHV-6-specific low avidity antibodies in serum or plasma (90); (iii) a positive PCR result from whole blood and IgG-negative plasma within 5 days of disease onset in children older than 3 months of age (to avoid problems with maternal antibodies; some recommend 6 months) (22); (iv) a PCR-positive blood and PCR-negative saliva taken during the acute phase of disease (27); (v) positive serum IgM and/or serum PCR, when both have been evaluated (to improve on the lower sensitivity of testing for each marker alone) (11); and (vi) detection of IgM antibodies in the absence of IgG antibodies. Assaying only for IgM is of limited value for this purpose, given that approximately 5% of adults are HHV-6 IgM positive at any time and the window of IgM positivity is poorly defined. Primary HHV-6 infection almost always occurs in children before 2 to 3 years of age. Thus, in cases when clinical symptoms are compatible with roseola (the illness most commonly associated with HHV-6 primary infection in young children), a serum specimen that tests positive for both IgM and IgG

could indicate primary infection if the blood was collected after the IgG response developed; avidity testing could clarify the diagnosis in this case. Quantitative PCR assays are preferred because they allow identification of individuals with extraordinarily high viral loads that may be due to germ line integrated viral DNA, which can conceivably lead to positive serum or plasma specimens that are diagnostically ambiguous (92). Further evaluations and standardization of diagnostic algorithms are needed.

Active Infections

The following criteria have been considered to be diagnostic for detecting active infections, which include both primary infections and reactivations: (i) virus isolation from PBMCs (the gold standard); (ii) a significant rise in antibody titers (>4-fold) between acute- and convalescent-phase serum or plasma specimens; (iii) a positive serum or plasma PCR result for HHV-6 DNA (the low sensitivity of this marker suggests that it should not be relied on as the only marker); (iv) quantitative PCR from whole blood or PBMCs with results above a defined threshold level (e.g., transplant patients routinely monitored to determine baseline HHV-6 viral load; a significant increase in viral load is indicative of reactivation); and (v) detection of spliced HHV-6 mRNA by RT-PCR (69). Yoshikawa et al. (96) suggest that a combination of RT-PCR detection of immediate early and late genes provides optimal sensitivity and specificity for confirming active HHV-6 infections. In many circumstances, the final diagnosis is strengthened by analysis of paired acute- and convalescent-phase specimens.

Individuals with Constitutively High Viral Loads

Approximately 1% of individuals harbor exceptionally high HHV-6 loads in their PBMCs (approximately one copy per cell equivalent of DNA), apparently due to germ line HHV-6 integration. No HHV-6-linked immunologic deficiency, disease, or unusual virologic activity has yet been observed in such individuals. This circumstance can be difficult to detect in acellular fluids but often leads to striking results when a cell-derived template is analyzed in a quantitative PCR assay. This reinforces the need to monitor serial specimens; if all are positive and the viral load is very high and constant, this may well represent integrated virus that could be irrelevant to the event being examined.

HHV-6 Testing in the Clinical Setting

Clinical testing for HHV-6 should be limited to circumstances for which there has been a well-characterized association between the disease and HHV-6 infection and when the results of a given test will be diagnostically informative. For example, HHV-6 IgG or IgM serology in an adult done at a single time point is of limited diagnostic value. Clinical scenarios where HHV-6 testing could be considered include primary infection in children under 3 years of age and primary infection or reactivation in immunocompromised individuals such as AIDS patients or transplantation patients, those with mononucleosis-like syndromes, and meningitis and encephalitis cases. Still relevant in some parts of the world, HHV-6 has been identified as the cause of rash illness incorrectly diagnosed as measles infection. The possibility of primary HHV-6 or HHV-7 infection should be considered when febrile and/or neurologic illness occurs shortly following routine immunizations; such illness may be incorrectly attributed to the vaccine (90).

Further studies are needed to improve our understanding of the associations between HHV-6 and disease and to improve the quality of HHV-6 diagnostic testing.

HHV-7

Taxonomy

Because of the relative similarity of its nucleotide sequences, genomic architecture, and biology with those of HHV-6A and HHV-6B, HHV-7 is included as a member of the *Roseolovirus* genus of the betaherpesvirus subfamily (75).

Description of the Agent

Like all herpesviruses, HHV-7 virions consist of four concentrically arranged major components: a double-stranded DNA genome (145 kb) that is contained in the core of an icosahedral capsid, which is surrounded by a proteinaceous tegument, all of which is surrounded by a lipid bilayer envelope that is studded with a variety of virally encoded proteins and a sprinkling of cellular proteins. HHV-7 genomes carry approximately 100 unique genes.

Epidemiology and Transmission

Epidemiology

HHV-7 seroprevalence in healthy adults in Europe, Japan, and the United States ranges from 60 to 90%. Following waning of maternal antibody, HHV-7 seroprevalence rapidly increases to over 50% by 2 years of age and then progresses toward the adult level over the remaining years of childhood.

Tissue Distribution

Although its in vitro host range is restricted to lymphocytes, HHV-7 has a broad cellular host range in vivo. One of its cellular receptors is the lymphocyte antigen, CD4; other receptors must be involved in the infection of other cell types. HHV-7 lytic antigens have been detected in salivary gland acini, lungs, skin, and mammary glands, and more sporadically in liver, kidney, and tonsils (48). HHV-7 DNA has been detected by PCR in PBMCs, salivary glands, and gastric mucosa. The virus has seldom been detected in brain tissues. Infectious HHV-7 has been occasionally isolated from PBMCs during roseola and is easily isolated from the saliva of healthy adults. HHV-7 shedding in the saliva is associated with younger age and low HHV-7-specific antibody titers (45). The ability to detect HHV-7 DNA in uncultured lymphocytes by PCR, along with the inability to detect infectious virus in the absence of T-cell activation, suggests that the virus is latent in PBMCs. Macrophages represent another potential reservoir (99).

Transmission

HHV-7 can be cultured from the saliva of ~75% of healthy adults, with viral loads frequently exceeding 10^6 genomes per ml. While the most plausible route of transmission is via saliva, HHV-7 has also been detected in breast milk, urine, and cervical secretions. In contrast to HHV-6, congenital transmission of HHV-7 has not been documented (41).

Clinical Significance

Primary Infection

HHV-7 causes perhaps 5% of roseola cases, as well as other febrile illness in children. In one small study, HHV-7 appeared to cause milder roseola than HHV-6 in terms of mean and maximum fever, fever duration, and duration of rash. However, other studies suggest a rate of central nervous system complications during HHV-7 primary infections equal to or higher than with HHV-6 (91). HHV-7 neurologic involvement includes hemiplegia and febrile seizures.

TABLE 3 Diagnostic methods for HHV-6, -7, and -8

Virus	Method	Approach	Advantages	Limitations	Clinical availability	Key reference(s)
HHV-6	Virus isolation	Cocultivate PBMCs with stimulated primary CBLs	Gold standard for active infection	Slow, need for ongoing access to umbilical cord blood	Not practical in the routine clinical lab	8, 9, 31, 72
	Antigen detection	Use of HHV-6A, -6B variant-common and variant-specific MAbs against p41/p38, gp116/54/64, gp82/105, 101K for IHC and culture confirmation	Specific, readily available reagents	Primarily described for research studies, not clinical testing	Commercially available MAbs	3
	Serology	IgM and IgG; NT, immunoblot, IFA, ACIF, ELISA; major antigen: p100/101K		Single IgG-positive results are uninformative; clinical significance of IgM in individuals >3 yr of age is questionable.	Practical for clinical labs; commercially available	11, 13
		Antibody avidity	Clear identification of primary infections	Best with acute and convalescent specimens	Avidity test not commercially available	90
	PCR	Qualitative and quantitative		Optimal target not determined; assays not standardized; quantitation threshold not determined or standardized	Practical for clinical labs; commercial assay available	Numerous methods reported in the literature
		PCR from PBMCs or plasma/serum		Not able to unequivocally differentiate latent from active infections for PBMC/whole-blood PCR.		11, 22, 27
		RT-PCR from PBMCs to detect active infections	Confirms active infection	Not standardized	Not commercially available	
		Herpesvirus multiplex	Convenient for the lab	Clinicians may not want testing done for all human herpesviruses because of interpretation and clinical utility issues.		46, 49, 78
HHV-7	Virus isolation	Cocultivation of PBMCs with stimulated primary CBLs; inoculation of CBLs with saliva from healthy and ill patients	Gold standard for active infections	Not practical in the routine clinical lab		8
	Antigen detection	Several, including against pp85; for IHC and culture confirmation			Commercially available MAbs	7
	Serology	IgM and IgG			Commercially available materials	7
		NT, immunoblot, IFA, ELISA; major antigen: pp85				10, 85
		Antibody avidity	Clear identification of primary infections	Best with acute and convalescent specimens		90
	PCR	Qualitative and quantitative PCR from PBMCs or plasma/serum			Practical in the clinical lab; commercial testing is available	6

	Herpesvirus multiplex	Convenient	Clinicians may not want testing done for all human herpesviruses because of interpretation and clinical utility issues.		46, 49, 78
HHV-8					
Virus isolation	Not available				
Antigen detection	MAbs against K8.1, ORF59, LANA; for IHC	Distinguish KS from clinical mimickers		MAbs are commercially available.	21, 43, 74, 80
Serology	Mostly IgG			Reagents, research kits, and testing are commercially available.	19, 66
	IFA based on unstimulated (latent) or stimulated (lytic) PEL cells; also immunoblot and ELISA				20, 23, 53, 61, 82, 83
	Neutralization assay				50
	Major lytic antigens: ORF65 and K8.1; major latent antigen: LANA-1				21, 43, 74, 80
PCR	Qualitative and quantitative				19, 63, 76
	Herpesvirus multiplex		Clinicians may not want testing done for all human herpesviruses because of interpretation and clinical utility issues.		46, 78

Immunocompromised Patients

HHV-7 becomes latent in lymphocytes following primary infection and can reactivate in immunocompromised hosts such as transplantation recipients and AIDS patients. In bone marrow, liver, and renal transplant patients, simultaneous CMV and HHV-7 activities have sometimes been associated with clinical events (see the corresponding HHV-6 section above), and a case of fatal HHV-7-associated encephalitis has been reported, but overall, HHV-7 infections are not frequent contributors to posttransplant illness.

Like HIV, HHV-7 uses CD4 as its major cellular receptor. In vitro, HHV-7 can inhibit HIV infection, possibly due to receptor interference. There is no clear evidence of a role for HHV-7 in AIDS progression.

Interstitial Pneumonia

HHV-7 DNA was detected more frequently and at higher loads in affected tissues from nonimmunocompromised patients with interstitial pneumonia (94). Further study is required to fully evaluate the significance of this observation.

Pityriasis Rosea

Data are inconsistent for an association between HHV-7 and pityriasis rosea (24).

Collection, Transport, and Storage of Specimens

The methods described above for HHV-6 are appropriate for HHV-7.

Direct Detection

By electron microscopy, HHV-7 virions have appearances typical of herpesviruses. One MAb (anti-HHV-7 pp85) is commercially available (Advanced Biotechnologies), and other MAbs are available upon request from some research laboratories (7). The most widely used qualitative PCR primer set for HHV-7 was described by Berneman et al. (6); others have also been described, but their performances have not been compared. Qualitative PCR for HHV-7 can be included in a herpesvirus multiplex setup (46, 49, 78). Quantitative PCR methods for HHV-7 include a LightCycler-based assay that discriminates among HHV-6A, HHV-6B, and HHV-7 (81). An HHV-7 RT-PCR system has not been described but could be useful for detecting active virus replication. Sources for commercial PCR testing for HHV-7 are listed in Table 2. Quantitated DNA for use as a PCR control is available from Advanced Biotechnologies.

Isolation Procedures

HHV-7 isolation can be accomplished using the methods described above for HHV-6 but is not used for routine clinical diagnosis. HHV-7 isolates initially propagated in primary cell culture or previously adapted HHV-7 strains (e.g., strain SB) can be grown in SupT1 cells in a manner similar to that used for the propagation of HHV-6 in continuous cells (8).

Identification

MAbs can be useful for confirming cell culture CPE when attempting virus isolation, especially to rule out CPE due to HHV-6, for IHC, and for immunoelectron microscopy. The PCR methods described above provide sensitive, specific, and rapid ways to unambiguously identify the virus.

Typing Systems

Biologically meaningful subtypes of HHV-7 have not been identified.

Serologic Tests

HHV-7 serologic assays include neutralization tests, immunoblot, immunoprecipitation, IFA (commercially available reagents), and ELISA (reviewed in reference 7). In one comparison of IFA, immunoblot, and ELISA, the overall performances of the three assays were quite similar, there being a small sensitivity advantage for the ELISA and a specificity advantage for the immunoblot assay (10). As described above, antibody avidity testing for both HHV-6 and HHV-7 allows differentiation between HHV-6 and HHV-7 primary infections as well as recent and past HHV-7 infections (90). Slides and control sera are available from Panbio Diagnostics and Advanced Biotechnologies.

Antiviral Susceptibilities

HHV-7 antiviral sensitivities are similar to those for HHV-6 and CMV, ganciclovir, foscarnet, and cidofovir being much more inhibitory than acyclovir (32). Drug-resistant HHV-7 has not been identified, but given the widespread use of ganciclovir for therapy of CMV and "CMV disease," it will likely occasionally emerge. Therapeutic regimens have not been defined; for transplant recipients they may ultimately be linked to strategies for dealing generically with betaherpesvirus activity.

Evaluation, Interpretation, and Reporting of Results

There are no circumstances that warrant routine single-agent monitoring for HHV-7 activity, but its inclusion in multiplex assays may prove to be of value in monitoring children with suspected encephalitis and/or febrile convulsions (especially in association with recent vaccination) (91) and transplant recipients. The main issues for HHV-7 diagnostic testing are distinguishing HHV-6 from HHV-7, differentiation of primary HHV-7 infection from reactivation, and distinguishing latent and active infections. Many of the same considerations for interpretation and reporting of results described for HHV-6 apply to HHV-7. Differentiating HHV-6 from HHV-7 can be done both by molecular analysis (e.g., PCR) and through the use of specific antibodies. Combinations of methods, as described for HHV-6, can also be used for HHV-7 laboratory diagnosis.

HHV-8

Taxonomy and Description of the Genus

HHV-8 is a member of the *Rhadinovirus* genus of the gammaherpesvirus subfamily. Gammaherpesviruses, which include EBV, are characterized by being able to replicate and establish latency in lymphoblastoid cells.

Description of the Agent

Like all herpesviruses, HHV-8 virions consist of four concentrically arranged major components: a core double-stranded DNA genome (165 kb) that is contained in the core of an icosahedral capsid, which is surrounded by a proteinaceous tegument, all of which is surrounded by a lipid bilayer envelope that is studded with a variety of virally encoded proteins and a sprinkling of cellular proteins. HHV-8 genomes carry approximately 80 unique genes.

Epidemiology and Transmission

Epidemiology

The global distribution of HHV-8 is uneven (reviewed in reference 65). In much of Africa, HHV-8 seroprevalence exceeds 50%, whereas in most of Europe, the United States, and Japan, seroprevalence is less than 10%. Seroprevalence is in the range of 30 to 50% in men who have sex with men (MSM) in the United States and Europe. The reasons for the population-based disparities are likely to be due to differences in child-rearing and family practices, sexual behaviors, and possibly viral and/or host genotypes. In Africa, the virus is frequently acquired early in life, in a maternally linked manner. In the United States, prevalence rises after adolescence. At least four major HHV-8 genotypes are in global circulation (100).

Tissue Distribution

Other than in the context of Kaposi's sarcoma (KS), HHV-8 DNA or antigens have seldom been detected in immunocompetent individuals, even if they are seropositive. The circumstance where the virus has been detected most often in immunocompetent individuals is in the saliva of HIV-negative MSM. In HIV-positive MSM, viral DNA can be detected in essentially all KS lesions, over 50% of saliva or oral fluid specimens, around 30% of PBMC specimens, about 10% of semen specimens, and less frequently in other types of specimens. In KS lesions, the virus is present mainly in spindle cells, mostly in a latent form, with scattered lytically infected cells. Although ordinarily restricted in its tissue distribution, the virus is capable of replicating in many cell types.

Latency and Persistence

In AIDS patients the virus has been detected in CD19-positive B cells. In PBMCs from patients with or at risk for KS, the HHV-8 concentration can be so low as to preclude reliable detection by PCR. The virus has been detected in a variety of tissues, especially in immunocompromised patients, but generally with methods that cannot discriminate between latent and lytic infections. Evidence for persistence of HHV-8 in immunocompetent seropositive individuals comes from organ transplant recipients who subsequently developed KS (16); at the time of their transplant, they most likely already harbored the virus that caused their KS.

HHV-8 latency has been extensively studied in cell culture systems that employ primary effusion lymphoma (PEL) cell lines that harbor HHV-8. In such cell lines, under ordinary culture conditions the virus is in a latent state in >95% of cells, with 1 to 5% of cells being in a lytic state. Treatment of such cultures with inducing agents such as a phorbol ester, sodium butyrate, or the HHV-8 lytic inducer gene encoded by open reading frame 50 (ORF50), rapidly induces lytic replication in 10 to 30% of cells. Only a handful of HHV-8 genes are transcribed during latency, including the ORF73 gene that encodes the major latency-associated nuclear antigen 1 (LANA-1), v-cyclin, and the highly expressed K12 (kaposin, also known as *nut*-1 and T0.7); transcripts of the latter are sensitive diagnostic targets.

Transmission

Most HHV-8 transmission is probably via oral fluids, although questions remain as to precisely how this might occur (44). Some transmission may also occur via breast milk and semen. In Africa, the virus is typically acquired in a maternally linked manner early in life, possibly via a traditional practice such as premastication of food or use of saliva to soothe bloodsucking-arthropod bites. Sexual transmission probably occurs in Africa but does not appear to be the major route. Among MSM, HHV-8 seropositivity is linked to a variety of sexual risk factors. Inconsistent linkages have been observed with various forms of anal sex (34, 62). In some studies, HHV-8 seropositivity has been linked to injection

drug use and blood transfusion (35); screening of blood products is under discussion but does not appear to be imminent. A suitable screening assay is not available.

Clinical Significance

Primary Infection

Little is known of primary HHV-8 infection. There are likely to be significant differences between healthy children, immunocompetent adults, and immunocompromised patients. HHV-8 DNA has been detected in febrile children in areas where the virus is endemic. Among 15 HIV-negative MSM who seroconverted to HHV-8, diarrhea, fatigue, localized rash, and lymphadenopathy were sometimes noted in association with primary infection (89). Seroconversion was contemporaneous with PCR positivity in PBMCs and development of cytotoxic T-lymphocyte responses to HHV-8 lytic proteins. For immunocompromised patients, acute illness has been associated with apparent HHV-8 primary infection, including KS, fever, arthralgia, lymphadenopathy, splenomegaly, and cytopenia (56, 70).

KS

The four major epidemiologic forms of KS are all associated with HHV-8: (i) African endemic KS, which primarily affects children with or without HIV infection; (ii) Mediterranean or classic KS, which affects older men from specific regions of Mediterranean Europe who have no known immunologic dysfunction; (iii) transplant-associated or idiopathic KS, which affects approximately 1% of solid organ transplant recipients in the United States; and (iv) AIDS-associated KS, which is the most common AIDS-associated neoplasm and the most aggressive form of the disease. KS incidence has decreased substantially but has not been eliminated since the advent of highly active antiretroviral therapy.

KS is characterized by reddish brown plaque or nodular lesions on the skin of the trunk or the extremities and in the oral cavity (oral KS), as well as on internal organs (visceral KS). The disease can be disfiguring, disabling, and life-threatening. KS lesions are characterized by networks of vascular slits, spindle-shaped cells of endothelial origin, extravasated red blood cells, and purplish deposits of hemosiderin. HHV-8 is present in the spindle cells, lytically in a small percentage and latently in the remainder. Immortalized KS cells have not been identified despite numerous attempts.

PELs

PELs are a rare (approximately 3% of AIDS-non-Hodgkin's lymphomas) but frequently aggressive subset of a class of lymphomas known as body-cavity-based lymphomas. PELs occur as lymphomatous effusions in body cavities, seldom disseminate beyond the cavity of origin, have cell morphologies between those of large-cell immunoblastic lymphoma and anaplastic lymphoma, express CD45 and associated antigens, have clonal immunoglobulin rearrangements suggesting B-cell origin but seldom express B-cell antigens, have infrequent c-*myc* rearrangements or alterations in other cellular oncogenes, are frequently infected with EBV, and harbor HHV-8 in every cell (17). These lymphomas have a poor prognosis.

MCD

Multicentric Castleman's disease (MCD) is a multicentric angiofollicular B-cell hyperplasia that occurs in germinal centers of lymph nodes. The disease has systemic manifestations that include fever, fatigue, recurrent lymphadenopathy, hepatosplenomegaly, bone marrow plasmacytosis, and polyclonal hypergammaglobulinemia. HHV-8-associated MCD occurs predominantly in HIV-infected individuals. Within the mantle zone of the germinal centers, HHV-8 is present in scattered B cells. HHV-8-associated MCD has a poor prognosis. Plasmablastic lymphoma has developed in patients who have the plasmablastic form of MCD.

Disease in Transplant Recipients

Organ transplant recipients have significantly elevated risk for developing KS (reviewed in reference 65). KS risk is highest in regions where HHV-8 seroprevalence is highest. In most cases, the virus causing the disease was probably reactivated from a prior infection in the transplant recipient, but graft-derived infections have been documented (5, 16, 56, 73). In renal transplant recipients, in addition to cutaneous KS, parenchymal KS infiltration has led directly to marrow aplasia with plasmacytosis, fever, splenomegaly, cytopenia, marrow failure, graft failure, and disseminated KS (56, 59). PELs (one in association with KS) have been reported following cardiac transplants (37, 47), as has apparently donor-derived fatal disseminated KS in liver transplant recipients (58).

The association between organ transplantation and adverse clinical manifestations of HHV-8 infections in the transplant setting defines an imperative to devise clinically useful approaches to their diagnosis, prevention, and treatment.

Other Diseases

HHV-8 has been associated with several other diseases. For some (e.g., angioimmunoblastic lymphoproliferative disease, oral plasmablastic lymphomas, and some cutaneous disease), further work is required to more firmly establish an etiologic association. In the case of multiple myeloma, the association with HHV-8 has been extensively studied, with the weight of the evidence being that the virus has little to do with the disease. Likewise, the initial observations of an association between HHV-8 and primary pulmonary hypertension have not been confirmed in subsequent studies by others. HHV-8 was detected in tissues from patients with idiopathic pulmonary fibrosis, but so were several other viruses. It remains to be determined whether the association is specific. An important part of establishing etiologic associations is consistency of association, a criterion that generally requires both agreement between laboratories and agreement when different populations are examined.

Collection, Transport, and Storage of Specimens

Conventional methods can be used for collection and storage of serum or plasma for serology. For preparation of lymphocyte DNA for PCR, blood should be collected in anticoagulant (not heparin) and PBMCs should be purified on a Ficoll gradient. Oral fluids can be collected for PCR by the methods described above for HHV-6. Biopsies are used for pathologic analyses that may include standard hematoxylin- and eosin-stained cytopathic examination, PCR on total DNA extracted from a section, in situ PCR, or IHC. Standard methods can be used for the preparation of such specimens.

Isolation Procedures

Reliable systems have not been developed for primary culture of HHV-8 from KS lesions. Ascitic fluids from PEL patients have been used as the source for deriving PEL cell lines.

Identification

HHV-8 is readily identified by PCR, hybridization with nucleic acid probes that target abundant transcripts (84, 86),

and MAbs. By electron microscopy, HHV-8 virions have a morphology similar to that of other herpesviruses, but this method is seldom applied to clinical materials.

The PCR primer sets described in the initial description of HHV-8 remain in wide use for qualitative PCR on specimens of all types (19). Nested PCR has been used, but the potential for false positives is higher and seems to outweigh the possible sensitivity increase (76). Numerous systems have been described for quantitative PCR. Because of the low frequency of circulating HHV-8-infected lymphocytes and low viral copy number in latently infected cells, important variables for PCR from blood specimens include the number of cells in the initial lysis and the total quantity of resulting DNA included in the PCR (63). Sources of commercial PCR testing are listed in Table 2. Quantitated DNA for use as a PCR control is available from Advanced Biotechnologies.

KS can be readily distinguished from its mimickers such as benign vasculoproliferative lesions and tumors that have a prominent spindle cell component by IHC employing a MAb to the HHV-8 LANA-1 encoded by ORF73 (21, 43, 74, 80). Antigen retrieval methods can influence sensitivity. IHC appears to be more specific for identifying KS than is PCR or RT-PCR, which has sometimes been positive for angiosarcoma specimens.

Typing Systems

HHV-8 genotyping is based predominantly on the sequence of the highly variable K1 gene sequence (100). HHV-8 genotypes have been associated with the global migration of the virus in association with human migration but have not been specifically associated with any of the major forms of the disease or conclusively associated with pathogenic variation.

Serologic Tests

Latent versus Lytic Antigens

EBV and HHV-8 share two biologic properties relevant to their serodiagnosis: (i) in vivo, they maintain their latent state in circulating lymphocytes and (ii) in vitro, they are propagated in cell lines in which the virus is present in a latent form in >95% of cells, but in which the lytic cycle can be induced in 10 to 30% of cells by treatment with an inducer such as a phorbol ester or sodium butyrate. Latency is regulated in part by chromatin-associated proteins that are significant antigens: EBNA in the case of EBV and LANA-1 for HHV-8. In addition, a handful of other HHV-8 proteins that are expressed only during the lytic cycle have been identified as significant serologic antigens. The most useful of these lytic antigens are encoded by ORF65 and ORFK8.1 (65). Sources for commercial antibody testing are listed in Table 2. IFA and ELISA antibody test kits are available from Advanced Biotechnologies and Biotrin.

IFA

The two main varieties of IFA in use for detection of HHV-8 antibodies are based on (i) uninduced PEL cell lines that target LANA-1 and (ii) lytically induced PEL cell lines. Latent fluorescence is primarily restricted to the nucleus, while lytic fluorescence is predominantly cytoplasmic. An important consideration is the serum dilution. Although a dilution of 1:10 has been used in some studies, in most cases this has led to unacceptable loss of specificity. Most laboratories have settled on dilutions in the range of 1:40 to 1:100 for IFA.

In addition to PEL cell lines that express complex mixtures of HHV-8 antigens, IFAs have been developed based on single defined antigens expressed via recombinant Semliki Forest viruses. Occasionally, false positives in the lytic IFA are due to antigenic cross-reactivity between the EBV and HHV-8 versions of the conserved herpesvirus protein, glycoprotein B.

Immunoblot

Immunoblot assays have been developed based on uninduced or induced PEL cells, as well as on purified bacterially or baculovirus-expressed LANA-1, ORFK8.1, and ORF65.

ELISAs

The most widely used HHV-8 ELISAs are based on purified virions (20, 23, 61), purified bacterially expressed ORF65 (83), and synthetic peptides based on ORF65 and ORFK8.1 sequences (53, 82). These assays have the obvious virtue of high throughput and objective evaluation of individual specimens relative to a defined cutoff but are less sensitive than lytic IFA.

IgG and IgM

Little attention has been paid to serologic methods for identifying and discriminating recent from past infection, such as detection of IgM or antibody avidity.

Neutralization Assays

A neutralizing antibody assay based on inhibiting infectivity of a recombinant HHV-8 that has been engineered to express green fluorescent protein in infected cells has been developed (50). The assay was used to demonstrate the potential importance of neutralizing antibody titers in preventing KS.

Sensitivity, Specificity, and Interassay Agreement

Assessment of the specificity and sensitivity of HHV-8 serologic assays has been complicated by several issues that revolve around the problem of unambiguously identifying individuals who are truly positive or negative for the virus. It has been a challenge to identify the most appropriate cutoff in the absence of true gold standard specimens. Healthy blood donors are often defined as HHV-8 negatives, although some of them are likely to be positive for the virus, and KS patients are frequently regarded as true positives, even though some may be seronegative. Specimens from individuals in groups of relatively greater or lesser risk for being positive or negative for the virus have been used to generate a receiver-operating characteristic (ROC) curve that allowed selection of assay cutoffs with various combinations of sensitivity versus specificity (61). A classification tree was used to define serostatus based on different interpretive algorithms applied to five assays (40); the best combined sensitivity and specificity was obtained by defining individuals as seropositive if they had strong reactivity in the ORFK8.1 enzyme immunoassay (EIA) or moderate reactivity in the same assay and were positive by a latent IFA. Latent class analytic methods were used to estimate the sensitivity and specificity of three assays (EIAs to ORF65 and ORFK8.1 and a latent IFA) in the absence of prior knowledge of truth (39). There was only modest agreement among the assays, with the ORFK8.1 EIA being the most sensitive and specific. Corchero et al. (28) bootstrapped gold standards by ROC analysis of results obtained at several serum dilutions in an IFA based on ORFK8.1 expressed via a recombinant Semliki Forest virus and then compared several assays on the basis of the area under ROC curves generated relative to the carefully defined standards; assays based on lytic antigens were superior to latent antigen assays. In an evaluation of 18 different assays, there was no clear delineation of assay quality between assays based on latent versus lytic antigens (82). The four assays with the best sensitivity

and specificity included a latent and a lytic IFA and EIAs based on bacterially expressed K8.1 or peptides derived from ORF65 and ORFK8.1. In a comparison of several laboratory-specific algorithms for HHV-8 serodiagnosis, some of which employed more than one assay to arrive at their final result, all approaches identified all KS patient specimens as being seropositive, and there was high agreement that most blood donor specimens were seronegative, but there was only modest agreement about which blood donor specimens were positive, with no approach being clearly superior (77).

Antiviral Susceptibilities and Therapy

The main forms of standard therapy for KS include highly active antiretroviral therapy (immune reconstitution and possibly reducing HIV cofactor effects), local and systemic cytotoxic and tissue-destructive regimens, and immune enhancement by alpha interferon (2, 4, 52, 87). HHV-8 is exquisitely sensitive to cidofovir and is also sensitive to ganciclovir and foscarnet. Although in small trials the therapeutic effect of these agents has been equivocal, in large retrospective analyses, foscarnet or ganciclovir used for controlling other herpesvirus infections has been associated with lower KS risk (60), possibly due to these agents being more effective in preventing than in treating KS. Therapeutic algorithms employ a stepped approach based on the extent of disease and rate of progression, with therapeutic options ranging from local to systemic to palliative.

Because cases of PEL and MCD are so rare, there have been no controlled therapeutic trials. Case reports suggest potential utility of anti-CD20 MAb therapy for both diseases (which may exacerbate KS [57]), ganciclovir for MCD (15), and cidofovir plus alpha interferon for PEL.

Evaluation, Interpretation, and Reporting of Results

We anticipate an expanded role for clinical microbiology and pathology laboratories in the management of HHV-8-related illness. A major part of the cohort of susceptibles and already affected individuals is currently under clinical scrutiny related to their HIV infection, and our understanding of the utility of various virologic and immunologic markers as prognostic indicators is rapidly expanding. In addition, consideration is being given to whether donors and recipients should be matched with respect to HHV-8 status or whether the HHV-8 status of the transplant recipients should be monitored to allow for heightened surveillance for KS and other HHV-8-associated disease.

KS

While KS has ordinarily been diagnosed on the basis of clinical or histologic presentation, as mentioned above, LANA-1-based IHC is of value in establishing a definitive diagnosis of KS. An area of active research is identification of markers for progression to KS (1, 38, 54). The major risk identified thus far is being dually positive for HHV-8 and HIV. There is movement toward a consensus that sensitive serologic tests are the best tools for identifying HHV-8-infected individuals from single specimens, with subsequent PCR monitoring of blood for the appearance of viral DNA as a harbinger of KS. This has not risen to the level of being a practice guideline.

PEL and MCD

Biopsies, cell smears, or lysates of either fluid or solid specimens from suspected cases of PEL or MCD can be analyzed for the presence of HHV-8 by any of the array of in situ molecular diagnostic tools described above.

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Adenoviruses*

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In 1953, Rowe and colleagues described an agent which they named "adenoid degeneration agent" that induced spontaneous degeneration of cultures prepared from adenoids of children (59a). In 1954, Hilleman and Werner cultivated a similar agent from respiratory samples from military recruits which they called "RI-67" (35a). The two viruses were subsequently shown to be related, and in 1956 the term adenovirus was proposed to acknowledge their initial source. Epidemiological studies soon identified the virus as the major cause of acute respiratory disease (ARD) in military recruits and of many other clinical entities. In the mid-1960s, adenoviruses were found to cause tumors in rodents, prompting studies that revealed fundamental processes of molecular biology but failed to link the virus convincingly to human cancer. Currently adenoviruses are again under scrutiny as vectors for gene therapy and as pathogens in immunocompromised patients.

TAXONOMY

Adenoviruses are widespread in nature and have been isolated from humans and many animals. They belong to the *Adenoviridae* family, which has been recently subdivided into four genera: *Aviadenovirus, Atadenovirus, Siadenovirus,* and *Mastadenovirus*. The *Mastadenovirus* genus, from the Greek "mastos," meaning breast, infects mammals. Six of the approximately 19 *Mastadenovirus* species (formerly called subgenera) infect humans and are designated A through F, with species B further subdivided into subspecies B1 and B2 (Table 1). Each human adenovirus species contains multiple serotypes, with 51 human serotypes distinguished to date (14).

DESCRIPTION OF THE AGENT

Adenoviruses are medium-sized, nonenveloped icosahedral viruses 70 to 90 nm in diameter (Fig. 1). Each particle contains a single linear, double-stranded DNA molecule of about 36 kb containing approximately 40 genes (13). Major transcription units include early genes E1A, E1B, E2, E3, and E4, genes expressed later in the early phase (IX and IVa2), and late genes (L1 to L5). One or more (depending on the serotype) virus-associated (VA) nontranslated genes are also described.

The protein capsid is formed by 252 capsomeres. The 240 hexons are grouped into 20 triangular faces, with one penton at each of the 12 vertices. Each penton consists of a base and the fiber, a rod-like projection of variable length with a terminal knob that interacts with cellular receptors. The hexon has antigenic sites common to all human adenoviruses. These sites reside within the capsid, so neutralizing (protective) antibodies are not formed. Hexons also contain type-specific sites which induce neutralizing antibodies. Fibers have type-specific and some species-specific antigenic determinants and are responsible for hemagglutination (HA) in vitro.

Adenoviruses interact with cells in at least three different ways. Productive infection occurs mostly in epithelial cells and begins by attachment of the fiber knobs to cell surface molecules such as CAR, the coxsackie-adenovirus receptor, although other molecules are used by some serotypes. Virus particles are then internalized, pentons are removed, and DNA cores move to the nucleus, where viral DNA replication ensues. Structural proteins synthesized in the cytoplasm then move to the nucleus and form capsids which encase the DNA and aggregate into crystalline arrays (Fig. 2A). From 10,000 to over a million viral particles are produced per cell, only 1 to 5% of which are infectious. Latent or persistent infections occur primarily in the lymphoid mucosal tissue at sites such as the tonsils and adenoids, where they are maintained by unknown mechanisms and infrequently produce virus (25). Transformation occurs primarily in rodent cells infected with species A serotypes, which integrate into the host genome and induce cellular replication without producing infectious virus.

EPIDEMIOLOGY AND TRANSMISSION

Adenovirus infections are common and ubiquitous. From 1 to 5% of all respiratory infections and 5 to 15% of acute diarrheal infections are due to adenoviruses. Children are especially susceptible. The prevalence of adenovirus respiratory infections in children ranges from 2 to 14% and may be higher during outbreaks (23, 74). Many (30 to 70%) respiratory illnesses in unvaccinated new military recruits are adenovirus associated as well. The incidence of all infections is higher in crowded closed settings such as day care centers, boarding schools, geriatric facilities, military training camps, and hospitals. Intrafamilial infections are common.

* This chapter contains information presented in chapter 91 by Paul D. Swenson, Göran Wadell, Annika Allard, and John C. Hierholzer in the eighth edition of this Manual.

TABLE 1 Properties of the 51 human adenovirus serotypes by species characteristics[a]

Species	Serotype(s)	Oncogenic potential	% G+C	HA		Fiber length (nm)
				Rhesus	Rat	
A	12, 18, 31	High	47–49	–	±	28–31
B1	3, 7, 16, 21, 50	Weak	50–52	+	–	9–11
B2	11, 14, 34, 35	Weak	50–52	+	–	9–11
C	1, 2, 5, 6	None	57–59	–	±	23–31
D	8–10, 13, 15, 17, 19, 20, 22–30, 32, 33, 36–39, 42–49, 51	None	57–60	±	+	12–13
E	4	None	58	–	±	17
F	40, 41	None	52	–	±	~29

[a] Modified from reference 35 with permission of the publisher.

Adenovirus infections are frequent in immunocompromised patients (34). Before highly active antiretroviral therapy (HAART) was introduced, adenoviruses were isolated from 7 to 29% of gastrointestinal and urinary tract specimens from human immunodeficiency virus (HIV)/AIDS patients. Even higher frequencies were observed using molecular methods (16). Most isolates were species D serotypes rarely detected in other situations (14). These viruses probably arose from reactivation of endogenous virus at mucosal sites in the gut, concurrent infection with other serotypes, and recombination, resulting in new serotypes. HAART has dramatically reduced the incidence of all infections, including adenovirus, in these

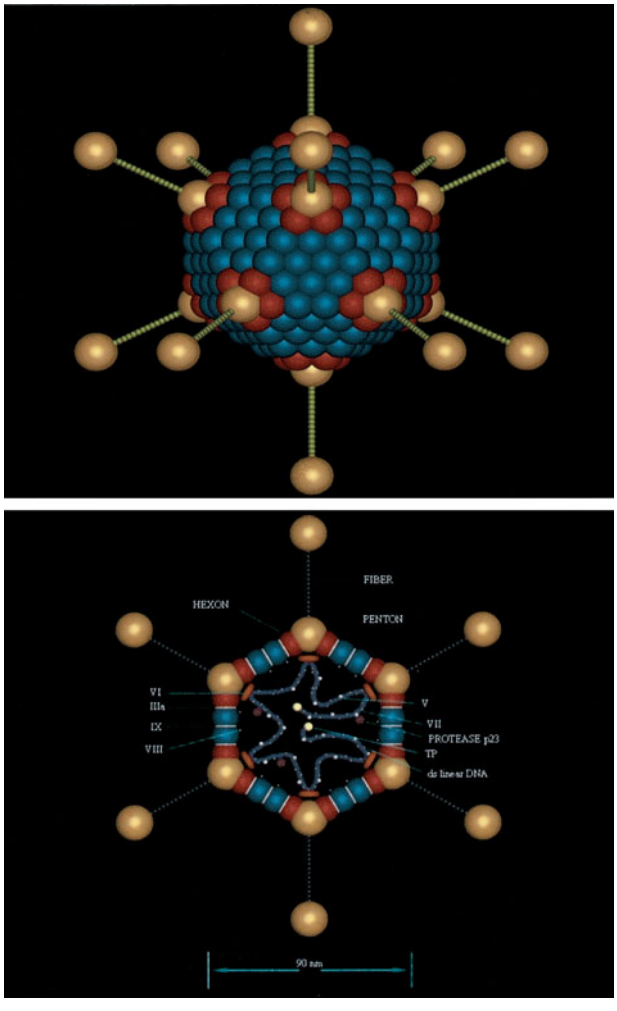


FIGURE 1 Model of adenovirus particle. (Top) Exterior; (bottom) interior.

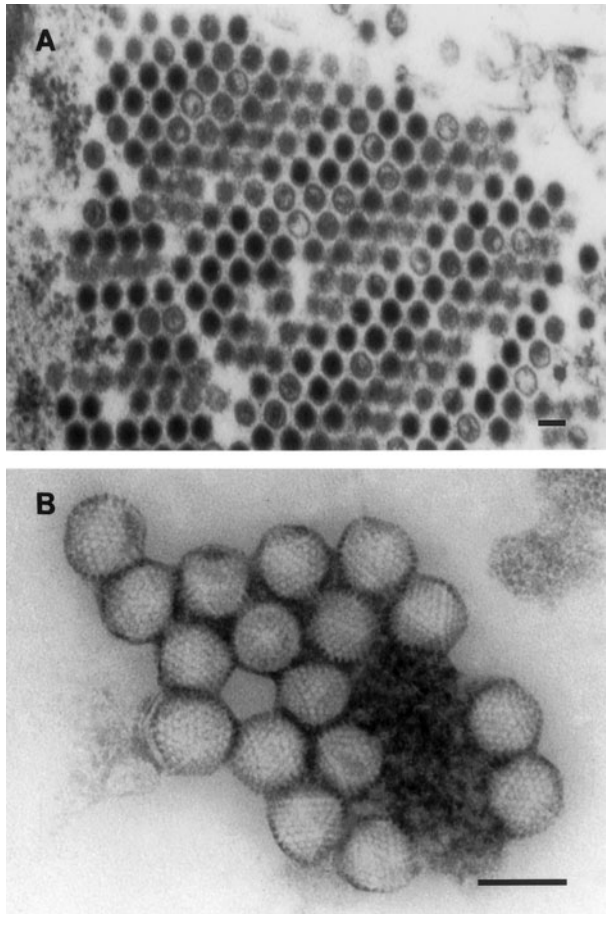


FIGURE 2 Ultrastructure of adenovirus particles. (A) Transmission EM of hepatocyte nucleus containing complete (dark) and empty (clear) adenovirus particles; (B) direct EM of a cluster of adenovirus particles in stool from a child with diarrhea. Bar = 100 nm.

patients. Adenovirus infections are, however, increasingly reported for solid-organ transplant and bone marrow transplant (BMT) recipients. The incidence is highest in pediatric BMT patients, with 5 to 47% of individuals affected depending on age and conditioning regimen. Most infections are probably due to reactivation of latent or persistent virus from mucosal sites. Viruses from species A, B, and C, the species most likely to establish latent or persistent infection, are commonly recovered.

Most adenovirus infections occur in the first few years of life. By age 10, a majority of individuals have been infected with one or more serotypes (23). About half of all infections are asymptomatic. There is no obvious difference in susceptibility by gender or ethnic group. Infections occur worldwide, with some differences in serotypes in other parts of the world. Disease patterns vary by serotype and type of host (Table 2). Respiratory infections are usually caused by species B, C, and E, with serotypes 1, 2, 5, and 6 causing endemic infections and serotypes 4, 7, 14, and 21 causing small epidemics, mostly in winter to early summer. Epidemics of ARD in military recruits typically occur in the winter and spring and are associated with serotypes 4 and 7. Large epidemics of keratoconjunctivitis are due mostly to serotypes 8, 9, and 37. Smaller outbreaks of serotypes 3, 4, and 7 occur in the summertime associated with contaminated swimming pool water. Adenovirus-associated gastroenteritis is endemic and is caused primarily by serotypes 40 and 41, also known as the "enteric adenoviruses." Sporadic and endemic infections occur year-round.

Transmission is primarily by the respiratory or fecal-oral routes. Airborne transmission occurs by small-droplet and, to a lesser extent, large-droplet aerosols. Fecal-oral spread probably accounts for most infections. Virus is also spread by contaminated fomites, fingers, ophthalmic solutions, and airborne particulates. The mode of transmission among new military recruits is thought to differ from that among civilians and similarly congregated college students (18). Spread is also facilitated by resistance of the virus to gastric secretions, bile, and pancreatic proteases, permitting passage of virus through the stomach and replication in the intestine. Prolonged virus shedding from various body sites aids transmission as well. Adenoviruses can be isolated from the throat and respiratory secretions of adults with upper respiratory tract infections for up to a week and from the eye for several weeks after recovery. Children shed nonenteric adenoviruses, especially the lower-numbered serotypes, for 3 to 6 weeks in the throat or stool following respiratory tract or generalized illness. Virus can be isolated intermittently from stools of children for 18 months or longer following an adenovirus infection, but this is uncommon (9). In contrast, enteric adenoviruses are rarely recovered more than a few weeks after recovery from gastroenteritis. No well-designed studies have examined shedding of adenoviruses in stools of immunocompetent adults. Immunocompromised patients shed virus longer than immunocompetent individuals.

TABLE 2 Adenovirus diseases, associated serotypes, hosts, and suitable specimens[a]

Disease	Associated serotype(s)		Frequent hosts	Specimens
	Frequent	Infrequent		
URI[b]	1–3, 5, 7	4, 6, 11, 14, 15, 18, 21, 29, 31	Infants, children	NP[c] aspirate or swab, throat swab
LRI[d]	3, 4, 7, 21	1, 2, 5, 7, 8, 11, 35	Infants, children, IP[e]	NP aspirate or swab, BAL sample, lung tissue
Pertussis syndrome	5	1–3, 12, 19	Children	Throat swab
ARD	4, 7	2, 3, 5, 8, 11, 14, 21, 35	Military recruits	Throat swab, BAL sample, lung tissue, NP aspirate or swab
Acute conjunctivitis	1–4, 7	6, 9–11, 15–17, 19, 20, 22, 37	Children	Conjunctival swab or scraping
Acute hemorrhagic conjunctivitis	11	2–8, 14, 15, 19, 37	Children	Conjunctival swab or scraping
Pharyngoconjunctival fever	3, 4, 7	1, 2, 5, 6, 8, 11–17, 19–21, 29, 37	Children	NP aspirate or swab, throat swab, conjunctival swab or scraping
Epidemic keratoconjunctivitis	8, 9, 37	2–5, 7, 10, 11, 13–17, 19, 21, 23, 29	Individuals of any age	Conjunctival swab or scraping
Gastroenteritis	40, 41	1–3, 5, 7, 12–18, 21, 25, 26, 29, 31	Children	Stool
HC	11	7, 21, 34, 35	Children, IP	Urine
Hepatitis	1–3, 5, 7	4, 31	Infants, children, IP	Liver tissue, blood
Myocarditis	7, 21		Children	Heart tissue, blood
Meningoencephalitis	7	1–3, 5, 6, 11, 12, 32	Children, IP	Brain tissue, CSF
Venereal disease	2, 37	1, 5, 7, 11, 18, 19, 31	Teens, adults	Lesion swab
Disseminated disease	1, 2, 5, 11, 34, 35	3, 6, 7, 14, 21, 29–31, 37–39, 43, 45	IP, newborns	Blood, BAL sample, urine, involved tissue

[a] Modified from reference 35 with permission of the publisher.
[b] URI, upper respiratory illness.
[c] NP, nasopharyngeal.
[d] LRI, lower respiratory illness.
[e] IP, immunocompromised persons.

CLINICAL SIGNIFICANCE

The spectrum of adenovirus-associated disease is broad due to the many serotypes and their distinct tissue tropisms, although there is some overlap (Table 2). Most infections affect the respiratory tract, eyes, and gastrointestinal tract, with lesser involvement of the urinary tract, heart, central nervous system, liver, pancreas, and genital tract. Disseminated disease is also reported.

Most respiratory tract infections occur early in life and are self-limited and mild. The incubation period is 2 to 14 days. Usual signs and symptoms are fever, nasal congestion, coryza, pharyngitis, cervical adenopathy, and cough, with or without otitis media. An exudative tonsillitis clinically indistinguishable from infection with group A streptococcus is described. Lower respiratory infections such as bronchitis, bronchiolitis, croup, and pneumonia can be severe and sometimes fatal, particularly in children under 2 years of age. Long-term sequelae can include bronchiectasis, bronchiolitis obliterans, and unilateral hyperlucent lung. Higher mortality rates are associated with some genotypes and when extrapulmonary manifestations occur. Adenoviruses have been associated with a pertussis-like syndrome, although the virus can also be isolated from *Bordetella pertussis*-positive patients as well.

Although adenovirus-associated ARD is uncommon in civilian adults, frequent outbreaks in young military recruits were noted beginning in 1953. Symptoms included febrile cold-like illness, pharyngitis with tonsillitis, bronchitis, and pneumonia. Hospitalization rates were as high as 50% in some outbreaks, and some fatalities occurred. Live adenovirus vaccines directed against serotypes 4 and 7, the most common serotypes involved, were introduced in 1980 and significantly reduced morbidity and mortality. In 1996, vaccine production was discontinued and the problem recurred. Although current outbreaks are not as severe as in the prevaccine era, ARD is still a significant problem among U.S military recruits. Reintroduction of the vaccine to the military is planned. Interestingly, adenovirus type 4 is uncommonly associated with respiratory disease in civilians. No vaccine is available for nonmilitary populations.

Ocular adenovirus infections are common. The most frequent manifestation is acute follicular conjunctivitis, which is usually superficial and resolves completely in a few weeks. Pharyngoconjunctival fever is a follicular conjunctivitis accompanied by upper respiratory tract symptoms, fever, lymphadenopathy, pharyngitis, and malaise. Epidemic keratoconjunctivitis is a more serious infection that starts with conjunctivitis but progresses to painful edema of the eyelids, sometimes followed by corneal erosions and infiltrates. Symptoms may resolve in 2 weeks, although reduced vision, photophobia, and foreign-body sensation may persist for months to years. Epidemics of acute hemorrhagic conjunctivitis similar to those caused by enterovirus are also described.

Gastroenteritis due to enteric serotypes 40 and 41 has an incubation period of 3 to 10 days. Diarrhea is usually watery and nonbloody, lacks fecal leukocytosis, and lasts for a mean of 10 days. Mild fever, vomiting, and abdominal pain may occur. Respiratory symptoms are noted in some studies. Immunocompetent children recover uneventfully, although infants with ileostomies or colostomies can have prolonged symptoms. Rare fatalities occur in immunocompromised patients. Gastrointestinal syndromes associated with nonenteric adenoviruses include intussusception, acute mesenteric lymphadenitis, and appendicitis.

Clinical manifestations in immunocompromised patients depend on the underlying disease or affected organ system, patient age, and virus serotype (34). Mortality rates of 12 to 70% are reported for pediatric solid-organ transplant or BMT recipients (11). Pneumonia, hepatitis, colitis, pancreatitis, and disseminated disease are observed. Hemorrhagic cystitis (HC) can be especially severe and may signal the start of dissemination (20). In liver transplant recipients, adenovirus typically causes hepatitis with mortality rates as high as 53%. In renal transplant recipients, the predominant symptom is HC, with a 17% fatality rate. In the post-HAART era, clinical manifestations in HIV/AIDS patients are uncommon until immune system deterioration occurs. Even terminally, the clinical significance of adenovirus detection in HIV/AIDS patients can be unclear due to infection with other opportunistic pathogens.

Less common clinical manifestations include severe and frequently fatal neonatal disease, meningoencephalitis and encephalitis, acute myocarditis in otherwise healthy individuals or associated with cardiac transplant rejection, and venereal diseases including genital lesions and urethritis (2, 62, 66, 71). The role of adenoviruses in fetal demise remains unclear (7). Adenovirus type 36 can cause obesity in animals, but the relevance of this finding for humans is uncertain.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Adenoviruses are readily detected from affected sites early in the course of illness. Suitable specimens depend on the patient's symptoms (Table 2). Cell-rich specimens such as nasopharyngeal aspirates or stool are usually preferred over swabs. When tissue is unavailable and deep-seated infection is suspected, specimens from multiple sites (e.g., respiratory, stool, and urine) or blood can be helpful (11, 61). To detect seroconversion, paired serum specimens should be collected, the first one during the acute illness and the second one 2 to 4 weeks later.

Recovery is optimal if specimens are collected as soon as possible after the onset of symptoms and kept cold (2 to 8°C) during transport. Infectivity is well stabilized by most commercial viral transport media (VTM), although studies comparing traditional and newer formulations are lacking. Some VTM, like Microtest M4 (Remel, Lenexa, Kans.), are compatible with molecular and antigen assays as well as culture. This medium also preserves infectivity of some serotypes for 5 days at ambient temperature (59). Specimen processing should occur within 2 to 3 days of collection whenever possible. Plasma or serum should be separated within a few hours after collection to avoid hemolysis and white blood cell degeneration. Specimens, virus isolates, or DNA extracts can be frozen at −70°C indefinitely with minimal loss of activity. Long-term storage in self-defrosting freezers and repeated freeze-thaw cycles will ultimately reduce infectivity and degrade virus DNA.

Adenoviruses are highly resistant to inactivation. Most serotypes are stable at 36°C for a week, at room temperature for several weeks, and at 4°C for several months. Infectivity is retained for several weeks on paper or in saline and for over a month on nonporous surfaces. Strict adherence to conventional safety practices such as use of personal protective equipment and biological safety cabinets, disinfection of work surfaces, and hand washing will minimize laboratory infections. Avoid hand-to-eye or hand-to-mouth contact due to the affinity of adenoviruses for mucosal and ocular tissue. Treatments that destroy infectivity include 1 min of a 1:5 dilution of household bleach, heating to 56°C for 30 min or 60°C for 2 min, and autoclaving. Serotype 4 is especially heat

resistant. Alcohol-based hand gels destroy infectivity, although up to 2 min of contact can be required. Variable inactivation occurs with iodine, UV light, and formaldehyde. Viral DNA can be detected long after infectivity is destroyed.

DIRECT DETECTION

Microscopy

Adenovirus-infected cells can be visualized by light microscopy as "smudge cells" in histologically stained tissues, fluid sediments, or cultures. These late-stage infected cells contain solitary, central, basophilic nuclear inclusions composed of viral particles. Other forms of inclusion are described but are less frequently observed. The inclusions of cytomegalovirus, herpes simplex virus, or polyomavirus can resemble adenoviral inclusions, so further evaluation is recommended to avoid misdiagnosis (41). In situ hybridization, immunohistochemistry, or PCR can identify adenovirus in fixed tissue.

The characteristic morphology of adenovirus particles permits detection by electron microscopy (EM) without the need for further identification. In typical transmission EM, fixed tissue fragments are thin sectioned, negatively stained, and then examined for crystalline arrays of virus particles usually found in the nucleus (Fig. 2A). In direct EM, fluid clarified by low-speed centrifugation is similarly stained and examined for clusters of particles (Fig. 2B). Sensitivity is improved by ultracentrifugation of virus onto EM grids (Airfuge; Beckman Coulter Inc., Fullerton, Calif.), by prior aggregation of virus with antihexon antibodies, or by other concentration techniques (30). Direct EM was historically used to detect adenoviruses in urine and respiratory secretions, but other methods are more sensitive and practical. Direct EM is still used at some centers to detect viruses in stool, including the enteric adenoviruses.

Antigen Detection

Antigen detection is widely used for diagnosis of adenoviral respiratory and gastrointestinal tract infections because it is rapid and reasonably sensitive. Most current assays identify conserved regions of the hexon protein and use monoclonal antibodies (MAbs). Immunofluorescence (IF) and enzyme immunoassay (EIA) are common approaches. Many products for antigen detection are commercially available (Table 3).

The major application of IF is detection of virus in respiratory tract specimens. Washes, aspirates, or swabs containing exfoliated ciliated columnar epithelial cells from the posterior nasopharynx should be collected. Touch preparations of fresh tissue or frozen or trypsin-treated, formalin-fixed tissue sections can also be used. Conjunctival or throat swabs should be evaluated by more sensitive techniques such as culture or PCR. A typical assay is performed by depositing spots of washed cells onto a glass slide, followed by acetone fixation, staining with fluorochrome-labeled MAbs, and examination under a fluorescence microscope. A satisfactory specimen should contain more than 50 virus-susceptible cells. Indirect IF can be used, but newer direct stains provide excellent and rapid results (40). Positive cells should display

TABLE 3 Representative commercial products for detection of adenovirus infection

Analyte	Method(s)	U.S. suppliers—products
Antigen	IF, immunohistochemistry	Argene—Anti-Adenovirus & Influenza, Parainfluenza;[a] Anti-Adenovirus,[a] Anti-Adenovirus 8[b]
		Diagnostic Hybrids—D3 DFA Respiratory Virus Screening & Identification Kit[a]
		Chemicon International—Light Diagnostics Respiratory Viral Screen & Identification DFA Kit,[a] Adenovirus Direct DFA,[a] Anti-Adenovirus 1, 1A, 2, 5, 6[b]
		Diagnostic Products Corp.— PathoDx Respiratory Virus Panel[a]
		Trinity Biotech—Anti-Adenovirus Antibody,[a] Respiratory Virus Kit[a]
	EIA	Meridian Bioscience—Premier Adenoclone,[a] Premier Adenoclone Type 40/41[a]
		Microgen Bioproducts— Adenoscreen EIA[a]
	IC	SA Scientific—Sure-Vue Adeno Test[a]
	LA	Microgen Bioproducts—Adenoscreen Latex Agglutination Test[a]
		Lifesign—Diarlex Rota-Adeno[a]
	In situ hybridization	Enzo Life Sciences—PathoGene Adenovirus DNA Probe Assay[b]
DNA	PCR	Argene—Adenovirus Consensus Kit[b]
		Chemicon International—Adenovirus OligoDetect[c]
		Prodesse—Adenoplex[b]
Virus	Neutralization	American Type Culture Collection—Anti-Adenovirus Antisera[b]
		Alfa Biotech SPA—Anti-Adenovirus Antisera[a]
Antibody	IF	Bion Enterprises—Adenovirus IFA Substrate Slides[a]
	EIA	Diagnostic Automation—Adenovirus IgG, IgM, IgA EIA[a]
		Scimedix Corp.—Anti-Adenovirus IgG/IgM/IgA EIAs[a]
		Virion Serion—Serion Elisa Classic Adenovirus IgG/IgA EIA[b]
	CF	Alfa Biotech SPA—Adenovirus CF Antigen Reagents[a]
		Virion Serion—Adenovirus CF Reagents[a]

[a] In vitro diagnostic use.
[b] Research use only.
[c] Analyte-specific reagent.

condensed nuclear or granular cytoplasmic staining (Fig. 3). Staining can be performed with an adenovirus-specific MAb or a pool of MAbs for adenovirus, respiratory syncytial virus, influenza A and B viruses, and parainfluenza virus (70). Dual infections can be identified using MAb pools. Pools are also economical if the laboratory sees little adenovirus, but confirmatory stains with the individual components are needed if the pool stain is positive. The sensitivity of adenovirus IF in respiratory specimens is 40 to 60% compared to culture. This is lower than the rate of detection of most other respiratory viruses. Higher values (70 to 75%) can be obtained using cytocentrifugation, which hastens drying and improves readability and specimen adequacy (40). The specificity of IF is very good (>99% in most studies).

The major application of EIA is detection of virus in stool. Genus-specific tests and type-specific assays for serotypes 40 and 41 are commercially available (Table 3). Most EIAs utilize microtiter plates and adenovirus-specific MAbs as capture and detector reagents (33). Color produced by an enzyme-substrate system conjugated to the detector MAb is then read visually or by spectrophotometer. The sensitivity of most enteric adenovirus EIAs compared to 293 cell culture or EM is >90% but can be lower if variants are circulating (63). The specificity is usually >97%. Genus-specific assays are sensitive (>95%) when stool is used. The sensitivity is lower (65 to 75%) with respiratory tract and eye specimens. Urine has the lowest detection rate (5, 26). Occasional false-positive reactions occur.

Other antigen detection tests include immunochromatography (IC), which is convenient when small numbers of specimens are tested. In IC, the specimen migrates laterally along a membrane strip, antigen binds to virus-specific antibodies, and a colored line develops within minutes. The sensitivity of a commercially available adenovirus IC assay with nasopharyngeal specimens tested at the bedside was 84% compared to culture and PCR, and 95% when the same specimens were retested in the laboratory (24). Another antigen detection assay is latex agglutination (LA), which is used primarily for genus-specific detection of adenovirus in stools. LA has a sensitivity similar to that of EIA, although up to 13% of specimens may yield uninterpretable results (28). Infrequently used antigen tests include time-resolved fluoroimmunoassay, radioimmunoassay (RIA), counterimmunoelectrophoresis (CIE), and complement fixation (CF).

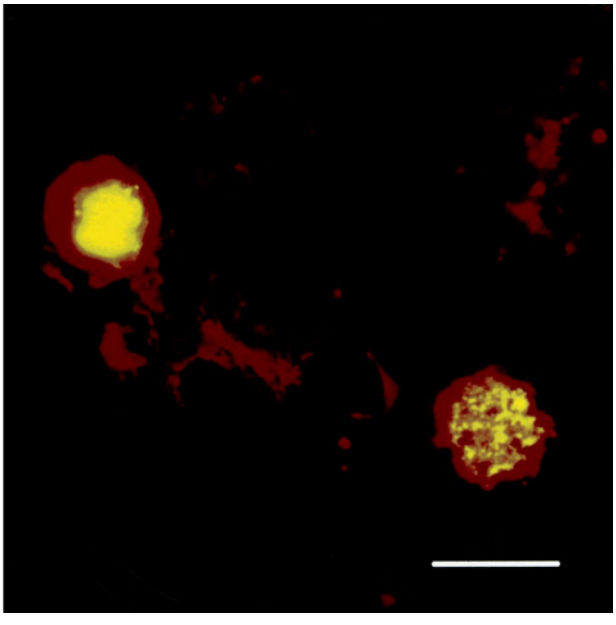


FIGURE 3 Adenovirus antigen demonstrated in two cells of a nasopharyngeal aspirate by direct IF using a rhodamine-conjugated antibody (yellow). Bar = 40 μm.

Nucleic Acid Detection Techniques

Detection of the viral genome is highly sensitive and especially applicable when noninfectious virus is present, when the viral load is too low to be detected by culture, or when results are needed quickly. Adenovirus DNA has been detected in virtually all clinical samples, so specimen selection depends largely on the associated disease (Table 2).

Many techniques have been used to detect adenovirus DNA. A simple early method was visualization of enteric adenovirus DNA as a 36-kb band on a gel after lysis of stools from children with diarrhea (51). Assays using extracted DNA and hybridization to isotope- or enzyme-labeled DNA probes increased the sensitivity, specificity, and applicability to other specimen types. Dot blot hybridization was often used for this purpose (39). In situ hybridization on tissue samples remains useful for diagnosis and studies of pathogenesis (48).

PCR is currently the most accepted detection method, with both conventional and real-time assays used. Sensitivities approach or exceed that of culture. Most assays use degenerate or nondegenerate primers from the hexon gene, the fiber gene, or the VA RNA I and II regions. The hexon gene consists of three different segments: a central variable region with seven hypervariable regions and two highly conserved regions flanking the central one. The conserved regions are most often used in generic PCR (12, 16, 32). The fiber gene contains regions that are conserved within and variable between species. Several PCRs with species-specific fiber gene primers are described (56). Multiplex primers flanking the VA RNA coding regions are also used (73).

Conventional adenovirus PCR typically takes 1 to 2 days, most of which is dedicated to detection. Methods for detection include ethidium bromide-stained gels, solid- or liquid-phase hybridization to labeled probes, time-resolved fluorometry, or hybridization to probes in microtiter plates. Single and multiplex PCRs are described. Multiplex conventional assays can detect adenovirus species A to F individually, together, or as a component of a panel for respiratory viruses (55, 56, 73). Plasma, urine, and stools of immunocompromised patients, ocular specimens from patients with conjunctivitis, throat swabs from ill military recruits and their environment, serum and respiratory tract specimens from healthy children with acute infection, heart biopsy samples, and polluted water have been tested (1, 16–19, 66, 73). Several conventional PCR kits are commercially available (Table 3).

Real-time PCRs provide qualitative and quantitative results within hours with less labor and fewer opportunities for contamination than conventional assays. Several real-time adenovirus PCRs have been developed, initially for BMT patients but recently for other patient populations as well (12, 15, 32, 43, 65). In general, more than one set of primers and several probes are necessary to detect all serotypes. Many specimen types have been assessed, but blood is the most frequently evaluated specimen in most laboratories. Plasma or serum is recommended to avoid variations due to low levels of viral DNA in white blood cells (WBC) of some healthy subjects and fluctuations in WBC counts, especially in BMT patients (12). Nevertheless, whole blood has been used in some studies.

Currently quantitative real-time adenovirus PCR has several limitations. First, universal standards are lacking for assay calibration and interlaboratory comparisons. Purified, titered adenovirus particles and DNA from several serotypes of species C can be purchased (Advanced Biotechnologies Inc., Columbia, Md.) but are not recommended as standards until their exact DNA concentration and stability are independently validated. Second, there is no universally accepted primer-probe combination for uniform detection and quantitation of all serotypes, although recent studies are promising in this regard (12, 15). Variable results among laboratories are therefore expected. A new European adenovirus PCR proficiency survey series available to U.S. subscribers (Quality Control Program for Molecular Diagnostics; European Union Concerted Program for Quality Control, Glasgow, Scotland) may soon provide data about optimum assay design. Until these issues are resolved, viral loads for clinical intervention should be determined locally. Meanwhile, it may be preferable to report viral kinetics (the relative change in adenoviral load over time) rather than absolute viral loads (42).

To verify a new adenovirus PCR, the limit of detection should be defined using several serotypes of each species, at a minimum. Wild isolates and prototypes should be tested to avoid false-negative results or misquantitation due to unanticipated polymorphisms (16). Dilutions of quantitated virus stocks spiked into negative samples can be used to generate standard curves. This approach can also be used to make positive controls. Plasmids containing inserts of the target region are also useful to create standard curves, but these should be added after extraction because loss can occur with some protocols. In general, the serotype best matching the primers and probe is used as the working standard. Analytic specificity should be evaluated by testing microorganisms likely to be present in the specimen types submitted. Clinical specificity should be carefully assessed because low levels of adenovirus DNA can be detected in a small proportion of specimens from healthy controls (22, 25, 32, 43). Published guidelines recommend testing sufficient known positive and negative specimens to have confidence in the test (54). Specimens for this purpose can be verified as adenovirus positive or negative by culture, pathology, careful clinical follow-up of the patient of origin, or a combination of methods. Archived specimens are frequently used for convenience and economy.

ISOLATION PROCEDURES

Isolation produces progeny for further studies and is reasonably sensitive. It therefore remains the "gold standard" but can be slow and plagued by overgrowth with unwanted organisms or cell death due to toxicity. All specimens are suitable for routine culture (Table 2), although enteric adenoviruses are optimally detected by nonculture methods. Blood rarely yields virus except by PCR, probably due to the presence of neutralizing antibody. Isolates can sometimes be obtained by culture of buffy coat or of WBC purified through Ficoll-Hypaque density gradients.

Adenoviruses can be isolated in conventional tube culture or centrifugation culture (CC). Growth is best in human epithelial cell lines. For culture of nonenteric serotypes, the A549 cell line is recommended because it supports adenoviruses and other viruses well (67). Other suitable cell lines are HEp-2, HeLa, and KB. Human diploid fibroblasts are less sensitive. Nonhuman cells such as Vero and primary monkey kidney cells containing simian virus 40 will recover adenovirus, although the yield is low. Recovery is not optimal in simian cells and human fibroblasts, but these cells are in widespread use and frequently detect adenoviruses. Enteric adenoviruses grow best but inconsistently in Graham 293 cells, a continuous line of adenovirus type 5-transformed HEK cells (72). Other serotypes replicate in these cells as well. Limited growth of enteric adenoviruses can occur in A549, HEp-2, KB, and other cell lines (57).

Inoculated tubes should be incubated at 35 to 36°C in stationary racks and read every other working day. Rolling offers little advantage. The speed with which cytopathic effect (CPE) develops depends on the cell type, serotype, and concentration of virus in the specimen. Epithelial cells typically develop adenovirus CPE in 2 to 7 days, although up to 28 days can be required in simian cells, in human fibroblasts, or with slow-growing serotypes. Most diagnostic laboratories retain tubes for 2 weeks. If 28 days is used, cells should be maintained in good condition by scraping them into the culture medium and subpassage onto fresh cells at 10 to 14 days (35). Cells with suspicious CPE that does not progress should be disrupted and reinoculated into sensitive cells such as A549.

Typical CPE usually begins at the edge of the monolayer and then progresses until grape-like aggregates of swollen, refractile cells form (Fig. 4B). Aggregation may not occur with subgroup D viruses. Other forms of CPE, such as web-like flattening with serotypes 3 and 7, are also described (45).

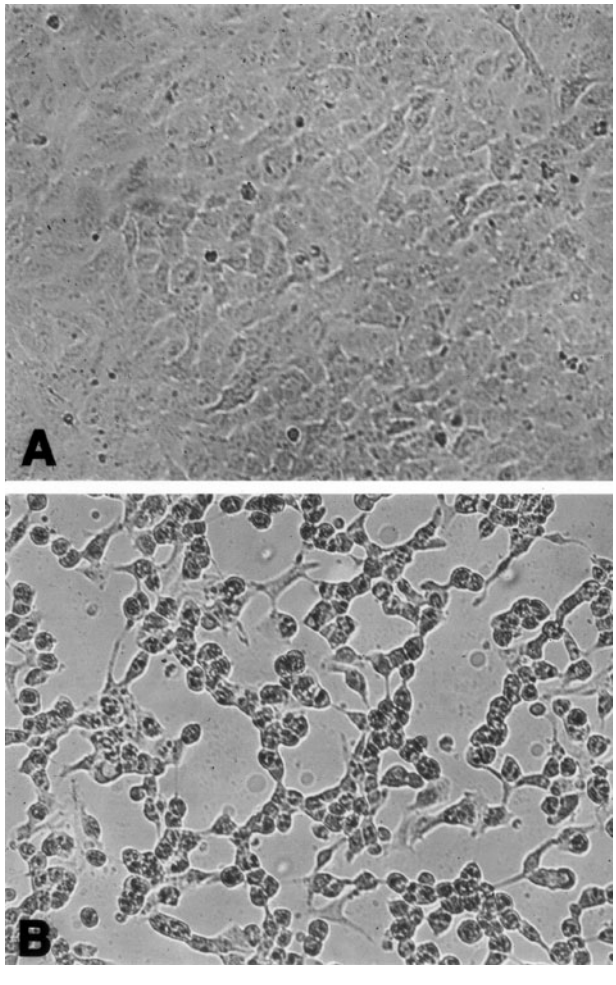


FIGURE 4 Adenovirus CPE in A549 cells. (A) Uninfected cells; (B) advanced adenovirus CPE. Magnification, ×100.

Glycolytic activity may also increase, resulting in excretion of acid and a color change of the medium. As infection progresses, cells may become highly granular and detach completely from the surface. The CPE of adenoviruses can be confused with that of herpes simplex virus or cytomegalovirus. Viable epithelial cells in bronchoalveolar lavage (BAL) specimens or in subpassages can produce colonies of rounded cells on the monolayer that also resemble adenovirus CPE. All presumptive adenovirus isolates should therefore be definitively identified.

For high-titered isolate preparations, infected cells and fluid should be frozen and thawed rapidly several times. Rapid CPE, also known as "lysis from without," can occur within hours after inoculation with concentrated extracts of infected cells. It is due to interaction between adenovirus free penton base and cellular adhesion molecules and can be prevented by further dilution of the inoculum or by using a different preparation.

Adenoviruses can be detected rapidly by CC. In this technique, specimens are centrifuged for 15 to 60 min onto cells growing on coverslips in shell vials or in microtiter plates. One to 5 days later, IF is performed to detect intracellular hexon antigen, typically before significant CPE develops (21). Adenovirus-specific antibody or a MAb pool to simultaneously detect adenoviruses and other respiratory viruses can be used (58). Isolates can be obtained if the culture medium is removed prior to staining. Detection of adenoviruses in CC stained at 2 days is only 50 to 85% compared to tube culture at 14 days. This detection rate is lower than for most other respiratory viruses. For maximal adenovirus recovery, tube culture or CC stained at 5 days is recommended. CC is insensitive for detection of enteric adenoviruses.

A mixture of A549 and mink lung cells has become popular to expand the type of respiratory viruses detected by individual CC. This mixture recovers influenza and parainfluenza viruses well, but adenovirus recovery is more variable. In an initial study, in-house-prepared CC of the mixture identified all adenovirus-containing specimens identified by A549 CC alone after overnight incubation (36). Most laboratories, however, use a commercially prepared mixture of the same cells (R-Mix; Diagnostic Hybrids Inc., Athens, Ohio). We and others find that only about half of all adenovirus-positive specimens identified by CC of A549, HEp-2, or RMK cells are detected by R-Mix CC, even after 2 days of incubation (76).

IDENTIFICATION

All isolates with adenovirus CPE should be definitively identified before a report is generated. For diagnostic work, genus-specific identification is usually sufficient. Most immunologic or molecular methods for direct detection are suitable for this purpose. Identification by IF is used most frequently because it is simple and rapid, and the reagents can be used for direct detection as well. Some smaller labs may prefer IC, but laboratories with larger test volumes may prefer EIA. LA and PCR can be used as well. Other procedures used historically include CIE, RIA, DNA hybridization, and CF. For identification by IF, cultures should be harvested when 25 to 50% of the cells show CPE. Depending on the cell line and assay used, more extensive degeneration may be required to obtain sufficient soluble material for analysis. On occasion CPE-positive variants or new serotypes cannot be identified. In this situation, EM of infected cells, HA, restriction enzyme analysis (REA), or DNA sequencing can aid identification.

TYPING SYSTEMS

Typing is used primarily for epidemiological investigations, for studies of pathogenesis, or to reveal the cause of an unusual or especially severe infection. Serologic or molecular typing can be performed. Serologic typing requires an isolate and detects differences in epitopes on the fiber gene and hexon gene. Molecular typing can be performed on isolates and on the original specimen. Differences in DNA sequences of the fiber gene and hexon gene are typically determined, although nontranscribed regions can be interrogated as well. The two systems differ fundamentally in target, so results do not always concur. Each provides a different view of virus structure.

Serologic typing provisionally determines the species of an isolate by HA (Table 1), or if the isolate is from stool, by a species F (serotype 40 or 41) EIA. Serotyping by HA inhibition (HAI) or serum neutralization (SN) is then performed, using antisera that define each serotype of that species. The HAI assay is easier, but SN is the primary arbiter of serotype. Most SN assays are tedious so modifications are made, such as the use of intersecting pools of antibodies to reduce the number of individual reactions. There are three major SN assays: a conventional 7-day test in human epithelial cells (or 293 cells for stool isolates); a 3-day test in monkey kidney cells, which is the most simple and rapid assay; and a 5-day microneutralization test using Vero cells (35). Interpretation is not always straightforward, as when HAI and SN fail to agree with intermediate species D variants containing the fiber gene of one serotype and the hexon gene of another. Yet serotyping remains the gold standard by which newer systems are evaluated. Other serologic methods include an immunoperoxidase technique, immune adherence HA, and indirect IF with serotype-specific antisera.

Molecular typing is increasingly performed because it is rapid, utilizes molecular equipment and expertise now in many laboratories, and does not require expensive and difficult-to-obtain antisera. The REA method was initially used. It can be applied to purified virus, virus-infected cells, or stools from children with diarrhea (3, 10). The starting material is first lysed and then digested with restriction endonucleases (e.g., SmaI), and then serotype- and/or genotype-specific band patterns are visualized after agarose gel electrophoresis. Unfortunately strains that defined the initial REA patterns are no longer circulating, so patterns of currently obtained adenoviruses may be uninterpretable. Coinfections can also cause ambiguous results. REA is still useful for presumptive identification of new serotypes, for identification of genotypes (e.g., 7h or 7d2) associated with severe disease, or to confirm results obtained by other means (14, 29). Other approaches include detection of single-stranded confirmation polymorphisms and heteroduplex mobility analysis (68).

Typing by PCR is now preferred by many laboratories. This method begins by amplification of extracted DNA from an isolate or purified virus, using a generic or multiplex species-specific PCR and hexon gene or fiber gene primers. The serotype is then indicated by measurement of product length, sizes or DNA sequence of fragments generated after cleavage with restriction enzymes, or DNA sequence. Early PCR-based methods required high concentrations of starting material. Newer methods can be applied directly to clinical specimens, although nested PCR may be required to obtain sufficient product for analysis (4). Some schemes detect only limited number of serotypes, whereas others are more comprehensive and can demonstrate coinfections (49, 60). Direct comparison among these methods is lacking.

SEROLOGIC TESTS

Most primary infections are accompanied by a fourfold or greater rise in adenovirus immunoglobulin G (IgG) and a less predictable IgA response. Virus-specific IgM appears in only 20 to 50% of cases (37, 50). The IgG response in older children and adults typically begins 7 to 10 days after infection, but it can be delayed for months in children and may never appear in immunocompromised individuals or in superficial infections. An IgM response can occur when virus reactivates. Both genus- and serotype-specific antibodies appear. Children often have a single serotype-specific response because their exposure to multiple serotypes is limited. Up to 25% of adults, however, raise heterologous antibodies, often to multiple serotypes within the same species as the infecting serotype. Neutralizing antibodies usually persist a decade or more in relatively undiminished titer, probably maintained by periodic reinfection, reactivation, and heterotypic anamnestic antibody responses. Complement-fixing antibodies typically disappear about a year after infection.

Serodiagnosis requires detection of an IgM response or documentation of at least a fourfold rise in IgG between acute- and convalescent-phase specimens. Acute- and convalescent-phase sera should be tested simultaneously for greatest accuracy. The choice of assay is based on its use. For clinical work, a generic test like CF or EIA for IgG or IgM is usually selected. Some reference laboratories offer these assays, and kits are commercially available as well (Table 3). Both CF and EIA use hexon antigen-rich preparations and detect genus-specific antibody responses. Historically CF was the most widely used generic test. It is well standardized, reagents can be readily acquired or made, and it is inexpensive, especially if antibodies to other pathogens are simultaneously assessed. A major drawback of CF is sensitivity. Fewer than half of all infections in children and immunized adults and 50 to 70% of infections overall are detected by CF. Sensitivity also varies with the infecting virus and serotype used as the antigen. EIA is therefore preferred for greater sensitivity and to detect different immunoglobulin classes (37).

Serotype-specific antibody tests are infrequently used for diagnostic work. They are valuable, however, to pinpoint serotypes responsible for a cluster of infections when viral isolates are unavailable, for diagnosis of illnesses typically associated with a limited number of serotypes, or for studies of the immune response. These tests are also more sensitive than CF and so can be used to assess otherwise-undetectable antibody rises in children or immunized military recruits. The assays used most frequently are HAI and SN. The HAI test is as highly standardized as the CF test and can be applied to other viruses as well. The SN assay remains the standard, with the 3-day test in monkey kidney cells or microneutralization preferred (35). Other serologic tests include indirect IF, CIE, LA, and RIA. Newer assays utilize genetically modified cells and fluorescent reporter molecules as surrogates for SN activity and may soon replace SN in serum surveys (69).

Overall, the insensitivity of the adenoviral IgM response and the need to demonstrate IgG seroconversion greatly limit the usefulness of serology for diagnostic purposes in individual patients. Interpretation is also problematic for immunocompromised individuals, in persistent infection, for adults due to heterotypic responses, and for children whose delayed antibody rises may coincide with other clinical problems. Despite its availability, serology should therefore be reserved for epidemiological investigations or to confirm associations between virus detection and unusual clinical outcomes.

ANTIMICROBIAL SUSCEPTIBILITIES

A variety of antivirals, including ganciclovir, vidarabine, ribavirin, and cidofovir, have been explored for treatment of adenovirus disease, especially in the immunocompromised host (46). Most reports are anecdotal or small scale and have yielded mixed results. There are still no prospective, randomized controlled trials of these drugs, nor is there a proven effective therapy. Most recent experience is with ribavirin and cidofovir.

Ribavirin is a broad-spectrum antiviral agent related to guanosine, with in vitro activity against RNA and DNA viruses, including adenovirus. It is well tolerated in the intravenous form, with reversible mild anemia the most common adverse effect. Efficacy appears best in treatment of adenovirus-associated HC, probably due to the high drug concentrations achievable in urine. Successful treatment of pneumonia, hepatitis, and enteritis in transplant recipients and severe disease in an immunocompetent neonate are described (46). Many failures and fatalities are also reported, even when the drug is used with intravenous gamma globulin. A factor perhaps contributing to this variability is an intrinsic antiviral resistance, since only species C serotypes are susceptible to ribavirin in vitro (50% inhibitory concentration, 48 to 108 μM) (52). If this limitation also occurs in vivo, the usefulness of ribavirin may be limited to treatment of a subset of adenovirus infections.

Cidofovir is an acyclic nucleoside phosphonate analogue used as a broad-spectrum antiviral agent. All adenovirus serotypes are susceptible in vitro in a narrow dose range (50% inhibitory concentration, 17 to 81 μM). Resistance can develop with serial passage (27, 52). Despite its significant side effects (nephrotoxicity, myelosuppression, and uveitis), cidofovir use has increased, especially for immunocompromised patients. Improvements were noted in 31 of 45 (69%) BMT patients with various clinical presentations and 10 of 14 (71%) BMT patients with HC (47, 53). Some investigators have suggested that surveillance of high-risk BMT patients by monitoring viral load kinetics and early preemptive treatment with cidofovir will be more effective than treatment after symptoms develop. An early trial of this approach was associated with good outcomes in five of eight immunocompromised patients, and plasma viral load predicted the clinical response (42). Some episodes of viremia, however, can spontaneously resolve without treatment, so other early indicators of clinical response may be needed (75).

Other potential treatments include reduction of immunosuppression or immunotherapy using donor lymphocyte infusion (DLI). Both approaches, with or without concomitantly administered antivirals, have improved the outcomes for BMT recipients with severe disease (8, 38). To avoid inducing graft-versus-host disease with DLI, virus-specific cytotoxic lymphocytes (CTL) have been produced in vitro. Most CTL clones have broad cross-reactivity, so serotyping of virus in individual cases may not be required (31). These strategies remain promising.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Clinical diagnosis of adenovirus infection is rarely accurate, so laboratory evaluation is usually required. Selection of tests should be guided by the patient's symptoms and immune status, the interval between disease onset and specimen collection, and laboratory expertise. Most infections can be detected within hours or several days of sampling by direct antigen tests, nucleic acid assays, or CC. Measurement of an

IgM response is infrequently productive, and tube cultures and IgG serology rarely provide results in time for clinical decision-making (64).

Interpretation of positive results is more challenging than test selection. Asymptomatic, persistent, or recurrent infections can occur, so detection of virus or a serologic response can be a coincidental finding. Accurate interpretation must take into account patient and specimen factors, the test method, and, in some circumstances, the viral serotype and quantity detected. This requires close communication between the laboratory and the clinician. In general, detection of adenovirus from an involved organ or large quantities of virus from a related specimen in a patient with an illness epidemiologically associated with adenovirus is valid evidence that the virus is the actual cause of the disease.

Respiratory tract infections due to adenovirus are often diagnosed by IF, which is typically more sensitive in children because they shed virus longer and in greater quantity than adults. The clinical and financial benefits of providing rapid IF results have been well demonstrated (6). Negative results do not rule out infection, so culture of the following IF-negative specimens is recommended: samples from patients hospitalized for lengthy periods (e.g., patients in intensive care), lower respiratory tract specimens such as BAL samples or lung tissue, samples from high-risk patients (e.g., neonates and transplant patients), and specimens from patients with especially severe or unusual presentations. A promising alternative to IF is PCR, but interpretation of positive PCR results in respiratory tract specimens is still uncertain (43, 65). Detection of viral DNA in blood by PCR, however, may become a helpful adjunct to diagnose severe respiratory tract disease in immunocompetent children (1, 65).

Adenoviral gastroenteritis in immunocompetent children is typically diagnosed by serotype 40- or 41-specific EIA of stool. Interpretation is usually straightforward because these infections are usually mild and self-limited, with virus infrequently shed after the symptomatic period. In contrast, nonenteric adenoviruses can be detected in stools of asymptomatic healthy or immunocompromised patients for months to years, with severe or fatal gastrointestinal disease resulting in the latter group. Detection of nonenteric adenoviruses in stool is readily performed by generic EIA, generic PCR, or culture. The major problem is interpretation of positive results, particularly for transplant patients, who can have concomitant graft-versus-host disease and adenovirus infection.

Recovery of adenoviruses from other sites can be technically challenging but somewhat easier to interpret because asymptomatic shedding is rare. Ocular adenovirus infections can be detected by EIA, but culture, PCR, and even serology are sometimes needed to make the diagnosis. PCR of urine is more sensitive than culture for diagnosis of adenovirus-associated HC (16). Adenovirus encephalitis and meningitis occur infrequently, so studies comparing traditional to newer methods of detection are lacking, but PCR of cerebrospinal fluid (CSF) is likely to be more sensitive than viral culture.

Diagnosis of adenovirus disease in immunocompromised patients is especially problematic due to their many copathogens, high rates of asymptomatic virus shedding, and protean manifestations of disease. Solid-organ transplant and BMT recipients are at highest risk of severe disease and are the usual focus of testing. Evaluation can include surveillance of throat, stool, or urine specimens for culture or PCR. Involvement is typically classified as infection, probable disease, or definite disease depending on the number of virus-positive sites and symptoms (11). A rapid and noninvasive alternative to culture is detection of adenovirus viremia by PCR of serum or plasma from high-risk BMT or solid-organ transplant patients. A reproducibly high positive result obtained by this method can now be recommended as an indicator of severe adenovirus disease in these patients, although caution is warranted because some viremic episodes resolve spontaneously (17, 32, 44, 61, 75). Other applications of PCR remain investigational. These include use of PCR for preemptive treatment of adenovirus disease, to monitor treatment, and for diagnosis of disease in immunocompetent patients. Studies are in progress to standardize PCR protocols and to further evaluate these potentially useful applications. Results are eagerly awaited.

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Human Papillomaviruses*

BRUCE K. PATTERSON

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TAXONOMY AND DESCRIPTION OF THE AGENT

Papillomaviruses are ubiquitous viruses that infect many animal species including humans. Over 118 papillomavirus types have been identified in a classification that is continuously adding new types. The papillomaviruses are now considered to be in a distinct taxonomic family, *Papillomaviridae,* and unrelated to the polyomaviruses (16). The taxonomic criteria for types, subtypes, and variants are based on L1 capsid gene sequence differences. A sequence difference of 10% constitutes a type, a difference of 2 to 10% constitutes a subtype, and a difference of >2% constitutes a variant (16). These double-stranded, supercoiled, circular DNA viruses have an icosahedral capsid consisting of 72 capsomeres ranging from 45 to 55 nm in diameter. Human papillomavirus (HPV) contains a double-stranded circular DNA genome that encodes eight open reading frames, which are transcribed as polycistronic messages (21). The gene products of HPV include the early (E) and the late (L) genes. There are eight early genes designated E1 to E8 and two late genes designated L1 and L2. E1 and E2 are responsible for DNA replication (55), while E2 has also been demonstrated to exert repressor activity on early viral promoters ultimately responsible for the expression of the E6 and E7 oncogenes (17, 51, 79). E4 has been implicated in the alteration of the cytoskeleton, and E5 encodes a membrane protein with weak transforming activity (12, 45, 50). The amino-terminal 21 amino acids of E8E2C represent a novel, transferable DNA replication repressor domain that controls the viral copy number during persistent infection (88). Little is known about the gene function of E3, although some groups are performing high-risk genotyping out of this region. L1 and L2 encode viral capsid proteins. In addition, the L1 gene contains the sequence heterogeneity used to type HPV. L1 is the major gene detected by molecular diagnostic assays, as is discussed below.

EPIDEMIOLOGY AND TRANSMISSION

HPV-related cervical cancer constitutes about 12% of cancers worldwide. Epidemiological studies over the past 30 years have led many to the conclusion that persistent infection with oncogenic types of HPV followed by HPV DNA integration into the cellular genome is a required precursor in the pathway leading to cervical neoplasia (7, 8, 9, 14, 15, 34, 35, 80, 81, 86). A worldwide multicenter epidemiologic study compared the presence and type of HPV in 1,918 cervical cancer patients and 1,928 matched control women without cancer (61). The prevalence of HPV in the cancer group was 90.7%, and the prevalence of HPV in the control group was 13.4%. Detection and typing of HPV were performed for all women, and the HPV types were categorized as high, intermediate (probable high risk), and low risk (Table 1). Cofactors previously thought to contribute to cervical cancer such as smoking, contraceptive use, and age of first menstrual cycle were not found to contribute to cervical cancer in this multicenter trial. Genetic factors, such as have been inferred by studies with twins, have yet to be identified (49).

Transmission of HPV from males to females occurs via virus shedding in the male followed by the infection of cervical epithelial basal cells by way of microabrasions. HPV is unique in its necessity to infect cells that are capable of proliferation. The HPV life cycle depends on the maturation of epithelial basal cells (21). Following infection of the basal epithelial layer, HPV establishes a nuclear episome with approximately 50 copies per cell. HPV episomes replicate with cellular DNA replication so that during cell division infected daughter cells migrate from the basal layer to the suprabasal layer, where the cells differentiate. After reaching the suprabasal layer, HPV-infected cells enter into S phase and produce hundreds to thousands of copies of HPV DNA per cell (40). Following viral DNA synthesis, viral proteins including capsid protein are produced and complete viral particles are released into the female genital tract as epithelium is shed. A schematic of lateral expansion and vertical differentiation of epithelial cells is shown in Fig. 1. HPV DNA replication and particle release accompany vertical differentiation.

CLINICAL SIGNIFICANCE

Cutaneous Infections

HPV infects epithelial tissues throughout the body including skin, larynx, and anogenital tissues. Cutaneous HPV infections affect both immunocompetent and immunocompromised individuals and can lead to both benign and malignant lesions (Table 1). Common warts, plantar warts, and Butcher's warts are caused by HPV and are exclusively

* This chapter contains information presented in chapter 99 by Qinghua Feng and Nancy B. Kiviat in the eighth edition of this Manual.

TABLE 1 Clinical conditions associated with various HPV types

Clinical significance	HPV type(s)
Skin	
Common warts	2, 4, 26, 27, 29, 57
Plantar warts	1, 2, 4
Flat warts	3, 10, 28, 49
Butcher's warts	7
Epidermodysplasia verruciformis[a]	2, 3, 5, 8, 9, 10, 12, 14, 15, 17, 19, 20, 25, 36, 37, 46, 47, 50
Nongenital mucosa	
Oral mucosa	13, 32
Recurrent respiratory papillomatosis	6, 11
Head and neck cancer	2, 6, 11, 16, 18, 30
Genital mucosa	
Genital warts (condyloma accuminata)	6, 11, 42, 43, 44, 54
Low-risk HPV	6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, CP6108
Intermediate risk	26, 53, 66
High-risk HPV	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82

[a] Inherited disorder of cell-mediated immunity.

benign in behavior. In addition to those conditions listed in Table 1, large cell acanthomas have been associated with HPV 6. Actinic keratosis is associated with a higher HPV DNA load of HPV types 5, 8, 15, 20, 24, or 36 than nonpigmented skin cancers (6, 97). HPV types belonging to the high-risk group were demonstrated in extragenital Bowen's disease, in melanoma, and in dysplastic nevi (107). HPV types associated with epidermoplasia verriciformis have also been recently demonstrated in cutaneous squamous cell carcinomas from both immunocompetent and immunocompromised individuals (70). Verrucous carcinoma of the scalp, a low-grade, well-differentiated form of squamous cell carcinoma, has also been associated with the high-risk HPV type 33 (62). In summary, evidence that supports a role for HPV in nonanogenital cancers is increasing (26).

Although HPV has been associated with nonanogenital forms of cancer, laboratory diagnosis of HPV is largely confined to genital HPV and its association with cervical cancer.

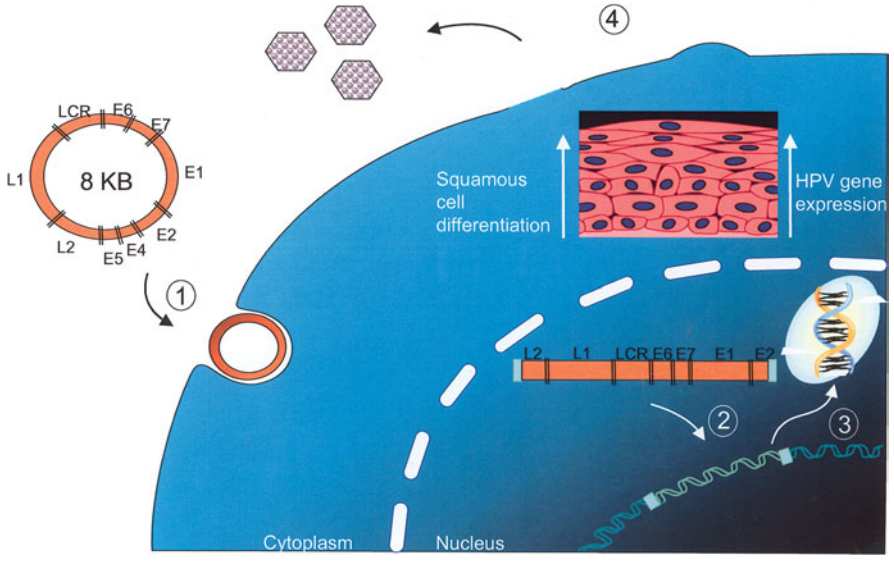


FIGURE 1 Schematic representation of HPV integration. This figure illustrates the events that most commonly lead to the transformation of cells infected by high-risk types of HPV. (1) HPV infection of epithelial cells. (2) HPV integration into the host chromosome; portions of E2 and L2 are deleted during this process. (3) Cell cycle dysregulation and cell proliferation. (4) Cell shedding and virus release. (Inset) Relationship between squamous cell differentiation and HPV gene expression (reviewed in references 21 and 109).

TABLE 2 Diagnostic terminology for cervical cytology and histology[a]

Cytologic diagnosis	Histologic diagnosis
WNL (within normal limits)	Normal (no abnormalities)
ASCUS (atypical squamous cells of undetermined significance)	Koilocytic change[b] (HPV-infected cells)
AGUS (atypical glandular cells of undetermined significance)	CIN 1 (cervical intraepithelial neoplasia—mild)
LSIL (low-grade squamous intraepithelial lesion)	CIN 2 (cervical intraepithelial neoplasia—moderate)
HSIL (high-grade squamous intraepithelial lesion)	CIN 3 (cervical intraepithelial neoplasia—severe)
SqCA (squamous cell carcinoma)	SCC (squamous cell carcinoma)

[a] Table based on the Bethesda system (105).
[b] Koilocytes represent HPV-infected cells with typical perinuclear halos.

During the past 50 years it has been well established that squamous cell cancers of the cervix arise as preinvasive squamous intraepithelial lesions (9, 18, 53, 54). A minority of these precursor lesions collectively referred to as cervical dysplasia progress over a number of years via one or more pathways to increasing states of cell cycle dysregulation, resulting in invasive cervical carcinoma. Other lesions may remain in the precursor state or, as for the majority of preneoplastic lesions, spontaneously regress as a result of immune control (31). The Bethesda system was established in the mid-1990s and revised in 2001 to classify cervical cancer precursor lesions into clearly defined cytological groups correlating with risk of progression to invasive disease as determined by histology (13, 78, 79, 105). Table 2 summarizes the terminology for cervical cytology specimens and cervical histology specimens and provides a context for the discussion of the role of HPV diagnostics in the cervical cancer screening algorithm (Fig. 2). Low-grade squamous intraepithelial lesions (LSIL) result from an infection by HPV, and ultimately, 80% of LSILs regress to within normal limits (WNL) cytology without any treatment (109). High-grade squamous intraepithelial lesions (HSIL) represent true cervical cancer precursors, and most untreated HSILs progress to invasive cancer over a period of years (109).

Pathogenesis

As previously mentioned, more than 99% of cervical squamous cell carcinomas are due to infection of squamous cells by oncogenic types of human papillomavirus (19, 108). Studies have also implicated HPV in the pathogenesis of adenocarcinoma of the uterine cervix (36). In high-risk HPV-associated lesions, transcriptional activity is always present (39, 41, 42, 92). The E6 and E7 oncoproteins as well as certain host factors are clearly involved in the transformation of cells and ultimate development of cervical cancer. The E6 protein causes degradation of p53 and BAK and activation of SRC family kinases in addition to the activation of telomerase. Similarly and synergistically, the E7 protein degrades the retinoblastoma protein (Rb) releasing the transcription factor E2F from inhibition and upregulating the cellular protein $p16^{INK4A}$ (4, 11, 32, 46, 52, 56, 57, 59, 60, 75, 76, 77, 84, 85, 98, 99). Further evidence supporting the role of E6 and E7 in the pathogenesis of cervical cancer comes from studies demonstrating that suppression of the E6/E7 promoter or inhibition of E6/E7 expression abrogates the proliferation of cancer cells in vitro (24, 72, 94). Integration of the HPV episome into the host chromosome commonly disrupts portions of the E2 gene, a negative regulator of E6/E7 expression, resulting in high levels of E6/E7 mRNA expression (17, 33,

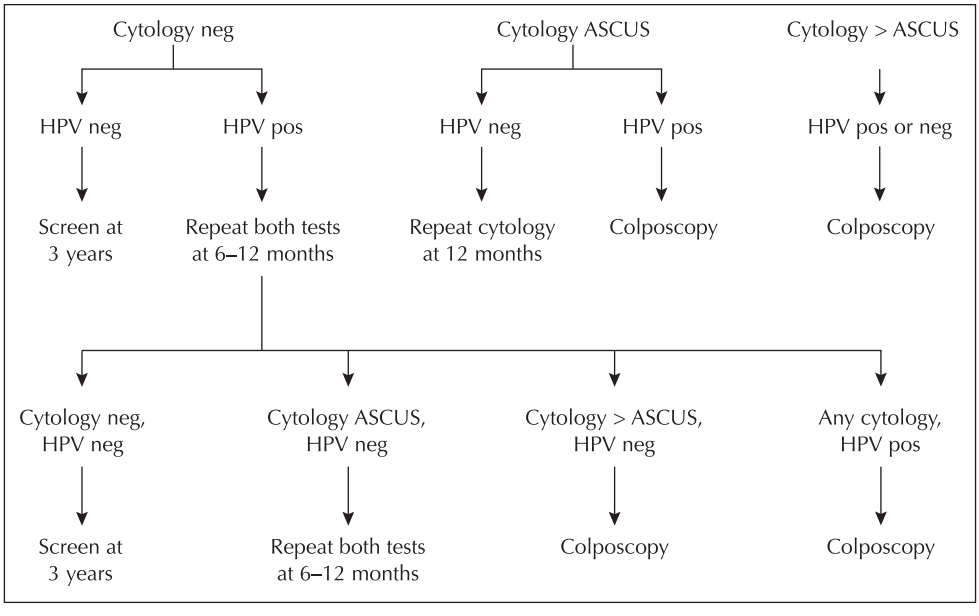


FIGURE 2 Cervical cancer screening algorithm (modified from reference 103). Neg, negative; pos, positive.

51, 73). From a diagnostic standpoint, the pattern of E6 and E7 gene expression also changes with the severity of the lesion; the level of E6 and E7 gene expression is increased in high-grade lesions compared to low-grade lesions, making the level of E6 and E7 gene expression potentially a functional discriminator between high-risk and low-risk HPV infections (58, 89). Similarly, the cellular marker $p16^{INK4A}$ is strongly associated with cancer and its precursors and serves as a diagnostic tool to screen for cervical cancer (37).

Data suggest that the HPV gene E5 also has cell-transforming capabilities (22, 25, 35, 68, 90). Expression of HPV-16 E5 protein in cultured keratinocytes resulted in an increase in the epidermal growth factor receptor on the cell surface (87). Receptor degradation is inhibited in cells expressing E5, resulting in increased recycling of receptors to the cell surface (87). Studies have indicated that anchorage-independent cell growth indicative of the transformed cell phenotype is induced in E5-transfected cells (43, 70). E5-transfected cells also demonstrated the ability to form tumors in nude mice while epidermal growth factor was capable of cooperating with the E5 gene to increase colony size (43). Of particular interest, the E5 protein from HPV-16 is expressed in LSIL, HSIL, and invasive cervical carcinoma epithelium (10).

HPV Vaccines

Much excitement is surrounding the publications of HPV vaccine trial results. HPV vaccines using L1 virus capsid-like particles (VLPs) and vaccines targeting cells expressing the HPV oncogenes (E6 and E7) have been extremely promising. Studies suggest that these VLP vaccines produce high anti-HPV antibody titers and HPV-specific T-cell responses (29, 69). Most importantly, an HPV-16 VLP vaccine demonstrated 100% protection from HPV-16 infection and related cervical intraepithelial neoplasia (CIN) in a study of 2,392 women (38). All of the nine cases of HPV-16-related cervical epithelial neoplasia in this study occurred in the placebo group (38). In another study involving a quadrivalent HPV vaccine directed against HPV 6, 11, 16, and 18 L1 genes, the study found a 90% decrease in the incidence of persistent infection or cervical abnormalities (93). Though promising, the duration of protection and infection by high-risk HPV types other than types 16 and 18 still remain as questions to be answered. Monitoring the efficacy of these vaccines has been performed using diagnostics directed against HPV L1 DNA.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

In the mid-1990s, new technologies were introduced that allowed the cervical cytology sample to be obtained using a brush that was then placed in a liquid preservative. These liquid preservatives are alcohol based and in general contain compounds that dissolve mucus and allow the cells to disaggregate in solution. Common to the different technologies that exist today is the creation of a slide that contains a monolayer of representative cells from the ecto and endocervix. One of the technologies (SurePath; TriPath Imaging Inc., Burlington, N.C.) for creating these cells involves moving the cell suspensions through the small orifice of a syringe which disaggregates cell clusters. Interfering debris and inflammatory cells are partially removed from the disaggregated sample by centrifugal sedimentation through the density reagent. After centrifugation, the centrifuge tubes containing cell pellets are placed on the PrepStain System. The other common technology (Thin Prep; Cytyc, Marlborough, Mass.) involves the mechanical application of a filter through the cell suspension until a monolayer is created on the filter paper. The filter is then pressed onto a treated slide and stained. Both of these technologies have improved the quality and uniformity of slides and reduced but not eliminated unsatisfactory slides (12, 100). As is discussed in subsequent sections, liquid-based cervical cytology has significantly aided the use of automated screening devices and the automation of advanced molecular and proteomic assays. Some studies suggest that liquid cytology preservatives can preserve RNA for up to 12 months (91). This has led to the use of liquid-based cervical cytology samples as well as swabs for HPV diagnostics (RNA and DNA). Detailed descriptions of the cytology process are included in this section to illustrate the potential pitfalls of molecular testing from these specimens. Sample carryover is a concern for both hybridization- and amplification-based HPV diagnostics when performed from these sample types.

DIRECT DETECTION

Microscopy

As molecular biology has advanced over the past 20 years, morphologic assessment of HPV infection has become inadequate for use in cervical cancer screening. Molecular diagnostics allowed laboratorians to detect HPV infection prior to morphologic changes, and most importantly, molecular biology allowed researchers to determine the direct role of HPV in the transformation of cells and the progression to cervical cancer. Recent advances in molecular technology have allowed the growing clinical use of these techniques to determine molecular changes that signal cellular transformation and later events that lead to cervical cancer.

Some clinicians advocate the utility of cervicography especially in low-resource settings (23). Cervicoscopy involves the use of directly applied 3 to 5% acetic acid solution or in some instances fluorescent dyes. Visualization of the cervix is then performed using a colposcope. Cross-sectional studies indicate that the sensitivity of visual inspection to detect high-grade lesions ranged from 66 to 96% with a specificity of 64 to 98%. Consequently, the positive predictive value ranged from 10 to 20% and the negative predictive value ranged from 92 to 97%. The conclusions of these studies suggested that the sensitivity of visual inspection was similar to that of cytology; however, the specificity was consistently lower than that of cytology (103, 104).

Antigen Detection

Detection of cervical biomarkers has been used more extensively in the clinical laboratory than the detection of HPV antigens. This typically involves the detection of host proteins that are dysregulated by HPV-mediated cellular transformation. Cellular transformation is driven by increasing expression of E6 and E7 and consequent inactivation of the tumor suppressor RB and p53 genes. These tumor suppressor genes interact with various cell cycle-regulating proteins. E7 inactivates RB, a negative regulator of the cyclin-dependent kinase inhibitor gene $p16^{INK4A}$. Increased expression of $p16^{INK4A}$ is a marker of dysplastic cervical cells (Fig. 3). The p16 marker is used clinically on both cells and tissues, though more frequently in the latter. This protein marker has a sensitivity of 82% and a specificity of 84% for the detection of atypical cervical lesions from LSIL to invasive cervical cancer (1, 28, 37). Other markers including proliferation antigens such as Ki-67, proliferating cell nuclear antigen, and the cell cycle (DNA replication)-dependent antigens cdc2, cdc6, and Mcm are being investigated but are not in widespread

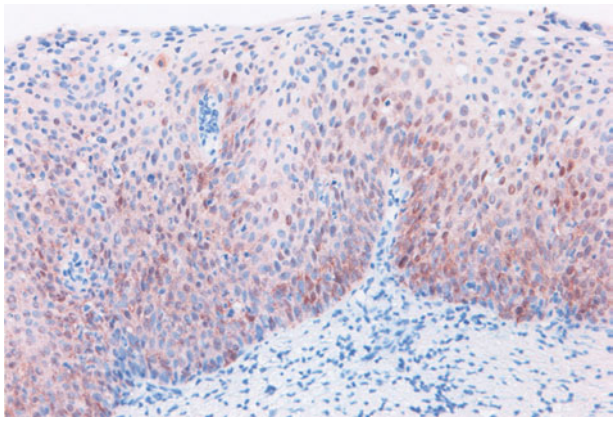


FIGURE 3 Photomicrograph of a high-grade cervical biopsy specimen demonstrating staining with the $p16^{INK4A}$ antibody (dark staining). Courtesy of Christina Kong, Stanford University School of Medicine.

clinical use (2, 52, 101). The MN transmembrane glycoprotein antigen has been studied extensively, and although the specificity of this marker was high, the sensitivity for abnormal cells was relatively low (44, 66). Epidermal growth factor receptor, transferrin receptor, and platelet-derived growth factor receptor are growth factor receptors thought to be involved in epithelial cell transformation (35). These receptors have been linked to the expression of HPV E5 protein (22, 35, 50, 71, 90). Other biomarkers predictive of HPV-driven cervical abnormalities are the focus of intense research at this time.

In Situ Hybridization for HPV L1 DNA

There are several automated platforms that perform in situ hybridization for HPV DNA on slides. The Ventana BenchMark Staining System combines immunohistochemistry and fluorescent/chromogenic in situ hybridization (Fig. 4). The INFORM HPV (Ventana Medical Systems, Inc., Tucson, Ariz.) assay detects high-risk HPV types 16, 18, 31, 33, 35, 45, 51, 56, 58, 59, 68, and 70 and low-risk HPV types

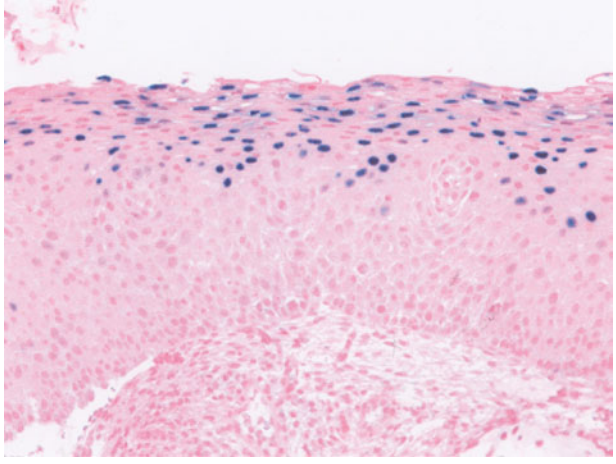


FIGURE 4 Photomicrograph of in situ hybridization performed in the clinical laboratory for high-risk HPV L1 DNA in a high-grade biopsy specimen. Courtesy of Christina Kong, Stanford University School of Medicine.

6, 11, 42, 43, and 44. The assay can be performed on both liquid-based Pap specimens and tissue biopsy specimens in less than 6 h. The sensitivity of the assay is 25 HPV copies per cell, and the localization of chromophore and the pattern of staining (homogeneous or punctate) may be indicative of either episomal or integrated forms of HPV DNA, respectively. Several studies have demonstrated that the assay has a positive predictive value of 60% for high-grade cervical lesions and a negative predictive value of 97% based on reported sensitivities of 86% and specificities of 90% (30).

Similarly, the GenPoint System (Dako, Glostrup, Denmark) uses a tyramide-based signal amplification strategy to detect biotinylated hybridization probes directed against HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. One of the advantages of this system is the ability of the kit to detect HPV in formalin-fixed, paraffin-embedded tissue sections or in a variety of cell preparations. Other studies report good specificity (>95%), although the sensitivity for CIN 2 or above is relatively poor (75%) (30). Newer instrumentation has improved on a very tedious manual staining procedure.

In Situ Hybridization for HPV E6 and E7 mRNA

Most HPV assays such as type-specific PCR and Hybrid Capture II (HCII) detect the presence of L1 DNA from oncogenic genotypes despite the fact that only a minority of women infected with oncogenic types of HPV progress to cancer (83). The pattern of E6 and E7 gene expression changes with the severity of the lesion. Namely, the level of E6 and E7 gene expression increases in high-grade lesions compared to low-grade lesions, making the level of E6 and E7 gene expression a functional discriminator between normal and neoplastic cells. The HPV OncoTect (Invirion Diagnostics, LLC) assay detects increased expression of E6 and E7 mRNA on a cell-by-cell basis with quantification by flow cytometry (Fig. 5). Cell-by-cell quantification eliminates the averaging of E6 and E7 mRNA gene copies on the total number of input cells that is the hallmark of solution-based detection such as PCR. The performance of this assay has been reported to be better than HPV DNA genotyping (HCII and type-specific PCR) for the detection of high-grade lesions (63).

Nucleic Acid

As described in this chapter, HPV nucleic acid (DNA) testing has been accepted in the cervical cancer screening algorithm (interim guidance, Fig. 2). Testing for HPV and/or biomarkers associated with HPV transformation of cells is the focus of intense investigation in the basic science and molecular diagnostics arenas. Testing for HPV presents a number of challenges discussed in this section.

The female genital tract contains a complex mixture of cells that provide a structural and functional barrier to a variety of infectious agents. The female genital tract is lined with epithelial cells, with stratified squamous epithelial cells in the vagina and ectocervix, changing to columnar epithelial cells in the endocervical canal, with the transition occurring in what is called the transformation zone (T-zone). The cervical and vaginal mucosa also contains T and B lymphocytes, macrophages, dendritic cells, Langerhans' cells, and polymorphonuclear neutrophils. In this complex mixture of cells, HPV is known to infect squamous cells of the vagina and ectocervix as well as columnar cells of the endocervix.

Detection of HPV nucleic acids, in particular the L1 gene (DNA), is the most common method employed today as part of the cervical cancer screening algorithm (Table 3). Methods include liquid hybridization, which is a signal amplification

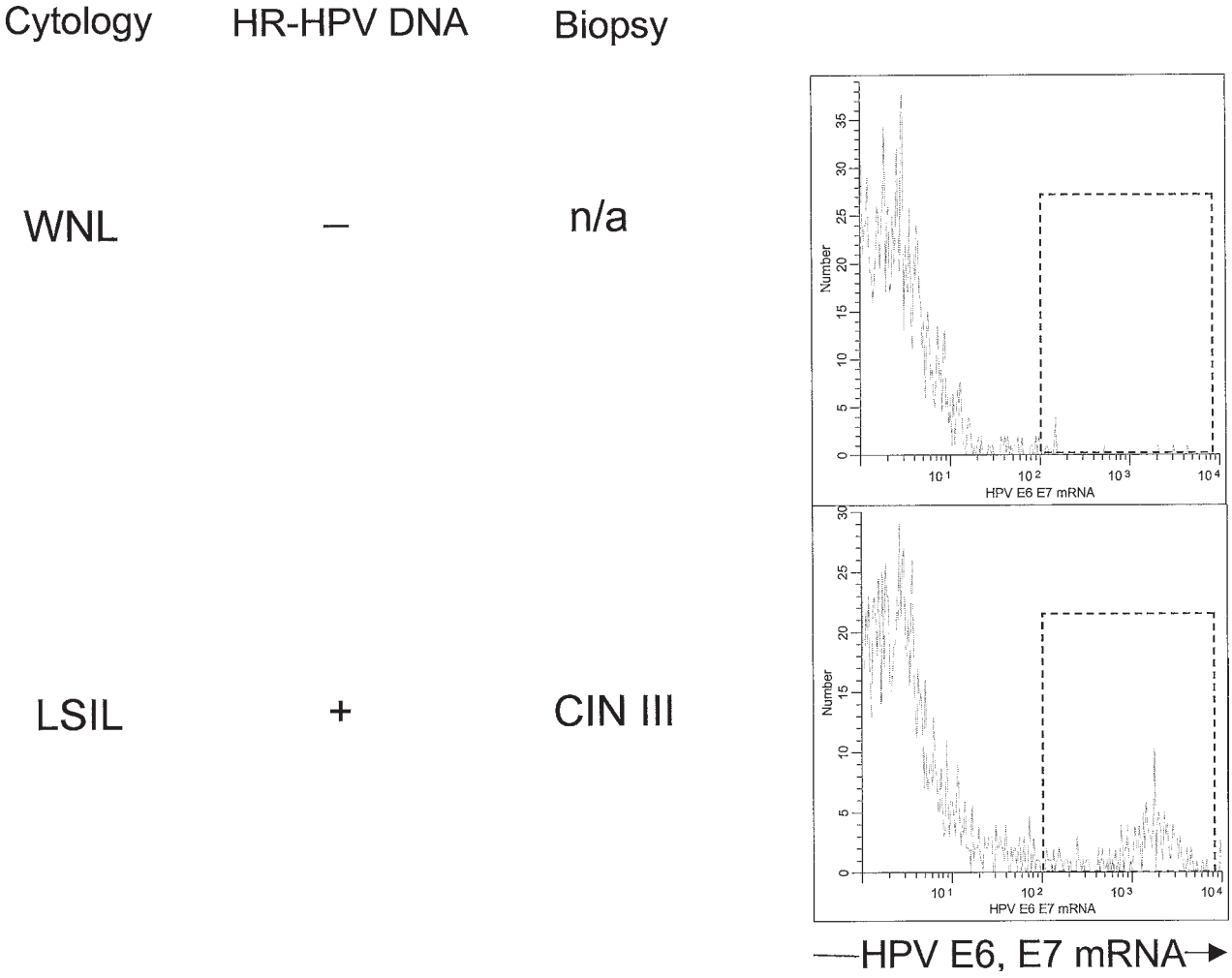


FIGURE 5 Representative histograms gated on light scatter (27) demonstrating HPV OncoTect (Invirion Diagnostics, LLC) staining of E6/E7 mRNA-expressing cells in liquid-based cervical cytology specimens (63). Samples were run on a personal capillary cytometer (PCA; Guava Technologies, Hayward, Calif.).

hybridization technology that involves the purification of DNA from cervical cytology specimens followed by hybridization to a complimentary RNA probe to form a DNA-RNA hybrid (47, 48). The Hybrid Capture Assay (HCII; Digene, Gaithersburg, Md.) High Risk probe detects high-risk HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 (61). Studies have demonstrated that the assay is extremely sensitive (93%), resulting in a high negative predictive value (99%) (48). However, the positive predictive value of the assay for CIN 2, CIN 3, or invasive cancer in the general population has been reported to be low (17 to 33%) due to the transient nature of most HPV infections (28). Cross-reactivity of the high-risk probe has been reported with some low-risk HPV types, and equivocal values contribute to indeterminate results in 5 to 10% of samples. The advantage of this assay is that it is FDA approved and there exists a large

TABLE 3 Assay types and target genes of commercially available HPV molecular diagnostic tests

Target type	Test name	Gene(s)	Format	Platform	Manufacturer
DNA	Hybrid Capture II	L1	Solution	Liquid hybridization	Digene Corporation, Gaithersburg, Md.
		L1	Solution	PCR	Roche Diagnostics, Indianapolis, Ind.
	INFORM HPV	L1	In situ	In situ hybridization	Ventana Medical Systems, Tucson, Ariz.
	GENPOINT HPV	L1	In situ	In situ hybridization	Dako USA, Carpinteria, Calif.
RNA	HPV Proofer	E6, E7	Solution	PCR	NorChip Klokkarstua, Hurum, Norway
	HPV OncoTect	E6, E7	In situ	In situ hybridization	Invirion Diagnostics, LLC, Oak Brook, Ill.

database of clinical studies supporting the assay's use in cervical cancer screening (5, 47, 48, 82, 83). The disadvantages of this assay are the large quantities of DNA that are required to increase the analytical sensitivity in addition to the relatively low specificity.

PCR has been classically used for HPV detection in the research laboratories employing the consensus PCR primers MYB09 and MYB11. Type-specific PCR assays based on the sequence heterogeneity in the L1 gene are currently in clinical trials for use in the diagnostic algorithm for cervical cancer screening. Commercially available PCR assays for HPV utilize two different formats for the detection of HPV types (Roche Diagnostics, Pleasanton, Calif.). One iteration of this assay is the microwell plate format that detects high-risk HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 and the line blot assay or linear array assays that detect high-risk HPV types 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 66, 67, 69, 70, 73, and 82 as well as low-risk types 6, 11, 40, 42, 54, 55, 57, 61, 62, 64, 71, 72, 81, 83, and 84. DNA is extracted from cervical cytology samples followed by PCR amplification using biotin-labeled primers targeting the L1 region of the HPV genome. The advantages of type-specific PCR are the sensitivity (10 to 20 HPV DNA copies) and the ability to report specific types (e.g., HPV 16 and HPV 18) rather than high-risk HPV positive. Recent studies have indicated that reporting specific HPV types such as HPV 16 may increase the positive predictive value for high-grade lesions on biopsy specimens.

HPV RNA Detection

The PreTect HPV-Proofer assay (NorChip) detects HPV types by targeting E6/E7 mRNA expression from five of the most common high-risk HPV types, i.e., 16, 18, 31, 33, and 45. The PreTect HPV-Proofer is a real-time detection strategy based on the nucleic acid sequence-based amplification technology. The five HPV types are detected using molecular beacons specific for genotypic sequence differences contained within the E6 and E7 genes. In a recent study, HC II (Digene) was positive in 95% of histologically determined high-grade lesions, and the HPV Proofer mRNA test was positive in 77% of the histologically confirmed high-grade lesions. HC II was more often positive in benign and low-grade lesions, at a statistically significant level, than HPV E6, E7 mRNA (45). Of particular interest in this study, high-grade histology was detected in 83% of women with normal cytology and positive mRNA test.

SEROLOGIC TESTS

Serologic diagnosis of exposure to HPV has, in general, been used infrequently in the clinical management of HPV infection. Humoral immune responses against HPV include both immunoglobulin G (IgG) and IgA responses. In general, IgG responses have been identified as markers of lifetime exposure to HPV while IgA responses have been proposed as a marker of persistent HPV infection (96). Other studies have demonstrated a decrease in IgA responses in women with precancerous and cancerous lesions (64). Cellular immune responses against HPV are detected late in the progression of HPV to cervical cancer. These immune responses consist of CD8-positive cytotoxic T cells directed against epitopes in the E7 protein. In addition to being detected late in disease, HPV-directed cytotoxic T cells vary widely from patient to patient with cervical cancer, and their potential as a marker of disease is poor given how late they appear in the disease process (20).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

To understand the clinical utility of HPV testing, one must also have an understanding of cytological and histological diagnoses of the uterine cervix given that, to date, HPV testing has not been approved for primary cervical cancer screening. Table 2 summarizes the terminology used for cytological and histological diagnoses of cervical cells and biopsy specimens, respectively. One of the most compelling and quoted statistics relating to the clinical significance of HPV testing is that >99% of cervical cancer is associated with the detection of HPV nucleic acids in the tumor specimen (95). Although this statistic strongly suggests that HPV is directly involved in cervical cancer, clinicians and laboratorians manage disease in the opposite direction. Accordingly clinicians and laboratorians are faced with a test that, for example, detects high-risk HPV in a patient. For a screening assay, the question arises "what are the chances that a woman with a high-risk type will develop a high-grade lesion or cancer?" In a large prospective study, the incidence of women with laboratory diagnosis of high-risk HPV who had CIN 3 or cancer was 4.4% after 45 months of follow-up. Conversely, women who were HPV high-risk DNA negative had an incidence of CIN 3 or cancer of 0.24% at 45 months and 0.87% at 10 years (83). Although these data support the use of HPV DNA testing, they also demonstrate that a large number of women who are high-risk HPV DNA positive do not develop cervical cancer or cervical cancer precursor lesions (CIN 2 or 3). Assays with the potential to improve the positive predictive value of HPV testing for the development of cervical cancer or cervical cancer precursor lesions are discussed below.

Over the past decade, HPV testing has been integrated into the cervical cancer screening algorithm. For this reason, HPV testing was incorporated into the new Bethesda System recommendations and integrated into current patient management recommendations (Fig. 3) (13, 105, 106). The recommendations were based on the combined use of cervical cytology and HPV DNA testing in large international studies of women over 30 years of age. In general, these studies demonstrated a sensitivity (for CIN 2 or higher) of 92% and a specificity of at least 95% (for CIN 2 or higher) (3, 5, 67, 74, 106). The two currently approved methods for using HPV testing are for the triage of samples diagnosed as ASCUS (abnormal squamous cells of unknown significance) and as an adjunct to the Pap smear. The goal of including HPV testing in this context is to triage or minimize the number of women who need further evaluation by colposcopy and biopsy, for example. HPV testing alone as performed today is not highly predictive of which women will definitely have a high-grade lesion considered to be CIN 2, CIN 3, or squamous cell carcinoma by biopsy. The sensitivity and specificity or more importantly the positive and negative predictive values are the most important determinants of how HPV testing is used in the cervical cancer screening algorithm.

In summary, the understanding of HPV pathogenesis and the role of HPV in cervical cancer has led to new diagnostic approaches to cervical cancer screening that should improve upon the long-standing success of the Papanicolaou smear. Cervical cancer screening in developing countries will also benefit from the many advances in HPV testing, albeit with a few modifications (102) and a focus on low cost and compatibility with instrumentation already in the field.

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Human Polyomaviruses*

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TAXONOMY

JC virus (JCV), BK virus (BKV), and simian virus 40 (SV40) are members of the family *Polyomaviridae* based on virion physical properties (icosahedral virion, 40 nm in diameter) and genome structure (5.1-kb DNA genome with closed, circular, supercoiled conformation). A number of human diseases have been associated with SV40; however, definitive causality has not yet been demonstrated. This chapter therefore focuses on JCV and BKV, the two viruses that have been demonstrated to unequivocally cause human illness. The initials of the patients from whom isolations were first made were used to name these viruses. JCV was cultured from autopsy brain tissue of a Hodgkin's lymphoma patient with cerebral white-matter lesions characteristic of the demyelinating disease progressive multifocal leukoencephalopathy (PML), and BKV was isolated from the urine of a renal transplant recipient (42, 83). The formal names applied to these viruses are *Polyomavirus hominis 1* (BKV) and *Polyomavirus hominis 2* (JCV).

DESCRIPTION OF THE AGENT

JCV and BKV have an icosahedral capsid made up of the three structural proteins VP1, VP2, and VP3. The outer shell of the capsid is made up of 72 capsomeres, each of which contains five VP1 molecules arranged symmetrically around either VP2 or VP3. The VP2 and VP3 structural proteins are believed to assist in transport of VP1 from the cytoplasm to the nucleus and further assist in virion assembly by ionic bonding to genomic nucleotides of the respective viruses (26). Electron microscopy studies showed that cloned VP1 molecules spontaneously aggregate to form virus-like particles with the icosahedral morphology of empty polyomavirus capsids (44, 98). JCV and BKV virus-like particles formed in this manner have been used as effective enzyme immunoassay antigens (111). The epitopes responsible for JCV and BKV neutralization and hemagglutination inhibition are located exclusively on the VP-l structural proteins and appear to be virus specific (46, 109).

The cell surface receptor for JCV and BKV is an α-2-6-linked sialic acid, and viral entry into cells occurs by clathrin-dependent, receptor-mediated endocytosis (37, 67, 85). Recently, a coreceptor for JCV binding has been described, the 5HT2A serotonin receptor (37). Both viruses are assembled within the nucleus.

DNA sequence analysis of BKV and JCV revealed approximately 75% homology at the DNA level and 68% homology at the amino acid level (40). The most significant genomic differences are in the noncoding nucleotides of their regulatory regions (RRs). RRs are located between the multifunctional nonstructural T protein and the agnoprotein coding regions. RRs have a number of different functions including regulation of transcription, genome replication, and host/tissue tropism including human glial, tonsillar stromal cells, and hematopoietic cells for JCV (12, 38, 104) and human tonsillar, pancreatic islet, kidney, and hematopoietic stem cells for BKV. RRs can contain single or tandemly repeated sequences, and they may contain sequence insertions. The mechanisms by which the singular and repeat structures arise are not fully understood but are believed to result from deletion and duplication of promoter/enhancer sequences during replication in the host (40, 71). The JCV RR contains several binding sites for the NF-1 family of transcription factors (40) that have been implicated in regulating cellular susceptibility to JCV infection (5, 75). JCV strains with RRs containing tandem repeats have been associated with PML and therefore appear to be more pathogenic (56, 71). The JCV regulatory region variant responsible for initiating primary infections in humans has not been definitively identified. After reactivation of latent infection in immunosuppressed hosts, JCV regulatory region nucleotide sequences apparently undergo rearrangements to produce variants that can be isolated from different tissues of the body (32, 40, 71). This also appears to be true of BKV, in which rearranged sequences were thought to have emerged from an archetype sequence (24). These viral variants have been associated with dissemination from urogenital organs or lymphoid tissue to other regions of the body, including lung, brain, and cerebrospinal fluid (CSF) (58, 100).

EPIDEMIOLOGY AND TRANSMISSION

Serological studies have confirmed the susceptibility of the majority of the human population to JCV and BKV infection. Seroconversion occurs in approximately 70% of the worldwide population between the ages of 5 and 8 (20, 61, 83). Many individuals maintain lifelong antibody titers to BKV and JCV. However, persistent, high levels of antibody to both viruses are uncommon. This observation suggests that JCV and BKV circulate independently.

* This chapter contains information presented in chapter 100 by Eugene O. Major, Maneth Gravell, and Jean Hou in the eighth edition of this Manual.

The primary route of JCV and BKV transmission is considered to be through respiratory inhalation or other readily accessible portals of viral entry, although supporting evidence is largely epidemiological or circumstantial (31, 101). Other possible routes of JCV or BKV transmission include transplacental passage and exposure to body fluids such as urine, blood, or oral secretions (31, 76, 86, 101).

CLINICAL SIGNIFICANCE

Following inhalation, the initial site of JCV infection has been proposed to be tonsillar stromal cells, due to their demonstrated susceptibility to infection (in vitro and in vivo) and to their anatomic location in the upper respiratory tract (73, 74). Although a specific correlation has not yet been made, BKV has also been demonstrated in tonsillar tissues of children suffering from upper respiratory tract infections (45) as well as in nasopharyngeal aspirates (101), suggesting respiratory inhalation as the initial viral entry point. B-lymphocytes have also been implicated as an initial target site of both JCV and BKV infection; however, they appear to be less susceptible than tonsillar stromal cells (73).

In individuals with normal immunity, primary infections are thought to be mild and usually asymptomatic, although fever and nonspecific upper respiratory symptoms may occur (45, 68). After acute infection, healthy individuals become latently infected and remain asymptomatic throughout life (14, 31, 68).

Evidence suggests that JCV and BKV may remain latent in a number of sites, notably in the kidney and cells of the lymphoid system (10, 30, 54, 73, 76). Recent evidence has also suggested a potential latent site for JCV in the colon (92). During periods of relative or absolute cellular immunodeficiency, JCV and BKV may reactivate in some latently infected individuals, producing asymptomatic viruria and in some instances severe or fatal disease (17, 31).

BKV causes cystitis (nonhemorrhagic or hemorrhagic) and ureteral stenosis in bone marrow and solid organ transplant recipients (7, 16, 27, 108). It also causes nephropathy (polyomavirus-associated nephropathy [PVAN]) in renal transplant recipients (19, 55, 91). The incidence of PVAN has increased from 1 to 5% in the era of intensive immunosuppression to suppress allograft rejection (22, 88). The prevalence of disease varies among centers from 1 to 10% (49). Most cases occur in the first year after transplantation, although approximately 25% of cases occur later (50). Rates of allograft loss range from 10 to 80% (49). Disease appears to result from reactivation of BK infection from the donor allograft (16).

In the correct clinical context, BK detection in urine can be suggestive of BK-induced hemorrhagic cystitis or ureteral stenosis (Table 1). BK replication is the primary risk factor associated with PVAN; therefore, detection of BK replication may be a useful screening measure to identify renal allograft patients at risk of disease (Table 1). Urine screening every 3 months in the first 2 years and annually thereafter up until 5 years posttransplant has been recommended (49) because BK viruria precedes viremia by 4 weeks and PVAN by a median of 12 weeks (50). Although nucleic acid detection in urine is more sensitive than cytology (49, 90), either method can be used for screening. Quantification of BK viral load in plasma or urine has been advocated to establish a presumptive diagnosis of PVAN (49).

PML usually presents as a triad of symptoms including motor dysfunction, visual deficits, and cognitive impairment. These can be manifested as hemiparesis, cortical blindness, hemianopsia, and progressive dementia (8, 68). Viruses resembling polyomaviruses had historically been visualized in white-matter lesions of PML patients; however, JCV was firmly established as the etiologic agent of this disease only after virus was consistently isolated from brain tissue of afflicted patients (81, 84, 93, 115).

PML is considered to be an opportunistic infection (14). It was a relatively rare disease before the AIDS pandemic, occurring initially in immunocompromised individuals with lymphoproliferative malignancies such as lymphoma and leukemia and later in solid organ transplant recipients receiving immunosuppressive therapy. The AIDS pandemic has caused a large increase in PML, and this disease is now seen in about 5% of all AIDS cases (15).

Histopathology results from lytic infection of oligodendrocytes that produces focal or multifocal demyelination in brain white matter (Fig. 1). In biopsy or autopsy tissues from PML patients, JCV antigens and DNA are consistently found in the demyelinated lesions by using immunohistochemistry or in situ DNA hybridization (3, 33).

Patient history, physical exam, and neuroradiology test results suggest the diagnosis of PML. The minimally invasive test of choice for confirmation of diagnosis is detection of

TABLE 1 Diagnostic tests for polyomavirus diseases

Virus	Disease	Specimen	Diagnostic test[a]	Comment
BKV	Hemorrhagic cystitis	Urine	Cytology	Decoy cells indicate polyomavirus replication
			NAAT	Qualitative PCR adequate; role for quantification unproven; negative predictive value is high
	PVAN	Urine	Cytology	Less sensitive than NAAT
			NAAT	Qualitative or quantitative format for screening; quantitative tests for presumptive diagnosis
		Plasma	NAAT	Quantitative tests for presumptive diagnosis
		Renal biopsy specimen	Histology, IHC	Definitive diagnostic tests
JCV	PML	CSF	NAAT	Qualitative PCR adequate; quantitative tests may be indicative of disease course
		Brain biopsy specimen	Histology, IHC, ISH, EM	Less invasive NAAT of CSF currently preferred over biopsy analyses, although biopsies are the most accurate

[a] Abbreviations: NAAT, nucleic acid amplification test; IHC, immunohistochemistry; ISH, in situ hybridization; EM, electron microscopy.

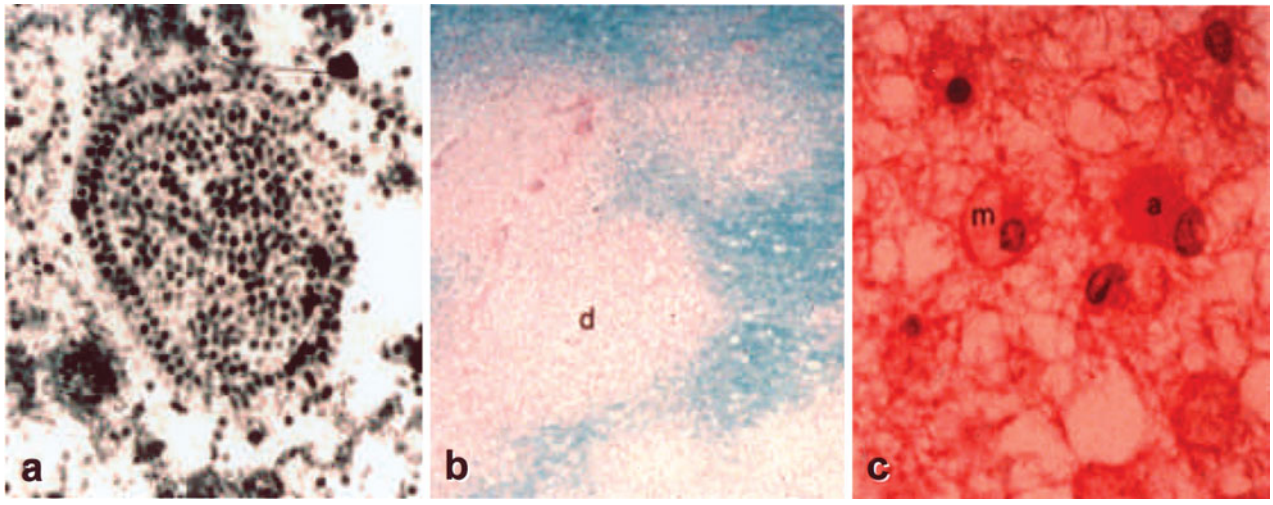


FIGURE 1 Representation of the major elements in the pathogenesis of PML. (a) Electron micrograph of brain tissue from a PML patient showing the assembly of JC virion particles in the nucleus of an infected oligodendrocyte. (b) Luxol fast blue stain of the white matter with demyelinated(d) plaque lesions in a patient with PML. (c) Hematoxylin stain of lesion showing the presence of a macrophage (m) and an astrocyte (smaller a).

JCV nucleic acids in CSF through DNA amplification methods such as PCR (Table 1) (43, 105). Electron microscopy, immunohistochemistry, and in situ hybridization (ISH) on biopsy specimens are used less commonly to establish the diagnosis.

The role of BKV and JCV as causative agents of human malignancies is controversial, at best. Detection of DNA sequences from both viruses in tumors has been variable, and currently there is insufficient evidence to unequivocally prove that these viruses are tumorigenic in humans.

SPECIMEN COLLECTION, TRANSPORT, AND STORAGE

Urine and blood can be tested for BKV. Collection methods for these specimens are reviewed in chapter 80. For nucleic acid detection by PCR amplification, urine can be tested without further processing. If virus is to be isolated, cellular debris is removed by low-speed centrifugation (1,500 × *g* for 15 min). The supernatant medium is then passed through a 0.2-μm-pore-size Nalgene filter (Nalge Co., Rochester, N.Y.), and the virus in the filtered supernatant medium is pelleted by ultracentrifugation (105,000 × *g* for 90 min). JCV or BKV pellets are resuspended and layered onto continuous 25 to 60% Optiprep (Life Technologies, Gaithersburg, Md.) gradients and banded by density gradient ultracentrifugation (160,000 × *g* for 16 h). Gradients are fractionated, and fractions are monitored for levels of JCV or BKV by hemagglutination of human type O erythrocytes. Urine can be stored for long periods at −80°C and used for future testing. Quantification of BKV in blood is performed using serum or plasma. There are currently no data on the effect of specimen type (serum versus plasma) on BKV load. Whole blood can be stored at 4°C prior to separation of serum or plasma. Serum and plasma can be stored at −80°C for extended periods of time. If isolation of BKV from peripheral blood is required, peripheral blood mononuclear cells should be isolated using suitable separation media (reviewed in chapter 80) and cocultured with susceptible cell lines as described below.

CSF can be tested for JCV nucleotide sequences. Collection methods for this specimen are reviewed in chapter 80. CSF samples can be frozen and stored for an extended period at –80°C.

Though invasive, biopsies can be useful in establishing the diagnosis of polyomavirus-induced disease. Prior to the advent of molecular detection, brain biopsy was used as the initial test, to demonstrate the characteristic histopathology of PML. The presence of JCV in affected brains was demonstrated by electron microscopy, immunohistochemistry, and ISH. These methods are still currently useful when the diagnosis is unclear (e.g., when clinical suspicion is high but CSF is negative for JCV DNA by PCR) or at autopsy. The diagnosis of PVAN is established unequivocally by examining renal biopsy specimens for histologic evidence of nephropathy and virus cytopathic effect. Immunohistochemistry is also routinely performed to definitively demonstrate the presence of polyomavirus antigens. Commercially available gelatin or sialinate-treated slides can improve adherence of tissues for use in ISH and immunocytochemistry (53, 54).

DIRECT DETECTION

Microscopy

A presumptive diagnosis of PML can be made based on histopathologic evidence of polyomavirus-infected oligodendrocytes. These cells are enlarged two- to threefold; their nuclei are also enlarged and contain large basophilic inclusions that displace chromatin. Associated histologic changes include perivascular mononuclear cell inflammation, enlarged irregular astrocytes with pleiomorphic nuclei, and foamy macrophages engulfing myelin debris (2, 9).

The diagnosis of PVAN is made by demonstrating viral cytopathic effect in renal tubular epithelium and urothelium. Enlarged basophilic inclusions and displaced chromatin are observed in infected epithelial cells. Tubular necrosis, atrophy, and fibrosis are observed (23, 35). The presence of infected epithelial cells or decoy cells in urine is suggestive of

polyomavirus infection of urinary epithelium. These cells have characteristic basophilic ground-glass nuclear inclusions (90).

Antigen Detection

There are currently no commercially available monoclonal antibodies that do not cross-react with JCV or BKV. However, a polyclonal antibody specific for JCV Vp1 has been developed by an independent laboratory and used to successfully identify JCV in PML brain tissue (57). Similarly, another laboratory-developed monoclonal antibody, Pab2003, has been used to identify JCV early protein, Large T, in the brain of PML patients (77). Polyclonal sera against BKV have been utilized to identify BKV antigens (Vp1 and agnoprotein) in human immunodeficiency virus-associated nephritis (19). Monoclonal antibodies against BKV Large T antigen have been used to detect virus in renal biopsy specimens (102). Unfortunately, there is a current lack of commercially available, specific monoclonal antibodies for diagnostic testing.

Nucleic Acid Detection

ISH

ISH can be used to detect polyomavirus DNA in tissue (Fig. 2) (53). It is used more commonly to diagnose JC syndromes (primarily PML) than diseases caused by BKV. ISH detects active viral DNA replication, an earlier event in the JCV or BKV life cycle than virion capsid formation. Cells undergoing latent infection by JCV or BKV may not produce sufficient viral antigen to be detected by immunocytochemical techniques. However, if sufficient viral DNA were present in these cells, it would still be detectable by ISH (21). Several reports have discussed the specificity and sensitivity advantages of using ISH over antigen detection by immunocytochemistry (3, 25, 95). Due to varying levels of viral transcription and protein synthesis occurring at different stages during infection, it is evident that ISH is a more sensitive method for viral detection than immunocytochemistry. Furthermore, the use of a specific DNA probe for BKV or JCV during DNA hybridization raises the level of specificity, with no cross-reactivity observed between viruses. Biotin-labeled DNA probes used in ISH assays are readily available commercially (Enzo Diagnostics, Inc., Farmingdale, N.Y.). Although biotin-labeled probes are not as sensitive as radiolabeled probes in detecting very low copy numbers of JCV DNA, they do detect as few as 100 copies of viral genomic DNA, an acceptable value for diagnostic use with patient samples.

Nucleic Acid Amplification Tests

Template Extraction

The method of nucleic acid extraction preceding PCR can affect the level of detection sensitivity. The utility of extraction lies mainly in the removal of any inhibitors in the clinical sample. CSF and urine may contain inhibitors of thermostable polymerases; although the CSF inhibitors remain unknown, some urine inhibitors have been identified (13). Furthermore, extraction increases the potential for better sensitivity by concentrating the target.

Manual extraction of urine is typically accomplished with chaotropic salt/silica membrane methods in commercially available kits such as QIAamp (QIAGEN, Valencia, Calif.), described for adenoviruses (36). This method is now widely used for extraction of other viruses in urine, including BKV (72, 80). The QIAamp Viral RNA kit (QIAGEN), which coextracts viral DNA, can be used to manually extract polyomaviruses from CSF. Automated extraction instruments (NucliSens and NucliSens MiniMag [BioMerieux, Durham, N.C.]; MagNAPure compact [Roche Diagnostics Corporation, Indianapolis, Ind.]; and BioRobot EZ1, QIAGEN) have the advantages of saving labor and potentially increasing throughput and can be used to extract BKV from urine (99, 103). Automated extraction of JCV from CSF has also been reported (MagnaPure; Roche Diagnostics Corporation). However, manual extraction appears to be 1 order of magnitude more sensitive than the automated method for JCV DNA extraction from CSF (113).

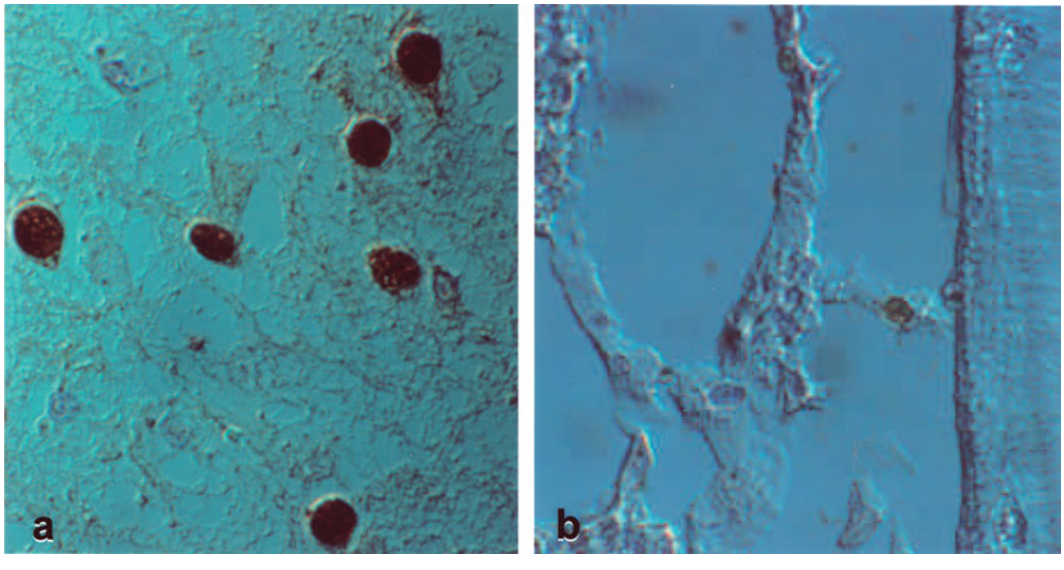


FIGURE 2 Photomicrographs of (a) brain and (b) bone marrow biopsy tissue and B lymphocytes with positive signals following in situ DNA hybridization using a JCV DNA biotin-labeled probe.

Internal Controls

Internal controls are important to identify failed amplification reactions. This is particularly important in urine specimen testing due to the possibility of residual inhibitors postextraction. In general, the internal controls are highly generalizable for JCV and BKV. Targets to ensure the presence of amplifiable DNA include RNaseP (72) and β-actin (103).

Standards

There are no commercially available assays or standards for polyomavirus detection and quantification. Laboratories must do their own internal verification studies to establish assay performance parameters. Independently developed standards are used for quality control and quality assurance once assays have been verified and implemented. Furthermore, they are critical as positive controls and as source material for proficiency testing. Collaborative efforts to develop internationally sanctioned and universally available standards for these purposes are underway.

Preparations of extracted BKV and JCV DNA from purified virus are commercially available (Advanced Biotechnologies, Inc., Columbia, Md.). Given the lack of "gold standard" methodology for polyomavirus quantification, laboratories should consider verifying concentrations of purchased nominal standards.

PCR Methods

PCR-based, qualitative BKV DNA detection assays can be useful in the diagnosis of hemorrhagic cystitis; quantitative assays are currently employed for PVAN screening (Table 1). Qualitative detection of JCV DNA in CSF by PCR has been a very useful, less invasive surrogate for biopsy (Table 1). The utility and predictive value of JCV DNA quantification are still under investigation. Some reports suggest that JCV loads in CSF correlate inversely with survival in patients who are not on antiretroviral therapy (18).

BKV and JCV DNA can be detected qualitatively with conventional or real-time PCR assays. PCR assay design for the polyomaviruses is complicated by the high degree of homology between their genome sequences. Regions within large T antigen and VP1 genes have been used as targets in a number of reported conventional (6, 94) and real-time (66, 94, 106) PCR assays since these sequences are sufficiently variable among different polyomaviruses.

After conventional PCR, products can be detected by electrophoresis through agarose gels containing ethidium bromide. Southern hybridization with radiolabeled DNA probes can also be performed (6, 94). This method has distinct advantages over simple electrophoresis including increased sensitivity and specificity.

In some studies, real-time PCR and conventional PCR-Southern detection have comparable sensitivities for the detection of polyomaviruses (94); in others it is reported to be 10- to 100-fold more sensitive than conventional PCR (72). Decreased labor intensiveness and increased throughput are additional advantages that have motivated many laboratories to switch detection methodologies. Finally, real-time PCR is currently the method of choice for polyomavirus DNA quantification due to its simplicity. Linear ranges of ≥ 7 $\log_{10}$ virus copies have been reported with these methods (97). For BKV, such high concentrations in plasma are uncommon; however, viral loads in urine frequently reach or exceed these levels.

A number of different approaches for the specific detection and quantification of different polyomavirus species have been reported including combinations of species-specific primers and probes (65, 72, 99, 106), genus-specific primers and species-specific probes (66), genus-specific primers followed by melting curve analysis to distinguish amplicons from different species (11), and species-specific primers and SYBR green (a nonspecific, fluorescent DNA intercalator) (89, 97). Most of the species-specific primer/probe strategies utilize TaqMan chemistry (described more fully in chapter 16). Published assays have used real-time PCR instruments with 96-well plate (Prism Sequence Detection System Models 7000, 7700, and 7900; Applied Biosystems, Foster City, Calif.) (48, 65, 72, 106) or capillary configurations (Light Cycler; Roche Diagnostics Corporation) (11, 90, 97). Excellent correlation of BKV quantification in analytical and clinical samples has been reported for the two types of instruments (1).

ISOLATION AND CULTURE PROCEDURES

JCV and BKV are not routinely isolated from clinical specimens because they have rather long growth cycles and a somewhat restricted host cell range. Permanent cell lines that support JCV and/or BKV replication have been developed; these cells can be used for isolation of these viruses from patient samples. Primary cultures derived from human embryonic tissues are also available. JCV grows best in cell cultures derived from human brain; virus replicates in astrocyte and precursor oligodendroglial cells. A permanent cell line, SVG, established from human fetal brain cells, can be used as an equivalent substitute for primary cultures derived from human fetal brain cells (69). The SVG cell line is highly susceptible to JCV infection and has been used for virus isolation and propagation. JCV does not infect human neurons in situ or neurons cultured in vitro. Even under optimum culture conditions and sufficient viral inoculum, JCV growth takes weeks, and multiple passages are sometimes required before progeny virus can be detected. Cytopathic effects in JCV-infected cultures are not easily recognized. They present as translucent cells with large nuclei that are ultimately shed from infected monolayers. JCV remains largely cell associated. Treatment of JCV-infected cell pellets with the nonionic detergent sodium deoxycholate releases JCV from cell association and negates inhibitors of JCV hemagglutination (78, 82).

BKV grows well and forms plaques in monolayers of primary cell cultures (human embryonic kidney, pancreatic islet, and human foreskin cells) and continuous cell lines (HeLa and Vero). BKV cytopathic effect includes nuclear enlargement and the gradual, continual peeling of cells from the monolayer. In infected cultures, BKV remains primarily cell associated; small amounts of virus are shed into the culture medium. Freeze-thaw cycles assist in releasing cell-associated BKV. Titers of JCV or BKV in harvests can be quickly determined by hemagglutination of human type "O" erythrocytes (78, 82).

SEROLOGIC TESTS

The majority of the world population is asymptomatically infected by JCV or BKV during early childhood. An immunoglobulin M antibody response is likely elicited upon initial infection by these viruses. However, due to the early age and the mild, nonspecific nature of JCV and BKV infections, serological assays have not been used to monitor their occurrence. Nor have serological assays been used to monitor the roles of recurrent infections or reactivation of latent infections in maintaining antibody titers in the adult population, or the class-specific antibody responses that may result from these infections. There is little evidence that recurrent infections occur and contribute significantly to maintenance

of antibody titers. Serology, however, has played an important role in understanding the epidemiology of human polyomaviruses and has contributed information on their worldwide distribution (20, 82).

Due to greater sensitivity and reproducibility, enzyme immunoassays have now largely replaced hemagglutination inhibition assays as the means of detecting antibodies to JCV and BKV. Two major developments have facilitated this change: the cloning of JCV and BKV VP1 proteins and the creation of a hybrid strain of JCV, JCV-SVE (Δ), with improved growth characteristics and expanded host cell range (41, 104). These two developments have made it much easier to obtain the antigen concentrations needed to perform enzyme immunoassays and other procedures requiring high antigen concentrations. Enzyme immunoassays to detect JCV- or BKV-specific IgM antibodies have been developed (62, 63). Despite 79% amino acid homology between JCV and BKV capsid proteins, a high degree of antibody cross-reactivity is not observed and independent infection by both viruses has been demonstrated serologically (46, 107).

ANTIVIRAL SUSCEPTIBILITIES

A number of drugs exhibit antiviral activity to JCV or BKV in vitro, including cytarabine, prednisone, vidarabine, fludarabine, topoisomerase inhibitors, camptothecin and alpha interferon. Only cytarabine has been examined as a potential treatment for PML in a multicenter clinical trial (45a). Although there did not appear to be an effect of ara-c on the progression of PML, subsequent analysis (112a) did correlate lower JCV viral loads in the CSF with longer survival times. Randomized controlled trials with other agents have not been performed, and accepted regimens for treatment of patients are not currently available (51). Furthermore, some severe side effects have been associated with their use. Treatment of polyomavirus-induced disease has therefore focused on modulation of immunosuppression, the underlying cause of polyomavirus reactivation.

The recently recommended BKV testing algorithm in renal transplant recipients is aimed at detecting PVAN at an early stage (49). The algorithm is based on the assumption that abrogation of BKV replication will more effectively preserve allograft function if intervention is initiated before significant renal destruction has occurred. Variable efficacy (<10 to >80% graft survival) has been observed after early reduction in immunosuppression using a number of different protocols (49). Low doses of the cytidine analog cidofovir and leflunomide, agents with in vitro antiviral activity, have been demonstrated to have some efficacy against PVAN (59, 60, 64, 106, 112). Decreased immunosuppression is contraindicated in patients with concomitant acute rejection; cidofovir combined with leflunomide is the only available treatment option for these patients. In case reports, cidofovir has also been used to treat BKV-induced hemorrhagic cystitis (47). Cidofovir has not been shown to be effective against replication of JCV in cell culture models (52).

Use of highly active antiretroviral therapy (HAART), including protease inhibitors, has been reported to prolong survival and significantly improve the health of some AIDS patients with PML (4, 29). This likely results from partial restoration of immunological function rather than from their specific action against JCV (109). Prolonged survival of HAART-treated PML patients has been shown to coincide with JCV clearance from the CSF, most likely due to partially restored intrathecal humoral responses (110). PML can progress in some AIDS patients despite effective HAART therapy (14). Inclusion of cidofovir in the drug regimen when HAART alone did not restore immune function has shown benefit to some PML patients (28, 96, 114).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Using BKV detection as the sole criterion for establishing the diagnosis of hemorrhagic cystitis is complicated by the fact that polyomaviruses have been detected in the urine of individuals without apparent disease (39, 70) including 10% of pregnant women, 30% of patients with renal tumors, bone marrow transplants, or renal transplants, 40 to 70% of the elderly, and many patients with human immunodeficiency virus type 1. These findings also preclude the use of viruria as a means of diagnosing acute polyomavirus infection (10, 12, 38). Recently, however, quantification of urine BKV concentrations has demonstrated that patients with hemorrhagic cystitis have greater viral loads in urine than asymptomatic patients, suggesting that optimal assay performance (sensitivity, specificity, positive predictive value, and negative predictive value) might be achieved through the application of quantitative cutoffs (65, 87).

Urine testing for BKV has been quite useful in the setting of PVAN following renal transplantation. The high negative predictive value of detection (>99%) allows effective exclusion of disease in appropriate patients (34, 35, 79). Positive urine test results require careful interpretation. Transient viruria has been observed following renal transplant; therefore, a single positive screening result is of little diagnostic value. Additional screening at regular intervals or confirmation by viral load measurement in peripheral blood or urine within 4 weeks has been recommended (49). Viral load assays (in urine or plasma) have been advocated to establish the presumptive diagnosis of PVAN. The positive predictive value of BK viral load testing is >90% when quantitative cutoffs are used (10^7 and 10^4 copies/ml in urine and plasma, respectively). However, since BKV quantification assays have not been standardized, individual laboratories need to establish and validate their own cutoffs. Renal biopsy should be performed after viral load measurement to establish a definitive diagnosis of PVAN (49).

Detection of JCV nucleotide sequences or virion particles in lesioned sites of the brain remains the definitive diagnostic indicator of PML. However, due to the invasive nature of biopsy, PCR assays of CSF have become the most frequently used alternative diagnostic tool. To date, JCV has been detected in the CSF of many individuals with PML (with or without AIDS), but not in the CSF of non-PML patients (105, 111). The ease, sensitivity, and specificity of JCV detection by PCR have alleviated the many difficulties of conventional virologic detection methods such as culture or serology.

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Human Parvoviruses*

JEANNE A. JORDAN

108

TAXONOMY

The *Parvoviridae* family includes the *Parvovirinae*, members of which infect vertebrates, and the *Densovirinae*, members of which infect insects. The *Parvoviridae* family consists of small, nonenveloped, single-stranded DNA viruses. The current classification of parvoviruses is based upon their host range and dependence on other viruses for their replication. The *Parvovirinae* subfamily has recently been subdivided into five genera: *Erythrovirus*, *Dependovirus*, *Parvovirus, Amdovirus,* and *Bocavirus* (3, 29, 87). *Bocavirus* has recently been identified as a cause of lower respiratory tract infections. However, this chapter focuses solely on the genus *Erythrovirus*, whose members replicate autonomously in erythroid progenitor cells, with a major emphasis on human parvovirus B19 (prototype strain, Au) (genotype 1). Cossart and colleagues first reported their discovery of B19 in 1975 after screening sera of blood donors for hepatitis B surface antigen using an Ouchterlony immunodiffusion technique (affected serum; number 19 in panel B) (25). Subsequent evaluation of this blood unit, using immune electron microscopy, revealed viral particles consistent in size and morphology with parvovirus. B19 is the predominant parvovirus pathogen in humans. The other, less common, and more recently described erythroviruses infecting humans include genotype 2 (prototype strain, LaLi) and genotype 3 (prototype strain, V9). Among B19 virus isolates, genetic diversity is low (<2%) at the DNA level. In contrast, there is significantly greater nucleotide divergence between genotype 1 (B19) and either genotype 2 or 3 (>11%), with slightly less divergence between genotypes 2 and 3 (~8%) (37, 73, 85). There is much less known about the transmission and epidemiology of genotypes 2 and 3 than for genotype 1.

DESCRIPTION OF THE AGENT

Many important observations regarding the B19 virion have been made using electron microscopy and X-ray crystallography (2, 21, 51). The structure of recombinant B19-like particles has been studied at ~3.5-Å resolution using the latter technology; the major virus capsid protein, VP2, has a structure like a jelly roll with a β barrel motif similar to that present in other icosahedral viruses (51). The diameter of the virus is approximately 26 nm. The complete virion has a molecular mass between 5.5×10^6 and 6.2×10^6 Da and a buoyant density in cesium chloride between 1.39 and 1.42 g/cm^3. The viral particle lacks an envelope, which contributes to the virus' tenacity to resist heat and solvent and detergent treatments, an important consideration when approaching the issue of virus inactivation in blood or blood products (22). The virion does not contain lipids or carbohydrates but does contain phospholipase A_2-like activity, which is required during host cell entry (19, 104).

The B19 capsid is comprised of two structural proteins, VP1 (the minor capsid protein [83 kDa]) and VP2 (the major capsid protein [58 kDa]), representing about 4 and 96% of the capsid, respectively (74). The major nonstructural protein is NS1 (71 kDa), a DNA binding protein involved in viral replication. The NS1 protein transactivates P_6, the single promoter present on the genome (79), and induces cellular apoptosis and activates the gene for the inflammatory cytokine interleukin-6 (62, 63, 65).

The B19 genome consists of a single-stranded 5.6-kb DNA genome; the 5′ and 3′ ends contain identical 365-nucleotide repeats (90), and there exist two large and two small open reading frames (ORFs) (86) (Fig. 1). The major left-hand ORF is translated into NS1, an early viral protein, while the major right-hand ORF is translated into the VP1 and VP2 structural proteins that are expressed later in infection. The antigenic domains of NS1 are located at its carboxy terminus (96). The VP1 and VP2 capsid proteins originate from the same ORF and are identical in sequence, except for an additional 227 amino acids at the amino terminus of VP1. Structurally, this unique region of VP1 is located on the exterior of the virion capsid and contains several neutralizing epitopes, most of which are linear in nature (69). In contrast, most neutralizing epitopes of VP2 are conformational (82). During replication, equal numbers of the plus and minus strands of DNA are packaged into separate virion particles, so that during DNA extraction, under the appropriate salt concentration, the complementary strands hybridize to form a double-stranded DNA structure (9). Zhi and colleagues have constructed an infectious clone of B19 (106).

B19 has an extremely limited cell tropism, replicating only in the nuclei of erythroid progenitor cells of the burst-forming unit and colony-forming unit (24, 89). B19 infection can have a profound effect on an individual, resulting in reticulocytopenia and an abnormally low hematocrit. The cellular receptor

*This chapter contains information presented in chapter 101 by Marialuisa Zerbini and Monica Musiani in the eighth edition of this Manual.

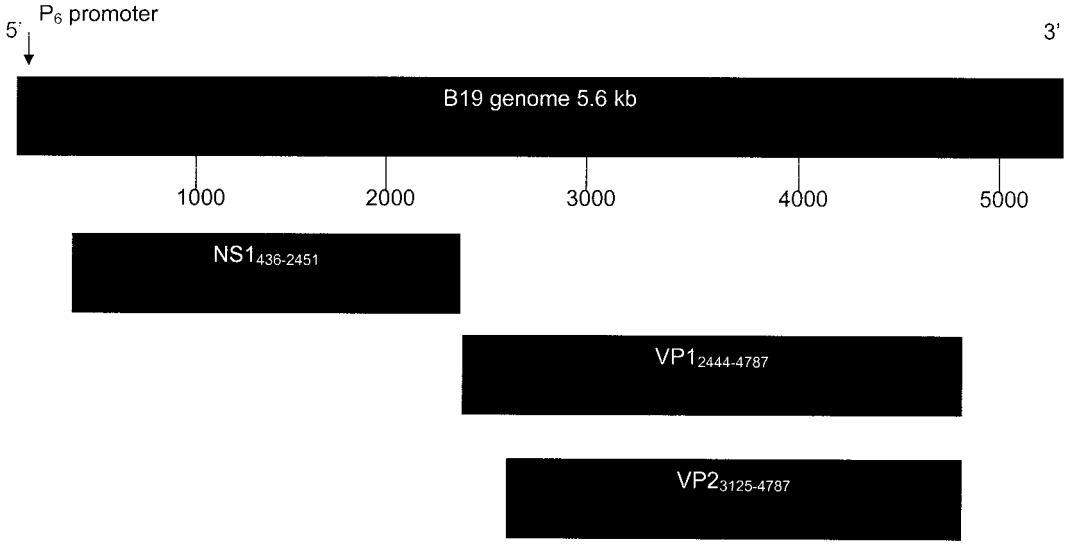


FIGURE 1 Schematic representation of human parvovirus B19 genome and transcription map (86), with the unique region of VP1 comprising bp 2444 to 3124.

that B19 binds to is the blood group P antigen, or globoside (11). Resistance in individuals to B19 infection can be attributed to a lack of the P antigen receptor on erythroid cells (13). The globoside receptor can also be found on other cell types, including cardiac myocytes, white blood cells, platelets, and trophoblasts, and may play a role in pathogenesis in these cell types (46, 97). A coreceptor has been identified that is necessary for virus entry into target cells, a process mediated by $\alpha5\beta1$ integrins (99). A permissive erythroid progenitor cell expresses high levels of both globoside and $\alpha5\beta1$ integrins, while the nonpermissive mature erythrocyte contains only globoside, lacking both $\alpha5\beta1$ integrins and a nucleus. Therefore, although globoside is necessary for infection, it is not sufficient for a cell to be permissive for B19 infection (98). Another key molecule required for B19 infectivity is phospholipase A_2, which is present in the VP1 unique region (19, 104). The enzyme cleaves phospholipids into arachidonic acid, a molecule used to synthesize both prostaglandins and leukotrienes, which play important roles in the inflammatory process.

EPIDEMIOLOGY AND TRANSMISSION

B19 is a common viral infection present worldwide. In temperate climates, epidemic manifestations occur more commonly in late winter, spring, or early summer. Epidemic peaks of B19 infection occur in cycles every 3 to 5 years. B19 infection is most common in young children and manifests itself as erythema infectiosum (EI) (fifth disease or slapped cheek syndrome) (6). In individuals who have disorders that shorten their red cell half-life, B19 infection results in transient aplastic crisis (TAC). Both EI and TAC manifestations are usually self-limiting illnesses. The incubation period for B19 infection is approximately 6 to 10 days.

B19 transmission most often occurs by the respiratory route through respiratory secretions and saliva that are expelled or released when an infected individual sneezes or coughs. Transmission is much more likely to occur among individuals in a household, day care, or crowded environment; this scenario places women with young children at high risk for acquiring infection, which can be problematic for the susceptible woman who becomes infected with B19 while pregnant. Vertical transmission of B19 occurs at a rate of approximately 30% and can result in transmission of the virus to her developing fetus. In the general population, the seroprevalence of B19-specific immunoglobulin G (IgG) rises steadily from 37% in children ages 1 to 5 years to 87% in individuals who are greater than 50 years (20). Between 50 and 60% of women in their reproductive years have circulating B19 IgG, leaving a significant percentage who do not (43).

B19 can also be transmitted parenterally from blood or blood products obtained from viremic donors (41, 81). The incidence of B19 in the blood of healthy donors ranges from 1 in 20,000 to 1 in 50,000 donors (41, 61). The risk of transmission is greater when the blood components are made from pooled units than with those made from single units, placing those individuals requiring repeated doses of any of these blood products at risk of becoming infected with B19 over time, including hemophiliacs, who require lifelong administration of clotting factor concentrate (15, 55, 81). Using nucleic acid amplification testing (NAAT), B19 DNA has been detected in numerous batches of albumin, factor VIII, factor IX, clotting factor concentrates, and immunoglobulin (10, 55). Recent observations in healthy volunteers suggest that acute B19 infection can occur from administration of blood components that contain $\geq 10^7$ genome equivalents/ml (geq/ml) of B19 DNA. In contrast, patients receiving $<10^4$ geq/ml have not shown evidence of virus transmission (15, 100). The presence of neutralizing activity in all of these pools may help to explain the lack of infectivity in pools with lower viral loads (15, 41).

CLINICAL SIGNIFICANCE

Symptoms associated with B19 infection can vary considerably, from asymptomatic to life threatening. In the immunocompetent individual, B19 infection is usually an acute self-limiting disease, while in those who are immunocompromised or who

have red cells with shortened half-lives, B19 infection can become chronic.

Much of what we first learned about B19 infection in the immunocompetent individual came from two studies out of the United Kingdom that were conducted using healthy adult volunteers (4, 78). These volunteers were infected intranasally with sera known to contain B19 particles. The infection in the B19-seronegative volunteers had two distinct phases, a nonspecific phase occurring at the end of the first week postinoculation and consisting of fever, headache, myalgia, chills, and/or itching, and a more specific phase occurring around day 17 or 18 postinoculation with symptoms including the absence of reticulocytes, followed by a significant drop in hemoglobin, along with a transient decrease in neutrophil, lymphocyte, and/or platelet counts. Viremia became detectable 6 days postinoculation, peaking on days 8 and 9. Viral levels rose to approximately 10^{10} to 10^{12} genome copies/ml of blood. B19-specific IgM antibodies became detectable 2 weeks postinoculation, while IgG antibodies appeared within days of detectable IgM antibodies (Fig. 2).

Anderson and colleagues first described EI in 1983 during an outbreak in a primary school in London, England (5). EI has a mean incubation period of approximately 17 to 19 days. Prodromal symptoms may include low-grade fever, headache, and one or more of the following: conjunctivitis, upper respiratory tract complaints, cough, myalgia, nausea, and/or diarrhea. The specific rash associated with B19 infection first appears on the face as a confluent maculopapular erythema on the cheeks, with relative perioral pallor. This manifestation provides the characteristic slapped-cheek appearance on the individual. The rash may appear next in a bilaterally symmetric fashion on the arms, legs, and trunk. Rarely are the palms of the hands and soles of the feet involved. The rash can wax and wane for an extended period, up to 3 weeks; a rise in body temperature, caused by fever, heat, exercise, or exposure to sunlight, can induce its reappearance.

TAC is an acute, self-limited cessation of the regenerative process within the erythropoietic cell lineage and is characterized by the abrupt onset of severe anemia that is associated with a dramatic decline in circulating reticulocytes. TAC was first described in 1981 as a clinical manifestation associated with B19 infection in children with sickle cell anemia and since then has been described to occur in a wide range of patients with underlying chronic hemolytic anemia or shortened red blood cell half-lives (14, 75).

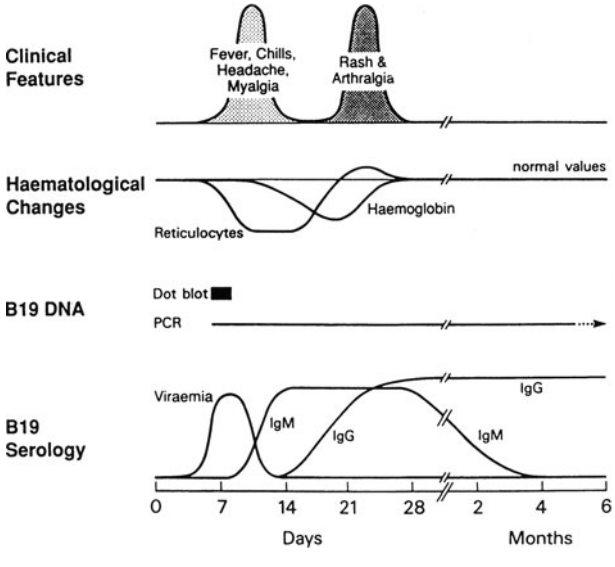


FIGURE 2 Clinical features, hematologic changes, B19 DNA presence, and serologic findings associated with B19 infection in a healthy individual (reprinted from reference 14).

One of the most important complications of B19 infection is its association with pregnancy loss (27, 94). Brown and colleagues first reported on the association of intrauterine B19 infection and fetal demise in 1984 (16). In a susceptible woman, infection with B19 during pregnancy can result in spontaneous abortion, stillbirth, severe fetal anemia, nonimmune hydrops fetalis, transfusion-dependent congenital anemia, hepatomegaly, or fetal death (12, 92). An inflammatory immune response and trophoblast apoptosis have been reported to occur in placentas from pregnancies with poor outcome due to B19 infection (45, 48). Although the rate of viral transmission across the placenta is relatively high, between 24 and 51% (102), the risk of fetal death is relatively low, between 1 and 10%, with the greatest risk being associated with infection occurring in or before the 20th week of gestation. B19 is responsible for 16 to 18% of all cases of idiopathic nonimmune hydrops fetalis (42). Although most cases of B19-induced fetal hydrops resolve spontaneously, the presentation can occur rapidly, within a few days, and result in fetal death. Weekly fetal ultrasonography monitoring for hydrops, for up to 14 weeks, is recommended for women who seroconvert to B19 positivity during pregnancy. The fetus is particularly vulnerable to B19 infection and developing hydrops during the second trimester, as this time correlates to the rapid expansion of red blood cells, including the erythrocyte precursor cell, the target cell for B19 infection.

B19 infection can be problematic for the immunocompromised patient, one with congenital or acquired immunodeficiency syndrome, a recipient of a solid-organ or bone marrow transplant, or a patient with a lymphoproliferative disorder or other malignancy, especially when receiving chemotherapy (33, 54). B19 is the leading cause of pure red cell aplasia in AIDS patients. An immunocompromised patient can present with acute B19 infection that does not resolve but becomes chronic; low- to medium-level viremia can persist for several months or even years, which can lead to selective aplasia of red blood cell precursors within the bone marrow, and occasionally to pancytopenia (71). Chronic infection can lead to persistent and severe anemia, due most likely to the patient's inability to mount an adequate immune response to clear the viremia. In these patients, clinical response to intravenous immunoglobulin (IVIg) is usually quite effective in eliminating the viremia (67).

In 1985, results from two large-scale studies were published describing the association between B19 infection and arthropathy (80, 101). A symmetric, polyarticular involvement of the joints can occur as a consequence of B19 infection in the presence or absence of the specific skin rash. Joint symptoms can occur in up to 33% of adults, being found more commonly in women than in men. Joint involvement is uncommon in children, occurring less than 10% of the time. The involved joints include the proximal interphalangeal joints of the hands and feet and, less frequently, the wrists, elbows, knees, and ankles. Swelling, warmth, redness, and stiffness can accompany the joint pain. These symptoms are usually self-limiting, remaining for a few weeks, but can persist for months or even years (72). The role of B19 in the etiopathogenesis of rheumatoid arthritis and chronic juvenile arthritis remains the subject of disagreement (18, 52).

The role of B19 has been firmly established for EI, TAC, nonimmune hydrops fetalis, chronic pure red cell aplasia, and arthropathy syndromes. However, less commonly recognized clinical manifestations have also been associated with B19, including glomerulonephritis, vasculitis, neurologic disease, myocarditis, hepatobiliary disease, rheumatic disease, hematologic disorders, and cutaneous eruptions apart from EI (93). The mechanism(s) for these various atypical clinical manifestations remains unknown, but has become a priority to elucidate.

Treatment and Prevention

Infection with B19 is usually a mild, self-limiting febrile illness and as such does not require specific therapy or treatment. Nonsteroidal anti-inflammatory drugs can provide relief to the symptomatic individual with arthritis or arthropathy. In cases where B19 infection is life threatening, treatment will vary with the clinical manifestations presented and the immune status of the patient (103). For instance, blood transfusion therapy can be used to provide temporary relief in patients with acute anemia. Severe fetal anemia, as a result of B19 infection, can be treated with one or more intrauterine transfusions of packed red blood cells. However, this treatment is not without its risks. Determining fetal anemia can now be accomplished noninvasively using Doppler measurements, which obviates funicentesis, fetal transfusion, and their associated risks if anemia is found to be absent (26, 66). IVIg therapy is generally limited to those patients suffering with chronic anemia due to B19 and has been used with much success in relieving anemia in these patients (67). Success with IVIg therapy has also been reported for treatment of juvenile polyarticular arthritis (95). However, it should be noted that as a pooled blood product, IVIg can contain B19 and therefore puts the susceptible patient at risk for acquiring B19 infection (28, 32, 35, 83).

Vaccine

Currently, preventing B19 infection is best accomplished through the use of good infection control practices to interrupt transmission, although developing a vaccine against B19 would be desirable. The most effective vaccine would contain both VP1 and VP2 antigens (8). Although VP1 comprises just 4% of the capsid, it contains important epitopes that illicit strong neutralizing antibody activity against the virus. Ideally, a vaccine should elicit both neutralizing-antibody production and a T-cell response (31, 91).

A recombinant human parvovirus B19 vaccine, composed of both VP1 and VP2 proteins that self-assemble into empty capsids resembling virus particles, was evaluated in a randomized, double-blind, phase 1 trial (7). The vaccine was given to 24 seronegative adults in a series of three doses over 6 months. The vaccine, formulated with MF59C.1 adjuvant, was found to be safe, well tolerated, and highly immunogenic. All 24 participants developed neutralizing antibodies (7). The results from this trial are promising for developing a vaccine that could protect against B19 infection and its associated risks.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Serum from whole blood, collected in a sterile tube lacking anticoagulant, is most suitable for serologic testing, although plasma, containing either EDTA or sodium citrate, can also be used. Whole-blood samples being transported to the laboratory should be maintained at 2 to 8°C; temperatures at or below freezing should be avoided during transport and storage to minimize red cell lysis. Serum or plasma, once separated from the cellular components of whole blood, can be stored for short periods at 2 to 8°C, or at −20 or −70°C for much more extended periods, before testing is performed.

Bone marrow aspirates, cord blood, amniotic fluid, and biopsy specimens of placenta and/or fetal tissues (liver, lung, and heart) are suitable for B19 DNA detection using in situ hybridization or NAAT, or for antigen detection using immunocytochemistry or immunohistochemistry. Bone marrow and cord blood samples should be collected in sterile tubes containing EDTA or sodium citrate. Heparin should be avoided for NAAT because of its inhibitory effect on the *Taq* polymerase unless DNA purification is included in the sample processing protocol. Amniotic fluid should be collected in a sterile tube lacking anticoagulant. Placental and fetal tissues can be placed directly into viral transport media or fixed using 10% buffered formalin. Cellular and fresh tissue samples should be maintained at 2 to 8°C for transport to the laboratory. Once again, freezing temperatures should be avoided for transport or storage prior to sample processing.

DIRECT DETECTION

Microscopic

Immune electron microscopy can be used to visualize the virus. However, this approach is not used routinely in the clinical laboratory for diagnosis.

Antigen

Immunocytochemical or Immunohistochemical Analyses

Cytospin preparations of bone marrow aspirates, cord blood, or amniotic fluid specimens can be analyzed by immunocytochemistry for the presence of B19-specific capsid antigen(s) using commercially available antibodies (49). These antibodies can also be used in an immunohistochemical analysis of formalin-fixed, paraffin-embedded placental or fetal tissues for viral antigens (49). However, these procedures are usually performed in an anatomical pathology laboratory or research laboratory, but not a clinical pathology laboratory.

Nucleic Acid

DNA In Situ Hybridization

The specimen types analyzed by immunocytochemistry or immunohistochemistry for viral antigen can be analyzed by in situ hybridization for B19 DNA (42). The DNA probes used for in situ hybridization consist of either short stretches of B19-specific oligonucleotides or a cloned plasmid containing a nearly full-length genome. Probes are most often labeled with either biotin or digoxigenin to avoid using a radioisotope. A colorimetric substrate is commonly used in conjunction with either alkaline phosphatase or peroxidase, whose products can be visualized using a light microscope. Increased analytical sensitivity can be achieved using a chemiluminescent substrate, but this strategy requires a high-performance luminometer and computer to be used in conjunction with an optical microscope.

NAAT

At present, NAAT is the most sensitive choice for detecting B19-specific DNA within a specimen, and is considered the test of choice for diagnosing infection in the immunocompromised or immunosuppressed individual, or in the fetus or

neonate, for whom using serologic tests would not be advisable. Nonhemolyzed serum or plasma samples can be analyzed for B19 DNA as a crude cell lysate without the need for DNA extraction and purification. However, DNA extraction and purification are recommended when analyzing hemolyzed serum or plasma, or highly cellular samples (bone marrow aspirates, cord blood, or amniotic fluid) or tissue specimens (fresh or formalin fixed) (105).

Numerous PCR-based primer and probe sets have been described for detecting B19-specific DNA (47, 68). It is important to note that most of these targets were designed to detect genotype 1, not genotype 2 or 3 necessarily. The earliest detection platforms consisted of agarose gel electrophoresis and Southern blot hybridization, or dot blot hybridization techniques, or enzyme immunoassay (EIA). More recently, numerous real-time PCR platforms have been developed in conjunction with fluorescence-labeled probes such as TaqMan, Molecular Beacons, or FRET probes (1, 34). This approach has many benefits, including speed, reduced risk of contamination, and ease of estimating viral load. Real-time PCR is readily adapted to either qualitative (endpoint analysis) or quantitative (viral load) detection. A quantitative assay for B19 DNA must demonstrate linearity over a wide range, as viral load can reach levels of 10^{12} geq/ml of blood during the acute phase of infection, but rapidly declines with the onset of a robust antibody production (70).

A U.S. Food and Drug Administration (FDA)-cleared NAAT assay is not currently available, although several manufacturers offer analyte-specific reagents for detecting B19 DNA. The LightCycler-Parvovirus B19 quantification kit (Roche Diagnostics, Indianapolis, Ind.) is highly sensitive for genotype 1 but is not suitable for detecting genotype 2 or one of the genotype 3 strains (39). In contrast, the RealArt Parvo B19 LC PCR (Roche Diagnostics) is suitable for detection, quantification, and differentiation of all three genotypes, albeit with lower sensitivity for certain strains (39). Because serology is insufficient for diagnosing B19 infection in the immunocompromised patient, it is imperative to assess the NAAT assay's performance for detecting the three genotypes. Investigator-designed real-time PCR assays targeting the NS1 gene or VP1 unique region have been successful in detecting genotypes 1 to 3 at similar sensitivities; these approaches use either consensus primer-based PCR followed by restriction fragment length polymorphism analysis or DNA sequencing, nested PCR, or multiplex PCR using genotype-specific primers and probes (39, 56, 84, 85).

ISOLATION PROCEDURES

Successful replication of B19 requires infection of an undifferentiated, actively replicating erythroid cell. Although freshly harvested bone marrow or fetal cord blood samples have been used successfully to propagate B19, it is impractical to use this approach routinely to culture B19 in the clinical laboratory.

IDENTIFICATION

This virus is not identified in the conventional sense using tissue culture but through molecular techniques that detect specific DNA sequences and by immunologic methods using antibodies that recognize specific viral capsid proteins.

TYPING SYSTEMS

DNA sequence analysis of the VP1 unique region or the NS1 gene can be used to differentiate among genotypes (39, 56, 84, 85).

SEROLOGIC TESTING

Serologic testing to detect B19-specific antibodies to viral capsid antigen remains the cornerstone in determining the immune status in immunocompetent individuals. In the practice of obstetrics, it is considered standard care for the physician to request B19-specific IgM and IgG serologic testing in the case of a pregnant woman with a history of exposure to a B19-infected individual. However, this approach would not be chosen when attempting to diagnose B19 infection in an immunocompromised individual, or in a fetus or neonate, due to the patient's limited ability to produce antibodies; instead, NAAT should be chosen for this purpose.

Most B19-specific serologic assays incorporate viral antigen(s) generated from recombinant expression vectors due to the limited availability of native B19 capsids. Both prokaryotic and eukaryotic expression systems have been used to produce viral capsid protein(s) (64) or self-assembled empty viral capsids (50). Most B19-specific serologic testing performed in the United States is done using one of two commercially available assays (17, 23). Biotrin International (Dublin, Ireland) manufactures B19-specific IgM and IgG EIAs for sale and distribution that have been cleared by the FDA; these kits use a μ capture EIA for B19-specific IgM antibody and a baculovirus-expressed conformational VP2 antigen for the B19-specific IgG EIA. The μ capture format is designed to minimize false-negative IgM results; microtiter wells are coated with anti-human IgM antisera, a process that allows for the binding of total IgM antibody in serum or plasma to the solid phase and thus reduces competition from IgG antibodies that can occur due to differences in concentration.

Focus Diagnostics Laboratory (Cypress, Calif.) is the other manufacturer of B19-specific IgM and IgG serologic tests, which are proprietary in nature but are known to incorporate only a linear (denatured) VP1 antigen purified from an *Escherichia coli* expression vector; these B19-specific IgM and IgG assays have not been FDA cleared.

It is well established that for better accuracy the B19-specific IgG EIA should incorporate a conformational capsid antigen (43, 53, 60). This is based on the fact that circulating IgG antibodies directed against linear epitopes of VP1 and VP2 gradually decline postinfection but that the IgG directed against conformational epitopes of those antigens are maintained long term.

EIAs measuring IgG avidity have also been developed. These assays are helpful in discriminating between primary and secondary infection but are not yet commercially available (77, 88). Standardizing B19 IgG results among laboratories and test systems can be achieved using an international standard (The 2nd International Standard, 2003; code 01/602; 77 IU per ampoule) (30). No such standard is available for B19 IgM determinations.

Commercially available indirect fluorescent-antibody (IFA) and Western blot assays can also be used to detect B19-specific IgM or IgG antibodies (59, 76). In the former, the antigen consists of a conformational VP1 protein expressed in insect cells using a recombinant baculovirus expression vector. Sera for IFA B19-specific IgM antibody testing should be pretreated with a suitable adsorbent reagent to prevent interference from rheumatoid factors (which generates a false-positive result) and reduce IgG competition (which produces a false-negative result). These assays can be used as a confirmatory assay in conjunction with an EIA that utilizes the linear, denatured VP1 antigen. In the latter assay, VP1, VP2, and NS1 antigens are used (38). However, this approach has its limitations, as the antigens are denatured, which may limit their diagnostic value.

In summary, unlike the significant variation in genomic sequence that exists among the three genotypes of *Erythrovirus*, there is a high degree of homology in the VP2 capsid protein for these three genotypes (85). This is expected to result in a high level of immunologic cross-reactivity of IgM and IgG. Therefore, choosing an EIA that incorporates a conformational VP2 antigen would be desirable for detecting all three genotypes of *Erythrovirus*.

ANTIVIRAL SUSCEPTIBILITIES

To date, there is no antiviral agent described for treatment of B19 infection.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Laboratory tests are essential when attempting to diagnose B19 infection due to its wide range of clinical presentations and patients affected. Determining whether to use serologic tests and/or NAAT is largely dependent upon the patient's immune status. Serologic tests should be used in diagnosing infection of or assessing immune status in the immunocompetent individual. In contrast, NAAT is the test of choice for screening the immunocompromised or immunosuppressed individual or diagnosing congenital or neonatal infections, as serologic screening would be unreliable.

Unlike serologic testing, where only serum or plasma samples are acceptable, NAAT for B19 DNA can be performed using a wide range of specimen types. The choice of specimen(s) is largely dependent upon the clinical manifestations of the patient, with each specimen type requiring validation before testing can be implemented clinically.

Assay design and antigen type(s) incorporated into a kit are important features to note when evaluating a commercial assay (44, 57). Capture EIAs employing native or recombinant antigens are excellent choices for detecting B19-specific immunoglobulin (43). Systems consisting of either *E. coli*-expressed or baculovirus-expressed B19 capsid antigens or both in combination have been described. Capture EIAs incorporating conformational antigens (nondenatured) only, or in combination with linear antigens (denatured), are superior to those utilizing denatured, linear antigens (denatured) alone (53). Several studies have demonstrated differences in B19 IgM and IgG reactivity against conformational and linear epitopes of VP1 and VP2 (53, 58, 60). Accurate B19 serologic diagnosis requires the presence of nondenatured, conformational antigens. Recombinant B19 nonstructural protein NS1 can also be expressed using either a prokaryotic or eukaryotic expression system. The presence of antibodies to NS1 is associated with persistent B19 infection and chronic arthritis (40).

Testing sera for the presence of B19-specific IgM antibodies is the most common approach for diagnosing acute infection in the immunocompetent individual (36). Detectable levels of B19-specific IgM can be found within 7 to 10 days of exposure to the virus; IgM antibodies remain measurable for 2 to 3 months before diminishing. Occasionally, IgM antibodies can persist for longer periods, 6 months or more. Therefore, their presence, especially at low levels for an extended period, is suggestive but not conclusive proof of recent infection. Acute infection can also be diagnosed by demonstrating a fourfold or greater rise in B19-specific IgG antibody titer in a patient's sera. However, this procedure requires two separate time points for sample collection and is considered impractical in most clinical situations.

Testing sera for the presence of B19-specific IgG antibodies can determine past or previous infection in the immunocompetent individual. Detecting the presence of IgG antibody, without IgM antibody, implies immunity in the patient. The antibody response to linear or denatured viral capsid proteins diminishes with time. In contrast, the antibody response to conformational capsid proteins persists for years.

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Poxviruses*

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Interest in poxviruses has undergone a resurgence in part because of bioterrorism response concerns (6, 35, 76) and because of the use of recombinant poxviruses for basic research, clinical therapeutics, and vaccines (38, 39, 43, 44, 61, 83, 85, 86). In preparing for the threat of smallpox, the U.S. government has developed a coordinated national response plan to refurbish relevant components of the public health infrastructure (6, 35, 47, 76, 83), including replenishing the vaccine and vaccinia immunoglobulin stockpiles, revitalizing poxvirus health education and epidemiologic training, developing antivirals and new diagnostics, and stimulating basic and recombinant poxvirus research. Additionally, periodic introductions of enzootic poxviruses continue to pose a risk to human health (2, 5, 7, 8, 15, 37, 40), as evidenced by the introduction of monkeypox into the United States in 2003 (74) and the ongoing apparent increased incidence of human monkeypox in the Democratic Republic of Congo (DRC, formerly Zaire) (5, 7, 8, 40, 62, 68) and other Congo Basin countries (46), as well as recurrences of vaccinia-like viruses infecting agricultural workers in Brazil (52, 63, 93).

TAXONOMY

All poxviruses described in this chapter belong to the family *Poxviridae* and subfamily *Chordopoxviridae* (61). The taxonomic structure of the family *Poxviridae* has been reviewed previously (59). The genuses and species of the viruses discussed in this chapter are shown in Table 1. DNA-based assays, including DNA sequencing, are the most precise methods for poxvirus genus, species, strain, and variant identification and differentiation (20, 23, 25, 27, 28, 44, 55). The G+C contents of orthopoxviruses, yatapoxviruses, *Molluscum contagiosum virus* (MCV), and parapoxviruses are ~33, ~32, ~60, and ~63%, respectively.

EPIDEMIOLOGY AND TRANSMISSION

All current, naturally occurring poxviruses that infect humans are sporadic zoonotic agents except the *Molluscipoxvirus*, MCV (71, 73, 94), which is transmitted strictly between humans. The zoonotic poxviruses include members of the genera *Orthopoxvirus* (monkeypox virus [40, 85], cowpox virus [4], and the vaccinia virus subspecies, including buffalopox virus [20, 53]), *Parapoxvirus* (Orf, milker's nodule, sealpox, and papulosa stomatitis viruses [11, 71, 75]), and *Yatapoxvirus* (tanapox virus [TPV], Yaba monkey tumor virus [YMTV], and Yaba-like disease virus [YLDV] [16, 39, 44, 79]). Orf virus and MCV infections are the most common poxvirus infections worldwide. These dermatologic lesions often can be readily identified, and laboratory confirmation of clinical diagnosis is often not utilized (22, 71, 72, 75).

Orthopoxviruses

Variola Virus

Variola virus, the cause of smallpox, has a strict human host range and no animal reservoir. The virus was most often transmitted between humans by large-droplet respiratory particles inhaled by susceptible persons who had close, face-to-face contact with an infectious person. It was spread less commonly by aerosol contact or by direct contact with the rash lesion contact or by sloughed crust material from the scab (30).

Monkeypox Virus

Human monkeypox was first reported in 1970 in the DRC. Since 1970, the disease has been seen in Liberia, the Ivory Coast, Sierra Leone, Nigeria, Benin, Cameroon, and Gabon, but most cases have been in the DRC. In the 1980s, serosurveys and virologic investigations in the DRC by the World Health Organization (WHO) indicated that (i) monkeys are sporadically infected, as are humans; (ii) three-fourths of cases, mainly those in children under 15 years of age, were from animal contact; (iii) the protective efficacy of vaccinia vaccination is about 85%; (iv) monkeypox virus has a broad host range, including squirrels (*Funisciurus* spp. and *Heliosciurus* spp.); and (v) human monkeypox has a secondary attack rate of 9% among unvaccinated contacts within households (i.e., it is much less transmissible than smallpox). In a recent outbreak in the DRC, about 250 serosubstantiated cases of monkeypox occurred among 0.5 million people in 78 villages from February 1996 to October 1997. Unlike those in the earlier outbreak, about three-fourths of the cases appeared to result from human-to-human transmission; however, the secondary attack rate of 8% among unvaccinated contacts within households appeared to be about the same as that found in the 1981-to-1986 surveillance (5, 7, 8, 37).

* This chapter contains information presented in chapter 105 by Inger K. Damon and Joseph J. Esposito in the eighth edition of this Manual.

TABLE 1 Taxonomy of poxviruses that infect humans

Genus	Species
Orthopoxvirus	Variola virus, vaccinia virus, cowpox virus, monkeypox virus
Parapoxvirus	Orf virus, milker's nodule virus, papulosa stomatitis virus
Yatapoxvirus	Tanapox virus, Yaba-like disease virus, Yaba monkey tumor virus
Molluscipoxvirus	Molluscum contagiosum virus

More recent case series have been reported from the DRC (54), and disease with a sustained chain of human-to-human transmission was reported in the Republic of Congo in 2003 (46).

The emergence of monkeypox in the United States provides another example of the ability of this zoonotic disease to exploit new ecologic niches. The North American prairie dog, diseased after exposure to an infected African rodent(s), subsequently infected and caused illness in U.S. human populations (9). The characterization of human disease in the United States, and its comparison with that classically described in the Congo Basin in the 1980s, enabled clinical and epidemiologic descriptions of distinct viral diseases.

Vaccinia Virus

Certain strains of vaccinia virus were used for human vaccination to globally eradicate smallpox (19, 22, 30, 32, 59). Since the early 1980s, vaccinia and certain other poxviruses have been used as recombinant vectors for the expression of a variety of proteins, including vaccine immunogens (38, 39, 43, 44, 61, 83, 85, 86). Infection can be transmitted to laboratory workers by accidental exposure, and significant pathology has been observed in unvaccinated individuals (48). Vaccination is therefore currently recommended for personnel working with live, replicative orthopoxviruses including vaccinia virus.

The origin of vaccinia virus is uncertain (59). Vaccinia virus infections are not generally regarded as naturally occurring, although vaccinee-to-cattle and cattle-to-human transmissions occurred on farms during the smallpox eradication campaign. Sporadic outbreaks of infection caused by the vaccinia virus subspecies buffalopox virus that involve transmission between milking buffalo, cattle, and people have been reported, mainly in India but also in Egypt, Bangladesh, Pakistan, and Indonesia. Vaccinia-like lesions have been observed on the animals' teats and the milkers' hands; milk is infectious. Biological data and limited DNA analyses of isolates from an outbreak in India in 1985 suggest that buffalopox virus may be derived from vaccinia virus strains transmitted from humans to livestock during the smallpox vaccination era (20, 53).

Quite interestingly, multiple distinct vaccinia viruses, possibly related to the vaccine strain used during smallpox eradication in Brazil, were recently found in cattle and their farm worker handlers in rural Rio de Janeiro (15, 81) as well as various locales within the Minas Gerais state (15, 52, 63, 93). Historic collections, when reevaluated with additional techniques, suggest that vaccinia-like viruses were previously isolated in the 1960s and 1970s (14). Inadvertent exposure to a vaccinia virus-vectored recombinant rabies virus vaccine dispersed to control rabies in wildlife has resulted in at least one instance of human infection; the bait was carried home by the family dog (80).

Cowpox Virus

Cowpox, sometimes a rare occupational infection of humans, can be acquired by contact with infected cows. Other animals, e.g., infected rats, pet cats, and zoo and circus elephants, have more often been sources of the disease. Cowpox virus is a rather diverse species and has been isolated from humans and a variety of animals in Europe and adjoining regions of Asia (2, 4). A serosurvey of wild animals in Great Britain found orthopoxvirus antibodies in a portion of bank and field voles and wood mice, which is consistent with small rodents' being reservoir hosts (3).

Yatapoxviruses

The epidemiology and natural history of yatapoxviruses are poorly understood. YMTV and YLDV infections have occurred in animal handlers (79); however, TPV is the main naturally occurring human pathogen in the *Yatapoxvirus* genus (31). Tanapox is an endemic zoonosis of equatorial Africa that is thought to be transmitted mechanically to humans by biting insects, especially during the rainy season (39). Recent reports have demonstrated that travelers to regions where the disease in endemic can be infected (16, 90).

Parapoxviruses

Three different parapoxvirus diseases occur in humans (31, 75), generally as occupational infections: milker's nodule (in dairy cattle, the disease is termed pseudocowpox or paravaccinia), Orf (in sheep and goats, the disease has been referred to as Orf, contagious ecthyma, contagious pustular dermatitis, contagious pustular stomatitis, and sore mouth), and papulosa stomatitis (in calves and beef cattle, the disease is termed bovine papular stomatitis). Parapoxvirus infections are transmitted to humans by direct contact with infected livestock through abraded skin on the hands and fingers, and ocular autoinoculation sometimes occurs (31, 71).

Molluscipoxvirus

MCV is the sole member of the genus *Molluscipoxvirus.* MCV appears to have a human-restricted host range, and it does not grow readily in culture. Molluscum contagiosum occurs worldwide. In children it is transmitted by direct skin contact, and sexual transmission occurs in adults (31, 71).

DESCRIPTION OF THE AGENTS

Virions are large and brick shaped (orthopoxviruses, yatapoxviruses, and molluscipoxvirus) or ovoid (parapoxviruses). Virions range in length from 220 to 450 nm and in width and depth from 140 to 260 nm. The appearances of virions under an electron microscope vary somewhat with sample preparation. By cryoelectron microscopy, in unstained, unfixed vitrified specimens, vaccinia virus appears as smooth, rounded rectangles; a uniform core is surrounded by a 30-nm-thick membrane. In conventional negatively stained thin sections, the core appears dumbbell shaped and is surrounded by a complex series of membranes. Lateral bodies of undefined function occupy the space between the outer membrane and the bar of the dumbbell. This feature may be a dehydration effect of negative staining.

Virus particles contain about half of the approximately 200 potential virus genome-encoded proteins. Virions consist of structural proteins and enzymes, including a virtually complete RNA polymerase system for primary transcription of viral genes (59). The genome is a 130- to 375-kbp (depending upon the genus) double-stranded DNA molecule that is

encapsidated in a nucleoprotein complex (nucleosome) inside the core. The genome is covalently closed at each end, and its ends are hairpin-like telomeres. Complete genome DNA sequences have been reported for several different poxviruses. GenBank entries are compiled at a dedicated website (http://www.poxvirus.org).

During virus replication (59), virion morphogenesis begins in the cytoplasm in areas known as cytoplasmic factories, where cellular organelles are largely absent. Thin-section electron microscopy observations of cells early after infection show crescent-shaped membrane structures, which progress to ovoid structures, called immature virions, which enclose a dense nucleoprotein complex. Primary transcription precedes the production of the crescents (cup-shaped in three dimensions) and the immature virons. Brick-shaped, membrane-covered mature virions (MVs; also known as intracellular mature virions [IMVs]) form as the viral core condenses.

A small portion of MVs may be further transported on microtubules from the viral factory and processed to acquire a bilayer tegument (envelope) of Golgi intermediate compartment membrane that contains specific viral proteins. The intracellular enveloped MV then moves along cellular microtubules to the cell surface, where the outermost membrane fuses with the cellular membrane to reveal a cell-associated enveloped virus on the cell surface. The cell-associated enveloped virus can prompt actin polymerization behind the virion, which may facilitate cell-to-cell infection with virus. Enveloped virions can also exit the cell to spread more distantly.

A virus receptor has not been identified. Enveloped and nonenveloped forms attach to cells differently; however, recent studies suggest that the nonenveloped particle (IMV) is the particle which enters, via fusion, into the host cell. A complex of viral proteins is believed to act as a fusion complex. The common result of entry is uncoating of the particle, release of viral contents into the cell, and initiation of virus-controlled transcription of early-class proteins. Recent reviews of virion entry, morphogenesis, and exiting processes have been published (60, 86–88).

CLINICAL SIGNIFICANCE

Orthopoxviruses

A global commission of the WHO declared smallpox eradicated in December 1979, and the declaration was sanctioned by the World Health Assembly in May 1980 (30). Human monkeypox, an emerging zoonotic smallpox-like disease caused by monkeypox virus, with recurrent (and likely endemic) disease in the Congo Basin countries of Africa, is now regarded as the most serious naturally occurring human poxvirus infection (5, 37, 40, 54). The emergence of monkeypox virus as a human pathogen in the United States in 2003 is a classic example of a pathogen's exploiting new ecologic niches and hosts. Ectromelia (also known as mousepox) virus is an orthopoxvirus that has been implicated in various human infections (97); however, such reports are not well founded because the virus can be a notorious contaminant of laboratory mice (17) used to produce reagents for diagnostics.

Variola Virus

Variola major virus strains produced a severe prodrome, fever, prostration, and a rash. Toxemia or other forms of systemic shock led to case fatality rates of up to 30%, with secondary attack rates of 30 to 80% among unvaccinated contacts within households. Variola minor virus strains (alastrim, amass, and kaffir viruses) produced less severe infection and case fatality rates of less than 1%, although secondary attack rates among unvaccinated contacts within households still ranged from 30 to 80%. DNA and biological data have indicated that alastrim variola minor viruses obtained from Europe and South America are similar to each other but distinct from the so-called African variola minor viruses, which appear to be variola major virus variants (19). The last naturally occurring smallpox case occurred in Somalia in October 1977, although a fatal laboratory-associated infection with variola major virus occurred at the University of Birmingham, Birmingham, England, in August 1978 (30).

Naturally acquired variola virus infection caused a systemic febrile rash illness. For ordinary smallpox, the most common clinical presentation, after an asymptomatic incubation period of 10 to 14 days (range, 7 to 17 days), was fever, quickly rising to about 103°F, sometimes with dermal petechiae. Associated constitutional symptoms included backache, headache, vomiting, and prostration. Within a day or two after incubation, a systemic rash appeared that was characteristically centrifugally distributed (i.e., lesions were present in greater numbers on the oral mucosa, face, and extremities than on the trunk). Initially, the rash lesions appeared macular, then papular, enlarging and progressing to a vesicle by day 4 to 5 and a pustule by day 7; lesions were encrusted and scabby by day 14 and sloughed off. Skin lesions were deep seated and were in the same stage of development on any one area of the body. Milder and more severe forms of the rash were also documented. Less severe manifestations (modified smallpox, or variola sine eruptione) occurred in some vaccinated individuals, whereas hemorrhagic or flat-pox types of smallpox occurred in patients with impaired immune responses.

Variola major smallpox was differentiated into four main clinical types: (i) ordinary smallpox (~90% of cases) produced viremia, fever, prostration, and rash; (ii) modified smallpox (5% of cases) produced a mild prodrome with few skin lesions in previously vaccinated people; (iii) flat smallpox (5% of cases) produced slowly developing focal lesions with generalized infection and an ~50% fatality rate; and (iv) hemorrhagic smallpox (<1% of cases) induced bleeding into the skin and the mucous membranes and was invariably fatal within a week of onset. A discrete type of the ordinary form resulted from alastrim variola minor infection (30).

Prior to its eradication, smallpox as a clinical entity was relatively easy to recognize, but other exanthematous illnesses were mistaken for this disease (30). For example, the rash of severe chickenpox, caused by varicella-zoster virus, was often misdiagnosed as that of smallpox. However, chickenpox produces a centripetally distributed rash and rarely appears on the palms and soles. In addition, in the case of chickenpox, prodromal fever and systemic manifestations are mild, if present at all. Chickenpox are superficial in nature, and lesions in different developmental stages may be present on the same area of the body. Other diseases confused with vesicular-stage smallpox included monkeypox, generalized vaccinia, disseminated herpes zoster, disseminated herpes simplex virus infection, drug reactions (eruptions), erythema multiforme, enteroviral infections, scabies, insect bites, impetigo, and molluscum contagiosum. Diseases confused with hemorrhagic smallpox included acute leukemia, meningococcemia, and idiopathic thrombocytopenic purpura. The Centers for Disease Control and Prevention (CDC), in collaboration with numerous professional organizations, has developed an algorithm for evaluating patients for smallpox. The algorithm

and additional information are available at http://www.cdc.gov/nip/smallpox and at www.bt.cdc.gov/EmContact/index.asp. Experience with the algorithm has been summarized previously (83).

Monkeypox Virus

Reviews of human monkeypox infection are available (5, 40). Monkeypox was first recognized by Von Magnus in Copenhagen in 1958 as an exanthem of primates in captivity. Later, the disease was seen in other captive animals, including primates in zoos and animal import centers.

The clinical appearance of human monkeypox, typified by the Congo Basin variant, is much like that of smallpox, with fever, a centrifugally distributed vesiculopustular rash (appearing also on the palms and soles), respiratory distress, and in some cases, death from systemic shock. Like variola virus, monkeypox virus appears to enter through skin abrasions or the mucosa of the upper respiratory tract, where it produces an enanthem and cough. During the primary viremia, the virus then migrates to regional lymph nodes, and during secondary viremia, it is disseminated throughout the body and the skin rash appears. During the prodrome, lymphadenopathy (generally inguinal) with fever and headache is common. Individual skin lesions develop through stages of macule, papule, vesicle, and pustule. Sequelae involve secondary infections, permanent scarring and pitting at the sites of the lesions, and sometimes alopecia and corneal opacities. Acute illness in the United States in 2003, caused by the "West African" variant, appeared generally more mild (49). Additional information on clinical manifestations of disease (36) is also available.

Vaccinia Virus

Humans have historically encountered vaccinia virus most commonly in the form of smallpox vaccine (now called vaccinia vaccine), a live-virus preparation that is cross-protective against other orthopoxvirus infections. The most recent recommendations of the Advisory Committee on Immunization Practices (ACIP) on vaccinia vaccination are available at http://www.cdc.gov/mmwr//preview/mmwrhtml/rr5010a1.htm (10). The ACIP recommends vaccination as a safeguard for laboratory and health care workers who are at high risk of orthopoxvirus infection. In the United States, the CDC Drug Service provides the vaccine after CDC approval of a formal request for this purpose by the administering physician. Vaccinia immunoglobulin is available to treat possible postvaccination complications, which can be severe. Although both products were previously licensed by the Food and Drug Administration, their use currently requires execution of an investigational new drug application because of changes in vaccine diluent formulation and a discoloration of the immunoglobulin.

Vaccination is done by using a multiple puncture technique that causes a local lesion, which develops and recedes in a distinctive manner in primary vaccinees during a 3-week period. At the site of percutaneous vaccination, a papule forms within 2 to 5 days, and the lesion reaches maximum size (about 1 cm in diameter) by 8 to 10 days postvaccination after evolving through vesicle and pustule stages; an areola may encircle the site. The pustule dries into a scab, which usually separates by 14 to 21 days after vaccination. In some vaccinated children, fevers with temperatures as high as about 100°F have occurred but have been uncommon in adults, and a regional lymphadenopathy has been observed.

Because of an increased risk for serious adverse events, such as eczema vaccinatum or vaccinia necrosum, the ACIP has stated that the vaccine is contraindicated for persons with eczema or immunocompromising conditions. The possible complications of vaccinia vaccination are described in the ACIP report (10). Despite attempts to prescreen potential vaccinees for contraindications, instances of generalized vaccinia rash, which may arise 10 to 14 days postvaccination, continue to be reported (42). On a clinical basis alone, it is often difficult to distinguish between generalized vaccinia, which represents virus presumably spread hematogenously, and a form of erythema multiforme, which may be immunologically mediated. Laboratory identification of virus within the disseminated rash may differentiate these conditions (42).

Cowpox Virus

In humans, cowpox lesions occur mainly on the fingers, with reddening and swelling. Autoinoculation of other parts of the body may occur, and severe systemic infections have been reported. Skin lesions are likened to those from a primary vaccinia virus vaccination. The site becomes papular, and a vesicle develops in 4 to 5 days. Healing takes about 3 weeks (2, 22, 31, 32).

Yatapoxviruses

The three members of the genus *Yatapoxvirus*, TPV, YLDV of monkeys, and YMTV, are serologically related (79). DNA maps of TPV and YLDV are extremely similar, suggesting that they are the same agent. However, these DNA maps are markedly different from YMTV DNA maps, even though the DNA from the three viruses cross hybridizes extensively (44).

TPV and YLDV infections in humans consist of a brief fever, followed by development of firm, elevated, round, maculopapular nodules which become necrotic and are distinct from the vesiculopustular lesions of orthopoxvirus infections. Generally, few lesions develop, and these occur primarily on the skin of the upper arms, face, neck, or trunk (39). Symptoms that occur prior to the appearance of lesions include fever, backache, and headache. Lesions umbilicate without pustulation during recovery from infection. They usually heal in 2 to 4 weeks. YMTV produces epidermal histiocytomas, tumor-like masses of histiocytic polygonal mononuclear cell infiltrates that advance to suppurative inflammation.

Parapoxviruses

Milker's nodule occurs as a reddened hemispheric papule that matures into a purplish, smooth, firm nodule varying up to 2 cm in diameter. The lesions usually are not painful and can persist for about 6 weeks. Human Orf virus infection is usually found on the fingers, hands, and arms but may also be found on the face and neck. Fever and swelling of draining lymph nodes may be present, and the lesions often ulcerate and are painful. Autoinoculation of the eye may lead to serious sequelae (71). Contact with (e.g., skinning of) certain wild animals, including deer, reindeer, chamois, and Japanese serow, has also been a source of human parapoxvirus infection. Technicians handling gray seals have contracted sealpox virus (11, 75).

Molluscipoxvirus

In children and teenagers, molluscum contagiosum lesions generally appear on the trunk, limbs (except the palms and soles), and face, where there may be ocular involvement. Infection is usually transmitted by direct skin contact. When MCV infection is transmitted sexually among teenagers and adults, the lesions are mostly on the lower abdomen, pubis, inner thighs, and genitalia. Lesions are pearly, flesh-colored,

raised, firm, umbilicated nodules, about 5 mm in diameter. The lesions tend to disseminate by autoinoculation. Prior to highly active antiretroviral therapy, MCV was an opportunistic pathogen in approximately 15% of AIDS patients in the United States. Restriction endonuclease mapping of isolates suggests that there are at least three MCV subtypes (73). Two predominant MCV subtypes, MCVI and MCVII, have been detected in a limited number of samples examined by restriction pattern and base sequence analyses, but no correlation of subtype with disease syndrome or geographic distribution has been confirmed (71–73). A rapid PCR and restriction fragment length polymorphism analysis using skin lesion material has been described previously for differentiating the MCV subtypes (69).

Clinical Utility of Laboratory Tests for Poxvirus Diagnosis

Poxvirus infections can often be distinguished by the appearances of rashes and associated dermatopathologies (18, 58). In addition, multiple different clinical laboratory tests can be useful for identifying and differentiating poxviruses, including electron microscopy, antigenic testing, nucleic acid detection, determination of virus growth features, and serology (12, 19, 20, 23, 31, 39, 44, 53, 95, 96). The utility of these test methods for the diagnosis of poxvirus infections is shown in Table 2.

COLLECTION, HANDLING, AND STORAGE OF SPECIMENS

A suspected case of smallpox should be immediately reported to the appropriate local or state health department. After review by the health department, the case should be immediately reported to the CDC if the diagnosis of smallpox is still suspected. Current international recommendations advise that work with variola virus be done using WHO-sanctioned biosafety level 4 laboratories. Two WHO collaborating centers (WHOCC) currently have the capability to handle smallpox specimens: one at the CDC in Atlanta, the other at the State Center for Virology and Biotechnology, Koltsovo, Russia. The WHOCC at the CDC also has containment facilities appropriate to work with monkeypox virus and other exotic poxviruses (e.g., TPV). Generally, clinical specimens suspected of containing other poxviruses (e.g., parapoxviruses and MCV) can be tested by experienced local staff using biosafety level 2 containment facilities and equipment.

Suitable specimens for laboratory testing of most suspected poxvirus infections are at least two to four scabs and/or material from vesicular lesions. Scabs can be separated from the underlying intact skin with a scalpel or a 26-gauge needle, and each specimen should be stored in separate containers to avoid cross-contamination. Coexistent infectious rash illnesses, including simultaneous chickenpox and monkeypox infections, have been noted (37).

Lesions should be sampled so that both the vesicle fluid and the overlying skin are collected. Once the overlying skin is lifted off and placed in a specimen container, the base of the vesicle should be vigorously swabbed with a wooden applicator or polyester or cotton swab. The viscous material can be applied onto a clean glass microscope slide and air dried. A "touch prep" can be prepared by pressing a clean slide onto the opened lesion by using a gradual pressing motion. If available, three electron microscope grids can be applied in succession (shiny side to the unroofed vesicle) to the lesion by using minimal, moderate, and moderate pressure. The glass slides and electron microscope grids should be allowed to air dry for about 10 min and then placed in a slide holder or a grid carrier box for transport to a laboratory.

Alternative lesion sampling processes, including storing material on appropriate filter paper types, are being evaluated. Sample storage in transport medium (as done, for example, with herpesviruses) is discouraged, since specimen dilution decreases the sensitivity of direct evaluation by electron microscopy. Specific recommendations for electron microscopy sampling and specimen processing can be found on the Internet (http://www.bt.cdc.gov/agent/smallpox/lab-testing/pdf/em-rash-protocol.pdf). Biopsy of lesions may also provide material suitable for direct viral evaluation or immunohistochemistry. A 3- to 4-mm-punch biopsy specimen can be made, and the specimen can be bisected, with half placed in formalin for immunohistochemical testing and the remainder placed in a specimen collection container. Blood and throat swabs obtained from suspected smallpox patients during the prodromal febrile phase and early in the rash phase were also a potential source of virus during the smallpox era (66).

Patient serum can also be obtained for serology to substantiate viral infection diagnoses or to infer a retrospective diagnosis. Paired acute- and convalescent-phase serum specimens can be of great value for diagnosis of infection. In this case, serum should be obtained as early as possible in the disease course and then 3 to 4 weeks later.

Most virus-containing specimens should be stored frozen at –20°C or on dry ice until samples reach their transport destination. Storage at standard refrigerator temperatures is acceptable for less than 7 days. Electron microscopy grids and formalin-fixed tissues should be kept at room temperature.

DIRECT DETECTION

Microscopy

Electron microscopy is a first-line method for laboratory diagnosis of poxvirus infections. Negative-stain electron microscopy of lesion samples was widely used during the

TABLE 2 Diagnostic tests for poxviruses[a]

Virus(es)	HP	EM	HA	NAT	Isolation	Serology
Orthopoxviruses	X	X	X	X	X[b]	X
Parapoxviruses	X	X		X	X[c]	X
Yatapoxviruses	X	X			X[c]	X
MCV	X	X		X		

[a] Abbreviations: HP, histopathology; EM, electron microscopy; HA, hemagglutination; NAT, nucleic acid test. X's indicate the utility of the tests for the specified viruses.
[b] Pock formation on CAM and tissue culture isolation are useful.
[c] Isolation in tissue culture only; viruses do not produce pocks on CAM.

smallpox eradication era. Because the clinical diagnosis of poxvirus infection is now infrequent, electron microscopy observations may provide one of the first clues into the cause of an unknown rash illness. Although electron microscopy can distinguish between orthopoxvirus and parapoxvirus morphologies, it cannot differentiate between species; selected serologic, biologic, and DNA-based assays must be used.

Orthopoxviruses have a distinctive brick-shaped, knobby morphology when examined with sodium phosphotungstate or other heavy-metal negative stains. Parapoxviruses appear ovoid with a spiraling criss-cross surface. The relative sensitivity of the negative-stain method was about 95% for detecting variola virus in smallpox lesions and about 75% for detecting vaccinia virus (66). Recent reports suggest that the sensitivity of such methods can be improved by directly pressing a prepared grid into the base of an unroofed skin lesion, as described above (33). Descriptions of methods for negative-stain evaluation and pictures of negative-stained particles are available on the Internet (http://www.bt.cdc.gov/agent/smallpox/lab-testing/pdf/em-rash-protocol.pdf) and elsewhere (22, 50, 66).

Poxviruses produce inclusions that have characteristic appearances when stained with May-Grunwald Giemsa and hematoxylin-eosin. Perinuclear basophilic or B-type cytoplasmic inclusions (virus factories or viroplasm) represent sites of virus replication. Certain species (e.g., the orthopoxvirus cowpox virus) produce acidophilic inclusions or A-type inclusions. Depending on the strain, A-type inclusions may (V^+) or may not (V^-) contain virions.

Antigen Detection

Orthopoxvirus is the only genus whose members produce a hemagglutinin (HA) antigen, which is detectable by hemadsorption or hemagglutination assays using chicken erythrocytes that are pretested to be suitable for such tests. Inhibition of hemagglutination and hemadsorption by patient serum is an indicator of orthopoxvirus infection (24, 26, 64–67); such antibodies may persist for 1 to 5 years after infection.

Nucleic Acid Detection

PCR analysis is used by the WHOCC at the CDC to detect orthopoxvirus DNA in samples. Single-gene PCR, followed by restriction fragment length polymorphism analysis or sequence analysis of the amplicon, permits species identification of orthopoxviruses. A number of different targets are used, including the HA gene which is unique for the genus *Orthopoxvirus*, the gene for the B cytokine response modifier (CrmB, one of several different tumor necrosis factor receptor homologs produced by orthopoxviruses), and the gene for the A-type inclusion body protein. In these assays, DNA that is present in any orthopoxvirus is amplified. The amplicon is digested with the appropriate restriction endonuclease, and digested fragments are electrophoresed. Fragment sizes are compared to reference restriction fragment length polymorphism profiles to discriminate species.

Perhaps the best-described locus for PCR detection and differentiation of orthopoxviruses is the HA DNA segment (77). This PCR method is based on the use of previously described primers EACP1 and EACP2 (for Old World orthopoxviruses) or NACP1 and NACP2 (for New World orthopoxviruses) with an ~900- or ~600-bp HA DNA amplicon to establish if the isolate is an orthopoxvirus. Subsequently, TaqI or RsaI digestion fragment analysis enables species identification.

Orthopoxviruses contain either a full-length or a truncated gene for the CrmB tumor necrosis factor receptor homolog. After the region is amplified with consensus primers (51), the amplicon size can be used to differentiate vaccinia virus (which contains a deletion in CrmB) from the other orthopoxviruses that infect humans. Primers VL2N and VL33, described by Loparev et al. (51), can be used for the amplification. Vaccinia viruses, including the subspecies buffalopox virus, show an ~1,200-bp amplicon, and monkeypox, variola, and cowpox viruses show an ~1300-bp amplicon. Restriction endonuclease digestion of the amplicon with NlaIII can be used to differentiate monkeypox, cowpox, and variola viruses; however, some isolates show more or less heterogeneous profiles by NlaIII digestion. Depending on the isolate, four to six fragments may be noted, but the sizes of the fragments can clearly differentiate species and may be adequately sensitive to distinguish strains and variants.

Amplification of the gene region for the A-type inclusion body protein is another useful technique to differentiate orthopoxviruses. Meyer et al. (56, 57) describe primers ATI-low-1 and ATI-up-1 to amplify 1,500- to 1,700-bp amplicons from the DNA of vaccinia, cowpox, or monkeypox viruses. In this method, amplicon identity is verified by using BglII or XbaI restriction fragment profiles.

Other poxvirus PCR diagnostic approaches include standard PCR methods to identify the parapoxvirus Orf virus (91). A random amplified polymorphic DNA fragment length polymorphism approach to orthopoxvirus species and strain discrimination is reported by Stemmler et al. (89). More recently described (45) is a method to discern PCR-amplified, fluorescence-labeled DNA fragments by hybridization to orthopoxvirus species-specific DNA immobilized on a microchip.

The high sensitivity of and rapid results from real-time PCR assays make them attractive for laboratory clinical diagnostic use (29). In the United States, many national reference laboratories, as well as the Laboratory Response Network laboratories, use this format of nucleic acid testing for rapid response to diagnose suspected orthopoxvirus infections and/or to rule out smallpox infections. The use of a probe or probes in this type of assay allows for the specificity; however, the extreme sensitivity of the assays can lead to false-positive contaminants from specimen carryover. Many of these methods for detection of orthopoxviruses (1, 29, 70, 84) and yatapoxviruses (98) have been described previously. Others are available at specific reference centers, including the CDC Poxvirus Program/WHOCC for Smallpox and Other Poxvirus Infections.

ISOLATION

Orthopoxviruses can be grown in a variety of established cell culture lines, including Vero, BS-C-1, CV-1, LLCMK-2 monkey kidney cells, human embryonic lung fibroblast cells, HeLa cells, chicken embryo fibroblast cells, and MRC-5 human diploid fibroblast cells, a line approved by the Food and Drug Administration for vaccine production (25). Methods for growing and discriminating the morphologies of orthopoxviruses on the chorioallantoic membranes (CAMs) of 12-day-old chicken embryos have been described previously (61, 66, 96). Orthopoxviruses are the only human poxviruses that produce pocks (95, 96) on the CAMs of fertile chicken eggs; pock morphology is useful for biologic species and variant differentiation. Parapoxviruses, yatapoxviruses, and MCV do not form pocks on the CAM, although avipoxviruses, leporipoxviruses, and capripoxviruses do so.

SEROLOGIC TESTS

Orthopoxviruses

When virus-containing clinical specimens are not available, antibody detection may be the only way to define the etiology of the disease. Serologic methods currently used to detect antibodies against human orthopoxviruses include enzyme-linked immunosorbent assays (ELISAs), the virus neutralization test (NT), Western blotting, and hemagglutination inhibition. Various protocols for poxvirus serologic testing used at the CDC are detailed elsewhere (12, 13, 24, 25, 31, 64–67, 78).

The recent description of an orthopoxvirus immunoglobulin M (IgM) assay offers great promise to enhance investigations of orthopoxvirus infection outbreaks, often semiretrospective in nature (41). This technique offers the advantage that it measures recent infection or illness with an orthopoxvirus. It is useful for evaluating disease incidence in epidemiologic surveillance studies. For example, during the 2003 U.S. monkeypox outbreak, the IgM capture assay demonstrated ~95% sensitivity and ~95% specificity for epidemiologically linked and laboratory test-confirmed patients when sera were obtained between days 4 and 56 post-rash onset. A low-grade response, termed "equivocal," awaits further research. This assay was also used to detect anti-orthopoxvirus IgM in the cerebrospinal fluid of an encephalitic patient with monkeypox (82). A recently reported peptide-based ELISA appears to show promise for specific identification of monkeypox virus-specific antibodies, perhaps even in the background of previous vaccination with vaccinia (34). This work awaits more extensive analysis.

Poxvirus genera can usually be identified and differentiated by NT with hyperimmune reference sera (12, 24, 26, 64–67). However, it can be difficult to identify the infecting species since poxviruses are antigenically closely related within a given genus. In the current state of bioterrorism response awareness, tests to evaluate residual protection from previous vaccination are being requested. It is important to note that there is no one routine immunologic test that defines a person's degree of protection against a poxvirus infection. Protection is genetically defined and requires a concert of cell-mediated and humoral immune responses. Recent work (21) suggests that humoral responses may be the critical component of recovery from and survival of a systemic orthopoxvirus infection. The presence of neutralizing antibodies generally indicates recovery from an infection, not always protracted protection from future infection. Neutralizing antibodies against variola, monkeypox, cowpox, or vaccinia viruses may be detectable as early as 6 days after infection or vaccination; the efficacy of the NT using sera of infected animals or humans may range from 50 to 95%. Neutralizing antibodies have been detected as long as 20 years after vaccinia vaccination or natural infection with other human orthopoxviruses.

In the NT, a fourfold rise in antibody titer between serum samples drawn during the acute and convalescent phases is usually considered diagnostic of poxvirus infection. When only one serum specimen is available from one phase of infection, confirmation of a clinical diagnosis may be difficult to impossible. Because orthopoxviruses are closely related, serum cross-absorption tests, such as those performed in immunofluorescent-antibody or immunodiffusion methods, have been used with variable success with patient and animal sera. Orthopoxvirus antigen cross-absorption assays have been performed using hyperimmune animal sera and have been utilized in serosurveys for animal or human monkeypox infection (40, 65, 67). False-positive results should be ruled out by using appropriate control sets of sera of known provenance.

The Western blot assay is performed essentially as described by Towbin et al. (92) and uses various antigens, including purified virus and sometimes the concentrate of culture fluid from infected cells maintained under medium that contains 1% or no serum supplement. Few laboratories are using this method, as reliable standardization has not been achieved.

Milker's Nodule, Orf, Tanapox, and Contagiosum Viruses

Serologic methods used at the CDC to help confirm parapoxvirus infections (milker's nodule and Orf) have included ELISAs (13) and Western blot assays that use various antigen preparations. Serologic testing for TPV infection by standard ELISA (13) with antigens obtained from concentrates of infected cell culture, by an indirect immunofluorescent-antibody test, and by NT has been moderately effective. Optimally, sera should be collected at the time of actual disease and 3 to 5 weeks or later after the presumed onset date. Because MCV cannot be readily grown in culture, no routine serologic test is available. Molluscum contagiosum is readily diagnosed clinically, often with the aid of electron microscopy and histopathologic testing performed by a local diagnostic facility.

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Hepatitis B and D Viruses

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Viral hepatitis is an ancient disease that was first described in the fifth century B.C. Since that time, epidemics of jaundice have been described, with the earliest recognized blood-borne outbreak of hepatitis occurring in Bremen, Germany, in 1883 among shipyard workers who received the smallpox vaccine. In 1947, MacCallum and Bauer (79) introduced the terms hepatitis A for infectious hepatitis and hepatitis B for "serum" hepatitis. It was recognized that the epidemiologies of the two diseases differed. The terms hepatitis A and hepatitis B were then adopted by the World Health Organization (WHO) in 1973 to distinguish the two distinct infectious agents of hepatitis. After the discovery of the Australia antigen in serum by Blumberg and colleagues (13–15) in 1965, the hepatitis B virus (HBV) was identified and characterized. The Australia antigen is now designated the hepatitis B surface antigen (HBsAg) and is associated with both acute and chronic hepatitis B infection. These and other discoveries in the late 20th century have led to explosive growth of information about HBV. Specific and sensitive serologic tests are now available for HBV which have led to a deeper understanding of the natural history of hepatitis B. This information also has aided the study of the pathogenesis and epidemiology of HBV, which eventually has led to the development of safe and effective vaccines for the prevention of HBV infection. In addition, this body of scientific work has fostered the development of antiviral therapies for treatment of patients with chronic HBV infection. It also became clear that not all posttransfusion hepatitis is associated with HBV infection. An additional agent(s) (hepatitis C virus) is responsible for nearly all of the remaining cases of posttransfusion hepatitis.

In 1977, a new viral antigen was recognized in hepatocytes from patients with chronic HBV infections (101). This new antigen was detected in the nuclei of hepatocytes but was antigenically distinct from HBsAg; however, it was always associated with HBV infection. Patients with this antigen responded to it with specific antibodies (102). Since it was an antigenically distinct protein, it was given the name delta antigen. In the 1980s, this protein was found to be associated with a novel, viruslike particle that was unable to replicate autonomously and formed viral particles only in the presence of HBV (116). This viruslike particle was named hepatitis deltavirus (HDV). It was then found that HDV "borrowed" HBsAg to form intact virions. Thus, without the presence of HBV, HDV cannot be packaged into viral particles. The incidence of HBV-HDV coinfection is high in certain parts of the Middle East, the Mediterranean, Africa, South America, and some South Pacific islands (64). Because HDV infection occurs only in people infected with HBV, measures taken to prevent HBV infection, such as immunization and avoidance of direct exposure to contaminated blood or needles, are also effective for the prevention of HDV infection.

HEPATITIS B VIRUS

Taxonomy

HBV, a partially double-stranded, enveloped DNA virus, is the prototype agent of the *Hepadnaviridae*. It is a member of group VII, which consists of DNA reverse-transcribing viruses. Viruses in the *Hepadnaviridae* have been isolated from woodchucks, ground squirrels, ducks, and herons (12, 126, 127). All viruses in this family are enveloped with circular, partially duplex DNA. They all have virion-associated polymerases with reverse transcriptase activities. All agents in the *Hepadnaviridae* have narrow host ranges, and to date, the mammalian hepadnaviruses have been difficult to grow in cell cultures (49, 93, 126).

HBV is an orthohepadnavirus along with the hepadnaviruses found in the ground squirrel and the woodchuck (127). The duck and heron HPVs are avihepadnaviruses (127).

Description of the Agent

Ultrastructural examination of the sera of HBV-infected patients reveals three distinct morphologic structures in various proportions (Fig. 1). The most numerous forms (by a factor of 10^4 to 10^6) are the small, pleomorphic, spherical, noninfectious particles measuring 17 to 25 nm in diameter. Particle counts of 10^{13}/ml have been detected in some sera. Tubular or filamentous forms of various lengths are also observed. A third particle, the HBV virion, has a lipid-containing outer envelope consisting of HBsAg that surrounds a nucleocapsid or core. The inner core of HBV contains a single molecule of partially double-stranded DNA, hepatitis B core antigen (HBcAg), hepatitis B e antigen (HBeAg), and DNA-dependent RNA polymerase (35). The sera of infected patients may contain as many as 10^{10} infectious virions per ml. The 17- to 25-nm-diameter spherical particles are composed entirely of HBsAg. The low buoyant densities of these

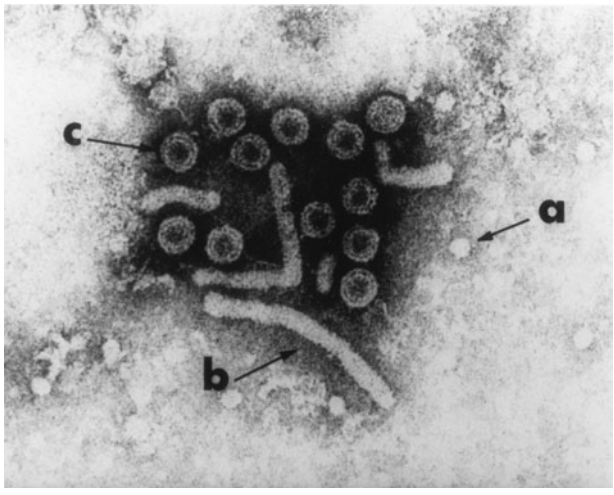


FIGURE 1 Electron micrograph of serum showing the presence of three distinct morphologic entities: 17- to 25-nm-diameter pleomorphic, spherical particles (a); tubular or filamentous forms with diameters similar to those of the small particles (b); and 42- to 47-nm-diameter double-shelled spherical particles representing the hepatitis B virion (Dane particle) (c). Magnification, ×85,008.

particles reflect the presence of lipids, which are presumably of cell membrane origin (47, 48). The tubular forms also consist of HBsAg.

HBV has a partially double-stranded, circular DNA genome that contains between 3,020 and 3,320 nucleotides. Neither of the circular strands is covalently closed. The genome is uniquely organized in that it contains overlapping genes. The genome has four open reading frames in the complete or minus-strand DNA. The genes encode structural proteins such as HBsAg and HBcAg, replicative proteins (polymerase and X protein), and regulatory elements. The genome is compact, and most of the genomic sequences are essential for productive infection. This compact organization restricts the development of mutations because changes are likely to result in a misreading of the code of an essential protein. However, recent studies have shown that there are genetic differences within the HBV genomes, creating at least eight different genotypes, some with subgenotypes (45, 91, 105).

After binding to hepatocytes, the HBV virion is taken up and uncoated. The partially double-stranded, relaxed circular DNA genome is converted into a covalently closed, circular DNA (cccDNA) template that takes a supercoiled form (Fig. 2). This cccDNA form serves as a template for transcription of the pregenomic RNA (pgRNA) and mRNA (72, 107). Within 24 h of infection, cccDNA can be detected in the liver (107). Interestingly, cccDNA accumulates only in the nucleus and is necessary for the successful initiation of

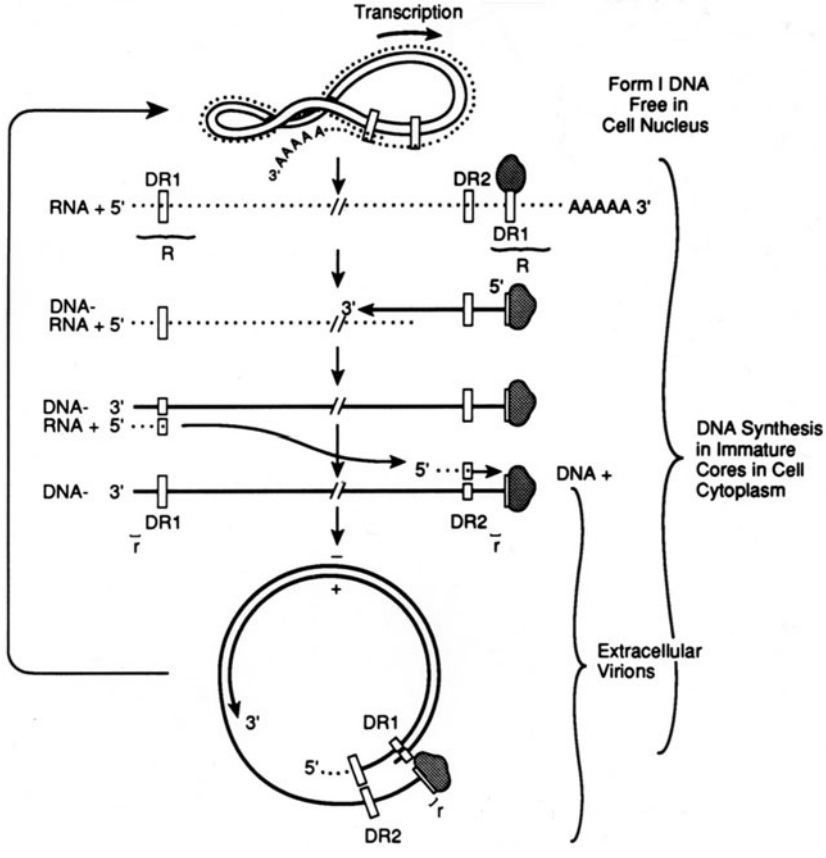


FIGURE 2 Scheme of HBV replication. Note the generation of the positive (+) and negative (−) DNA strands from an RNA intermediate. DR1, direct repeat sequence 1; DR2, direct repeat sequence 2; R, redundancy; r, 5′ and 3′ redundancy sequence. (Reprinted from reference 103 with permission of the publisher.)

HBV replication. The pgRNA that is transcribed from the cccDNA in the nucleus locates to the cytoplasm of the infected cell. In the cytoplasm, pgRNA serves as the template for the reverse transcription enzyme (polymerase) and for the HBcAg. The polymerase then converts the pgRNA into a new circular DNA molecule. Early in infection, some newly synthesized genomes from the cytoplasm circulate back to the nucleus to build up and maintain the pool of cccDNA.

Every nucleotide of the HBV genome is in a coding region, and more than half of the genome is translated in more than one open reading frame. Four viral mRNA transcripts are involved in the translation of HBV proteins. These viral mRNAs are transcribed from various promoter regions on the cccDNA template (Fig. 2). The longest is 3.5 kb and acts as the template for genome replication (pgRNA), as well as translates precore (HBeAg), HBcAg, and polymerase proteins. The 2.4-kb transcript encodes the pre-S1 protein (39 kDa), pre-S2 protein (33 kDa), and S protein (24 kDa; also known as HBsAg). A third transcript (2.1 kb) encodes the pre-S2 protein and the S protein. The final mRNA (0.7 kb) codes for the X protein that is responsible for transactivating transcription. Mature virions are produced by reverse transcription of RNA into a circular DNA molecule. The long RNA transcript (3.5 kb) and the polymerase protein are packaged into mature core particles, and the reverse transcriptase enzyme (polymerase) synthesizes a new viral DNA genome. These particles are then packed into the surface proteins in the endoplasmic reticulum and exported from the cell. The HBV surface proteins such as HBsAg are also released from infected cells as spherical and filamentous particles devoid of HBV genome and core proteins. HBV DNA replication through an intermediate reverse transcription step is unique for human DNA viruses. However, this mechanism is also found in nonprimate hepadnaviruses and the cauliflower mosaic virus.

HBcAg and HBeAg differ antigenically from each other. As noted previously, HBsAg is produced in copious amounts and circulates in the blood of infected individuals as spherical and tubular particles. HBsAg can be detected in serum 30 to 60 days after initial exposure to HBV and may persist for variable periods (39, 81). The HBcAg is a polypeptide with a molecular size of 22,000 Da. The HBeAg is approximately 16,000 Da and is derived by the proteolytic cleavage of the larger, 24,000-Da precore/core protein. Host cellular proteases cleave the protein during passage through the Golgi complex and subsequently secrete the HBeAg into the bloodstream. HBeAg appears to lack the viral DNA-binding domain. HBeAg either circulates freely in the blood as a soluble protein or is bound to albumin, α-1-antitrypsin, or immunoglobulin. It is a reliable marker for the presence of intact virions and thus for infectivity. A mutation at the end of the precore region leading to a translational stop codon yields a variant that is unable to produce HBeAg: such precore mutants may contribute to the pathogenesis of chronic hepatitis B and fulminant hepatitis (52, 105). Other HBV gene mutations have been observed in the core, core promoter, envelope, and polymerase regions. The envelope protein variants may become relevant if they are able to escape vaccine-induced antibodies to HBsAg (anti-HBs) or lead to failure of hepatitis B immune globulin (HBIG) therapy after transplantation. Likewise, the mutations in the polymerase regions may lead to resistance to antiviral agents.

HBV infection in humans is diagnosed by detecting anti-HBs, antibodies to HBcAg (anti-HBc), and antibodies to HBeAg (anti-HBe) as well as the various HBV antigens. The terminology used to describe the various antigens and antibodies associated with HBV is given in Table 1.

Epidemiology and Transmission

Hepatitis B infection is a global public health problem and is one of the most common infectious diseases in the world. The WHO estimates that more than one-third of the world population has been infected with HBV, with the majority of HBV carriers living in Asia or Africa (http://www.who.int/gpv-surv/graphics/htmls/hepbprev.htm). The WHO estimates that these infections cause more than 1 million deaths per year. Figure 3 shows the prevalence of HBV infection in different parts of the world. Almost half of the world's population resides in Asia and Africa, where the prevalence of chronic HBV infection is high (>8%). In North America, western parts of South America, Australia, and Western Europe, the prevalence of chronic HBV infection is lower (<2%). Thus, much of the burden of HBV infection is carried by the developing world.

The only natural host for HBV is humans. The major modes of HBV transmission are vertical transmission (mother to child perinatally), sexual activity (both heterosexual and male homosexual), injection drug use, and other types of physical contact with infected bodily fluids (occupational exposure, exposure to contaminated blood products,

TABLE 1 Nomenclature for hepatitis B virus

Term	Abbreviation(s)	Description
Hepatitis B virus	HBV	42-nm-diam double-shelled particle that consists of a 7-nm-diam outer shell and a 27-nm-diam inner core. Core contains a small, circular, partially double-stranded DNA molecule and DNA polymerase activity. Originally called the Dane particle. This is the prototype for the family *Hepadnaviridae*.
Hepatitis B surface antigen	HBsAg	Complex antigen found on the surface of HBV and on 20-nm-diam particles and tubular forms. Formerly designated Australia (Au) antigen or hepatitis-associated antigen (HAA).
Hepatitis B core antigen	HBcAg	Antigen associated with 27-nm-diam core of HBV.
Hepatitis B e antigen	HBeAg	Protein that results from the proteolytic cleavage of the precore/core protein by cellular proteases and is secreted as soluble protein in serum.
Antibodies to HBsAg, HBcAg, and HBeAg	Anti-HBs, anti-HBc, and anti-HBe, respectively	Specific antibodies produced in response to their respective antigens.

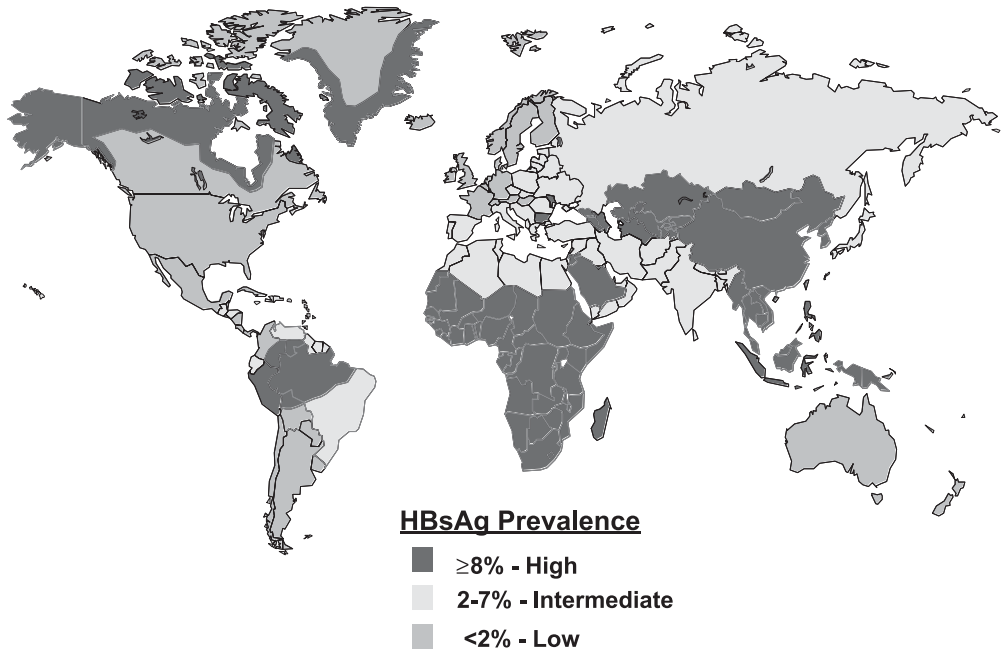


FIGURE 3 Worldwide prevalence of HBsAg-positive individuals. Data are from the CDC, 2005. (CDC website: http://www.cdc.gov/ncidod/diseases/hepatitis/slideset/hep_b/9.)

and person-to-person contact). HBV is not transmitted by aerosols, feces, or contaminated food, water, or insects. HBV can survive in the environment for prolonged periods of time outside the body. Individuals who are HBeAg positive have large numbers of virions in their blood ($>10^7$/ml) and can contaminate the environment from open sores or cuts (17, 99). Thus, transmission can occur through close person-to-person contact, most likely through open sores (84, 99). This type of transmission may occur more often in children in areas where the virus is hyperendemic and is the basis for using standard precautions in the health care setting (7, 18).

The HBV carrier rate is related to the age at which infection occurs. In some Asian and African countries, the HBV carrier rate is as high as 20% because most of the infections occur at birth or during early childhood, when the risk of chronic infection is greatest. In the United States, more than 1.25 million people are thought to be chronically infected with HBV (http://www.cdc.gov/ncidod/diseases/hepatitis/b/fact.htm). The number of new infections per year in the United States has declined from an estimated 260,000 in the 1980s to 73,000 in 2003. The greatest decline has occurred in children and adolescents and seems to be due to the increased use of hepatitis B vaccination (26, 70, 71, 85). The highest rate of disease occurs in 20- to 49-year-olds.

In the United States the lifetime risk of HBV infection is approximately 5%, with most infections occurring among well-defined risk groups. The high-risk groups historically include intravenous drug users, persons who have sexual relations with multiple partners (heterosexuals and homosexual males), hemophiliacs, hemodialysis patients and staff, inmates of correctional facilities, individuals with occupational and household exposure to infected body fluids, and institutionalized patients with developmental disabilities (80, 81). In the past, approximately one-third of the chronic infections in the United States were acquired through perinatal exposure (23). In 1994, the Centers for Disease Control and Prevention (CDC) estimated that 22,000 infants were born in the United States each year to chronically infected mothers (23); however, by 2003 the CDC showed that 92.4% of all children aged 19 to 35 months had received three or more doses of the hepatitis B vaccine (27). This reported vaccination rate should continue to reduce the number of hepatitis B infections. In 1995, the CDC expanded the hepatitis B vaccination program to include all adolescents aged 11 to 12 years (25). In 1999, the CDC included all children of less than 18 years of age who had not been vaccinated previously (25). Over the next 10 years, this comprehensive vaccination program should lead to a dramatic reduction in the number of individuals with hepatitis B infection (26, 27, 125).

Based on serologic data, the WHO has estimated that more than 2 billion people have been infected with HBV (56). Worldwide, 350 million people are estimated to be chronically infected with HBV and therefore at risk for cirrhosis and liver cancer. Annual deaths worldwide related to HBV infection are estimated to range from 500,000 to 700,000. Thus, the consequences of acute and chronic HBV infection are a major public health problem. Reports of HBV infections to public health authorities are mandatory in most countries, but unfortunately underreporting has seriously affected the efforts to raise awareness of the massive global burden of HBV infections (http://www.who.int/health-topics/hepatitis.htm). Public health education, infection control policies in hospitals and clinics, and, most important, the availability of an effective vaccine have already led to a dramatically expanded use of the hepatitis B vaccine.

Clinical Significance

Hepatitis B is a viral infection of the liver that can have variable clinical features. Symptoms may range from mild and transient to severe and prolonged. Patients may recover completely from infection, be overwhelmed by fulminant hepatitis that leads to mortality (<1.5% of hospitalized patients), or develop a chronic infection that may evolve into cirrhosis.

HBV infection can be divided into four stages that vary in duration (33, 67). The first stage (stage I) occurs during active viral replication when no immune response is detectable. The second stage (stage II) occurs when an immunologic response to the virus leads to inflammation and hepatic tissue damage, often detected as symptomatic hepatitis. At times patients with an inflammatory infection can be asymptomatic. During the third stage (stage III), the virus-infected cells are cleared and the HBeAg is no longer detected. However, HBsAg will still be present. In the fourth stage (stage IV), full immunity to HBV has developed and HBsAg has been cleared. Infected patients who do not progress beyond stage I or II become chronic HBV carriers and thus are at higher risk for the development of cirrhosis and/or hepatocellular carcinoma (HCC) (77).

Acute Hepatitis B

The incubation period for acute hepatitis B ranges from 6 weeks to 6 months. This period corresponds to stage I of the disease. The incubation period may be shortened when a patient is exposed to a large viral inoculum or infected by the percutaneous route. The administration of high-titer antibody specific for HBsAg (hepatitis B immune globulin [HBIG]) shortly after exposure prolongs the incubation period and may prevent infection. The clinical manifestations of acute HBV infection are highly age dependent. Nearly all infected newborns are asymptomatic. In children 1 to 5 years of age, only 5 to 15% have clinical manifestations. Approximately 10 to 30% of older children and adults become symptomatic (70, 74).

The clinical symptoms of acute HBV infection are flulike. Some patients experience physical signs such as jaundice, dark urine, clay-colored stools, and hepatomegaly (103). The prodromal, preicteric phase of hepatitis B tends to be more insidious and prolonged than that of hepatitis A, lasting from 1 to 2 weeks in most patients. This phase is stage II and is characterized by malaise, low-grade fever (<39.5°C), fatigue, myalgia, anorexia, nausea, and vomiting. Some patients experience weight loss and right-upper-quadrant pain associated with hepatomegaly.

The icteric phase of acute viral hepatitis is often accompanied by the appearance of dark urine followed by the development of clinical jaundice. With the emergence of jaundice, symptoms improve and fever usually subsides. The icteric phase lasts, on average, about 1 month. In 10 to 20% of HBV-infected patients, extrahepatic manifestations of the disease develop, most likely mediated by virus-specific immune complex injury. They include a serum sickness-like syndrome, acute necrotizing vasculitis (polyarteritis nodosa), and membranous glomerulonephritis.

The clinical diagnosis of acute hepatitis B is based on the results of liver enzyme testing and the detection of viral antigens, antibodies, and nucleic acid. The total bilirubin concentration during acute hepatitis B is usually <10 mg/dl (normal range, 0.20 to 1.50 mg/dl) but may occasionally exceed 20 mg/dl. Prolongation of the prothrombin time may reflect early and potentially serious derangements of liver function which may signify the development of fulminant hepatic failure. Fulminant hepatic failure occurs in <1% of patients who develop symptomatic, acute HBV infection. Evidence suggests that these patients have an overexuberant immune response to the HBV infection, resulting in massive necrosis of infected hepatocytes. The immune response to HBcAg (immunoglobulin M [IgM] anti-HBc) is almost always positive; however, HBsAg and HBeAg are not usually detectable. Mortality associated with fulminant hepatic failure is high without liver transplantation. After liver transplantation, HBV reinfection of the new liver is universal, resulting in significant HBV-induced liver injury in a number of patients (94). HBIG or antiviral therapy appears to modulate these outcomes.

Pathologic features of acute hepatitis B include both degenerative and regenerative parenchymal changes that lead to lobular disarray. HLA class I- and II-restricted cytotoxic T lymphocytes (CTLs) appear to play a major role in facilitating viral clearance during acute HBV infection. On the other hand, they are also the agents of hepatocellular injury by recognizing HBV nucleocapsid epitopes expressed on the surfaces of infected hepatocytes (10, 29, 30).

Chronic Hepatitis

Patients who remain HBsAg positive for at least 6 months are considered to have chronic HBV infection (70, 77). These patients are likely to remain positive indefinitely and thus remain at stage II of the infection. The consensus definition for chronic hepatitis B is found in the practice guidelines published by the American Association for the Study of Liver Diseases (77). Symptoms of chronic hepatitis B are generally vague and nonspecific. Patients may have some elevation in serum aminotransferase levels. In addition, HBV virions, DNA polymerase activity, and HBV DNA can be detected in blood, indicating active viral replication. A liver biopsy shows active inflammation with hepatocellular necrosis and may show fibrosis. Symptoms generally become more pronounced when cirrhosis develops. HCC develops in 12 to 55% of patients with chronic HBV infection and cirrhosis (43).

Studies from Japan have shown that 29% of HBsAg-positive patients will spontaneously revert to being HBsAg negative after approximately 12 years (59). This reversion was seen more frequently in patients 40 years old or older. The HBV DNA was detected in the sera of only 6% of the patients who reverted to an HBsAg-negative status, indicating a reduction in viral replication. The factors that determine resolution or chronicity remain largely unknown. In general, those infected early in life (<3 years) are more likely to become chronic carriers than older individuals. Individuals infected in the perinatal period have high serum HBV DNA levels but normal alanine aminotransferase (ALT) levels. HBeAg persists longer in these younger persons (74). Interestingly, the majority of these individuals (~90%) have a chronic infection without symptoms. In contrast, the potential for chronicity in immunocompetent older children and adults ranges from 6 to 10%. These patients usually have symptoms during the acute infection.

Histologic examination of liver tissue is useful to differentiate acute viral hepatitis from chronic hepatitis and cirrhosis. Chronic hepatitis B is associated with HCC, especially in males living in Asia and Africa. The incidence of death from liver cancer and cirrhosis increases as a direct function of age; however, cirrhosis is not always a requirement for the development of HCC due to HBV infection (43).

Pathogenesis

Both acute and chronic HBV infections are associated with hepatocellular necrosis as well as inflammatory and regenerative responses. Some evidence suggests that HBV may not be directly cytopathic to hepatocytes and that the host's cellular immune response to infected cells correlates with the severity of clinical disease (30). In most patients with acute HBV infection, recovery is dependent on a B-cell response that leads to production of antibodies to pre-S and S antigens. These patients also have a vigorous CTL response that is reactive to multiple epitopes in the HBV envelope, nucleocapsid, and

polymerase proteins (89). The HBV-related liver damage is attributable to this CTL response directed against HBV-infected hepatocytes. Patients who develop fulminant hepatitis due to HBV infection frequently have no evidence of HBV replication. The disease course is thought to be due to the rapid and aggressive immune clearance of HBV-infected hepatocytes (74). Patients with chronic HBV infection usually are deficient in CTLs directed against HBV proteins (30). Individuals with perinatally acquired HBV infection appear to have immune tolerance to HBV antigens, which may account for the high rate of chronic infection in the group. They do not seem to have severe liver disease in spite of the presence of high levels of virus (73, 77).

There is a strong etiologic association between chronic HBV infection and the development of HCC (43, 75). Currently, the mechanism by which HBV is oncogenic is still under investigation. Multiple factors are likely to contribute to the development of HCC. The majority of patients (80 to 90%) with HBV infection and HCC have integrated HBV DNA in hepatocytes. This integrated viral DNA may activate cellular proto-oncogenes or suppress growth regulation genes (43). Some of the gene products of HBV, such as the X protein and the truncated pre-S/S protein, seem to be potent transactivators of host cellular genes (19, 78). Recent studies have shown that the HBV DNA from HCC tissue has a high rate of mutations in the X gene (44). These mutations were noted to induce functional alteration that affects the transactivating ability of the X protein. Future studies are needed to determine whether diagnostic tests can be used to detect these mutations and predict the occurrence of HCC. In addition to these expressed HBV products, other factors are associated with HCC, such as smoking, consumption of alcohol, other hepatotropic viruses (e.g., hepatitis C virus), and mycotoxins (aflatoxin) (43). Thus, it seems that, like that of other human cancers, the development of HCC is likely to be multifactorial, involving several mutagenic events and HBV infection.

Collection, Transport, and Storage of Specimens

Except for HBV DNA, the stability of serologic markers that develop following HBV infection eliminates the need for extraordinary collection and storage procedures. Serum or plasma should be separated from blood samples within 24 h and stored at 2 to 8°C if testing is to take place within 5 days. Should longer delays be anticipated, the processed specimens must be frozen. Because heparinized or severely hemolyzed blood may occasionally cause false-positive enzyme immunoassay (EIA) responses, such samples should be avoided. Although HBV serologic markers are stable for years at −70°C, repetitive freezing and thawing may lead to substantial losses in concentration.

For nucleic acid analyses, specimens should be processed within 6 h and either tested within 24 h or stored at −70°C or lower. Plasma recovered from blood collected in tubes containing EDTA (lavender-top tubes) or in tubes containing anticoagulant citrate dextrose (yellow-top tubes) is preferred for genome amplification techniques (e.g., PCR). Conversely, heparinized plasma is considered unacceptable, because heparin interferes with some enzymes involved in amplification of the nucleic acid (6, 55), causing false-negative results. Heparin also inhibits reverse transcription (119). The above-described recommendations are general, conservative guidelines that may not conform to those specified by reference laboratories offering HBV testing. Manufacturers' recommendations for specimen type and handling should be followed for all serologic and molecular testing.

Laboratory personnel should regard all specimens collected from hepatitis B patients as potentially dangerous. As shown in several studies, the HBV is stable for prolonged periods outside the human host. Additionally, patients who are HBeAg positive will have a high viral load (10^7 to 10^9) that can contaminate the laboratory environment (7, 16, 17). Frequent disinfection of laboratory benches is important to prevent this type of exposure and to prevent cross-contamination of samples and/or equipment. The Occupational Safety and Health Administration standards for occupational exposure to blood-borne pathogens (6a) are designed to protect employees exposed to blood and other potentially infectious materials. Compliance is mandatory. At the core of the standard is the concept of standard precautions which mandate that all human blood and certain human body fluids be treated as if they are known to be infectious. The Occupational Safety and Health Administration requires that all employees who have occupational exposure be offered the hepatitis B vaccine at no cost.

Direct Detection

Detection of HBV Antigens

Currently, two HBV antigens are used to diagnose HBV infections. HBsAg can be detected in the sera of both patients with acute and those with chronic infections. It can also be detected for a short period of time after hepatitis B vaccination, which sometimes complicates the interpretation of HBV serology results. The other HBV antigen detectable in serum is HBeAg, which is commonly used as a marker for active viral proliferation and infectivity (70). A recent long-term follow-up study shows that chronic carriers with both HBsAg and HBeAg have a significantly greater risk for HCC than patients who have HBsAg alone or who are negative for HBV antigens (121). The test methodologies are similar in detail and are discussed in "Serological Tests" below.

Nucleic Acid Detection Techniques

HBV DNA is a marker for viral replication. Consequently, its detection plays an important role in the evaluation and therapeutic monitoring of HBV infection (8, 11, 22, 77). HBV DNA assays have been shown to be useful in clinical staging, monitoring of therapy, and detection of relapses after and during therapy. However, HBV DNA testing should be limited to those patients with chronic HBV infection being considered for treatment and to evaluation of response to treatment. Testing for HBV DNA is not recommended in the work-ups of all HBsAg-positive patients (77). A value of $>10^5$ genomic equivalents was established as a diagnostic criterion for chronic hepatitis B (76). The difficulty with this threshold has been the lack of standardization among the various commercial assays used for HBV DNA quantitation. Moreover, some chronic carrier patients will have fluctuating HBV DNA levels. These issues have confounded attempts to determine the impact of HBV DNA levels on the progression of liver disease (77).

Dot Blot Hybridization

The analysis of HBV DNA sequences was first performed using dot blot hybridization with human serum and tissue samples (88, 123). However, this method has been replaced with more sensitive methods such as amplification of HBV DNA. The dot blot hybridization technique is capable of detecting 0.1 to 1.0 pg (28,000 to 280,000 genomic equivalents) of HBV DNA/ml after 5 days of autoradiography, and

TABLE 2 Molecular assays used to detect HBV nucleic acid in human specimens

Assay name	Method	Detection signal	Target HBV gene(s)	Range of detection (genome copies/ml)	Minimum vol (μl) of specimen
Digene Hybrid Capture II	RNA probe binds to target DNA; captured on solid phase by antibody	Chemiluminescence	Specific for HBV *ad* and *ay* subtypes; uses entire genome probe	2×10^5–2×10^9	30
Digene Hybrid Capture Ultra-Sensitive	RNA probe binds to target DNA; captured on solid phase by antibody	Chemiluminescence	Specific for HBV *ad* and *ay* subtypes	5×10^3–6×10^7	1,000 (concentrated by ultracentrifugation)
Quantitative HBV Amplicor	Amplification by PCR with biotin-labeled primers	Absorbance of enzymatic activity	Precore/core region	200–>2×10^5	100 (processed and used as an equivalent of 50)
Versant HBV DNA3.0	Binding of target to solid-phase probe; amplification of probe by binding of branched DNA chains	Chemiluminescence	Capture and extender probes throughout entire HBV genome	2×10^3–1×10^8	50
Home-brew PCR	Amplification of target by using real-time or traditional PCR	Enzymatic activity, chemiluminescence, or fluorescence	Varies	Varies	10–2,000

the amplification methods can detect levels as low as 0.001 pg (200 to 400 genomic equivalents) of HBV DNA/ml within a few hours (77). Thus, due to the length of the procedure, the long turnaround time, and the method's lower sensitivity, dot blot hybridization is not the preferred molecular method to detect HBV DNA in patient specimens. It has been replaced by the quantitative methods listed in Table 2.

Commercial Assays

Several commercial assays have been developed to detect HBV DNA in serum. HBV DNA can be detected by amplifying a target gene or by capturing the target DNA and amplifying the specific probe that detects the target. Currently, several techniques are available to detect and/or quantify HBV DNA in patient specimens (Table 2). As of late 2005, only one HBV DNA assay had been approved by the Food and Drug Administration (FDA), namely, the COBAS Ampliscreen HBV test, a qualitative test approved only for screening of blood donors.

The assays used for the quantitative evaluation of HBV DNA include the target amplification techniques such as PCR (COBAS Amplicor HBV Monitor test [Roche Diagnostics, Indianapolis, Ind.] and HBV SuperQuant [LabCorp, Burlington, N.C.]), liquid-phase hybridization (Digene Hybrid Capture II, distributed by Abbott Laboratories, Abbott Park, Ill.), and a signal amplification, branched-DNA assay (Versant HBV DNA [Bayer HealthCare, Tarrytown, N.Y.]) (34, 53, 54). A number of other assays are being developed such as the LCx HBV DNA quantitative assay (Abbott Laboratories), a transcription-mediated amplification assay for HBV DNA (Bayer HealthCare), molecular beacons (Abbott Laboratories), and a microarray method (2, 54, 60, 124).

The commercial assays currently available for quantitation are for research use only and have not been approved by the FDA for use in diagnosis. However, all of the quantitative assays for HBV DNA have been used to monitor the status of HBV infection before and after antiviral treatment and show promising results (8, 11, 66, 70, 95, 109).

Roche Diagnostics has a target amplification assay with the trade name COBAS Amplicor HBV Monitor test. The sensitivity of this test is claimed to be 60 IU of HBV/ml, and the linear dynamic range is 60 to 38,000 IU of HBV/ml. This assay can reproducibly detect the HBV viral load in patient serum or plasma (Table 2) (34, 62, 108). The assay uses synthetic biotin-labeled oligonucleotide primers that are complementary to a conserved sequence within the precore/core gene. The HBV DNA from patient serum is amplified by using the typical cycles of PCR, and quantitation is accomplished by comparison to standards incorporated into each patient sample. The standard is carried through all steps of the assay. The PCR amplicons are detected colorimetrically after hybridization to specific probes bound to microparticles. The automated COBAS Amplicor analyzer performs all the amplification, hybridization, and detection steps for this assay. The instrument calculates the level of HBV DNA in the patient's sample by comparing it to the quantitative standard of known copy number. Another PCR assay used to quantitate HBV DNA has been developed using the LightCycler (Roche Diagnostics). This assay can quantitate HBV DNA from serum over a wide viral load range (10 to 110,000,000 IU of HBV/ml). Results are rapid since the amplification and detection can be completed simultaneously in as little as 2.5 h (54, 66).

A third assay for quantitating HBV DNA by PCR is the HBV NGI SuperQuant, available only from LabCorp. This PCR assay was originally developed by the National Genetics Institute and has a broad dynamic range from 100 to 5×10^9 genome copies/ml.

Another assay, the Digene Hybrid Capture, uses liquid-phase hybridization for the quantitation of HBV DNA. The first step is the covalent binding of a full-length RNA probe to denatured HBV DNA. Then the RNA-DNA hybrid is captured on a solid phase by using antibody specific for RNA-DNA hybrids. Another anti-RNA-DNA antibody conjugated to alkaline phosphatase is used to generate a chemiluminescent signal. The Digene Hybrid Capture assay

is designed to quantitate HBV DNA concentrations ranging from 1.42×10^5 to 1.7×10^9 copies/ml. The Digene Hybrid Capture II is an ultra-sensitive assay that will quantitate HBV DNA concentrations ranging from 4.7×10^3 to 5.6×10^7 copies/ml. This increased sensitivity is gained by concentrating 1 ml of serum by ultracentrifugation (53, 62, 108).

A commercial assay that uses the signal amplification technique is the Bayer Quantiplex/Versant HBV DNA assay which uses branched-DNA technology (53, 62, 108). In this assay, synthetic oligodeoxyribonucleotides containing unique primary segments designed to hybridize to the minus-sense strand of HBV DNA are covalently attached to a secondary fragment through branch points. These target probes hybridize to HBV DNA and to complementary synthetic fragments bound to a solid phase. On another level, the secondary fragments of the target probe direct the binding of multiple copies of synthetic branched DNA amplifier molecules that contain repeated nucleotide sequences that hybridize with alkaline phosphatase-labeled probes. The detection scheme relies on chemiluminescence emission catalyzed by alkaline phosphatase from a dioxetane substrate. This assay will detect as few as 700,000 HBV DNA copies/ml and up to 5×10^9 copies/ml (53, 62, 108).

Several other assays for the quantitation of HBV DNA are being developed but are not on the market. Abbott Laboratories is developing a ligase chain reaction (LCx HBV DNA quantitative assay)-based assay. A transcription-mediated amplification assay has been developed by Bayer HealthCare. An intriguing new technology that is being applied to the quantitation of HBV DNA is the use of microarray technology (60). This technique may be useful for quantitation of HBV DNA and simultaneous genotyping of the virus and detection of mutations associated with antiviral resistance. However, the routine use of microarray technology will require automation of the assay and thus new instrumentation. Another new test under evaluation will rapidly detect mutations in HBV associated with antiviral resistance. The line probe assay (INNO-LiPA HBV DR) is manufactured by Innogenetics, N.V., in Gent, Belgium. This assay uses numerous specific probes designed to detect known mutations associated with antiviral resistance in the HBV polymerase gene (1, 111). The probes are applied to nitrocellulose membranes and then incubated with biotinylated PCR products generated from the HBV in patients' sera. The HBV PCR product specifically hybridizes to the probes by reverse hybridization. The hybridized product is detected by color development after addition of streptavidin-alkaline phosphatase. This assay provides a relatively rapid analysis of HBV mutations in a mixed population that is developing mutations associated with resistance. The assay is easy to use and permits the detection of known HBV mutations. However, it is unable to detect new mutations associated with antiviral resistance.

A previously mentioned concern with the quantitation of HBV DNA is the poor interassay standardization among the commercial assays. Thus, results from quantification of HBV DNA by different assays can vary even when the same specimen is tested (53, 62, 66). Intra-assay variation also exists such that repeat testing of the same sample may result in differences depending on the methodology. Thus, the WHO has established international standards for HBV quantitation units (104). All commercial assays for HBV DNA should be evaluated by the manufacturers by using the WHO HBV International Standard 97/746 that can be serially diluted in HBV-negative human plasma as part of the validation process. The goal of the WHO is to have all commercial HBV DNA quantitative assay results reported as international units of HBV DNA (104). At the current time, it is important for laboratories to use consistent methodologies for all patient samples. Variations in HBV DNA levels that are less than threefold (i.e., half a log) can be related to the intrinsic variability of the assays and must not be taken into account. In contrast, variations of more than threefold can reliably be considered as reflecting significant changes in HBV load.

Isolation Procedures

Cell Cultures and Animal Models

Despite evidence for the growth of HBV in primary cultures of healthy adult or fetal human hepatocytes (49, 93), serial propagation over a prolonged period has not been accomplished. Thus, culture of HBV as a diagnostic test is not available.

Animal models have played an essential role in studies of viral inactivation, vaccine safety and efficacy, disinfection kinetics, infectivity determinations, and immunopathology and in seroepidemiologic investigations. Most have used chimpanzees and other high-order primates since they are highly susceptible to experimental infection with hepatitis B. The pattern of infection is similar to that observed in humans except that the disease is clinically milder.

Identification

Culture methods for HBV are not used in the clinical laboratory for diagnostic purposes. Thus, identification of HBV is based on serologic and molecular methods.

Typing Systems

HBV has been grouped into eight genotypes designated A through H (61, 91). These genotypes have been associated with clinical and serologic variation in patients infected with HBV (61, 91, 105). The first typing of HBV used serologic analysis to distinguish antigenic heterogeneity of the HBsAg. Nine HBsAg subtypes have been designated: *ayw1, ayw2, ayw3, ayw4, ayr, adw2, adw4q*$^-$, *adrq*$^+$, and *adrq*$^-$ (91). These antigenic differences led to the genetic typing and eventually to the genotyping of HBV isolates into eight distinct groups that differ by more than 8% from one another (91, 92). Subsequent reports have segregated the genotypes into subgroups with distinct geographical origins (5, 63, 91). The strains in genotype A derive primarily from Europe, India, Africa, and North America. There are two subgenotypes within genotype A, designated A1 and A2, which are found primarily in South Africa. Genotype B occurs mainly in China, Japan, and southeast Asia. There are four subgenotypes in this group, designated B1 through B4. The B1 subgenotype is found primarily in Japan, whereas in China the major subgenotype is B2. Subgenotype B3 was identified in Indonesia, and subgenotype B4 is detected primarily in Vietnam. Both genotype A and B isolates have primarily the *adw2* epitope, with a few exceptions such as B4 and some A1 isolates that have the *ayw1* epitope. At this time, the genotype C has four subgenotypes, C1 through C4, which are found primarily in Asia, Polynesia, Australia, and the Pacific Islands. The C1 subgenotype was detected in isolates from Japan, Korea, and China. The C2 subgenotype is found in China and southeast Asia. The C3 subgenotype was isolated from a very specific population of aboriginals from New Zealand to Polynesia. The C4 subgenotype comprises only two strains from aborigines in northeast Australia. Genotype D is widespread across Europe, Asia, and Africa and has been divided into four subgenotypes. This genotype is not restricted to a specific geographic region; rather, it is found

worldwide. Most isolates from the Middle East are of the D1 subgenotype, and HBV isolates from South Africa and Alaska are primarily D3. Genotype E is found only in West Africa and currently has no subgenotypes. All the HBV strains that are genotype E have the *ayw4* antigen. Genotype F strains are divided into two subgenotypes, called F1 and F2. F1 strains are found primarily in Central America, and F2 strains are primarily from South America. Genotypes G and H are not subgenotyped. G strains originate from the United States, Mexico, and Europe, and H strains are from Central America and California.

Genotyping of HBV may become important in disease management. Recent studies have evaluated the epidemiology of HBV genotypes and their association with progression to liver disease and HCC (31, 32, 38, 96, 97, 112, 122). Most of the studies have been conducted in Asian countries because of the large number of patients in these countries with chronic HBV infection. A number of studies have shown that patients infected with HBV genotype C are HBeAg positive for a longer period of time, have more active liver disease, and progress to cirrhosis more rapidly than patients infected with HBV genotype B (31, 32, 38, 96). Additional studies have shown that HBV-infected patients treated with pegylated interferon have a better response if they are infected with genotype A than if they are infected with HBV genotype D (4, 40). However, these preliminary studies on the impact of HBV genotypes and antiviral therapy need to be expanded before definitive conclusions can be made.

Serologic Tests

Detection of HBV Antigens and Antibodies

Serologic assays with high levels of sensitivity, specificity, and reproducibility have been developed to detect HBV antigens and antibodies. Table 3 lists the manufacturers of commercially available assays, the detection method used in each assay, and the amount of specimen needed. Some of the assays have been licensed or cleared by the FDA, and others are available outside the United States. The FDA website (http://www.fda.gov) can be checked to determine which assays are approved for use in the United States. EIAs dominate the diagnostic marketplace because the introduction of EIA kits some 20 years ago obviated the need to handle radioisotopes and provided kits with significantly longer outdates than their radioimmunoassay (RIA) counterparts. These solid-phase assays use either the sandwich principle or competitive binding. Originally, the solid phase was either a polystyrene bead or a microtiter well. More recently, microparticles have begun to replace beads in many assays, and wells of different compositions and sizes are now being used with automated platforms.

Antigen or antibody capture and detection reagents have also evolved over time. Early assays used hyperimmune animal sera or human immune sera which were later replaced by mouse monoclonal antibodies exclusively or by a combination of hyperimmune sera and monoclonal antibodies. Likewise, native, purified antigens have given way to recombinant antigens in some assays. Whereas detector antigens or antibodies labeled with horseradish peroxidase and colorimetric endpoints are still available in some assays, other detection systems such as fluorescence polarization, chemiluminescence, and now electrochemiluminescence are replacing colorimetric detection.

In some assays, testing of reactive samples must be repeated in duplicate in an independent run to confirm the results. If one or both of the duplicate results are reactive, the sample is said to be repeatably reactive. According to the manufacturers' package inserts, samples repeatably reactive for HBsAg should be subjected to specificity testing with the confirmatory kit provided by the manufacturer of the screening test that identified the reactive samples. All confirmatory assays are based on the principle of specific antibody neutralization. All of the assays utilize human anti-HBs and normal human plasma devoid of anti-HBs. Repeatably reactive samples are incubated in duplicate in the presence of human anti-HBs and in duplicate in the presence of the anti-HBs-negative human plasma. If HBsAg is present in the sample, the anti-HBs-containing reagent binds to it, blocks its binding to the anti-HBs capture antibody on the solid phase, and yields a negative result in the confirmatory test. Conversely, since no anti-HBs is present in the normal human serum, HBsAg will bind to the capture antibody on the solid phase and yield a positive test result. By using the detection values given by the sample in the presence and absence of anti-HBs, the amount of signal reduction can be calculated. If the percent reduction reaches a level defined by the test manufacturer, usually >50%, the sample is considered to be positive for HBsAg. In the diagnostic setting where the panel testing is performed, confirmatory testing for HBsAg-reactive, IgM anti-HBc-positive samples is often not done.

The older assays (bead or well based) can detect HBsAg in the range of 0.25 to 0.5 ng/ml. Newer assays are capable of detecting subnanogram amounts of HBsAg with no trade-off in specificity. For most diagnostic applications, the increased sensitivity does not result in a significant increase in detectability because of the copious amounts of HBsAg present in the blood of individuals who are either acutely or chronically infected with HBV. However, some of the newer automated assays have a reduced time for detection and require less technical personnel time.

At present, 10 commercial assays are available for detecting HBsAg (Table 3). All are solid-phase tests based on the sandwich principle. Four are EIAs (AUSZYME monoclonal EIA [Abbott Laboratories], Genetic Systems HBsAg EIA 3.0 [Bio-Rad Laboratories], ETI-MAK-2 PLUS [DiaSorin], and Ortho HBsAg System 3 [Ortho-Clinical Diagnostics]), four are chemiluminescent EIAs (CEIAs; PRISM HBsAg [Abbott Laboratories], ADVIA Centaur HBsAg total assay [Bayer HealthCare], Immulite 2000 HBsAg, [Diagnostic Products Corporation], and VITROS HBsAg assay [Ortho-Clinical Diagnostics]), one is an enzyme-linked fluorescent assay (ELFA; AxSYM HBsAg [Abbott Laboratories]), and one is an electrochemiluminescence EIA (ECL-EIA; Elecsys HBsAg [Roche Diagnostics]). Not all of the assays listed in Table 3 are approved for diagnostic use in the United States, and only four (Abbott AUSZYME, Abbott PRISM HBsAg, Genetic Systems HBsAg EIA 3.0, and Ortho HBsAg System 3) are approved for blood screening.

In all of the assays to detect anti-HBs, purified human HBsAg is bound to a solid phase to capture anti-HBs present in the test specimen. The DiaSorin test uses recombinant HBsAg as the solid-phase antigen with a human-sourced HBsAg as the labeled probe. All of the other anti-HBs tests currently licensed for use in the United States use HBsAg purified from human plasma. Newer tests, soon to be licensed, e.g., Abbott AxSYM and Architect, will use recombinant HBsAg in lieu of human-sourced material. After capture, the human antibody is then detected by using a variety of detection systems listed in Table 3. Both the *ad* and *ay* subtypes of human HBsAg are represented in the detection steps of all the assays.

TABLE 3 U.S. commercial systems for serologic testing of HBV

Marker and manufacturer	Name of assay	Detection method	Minimum vol (μl) of specimen
HBsAg			
Abbott Laboratories	AUSZYME monoclonal EIA	EIA	200
	AxSYM HBsAg	ELFA	100
	PRISM HBsAg	CEIA	400
Bayer HealthCare	ADVIA Centaur HBsAg total assay	CEIA	100
Bio-Rad Laboratories	Genetic Systems HBsAg EIA 3.0	EIA	100
Diagnostic Products Corporation	Immulite 2000 HBsAg	CEIA	100
DiaSorin	ETI-MAK-2 PLUS (HBsAg) ELISA	EIA	100
Ortho-Clinical Diagnostics	VITROS HBsAg assay	CEIA	80
Ortho	HBsAg System B	EIA	150
Roche Diagnostics	Elecsys HBsAg	ECL-EIA	50
Anti-HBs			
Abbott Laboratories	AUSAB EIA	EIA	200
Bayer HealthCare	ADVIA Centaur anti-HBs assay	CEIA	100
Diagnostic Products Corporation	Immulite 2000 anti-HBs	CEIA	50
DiaSorin	ETI-AB-AUK PLUS (anti-HBs) ELISA	EIA	100
Ortho-Clinical Diagnostics	VITROS anti-HBs assay (quantitative)	CEIA	80
Roche Diagnostics	Elecsys anti-HBs	ECL-EIA	40
Anti-HBc			
Abbott Laboratories	CORZYME EIA	EIA	100
	PRISM HBcore	CEIA	400
Bayer HealthCare	ADVIA Centaur HBc total assay	CEIA	50
Diagnostic Products Corporation	Immulite 2000 anti-HBc	CEIA	50
DiaSorin	ETI-AB-COREK PLUS (anti-HBc) ELISA	EIA	50
Ortho-Clinical Diagnostics	Ortho HBc ELISA test system	EIA	10
	VITROS anti-HBc assay	CEIA	50
IgM anti-HBc			
Abbott Laboratories	CORZYME-M (recombinant protein)	EIA	
	IMx CORE-M MEIA	EIA	150
Bayer HealthCare	ADVIA Centaur HBc IgM assay	CEIA	15
Diagnostic Products Corporation	Immulite 2000 anti-HBc IgM	CEIA	10
DiaSorin	ETI-CORE-IgMK PLUS (IgM anti-HBc) ELISA	EIA	100
Ortho-Clinical Diagnostics	VITROS anti-HBc IgM assay	CEIA	10
Anti-HBe			
DiaSorin	ETI-AB-EBK PLUS (anti-HBe) ELISA	EIA	50
Roche Diagnostics	Elecsys anti-HBe	ECL-EIA	35
HBeAg			
DiaSorin	ETI-EBK PLUS (HBeAg) ELISA	EIA	100
Roche Diagnostics	Elecsys HBeAg	ECL-EIA	35

As hepatitis B vaccination has become more widespread, the use of anti-HBs to monitor vaccine success rates has increased. Both the WHO and the CDC recognize a level of anti-HBs at 10 mIU/ml as being protective. The CDC recommends postvaccination testing for anti-HBs for health care workers who have blood or patient contact (24). Although the cutoffs of some currently available anti-HBs tests are near this level of detection, others detect anti-HBs at lower concentrations. Hence, a positive test does not necessarily indicate immunity according to WHO and CDC criteria. Anti-HBs levels decline over time, and approximately 60% of individuals who respond to vaccination lose detectable levels of antibodies over 10 to 12 years (110). Studies have demonstrated that vaccine-induced immunity continues to prevent clinical disease despite declining levels of antibody. As a result, the CDC states that "periodic serologic testing to monitor antibody concentrations after completion of the three-dose series is not recommended" (24).

Six commercial anti-HBc kits are available (Table 3). Three are EIAs (CORZYME, [Abbott], ETI-AB-COREK PLUS [DiaSorin], and Ortho HBc ELISA test system [Ortho-Clinical Diagnostics]), and three are CEIAs (ADVIA Centaur HBc total assay [Bayer HealthCare], Immulite 2000 anti-HBc [Diagnostic Products Corporation], and VITROS anti-HBc assay [Ortho-Clinical Diagnostics]). Some tests to detect anti-HBc are based on competitive binding, and all use recombinant HBcAg. In some of the assays, binding of the patient's anti-HBc is in competition with that of a monoclonal anti-HBc. In other assays, recombinant HBcAg is labeled with two different detectors to assess competitive anti-HBc binding. In

most assays, the specimens containing anti-HBc lead to signal suppression that is proportional to the amount of anti-HBc present. Other assays use the traditional sandwich EIA. The sandwich-based EIA for anti-HBc is carried out in HBcAg-coated microtiter wells to which patient anti-HBc binds. After residual sample is washed away, bound anti-HBc is detected by enzyme-labeled murine monoclonals with specificities for IgG and IgM. Values generated by positive and negative controls are used to calculate the assay cutoff and to establish run validity.

Some anti-HBc kits have testing algorithms similar to the ones required by HBsAg and anti-HBs tests. That is, duplicates of an initially reactive sample must be tested in a second, independent run. If one or both duplicates are reactive, the sample is repeatably reactive, or positive. However, results generated by recently approved automated assays do not require repeat testing unless results fall into a gray zone around the test cutoff value. In addition to their use for the diagnosis of prior or ongoing HBV infection, Abbott CORZYME and Ortho HBc ELISA test system kits may be used for blood donor screening. The Abbott PRISM anti-HBc is primarily a blood donor screening test; however, it can also be used for patient diagnosis. The other anti-HBc assays are not approved for blood donor screening.

Table 3 also gives details on six commercial kits available for the detection of IgM anti-HBc, three EIAs (CORZYME-M and IMx CORE-M MEIA [Abbott] and ETI-CORE-IgMK PLUS [DiaSorin]) and three CEIAs (ADVIA Centaur HBc IgM assay, [Bayer HealthCare], Immulite 2000 anti-HBc IgM [Diagnostic Products Corporation], and VITROS anti-HBc IgM assay [Ortho-Clinical Diagnostics]. Anti-human IgM is bound to the solid phase and used to capture IgM class antibody in the patient's serum, including IgM anti-HBc if present. Recombinant HBcAg is then added and binds to IgM anti-HBc. Enzyme-conjugated human anti-HBc is then added and in turn binds to HBcAg to complete the multilayer sandwich. The signal from the conjugated human anti-HBc is detected and evaluated. Both negative and positive controls contribute to the cutoff calculation in the bead-based CORZYME-M EIA, whereas a calibrator(s) establishes the cutoff in the other assays. Run validity is established with negative and positive controls.

Two HBeAg and two anti-HBe assays are available from two manufacturers (ETI-AB-EBK PLUS [anti-HBe] ELISA and ETI-EBK PLUS [HBeAg] ELISA [DiaSorin] and Elecsys anti-HBe and Elecsys HBeAg [Roche Diagnostics]). Both manufacturers use direct sandwich assays to detect HBeAg where anti-HBe bound to the solid phase captures HBeAg in the patient's specimen. Detection of bound antigen is accomplished by using enzyme-labeled human anti-HBe. All the assays establish run validity through the use of both positive and negative controls.

Competitive binding assays serve as the basis for anti-HBe detection in each of the available assays. The solid-phase anti-HBe reagents are the same ones described above for the detection of HBeAg. To detect anti-HBe, the patient sample and a measured amount of labeled recombinant HBeAg are added simultaneously to the solid phase. If anti-HBe is present in the patient's sample, it competes with solid-phase anti-HBe for the limited number of binding sites on the added HBeAg. Positive and negative control values are used in cutoff and run validity calculations. As with other competitive assays, and in contrast to direct sandwich assays, samples containing anti-HBe yield low detection outputs, i.e., below the respective assay cutoffs. The HBeAg and anti-HBe tests may be used for diagnosis and monitoring.

Antiviral Susceptibilities

At the current time, no specific treatment is available to patients with acute HBV infection except for supportive and symptomatic care (46, 70). However, the treatment of chronic HBV infection has changed in the last few years from bed rest to effective antiviral therapies. The major goal of treating chronic HBV infection is to suppress viral replication and halt the progression of liver damage before it leads to cirrhosis and/or HCC (36, 75, 77, 98). Although numerous antiviral and immunomodulatory therapies have been investigated in the last 30 years, only three approved therapies are available in the United States. These are alpha interferon (IFN-α); lamivudine (Epivir HBV), a nucleoside analogue; and adefovir dipivoxil, an adenosine monophosphate nucleotide analogue (36, 77, 98). Interferon was the first therapy approved for chronic HBV infection; thus, more information regarding the outcome of this treatment is available. The long-term outcome of IFN-α treatment is promising in that, of the HBeAg-positive patients who respond to this therapy, 80 to 90% remain negative for HBeAg after 4 to 8 years (3, 77, 98). However, HBV DNA is still detected in most of the patients when tested by PCR. Several studies have shown that 77% of patients receiving lamivudine therapy are HBeAg negative after 3 years (37, 106). Unfortunately, resistance to lamivudine has been detected in patients given this drug (9, 58). Resistance is manifested as the reappearance of HBV DNA in serum as detected by using a nonamplification assay. Of great interest are the studies showing that adefovir dipivoxil treatment results in a greater than 3 $\log_{10}$ reduction in HBV DNA levels after 2 years regardless of HBeAg status (51, 83). However, there have been reports of adefovir dipivoxil resistance developing in a small percentage of patients treated with this drug (51, 69, 82). A number of other antiviral agents, such as famciclovir, entecavir, emtricitabine, telbivudine, clevudine, and tenofovir, as well as various combinations of antiviral drugs, are currently being tested for possible use in the treatment of chronic HBV infection (21, 82).

Evaluation, Interpretation, and Reporting of Results

As shown in Fig. 4 and 5 and summarized in Table 4, the presence of HBsAg in serum indicates either acute or chronic active HBV infection. In a typical acute HBV infection,

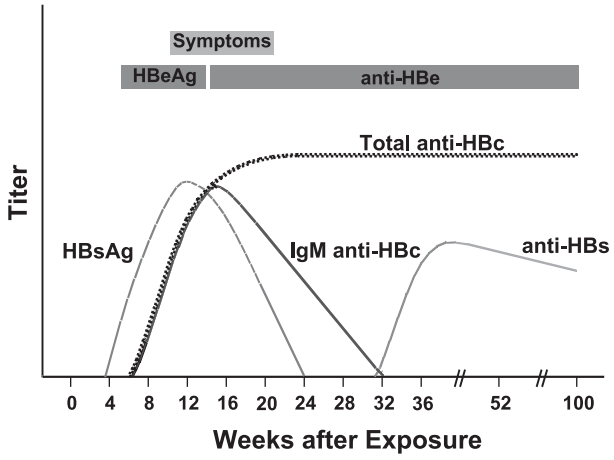


FIGURE 4 Typical sequence of serologic markers in patients with acute hepatitis B with resolution of symptoms (CDC website: http://www.cdc.gov/ncidod/diseases/hepatitis/slideset/hep_b/slide_3.htm.)

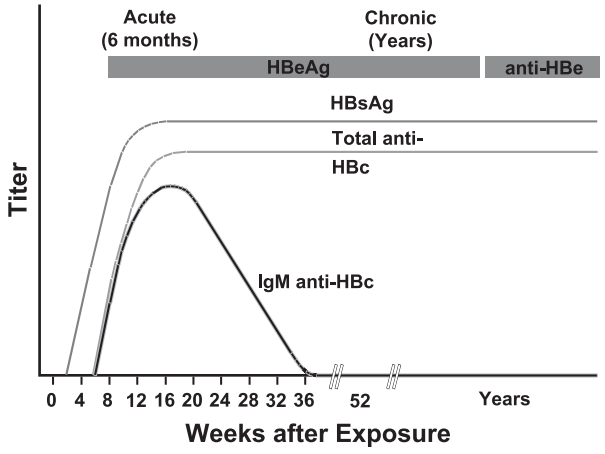


FIGURE 5 Typical sequence of serologic markers in patients with hepatitis B that progresses to a chronic course. In patients with chronic HBV infection, both HBsAg and IgG anti-HBc remain persistently detectable, generally for life. HBeAg is variably present in these patients. (CDC website: http://www.cdc.gov/ncidod/diseases/hepatitis/slideset/hep_b/slide_4.htm.)

HBsAg will be detected 2 to 4 weeks before the ALT level becomes abnormal and 3 to 5 weeks before symptoms or jaundice develops. The presence of HBV DNA precedes the appearance of HBsAg in the serum by 3 to 4 weeks. Anti-HBc, primarily of the IgM class, usually appears when the ALT level begins to increase. The presence of IgM-specific anti-HBc at a relatively high titer (>1:1,000) is evidence of an acute infection. These elevated IgM titers decline regardless of whether the clinical disease resolves or becomes chronic. Figure 5 illustrates the typical serologic pattern that occurs in a patient who becomes chronically infected. Sera from 5 to 15% of chronic hepatitis B patients may be positive by the IgM anti-HBc assay during reactivation of their disease; however, the IgM-specific anti-HBc levels are generally low, e.g., near the cutoff level for the test.

Because the total anti-HBc test is invariably positive when HBsAg is present in a clinically ill patient, this test can be used to validate the HBsAg reaction. In perhaps 5 to 10% of patients with acute hepatitis B (especially those with fulminant disease) and more frequently during early convalescence, HBsAg may be undetectable in sera.

One diagnostic dilemma is the patient with only anti-HBc detected over time without detectable anti-HBs and HBsAg. A recent workshop addressed this situation and found that in geographic areas where HBV endemicity is low, the rate of patients with only anti-HBc is 10 to 20% of all patients with HBV markers (50). About 10% of these patients had detectable HBV DNA when tested by using a PCR assay. Patients with only anti-HBc may be individuals in the "window" period and may be resolving acute HBV infection. In other patients, this result may reflect past infection that resolved or may represent an unresolved, low-grade HBV chronic infection. A recent case report has shown that a patient with only the anti-HBc marker reactivated HBV infection during immunosuppression due to human immunodeficiency virus disease (28). Therefore, it is important to consider that infections in some individuals with only anti-HBc as a marker for HBV infection may reactivate during immunosuppression. Moreover, these individuals should not be considered as blood donors.

The presence of anti-HBc alone is plausible evidence for an active or past HBV infection. Some individuals who have recovered from hepatitis B with the development of anti-HBs and anti-HBc may eventually lose their anti-HBs, resulting in a solitary anti-HBc response. Challenging a patient displaying this profile with hepatitis B vaccine leads to an anamnestic response 2 weeks later (anti-HBs level, >20 mIU/ml), whereas an HBsAg-negative carrier fails to respond in this time interval. The detection of anti-HBc alone may also represent a false-positive test result. Thus, it may be prudent to repeat the test with a newly acquired specimen.

An additional consideration in a patient with only anti-HBc as a specific serologic marker of HBV infection is the presence of a mutant HBV. A number of reports have described HBV mutant strains that resulted in conformational changes in the HBsAg or the HBeAg (117, 118). These mutants are usually not detected by commercial HBsAg or HBeAg kits. Thus, patients with unusual serologic patterns such as (i) anti-HBc reactivity only, (ii) a positive HBeAg result but a negative HBsAg result, and (iii) positive anti-HBc and anti-HBs results but a negative HBsAg result (similar to the serologic pattern that is observed in patients with a past infection) may be considered to be potentially infected with a mutant HBV. In these circumstances, the

TABLE 4 HBV markers in different stages of infections and convalescence[a]

Stage of infection	HBV DNA	HBsAg	HBeAg	Anti-HBc		Anti-HBe	Anti-HBs
				Total	IgM		
Early incubation	+	−	−	−	−	−	−
Late incubation	+	+	+ or −	−	−	−	−
Acute infection	+	+	+	+	+	−	−
HBsAg-negative acute infection	−	−	−	+	+	−	−
Chronic infection	+	+	+	++	+ or −	−	−
Healthy HBsAg carrier	−	+	−	++	+ or −	+	−
Recent infection[b]	+ or −	−	−	++	+	+	+ or ++
Remote infection[c]	−	−	−	+	−	−	+ or −
Vaccination response	−	−	−	−	−	−	+

[a] Modified from reference 103. ++, marker almost always present; +, generally present; −, typically not present.
[b] This description can be applied to early convalescence and to individuals who remain HBV DNA positive for prolonged periods in the absence of HBsAg.
[c] This description can be applied to individuals with anti-HBc in the absence of other serologic markers, including DNA. These patients may or may not have anti-HBs. There is evidence that these patients may reactivate HBV infection during immunosuppression (28).

detection of a high number of HBV DNA copies in plasma will help resolve this serologic interpretation. In the absence of anti-HBc and HBsAg, past or present hepatitis B disease can almost always be excluded. However, in rare cases HBV DNA has been detected in the sera and/or livers of some HBsAg-negative patients with or without other HBV serologic markers (20). Finally, due to the increased sensitivity of PCR, HBV DNA can be detected in the livers of some patients who have recovered completely from their infections and thus may be able to reactivate the disease during immunosuppression (20).

Anti-HBs usually becomes detectable immediately after the disappearance of HBsAg. Some patients have become anti-HBs positive several weeks after the loss of HBsAg. Anti-HBs is produced much earlier but is not observed because of the formation of immune complexes in the presence of excess HBsAg (113). Anti-HBs, with or without anti-HBc, signifies immunity to reinfection and indicates adequate protection after hepatitis B vaccination. Anti-HBs without anti-HBc develops in persons who receive the hepatitis B vaccine (which contains only HBsAg), and levels of ≥10 IU/ml are considered protective. However, many of the commercial assays can detect levels much lower than 10 IU/ml. It has been proposed that detection of anti-HBc, not detection of anti-HBs, be used as a screening test to determine susceptibility to HBV for the purpose of recommending hepatitis B vaccination. Depending on the circumstances, passive transfer of anti-HBs or anti-HBc may be observed in patients receiving clotting factor concentrates derived from human plasma, in patients after immunoglobulin administration, and in neonates of mothers with current or past hepatitis B. Since blood donations are pretested for HBsAg and total anti-HBc, passive transfer of antibodies following transfusions of blood is unlikely unless the donor has been vaccinated (donors are not tested for anti-HBs). Recognition of these possibilities will avoid an erroneous determination of HBV exposure or infection, because passive antibodies disappear gradually over 3 to 6 months, whereas actively produced antibodies are remarkably stable over many years.

Patients with chronic HBV infection should be tested for HBeAg, anti-HBe, or HBV DNA to determine their level of HBV replication and their potential for infectivity. HBeAg-positive specimens usually contain high concentrations of HBV DNA in contrast to anti-HBe-positive samples, in which the number of hepatitis B virions is markedly reduced. Patients who are HBeAg positive are more likely to transmit hepatitis B sexually, percutaneously, or perinatally. Seroconversion of a patient with HBeAg to an anti-HBe status with loss of HBV DNA is a favorable prognostic sign either after treatment with antiviral agents or when it occurs spontaneously.

HEPATITIS D VIRUS

Taxonomy

HDV is an unclassified, roughly spherical particle which is smaller than HBV, with a diameter ranging from 36 to 43 nm (103). The genome of HDV was sequenced in 1986 and found to comprise single-stranded RNA (116). The HBV genome is not homologous with the HBV genome and is not organized like a hepadnavirus genome. HDV is classified as a deltavirus (64, 87).

Description of the Agent

HDV is considered a satellite virus of HBV but biologically has no resemblance to HBV and is in a separate genus (64, 87). HDV contains a circular RNA genome that has substantial internal complementarity resulting in an unbranched, rodlike structure reminiscent of viroids or plant RNA satellite viruses. The genome is replicated by the host RNA polymerase II rather than by an HDV-encoded RNA polymerase. Replication of HDV RNA involves a rolling circle mechanism with self-cleavage. The HDV RNA is surrounded by a unique nucleocapsid protein of 68,000 Da called the HDV antigen (HDAg), which itself is surrounded by an envelope consisting of HBsAg. The HDAg, a nonglycosylated phosphoprotein, is the only product of the HDV genome. It consists of two proteins, a 195-amino-acid, 24-kDa protein and a larger, 214-amino-acid, 27-kDa protein, that play a role in the assembly of the virus (114, 115).

Epidemiology and Transmission

The incidence of HDV infection is very high in the Mediterranean Basin, the Middle East, Central Asia, West Africa, parts of South America, and some South Pacific islands (64). In the United States, most cases of HDV infection are observed in populations frequently exposed to blood-contaminated needles and blood products, such as injection drug users and hemophiliacs. Because hemophiliacs are now effectively vaccinated against hepatitis B and recombinant clotting factor concentrates are replacing plasma-derived products, hemophiliacs are no longer at risk for HDV infection. Secondary cases have been recorded in sexual partners of drug users, and such cases can amplify the spread of HDV infection within population groups other than those recognized as being at high risk for HDV infection. Person-to-person spread, including spread through household contact, is an important mode of transmission in Mediterranean countries. In South American populations with high HBV carrier rates, recurring epidemics of clinically severe acute hepatitis D have been observed (Fig. 3 and 6) (87).

Clinical Significance

Because HDV infection can become established only in persons with HBV infection, acute HDV infection can occur simultaneously with acute HBV infection (coinfection) or HDV infection can become superimposed upon an already established chronic HBV infection (superinfection).

HDV and HBV Coinfection

Coinfection with HBV and HDV usually causes an acute illness indistinguishable from acute HBV infection alone. The likelihood of fulminant hepatitis can be as high as 5% in coinfected patients. A biphasic course is sometimes observed during coinfection. HDV infection does not increase the rate of chronic hepatitis B carriers. Acute HDV infection generally occurs after a 3- to 7-week incubation period. The initial symptoms are fatigue, anorexia, and nausea that occur for 3 to 7 days. During this phase, liver enzymes become abnormal, which then leads to the typical appearance of jaundice and the onset of the icteric phase. Fatigue and nausea persist, with clay-colored stools and dark urine. In patients with acute, self-limited infection, convalescence begins with the disappearance of clinical symptoms. Fatigue may persist for longer periods of time (46, 87).

HDV Superinfection

HDV superinfection occurs when an HBV carrier is infected with HDV. Superinfection with HDV most often leads to severe, acute hepatitis with a short incubation period. Acquiring HDV in this fashion leads to chronic hepatitis D in up to 80% of cases. Chronic hepatitis D from superinfections



FIGURE 6 Worldwide prevalence of HDV-positive individuals. Data are from the CDC, 2005. (CDC website: http://www.cdc.gov/ncidod/diseases/hepatitis/slideset/hep_d/slide_6.htm.)

can lead to cirrhosis or to fulminant hepatitis (100). Fulminant hepatitis occurs more frequently after HDV superinfection than after other types of viral hepatitis. During acute HDV infection, the production of HBsAg and HBV DNA is inhibited.

Fulminant HDV infection is characterized by a serious clinical presentation resulting from encephalopathy. This complication can lead to confusion, changes in personality, problems with sleep, difficulty concentrating, and loss of consciousness. The mortality rate of fulminant hepatitis D can be as high as 80% (100). Patients with hepatitis D who have hepatic decompensation are candidates for liver transplantation, the outcome of which is better than that for patients with chronic hepatitis B alone (46).

A large percentage of patients with chronic HDV infection (60 to 70%) develop cirrhosis, usually after 5 to 10 years. However, in some patients cirrhosis can appear as early as 2 years after the onset of infection. Mortality due to hepatic failure is high among these patients. HCC develops with the same frequency in chronically infected HDV patients as in patients with HBV infection alone. The overall mortality rate for HDV infections is between 2 and 20%, which is 10 times higher than that for HBV infection alone.

Some success has been achieved in treating chronically HDV-infected patients with IFN-α. High-dose, long-term therapy is required, but relapses are common after therapy is stopped (41, 42). Recently, lamivudine has been used in patients with chronic hepatitis D (90).

Collection, Transport, and Storage of Specimens

The recommendations for the collection of specimens are similar to those for HBV. Serum or plasma should be separated and stored at 2 to 8°C, and testing should be performed within 5 days. Severely hemolyzed blood should be avoided due to the possibility of false-positive results. Repetitive freezing and thawing of specimens may lead to a loss in accurate detection. Specimens for nucleic acid analyses should be processed within 6 h, and if not tested within 24 h, they can be stored at −70°C or lower. The above-described recommendations are general, conservative guidelines that may not conform to those specified by reference laboratories offering HDV testing.

Direct Detection

Microscopy

HDAg can be detected directly in biopsy material by using microscopy to visualize specific staining using antibody specific for HDV antigen. Immunohistochemical staining of liver biopsy material for intrahepatic HDAg is a reliable way to demonstrate ongoing HDV replication. However, this method does not lend itself to simple, rapid diagnostic testing and requires that a liver biopsy be done. The anti-HDV is conjugated to fluorescein or peroxidase. Anti-HDV is derived from serum or plasma containing a high titer of anti-HDV accompanied by a low level of anti-HBc. The latter can be reduced or eliminated by dilution. An anti-HDV-negative, anti-HBc-positive control probe must be used in parallel with the anti-HDV probe to validate the anti-HDV specificity of staining. Cryostat sections are the optimum specimens. However, HDAg is stable for formalin fixation and paraffin embedding and therefore can be studied in stored paraffin-embedded tissue after digestion of the section with trypsin or pronase (57).

Antigen Tests

In the United States, commercial kits to detect HDAg in peripheral blood samples have never come to the market because of limited demand for this test. HDAg EIA services are available from a few clinical reference laboratories.

HDVAg can also be detected by Western blotting. More sensitive than EIA, Western blotting is reactive for more than 70% of patients with chronic hepatitis D. Because of its technical difficulty, Western blotting has not been used as a diagnostic test; rather, it remains a technique confined to a limited number of research laboratories. Methods used to detect HDV antibody in serum are described below in "Serologic Tests for HDV."

Nucleic Acid Detection Techniques

HDV RNA can be detected in peripheral blood samples by using real-time, reverse transcription-PCR (42, 86, 120). This technique has provided a more precise understanding of HDV infections. The levels of HDV RNA in the sera of patients with chronic hepatitis tend to be higher than the levels in asymptomatic carriers (120). Levels of HDV RNA are reduced in patients treated with IFN-α (42, 68). These reductions in HDV RNA levels are associated with improvements in liver fibrosis. Thus, the reverse transcription-PCR assay can be used to monitor response to antiviral therapy. In the United States, the assay is performed by a few reference laboratories.

SEROLOGIC TESTS FOR HDV

Antibody Tests

Tests for anti-HDV require a source of HDAg. Previously, HDAg has been obtained from HDAg-positive human (postmortem) or chimpanzee livers or serum. A more reliable source is now available from the livers of HDAg-positive woodchucks.

Commercial kits to detect IgM anti-HDV are unavailable in the United States, but testing can be accessed through a limited number of reference laboratories in the form of IgM antibody capture EIAs.

Commercial kits to detect total anti-HDV appeared in the United States in the early 1980s, first as an RIA and then as an EIA which supplanted the RIA. Because demand for anti-HDV testing has been historically low, the EIA kit was withdrawn from the U.S. market. However, testing for total anti-HDV can be obtained from the same reference laboratories offering tests for HDAg and IgM and anti-HDV.

Evaluation, Interpretation, and Reporting of Results

Any individual who is positive for HBsAg may have HDV infection, especially if the patient is from an area in which HDV infections are prevalent (Fig. 6). The diagnosis of both acute and chronic HDV infections uses the detection of HDV-specific antibodies and antigen as well as the detection of HDV RNA (86, 87, 120). The detection of total anti-HDV is sufficient for making the diagnosis of HDV infection. Anti-HDV serology should be considered when the patient is HBsAg positive, has elevated transaminase levels, and is from a country where HDV infection is prevalent. However, anti-HDV disappears shortly after an acute infection (coinfection with HBV) and may not be a consistent marker to indicate past infection (Fig. 7). Only those patients that have positive serology results for HDAg or anti-HDV should be tested for the presence of HDV RNA.

The interpretation of HDV infection diagnostic tests and their correlation with disease conditions are discussed below. The majority of this material has been adapted from the WHO website (http://www.who.int/csr/disease/hepatitis/whocdscsrncs20011/en/index3.html).

The molecular and serologic natural history of acute HBV-HDV coinfection is graphically depicted in Fig. 7. During a coinfection, HBsAg and HBV DNA can be detected in serum during the incubation period. At the onset of clinical disease, anti-HBc, HDV IgM, and HDAg (not shown due to its transient and low level if present) can be detected in serum. Late in the acute phase, anti-HDV antibodies develop and then decline to subdetectable levels when clinical symptoms resolve. With the appearance of anti-HDV, HDAg disappears. During early convalescence,

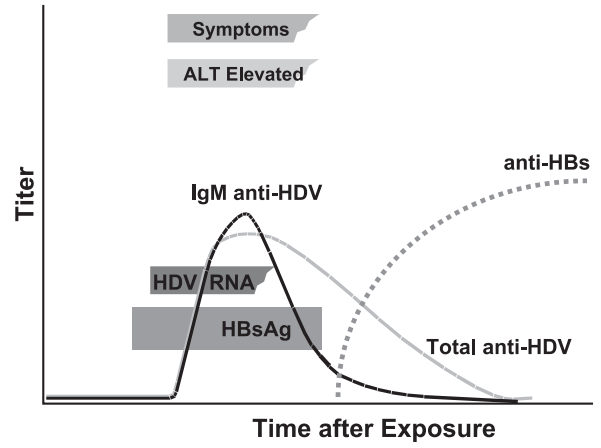


FIGURE 7 Serologic course of HDV infection when the virus is acquired as a coinfection with HBV. (CDC website: http://www.cdc.gov/ncidod/diseases/hepatitis/slideset/hep_d/slide_4.htm.)

viral replication (HDV RNA) cannot be detected and the level of anti-HDV begins to decline. The serologic markers of HDV infection usually disappear within months after recovery from acute disease. Since antibodies to HDV do not always persist after the acute infection is cleared, detection of past HDV infection is not easy to confirm. In contrast, when an HBsAg carrier acquires a superinfection with HDV, there is a loss of IgM anti-HBc and anti-HDV (IgM and total) is detected. The molecular and serologic history of HBV-HDV superinfection is graphically depicted in Fig. 8. Both HBsAg and HDV RNA appear in serum early during the infection. Chronic HDV infection is associated with the ongoing detection of HDV RNA, IgM anti-HDV, and total anti-HDV, and these markers persist indefinitely. The usual outcome of an HDV superinfection is a persistent, chronic infection. The detection of HDV RNA levels in serum can assist in evaluating the stage of HDV infection and the response to therapy.

Considerable progress has been made in the understanding of HDV epidemiology. Additionally, there is more information on the molecular characteristics of this satellite virus and its association with HBV (64, 86). The evolution of knowledge about the clinical consequences of HDV-HBV

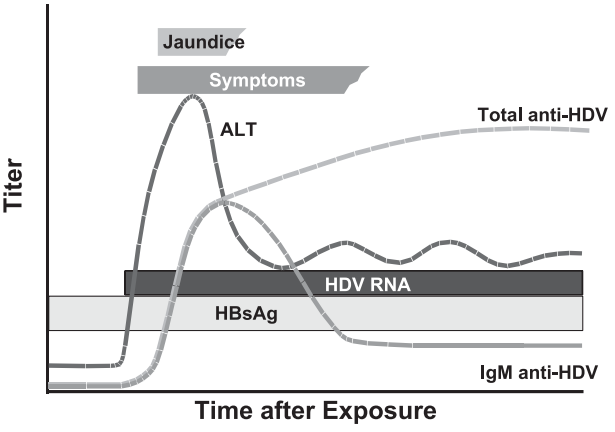


FIGURE 8 Typical serologic course of HDV superinfection in patients with chronic HBV infection. (CDC website: http://www.cdc.gov/ncidod/diseases/hepatitis/slideset/hep_d/slide_5.htm.)

coinfections and HDV superinfection of HBV carriers coupled with novel rapid technologies will inevitably lead to the development of new assays that will facilitate the diagnosis and monitoring of HDV infections.

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Transmissible Spongiform Encephalopathies*

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TAXONOMY

The "Protein-Only" Hypothesis

The most widely accepted hypothesis on the nature of the infectious agent causing transmissible spongiform encephalopathies (TSEs), which was termed prion by Stanley B. Prusiner (35), predicates that it consists essentially of a scrapie-like prion protein (PrP^{Sc}), an abnormally folded, protease-resistant, beta-sheet-rich isoform of a normal cellular prion protein (PrP^{C}). According to this theory, the prion does not contain any informational nucleic acids, and its infectivity propagates simply by recruitment and "autocatalytic" conformational conversion of cellular prion protein into disease-associated PrP^{Sc} (5).

A large body of experimental and epidemiological evidence is compatible with the protein-only hypothesis, and very stringently designed experiments have failed to disprove it. It would go well beyond the scope of this chapter to review all efforts that have been undertaken to this effect. Perhaps most impressively, knockout mice carrying a homozygous deletion of the murine *Prnp* gene, which encodes PrP^{C} (*Prnp*$^{0/0}$ mice), fail to develop disease upon inoculation with infectious brain homogenate (10), and their brains do not carry prion infectivity (39). Reintroduction of *Prnp* by transgenesis—even in a shortened, redacted form—restores infectibility and prion replication in *Prnp*$^{0/0}$ mice (14, 15, 40, 41). In addition, all familial cases of human TSEs are characterized by mutations in the human PrP^{C} gene (designated *PRNP*) (4, 36). Two recent studies go a long way toward settling the score as to the nature of the infectious agent, by demonstrating that prions may be synthesized in cell-free systems (11, 26).

DESCRIPTION OF THE AGENT

According to the protein-only hypothesis, the abnormal form of PrP^{C}, PrP^{Sc}, represents the infectious agent. Even if the protein-only hypothesis has not formally been proven, all studies agree that PrP^{Sc} represents an essential component of prion infectivity. The three-dimensional structure of PrP^{C} has been solved by nuclear magnetic resonance spectroscopy; similar analyses have not been possible for PrP^{Sc} (23, 37). Studies employing Fourier transform infrared spectroscopy have demonstrated that PrP^{Sc} contains more beta-sheets (about 40%) and fewer alpha-helical structures (about 30%) than PrP^{C} (30).

EPIDEMIOLOGY AND TRANSMISSION

Human prion diseases manifest as sporadic, genetic, and acquired disorders. They are referred to as sporadic Creutzfeld-Jakob disease (sCJD), familial or genetic CJD (gCJD), iatrogenic CJD (iCJD), and variant CJD (vCJD).

sCJD

sCJD is a rapidly progressive dementia, usually leading to death within 6 months of disease onset (16). The cause of sCJD remains enigmatic. To date no obvious risk factors have been identified. Because of the short mean duration of this disease, incidence and mortality rates for sCJD are similar; thus, mortality rates are routinely used to describe the epidemiology of this disease. The overall annual mortality rate from sCJD in a panel of European and non-European countries, including Australia and Canada, is 1.39 cases per million for the period between 1999 and 2002 (25). Mortality rates are fairly constant, both over time and between countries, ranging from 0.41 cases per million in 1994 in Spain to 2.63 cases per million in 2002 in Switzerland (18). Unlike other dementia-causing diseases such as Alzheimer's and Parkinson's diseases, in which incidence rises with age, the peak incidence is between 55 and 65 years of age (18).

gCJD

gCJD can be subdivided into three phenotypes: familial CJD, Gerstmann-Sträussler-Scheinker syndrome (GSS), and fatal familial insomnia (FFI). The mode of inheritance in all of these diseases, which cosegregate with mutations in *PRNP,* is autosomal dominant (19). The overall annual mortality rate for gCJD in a panel of European and non-European countries, including Australia and Canada, is 0.17 case per million for the time period 1999 to 2002. Between 1999 and 2002, mortality rates for gCJD were markedly different in various countries, ranging from 0.02 in The Netherlands to 1.07 in Slovakia (25).

* This chapter contains information presented in chapter 106 by David M. Asher in the eighth edition of this Manual.

iCJD

iCJD is caused by prion exposure during neurosurgical procedures such as implantation of human dura mater, corneal graft implantation, or treatment with human cadaveric pituitary extracts (7). iCJD is rare, with fewer than 300 published cases. The majority of cases were caused by implantation of dura mater and injection of pituitary growth hormone (7). Recent epidemiological data confirm the observation that iCJD mainly affects individuals younger than 39 years (25).

vCJD

vCJD, a relatively new member of the human prion diseases, was first reported in 1996 (46). Biochemical, neuropathological, and transmission studies have substantiated the concern that vCJD represents transmission of bovine spongiform encephalopathy (BSE) prions to humans (1, 3, 8, 21). The incidence of vCJD in the United Kingdom rose each year from 1996 to 2001, evoking fears of a large upcoming epidemic. Since 2001, however, the incidence of vCJD in the United Kingdom has appeared to be stabilizing, and only a small number of other countries have seen isolated cases. Although predictions on the future of the vCJD epidemic are still flawed with imprecision, there is growing evidence that the total number of vCJD victims will be limited (45). vCJD has a distinct clinicopathological profile, including age of onset. vCJD victims are much younger than sCJD patients (median age at death: 29 years) (25).

CLINICAL SIGNIFICANCE

Initial symptoms of sCJD include cognitive deficits, sleep disturbances, and behavioral abnormalities. As the disease progresses, other clinical features such as extrapyramidal and pyramidal symptoms, ataxia, and visual disturbances become obvious and the patients usually develop myoclonus (16). Terminally affected sCJD patients typically develop a state of akinetic mutism prior to death. The disease course is usually short, the mean duration of the illness being 4 to 5 months (Table 1).

The clinical presentation of gCJD varies with the underlying mutation. Some mutations present with a clinical picture that is similar to that of sCJD. The age at onset tends to be younger and the disease duration longer than for sCJD. FFI and GSS represent exceptions. FFI has a unique clinical course which is characterized by profound disruption of the normal sleep-wake cycle, insomnia, and sympathetic overactivity (29), whereas GSS presents with a progressive cerebellar ataxia.

In iCJD, the site of prion exposure seems to dictate the time between exposure and onset of prion disease-related symptoms. Direct intracerebral exposure to prions and implantation of prion-contaminated dura, for example, are associated with short incubation periods (16 to 28 months), whereas exposure to prions at sites outside the central nervous system (CNS) results in long incubation times, ranging from 5 to 30 years (13). Furthermore, there is evidence that the route of prion exposure influences the clinical presentation. Dura mater or growth hormone-related cases of iCJD present with a predominantly ataxic phenotype, whereas dementia was the initial symptom in cases in which prions were directly introduced into the CNS.

The fact that vCJD carries a distinct clinical profile has facilitated the formulation of diagnostic criteria. As stated above, vCJD victims are much younger than sCJD patients. Furthermore, initial features and illness duration are relatively specific, with initial psychiatric symptoms and a median illness duration of 14 months (Table 1). It has been hard to estimate incubation times for vCJD due to the fact that the exact time points of prion exposure are not defined.

The diagnosis of human prion diseases is based on the appraisal of clinical signs and symptoms (described below) and a number of auxiliary examinations (13). Electroencephalography (EEG) has historically been used to substantiate the diagnosis of a human prion disease. The usefulness of EEG has been questioned due to its limited sensitivity (49). Recent advances in neuroimaging, especially in magnetic resonance imaging (MRI), have revealed that different human prion diseases have specific patterns (44). For vCJD, the "pulvinar sign," a high T2 MRI signal in the posterior thalamus, seems to be unique and is present in about 75% of patients with vCJD (48). For sCJD, fluid-attenuated inversion recovery and diffusion-weighted MRI sequences are associated with high sensitivity and specificity and may represent a relatively noninvasive method to corroborate the diagnosis of a human prion disease (38).

An impressive wealth of molecules have been proposed as potential antiprion compounds based on in vitro data

TABLE 1 Human prion diseases: clinical features

Human prion disease	Clinical features		
	Age (yr) at onset (mean)	Disease duration (mean)	Leading clinical symptoms
sCJD	60–70	6 mo (1–35 mo)	Progressive dementia and neurological signs, e.g., myoclonus, cerebellar ataxia, visual problems, extrapyramidal symptoms
Inherited CJD			
gCJD	50–60	6 mo (2–41 mo)	Clinical symptoms similar to sCJD
GSS	50–60	5–6 yr (3 mo–13 yr)	Cerebellar dysfunction (ataxia, nystagmus, dysarthria)
FFI	50 (20–63)	13–15 mo (6–42 mo)	Insomnia Autonomic dysfunction
Acquired CJD			
vCJD	26 (12–74)	14 mo (6–24 mo)	Early psychiatric symptoms (depression, anxiety, social withdrawal), dysesthesia; later neurological deficits and cognitive decline
iCJD	—[a]	Similar to sCJD	Clinical symptoms similar to sCJD

[a] —, age at onset depending on iatrogenic exposure: incubation period, 1 to 30 yr.

(12, 33, 34, 42). Disappointingly, none of these compounds have been effective for actual therapy of prion disease in vivo. Transgenic expression of an immunoglobulin Fcγ domain fused to PrP^{C} drastically delays prion disease onset after peripheral and intracerebral prion challenge (27), raising the possibility that soluble prion protein mutants may be potential prionostatic compounds. Immunotherapy with anti-PrP antibodies has been shown to be effective in model systems (20) and may represent an alternative therapeutic approach.

COLLECTION, STORAGE, AND TRANSPORT OF SPECIMENS

General Considerations

PrP^{Sc}, the form of the infectious agent that causes prion diseases, is present in an oligomeric or polymeric form. The tight association of PrP^{Sc} molecules in aggregates may be responsible for the extreme stability of PrP^{Sc}. Prion infectivity is thus resistant to a number of techniques customarily used for decontamination of conventional infectious agents. In clinical laboratories, personal protective clothing such as disposable gowns, gloves, and barrier protection for mucous membranes (eye protection or full-face visor) is recommended when working with potentially contaminated specimens. In addition, strict adherence to standard working procedures aimed at minimizing the chance of penetrating injuries is essential. Handling of potentially infectious material requires certain additional precautions as described below.

Laboratory Handling of Potentially Contaminated Specimens

The highest titers of prion infectivity can be found in CNS tissues (brain, spinal cord, and cranial nerves), but nonneural tissues such as lymphatic tissue or muscle may contain infectious prions. Prions are not inactivated by formalin. A procedure recommended by the College of American Pathologists (http://www.cap.org) for safe handling of tissues is formalin fixation for at least 10 days, followed by formic acid treatment (50 to 100 ml of 95 to 100% [vol/vol]) for 1 h, and formalin fixation for 2 days. Tissues can then be embedded. Formic acid inactivates formalin-treated prions but has minimal effect on the quality of histology. Disposable histologic equipment should be used whenever possible.

Blood and bone marrow are presumed to contain low levels of infectivity (http://www.advisorybodies.doh.gov.uk/acdp/tseguidance/). These specimens can be handled safely under biosafety level 2 conditions by adhering to universal precautions for prevention of transmission of blood-borne pathogens. These specimens can be tested in automated analyzers found throughout clinical laboratories if instruments are enclosed, if they can contain spillage, and if waste can be disposed of safely. Maintenance and emergency procedures that protect the user from exposure should be outlined in laboratory standard operating procedures and implemented. Manual processing (specimen decanting, for example) should be performed inside a negative-pressure laminar-flow hood in a contained environment.

Prions can be substantially but not completely inactivated by physical exposure to steam or dry heat at high temperatures. Disposable laboratory equipment should be used whenever possible. Potentially contaminated laboratory waste should be autoclaved (134 to 137°C, 20 min) and then incinerated (http://www.advisorybodies.doh.gov.uk/acdp/tseguidance/). Chemical exposure to high concentrations of either sodium hypochlorite (>5.25% solution, freshly prepared) or sodium hydroxide (1 N solution) for 1 h is effective against prions and can be used to disinfect spills. Reusable laboratory material should be immersed in a freshly prepared >5.25% solution of sodium hypochlorite, or a 1 N solution of sodium hydroxide for 1 h, and then rinsed with water before being packaged for autoclave sterilization at 134°C for at least 20 min (http://www.advisorybodies.doh.gov.uk/acdp/tseguidance/).

Collection of Samples

Whole blood may be used for isolation of DNA for genetic analysis (exclusion of gCJD and detection of the *PRNP* codon 129 status). Blood should be shipped in a chilled container. Currently, an approved blood-based screening test for human prion diseases is not available. Cerebrospinal fluid (CSF) is routinely used to monitor elevation of nonspecific neuronal injury markers. CSF should be shipped at 4°C. Tissue may be used for histopathology and biochemical examination. Brain biopsies can be recommended only in order to exclude the diagnosis of diseases for which therapeutic options are available. Nonneural tissues (lymphatic tissue or muscle) may be useful in order to confirm the suspicion of vCJD. Tissue should be fixed in formalin for histologic assessment as described above and snap-frozen for Western blotting. Formalin-fixed material may be shipped at room temperature, whereas snap-frozen material must be shipped on dry ice.

Transport of Samples

Shipping of infectious material via water, land (road or train), and air must comply with the "Recommendations of the United Nations Committee of Experts on the Transport of Dangerous Goods." In these guidelines, human prions are listed in category 6 (Toxic and Infectious Substances), division 2 (Infectious Substances). The code numbers UN 2900 apply for this type of pathogen. Certified shipping containers must be used.

DIRECT DETECTION

Microscopy

Although it is not routinely recommended for the diagnosis of human prion diseases, histologic examination of CNS tissue can be diagnostically helpful premortem. Routine hematoxylin-eosin stains are used to interpret the vacuolization patterns, whereas immunohistochemical demonstration of PrP is necessary in order to determine PrP deposition patterns (Fig. 1). Postmortem, defined regions within the CNS (cerebellum and thalamus) and nonneuronal tissues can be sampled to demonstrate distinct PrP deposition patterns (9). Histologically, prion diseases are characterized by spongiform changes, astrogliosis, and neuronal loss. These changes are most evident in the cerebral cortex and in the cerebellum. The prominent cerebellar involvement is typical for prion diseases and clearly separates this group of diseases from other dementia-causing illnesses such as Alzheimer's disease or diffuse Lewy body disease. White-matter changes, which may be present in some cases, are thought to constitute secondary changes. In vCJD and in about 10 to 20% of sCJD patients, prion plaques are a prominent feature and may be demonstrated by Congo red staining or immunohistochemistry. Birefringence under polarized light, usually a characteristic feature of amyloid plaques, may be absent due to harsh pretreatment of fixed tissue.

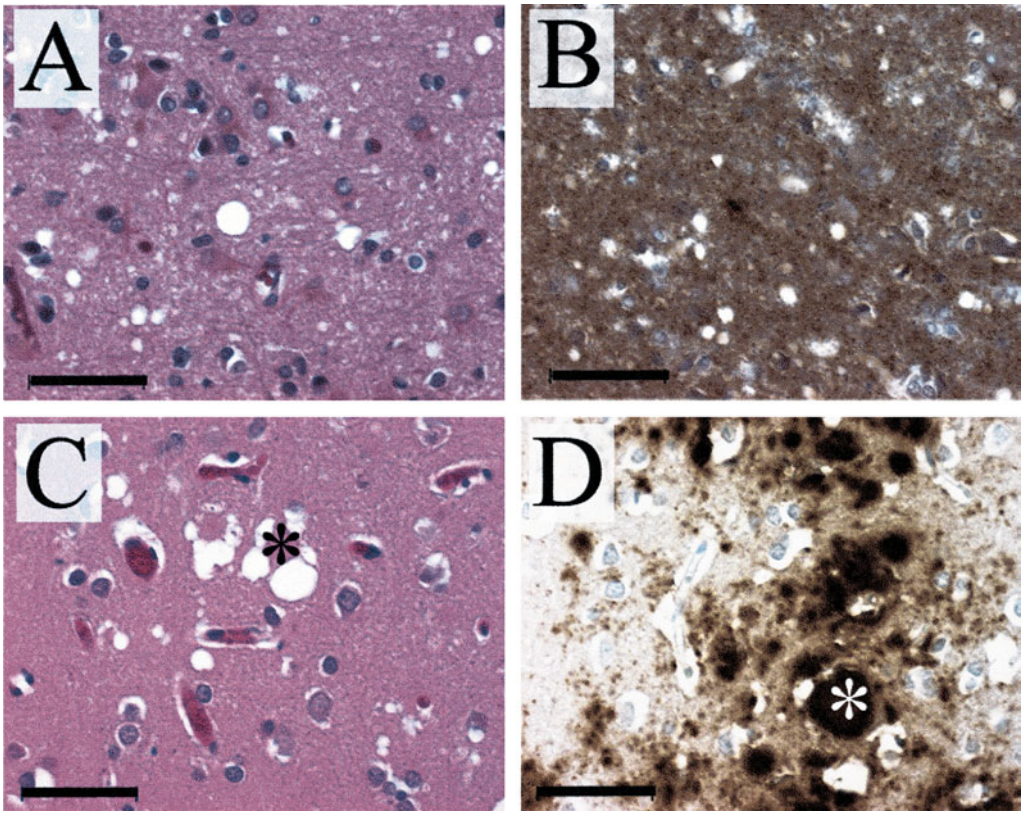


FIGURE 1 Histological features of prion diseases. The panels depict CNS parenchyma of sCJD (A and B) and vCJD (C and D) showing astrogliosis and widespread spongiform changes. PrP depositions are synaptic (A and B) and in the form of florid plaques (asterisk, C and D). Panels A and C are hematoxylin-eosin stains, and panels B and D are immunohistochemical stainings for PrP (scale bar = 50 μm).

Antigen Detection of PrP^{Sc} by Western Blotting

Western blotting is routinely performed on unfixed tissue originating from the CNS. Generally, this test is undertaken in a postmortem setting. In rare cases where a brain biopsy is justified, a biopsy specimen may be used.

The basis of biochemical characterization of PrP^{Sc} resides in its relative resistance to proteolytic degradation. Whereas PrP^{C} is entirely digested by proteinase K, identical treatment of PrP^{Sc} leads to removal of a variable number of N-terminal amino acids. Western blotting of digested PrP^{Sc} reveals three distinct bands, corresponding to di-, mono-, and unglycosylated forms (31). The molecular classification of PrP^{Sc} takes two parameters into account: the molecular weight of unglycosylated PrP^{Sc} and the relative amounts of PrP^{Sc} di-, mono-, and unglycosylated forms. The resulting information is then used to establish the "type" of PrP^{Sc} according to proposed schemes (22, 32) (Fig. 2). Depending on the exact conditions under which the protease digestion and the Western blotting procedure are performed, between three and six different PrP^{Sc} types can be distinguished (22, 28). Distinct PrP^{Sc} types are thought to represent the molecular correlates of distinct prion strains. The fact that the PrP^{Sc} types found in vCJD patients and in BSE-diseased cattle are identical is one of the main arguments supporting the theory that BSE prions are responsible for the vCJD epidemic in humans (21). It is unknown how glycotype ratios can be faithfully maintained during prion replication; however, experiments with yeast prions indicate that this occurs in a synthetic prion replication system (24, 43). This phenomenon may be related to the quaternary structure of prion aggregates (2).

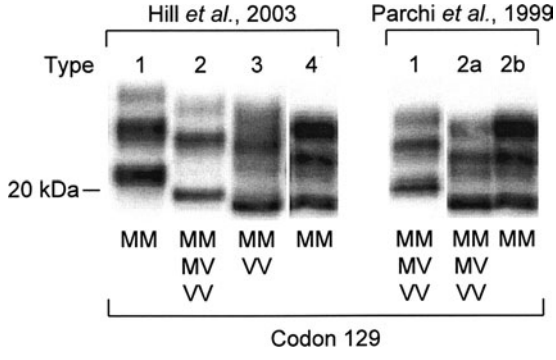


FIGURE 2 Western blot analysis of PrP^{Sc}. Depicted are PrP^{Sc} types according to two proposed schemes which discriminate PrP^{Sc} types based on the mobility of the unglycosylated band of PrP^{Sc} and the signal intensity of the PrP^{Sc} di-, mono-, and unglycosylated forms. One scheme (22) differentiates four principal PrP^{Sc} types (1 to 4) (22). Three principal PrP^{Sc} types (1, 2a, and 2b) are proposed in the second scheme (32).

Nucleic Acid: Genetic Testing

For genetic testing, DNA is extracted from whole blood using routine methods. The entire open reading frame may be amplified for sequencing using PCR (17). Sequencing of *PRNP* allows for the exclusion of gCJD (47). More than 29 disease-causing insertional or point mutations in *PRNP* have been identified to date (Table 2). In addition, there are several *PRNP* polymorphisms, one of which (codon 129M/V) may have a disease-modifying function. Homozygosity for methionine at codon 129 may constitute a risk factor for the development of prion disease (6): methionine homozygotes are clearly overrepresented among sCJD patients, and all individuals affected by vCJD are homozygous for methionine at codon 129. In addition, this polymorphism has a considerable effect on the clinical, biochemical, and neuropathological presentation of prion-diseased individuals (6).

SEROLOGIC TESTS

To date there are no serologic screening or confirmatory tests for prion diseases. Rather, several studies had evaluated the usefulness of surrogate markers, such as the 14-3-3 and tau proteins, that are found in CSF as a consequence of neuronal death. These proteins are ubiquitously expressed. 14-3-3 participates in protein kinase signaling and is involved in neuronal migration. Tau is a phosphoprotein that promotes the assembly and stability of neuronal axons by binding to microtubules. Elevation of these proteins is relatively nonspecific and can be seen in other conditions such as encephalitis, cerebral infarction, and paraneoplastic neurological disorders. Satisfactory sensitivity and specificity (over 90%) can be achieved only in selected cohorts (49). Results from these tests, in combination with clinical data, may help at best to strengthen the diagnosis of a probable human prion disease. While 14-3-3 is routinely tested using a Western blot, tau may be measured in an enzyme-linked immunosorbent assay format (49).

TABLE 2 Disease causing mutations in *PRNP*

Mutations that cause GSS	Mutations that cause a disease that is clinically similar to sCJD	Mutations that cause FFI
P102L	I138M	D178N (codon 129 M)
P105L	G142S	
A117V	D178N (codon 129 V)	
G131V	V180I	
F198S	V180I + M232R	
D202N	T183A	
Q212P	H187R	
Q217R	T188R	
M232T	T188A	
192-bp insertion	T188K	
	E196K	
	E200K	
	V203I	
	R208H	
	V210I	
	E211Q	
	M232R	
	P238S	
	24-, 48-, 96-, 120-, 144-, 168-, 216-bp insertions	

TABLE 3 Useful diagnostic tests for TSEs

Disorder[a,b]	Useful supplemental diagnostic tests and specimens[a]
sCJD	Histology, Western blotting, genetic testing for exclusion of gCJD, auxiliary tests such as determination of 14-3-3 or tau protein in the CSF
gCJD	Genetic testing, histology, Western blotting, auxiliary tests such as determination of 14-3-3 or tau protein in the CSF
vCJD	Histology, Western blotting (lymphoid tissue and CNS tissue), genetic testing for exclusion of gCJD, auxiliary tests such as determination of 14-3-3 or tau protein in the CSF

[a] Clinical presentation is of primary diagnostic importance.
[b] Neuroimaging studies yield distinct results and can be useful.

EVALUATION, REPORTING, AND INTERPRETATION OF RESULTS

The diagnosis of human prion disorders can be difficult to establish but is best approached through careful consideration of clinical presentation and other clinical studies such as neuroimaging. Brain biopsy and histologic assessment can be performed to rule out treatable disorders. Detection of spongiform changes and deposition of PrP^{Sc} in affected areas can help establish the diagnosis of prion disorders. The absence of these changes (for example, due to sampling bias) is not helpful in the exclusion of these disorders. Less invasive tests such as detection of surrogate markers (tau and 14-3-3) in CSF can be helpful when considered in the context of available clinical neuroimaging information. Genetic testing can be performed to establish the diagnosis of genetic prion disorders. The constellation of tests that can be helpful in the diagnosis of each of these disorders is shown in Table 3. All testing other than neuropathology and sequencing of *PRNP* is usually performed on a research basis.

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ANTIVIRAL AGENTS AND SUSCEPTIBILITY TEST METHODS

VII

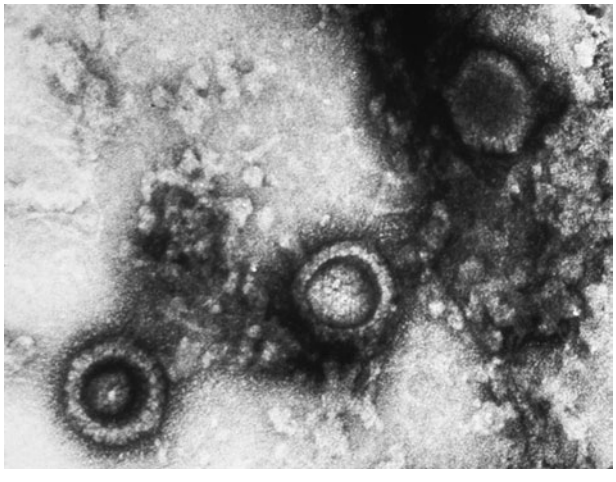
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Transmission electron micrograph of herpes simplex virus (from CDC image library).

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Antiviral Agents*

NELL S. LURAIN AND KENNETH D. THOMPSON

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The use of antiviral agents for the treatment of viral diseases continues to expand. Most of the agents currently approved by the Food and Drug Administration (FDA) are active against one or more of the following viruses: human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2), hepatitis viruses B and C (HBV and HCV), the human herpesviruses, and influenza viruses A and B. This chapter is organized according to these virus groups with cross-referencing for agents with activity against more than one group of viruses. The major targets of these agents are viral replication enzymes, proteases, and entry and exit pathways (27, 31, 39, 93, 112).

AGENTS ACTIVE AGAINST HUMAN IMMUNODEFICIENCY VIRUS TYPES 1 AND 2

There are now four classes of antiviral agents for the treatment of HIV-1 infection: (i) nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs and NtRTIs), (ii) non-nucleoside reverse transcriptase inhibitors (NNRTIs), (iii) protease inhibitors (PIs), and (iv) entry or fusion inhibitors. Current information on each drug is available through the AIDSinfo website (http://AIDSinfo.nih.gov), which has separate guidelines for the use of approved antiretroviral agents in (i) adolescents and adults, (ii) pediatric patients, and (iii) pregnant HIV-1-infected women (33, 61, 107). These guidelines describe the agents along with dosages, adverse effects, and drug interactions. Working groups for each of these patient populations regularly update the guidelines. Additional prescribing information can be obtained from the individual pharmaceutical company websites. Changes in recommended drug doses, as well as observed adverse effects and drug interactions, occur frequently, making it necessary to consult the most up-to-date sources.

Antiretroviral agents are administered in combinations of different drug classes termed highly active antiretroviral therapy (HAART) to maximize efficacy and to minimize the induction of drug resistance. Initially, HAART was defined as two NRTIs and a PI; however, HAART is now generally regarded as any combination regimen designed to achieve the goal of complete virus suppression. These regimens comprise a minimum of three drugs and are usually either NNRTI-based (two NRTIs or NtRTIs plus one NNRTI) or PI-based (two NRTIs or NtRTIs plus one or more PIs). Monotherapy is restricted to the prevention of perinatal transmission of HIV. The drugs most often used for this purpose are zidovudine (ZDV or AZT) and nevirapine (NVP) (33).

There are currently 21 approved antiretroviral drugs with numerous possible combinations for treatment regimens. Recommended regimens for adults and adolescents are given in the guidelines (33) for treatment-naïve and treatment-experienced patients. The large number of drugs creates a tremendous potential for drug interactions among the different classes as well as interactions with other types of drugs prescribed for conditions associated with HIV infection. Close monitoring of these complex interactions is required to avoid detrimental changes in drug levels and/or toxicity.

Table 1 summarizes the structures, mechanisms of action, and major adverse effects of the individual drugs and drug combinations approved by the FDA. Drug interactions described below for each drug are only highlights of potential interactions. Frequent updates and more comprehensive information can be obtained from the AIDSinfo website listed above.

Nucleoside and Nucleotide Reverse Transcriptase Inhibitors

NRTIs and NtRTIs are not active as administered but must be phosphorylated by cellular kinases into the nucleoside triphosphate form, which lacks a 3-prime hydroxyl group for DNA chain elongation (3). These compounds act as competitive inhibitors of the viral reverse transcriptase (RT), which results in chain termination. The NRTIs require triphosphorylation, while the NtRTIs require only diphosphorylation.

These drugs are active against both the HIV-1 and HIV-2 RTs (133), and they are used in combination regimens with either NNRTIs or PIs. Several of them also are active against the HBV DNA polymerase, which has RT activity (see "Agents Active against Hepatitis B Virus" below) (127, 137). Lactic acidosis with hepatic steatosis is a rare but very serious adverse effect associated with all members of this class. These toxic effects of NRTIs and NtRTIs appear to be the result of inhibition of the mitochondrial DNA polymerase γ (14, 26).

*This chapter contains information presented in chapter 107 by Ella M. Swierkosz in the eighth edition of this Manual.

TABLE 1 Antiviral agents for HIV

Antiviral agent (abbreviation)	Trade name, pharmaceutical company[a]	Structure	Mechanism of action	Major adverse effects
NRTIs and NtRTIs				
Zidovudine (ZDV or AZT)	Retrovir, GSK	3′-Azido-2′,3′- dideoxythymidine	Converted to triphosphate analogue of dTTP by cellular kinases; competitive inhibitor of RT viral DNA chain termination	Bone marrow suppression, GI symptoms,[b] headache, insomnia, lactic acidosis and severe hepatosplenomegaly with steatosis
Didanosine (ddI)	Videx, BMS	2′,3′-Dideoxyinosine (adenosine analogue)	Converted to dideoxytriphosphate analogue of dATP by cellular kinases; activity similar to that of ZDV	Pancreatitis, peripheral neuropathy, nausea, diarrhea, lactic acidosis and severe hepatosplenomegaly with steatosis
Zalcitabine (ddC)	Hivid, Roche	2′,3′-Dideoxycytidine	Converted to ddCTP analogue of dCTP by cellular kinases; activity similar to that of ZDV	Peripheral neuropathy, pancreatitis, stomatitis, lactic acidosis and severe hepatosplenomegaly with steatosis
Stavudine (d4T)	Zerit, BMS	2′,3′-Didehydro-2′,3′-dideoxythymidine	Converted to triphosphate analogue of dTTP by cellular kinases; activity similar to that of ZDV	Peripheral neuropathy, lipodystrophy, motor weakness, lactic acidosis and severe hepatosplenomegaly with steatosis
Lamivudine (3TC)	Epivir, GSK	Dideoxycytidine analogue	Converted to triphosphate analogue of dCTP by cellular kinases; activity similar to that of ZDV	Minimal toxicity, lactic acidosis and severe hepatosplenomegaly with steatosis posttreatment exacerbation of hepatitis B coinfection
Abacavir (ABC)	Ziagen, GSK	Carbocyclic guanosine analogue	Converted to triphosphate analogue of dGTP by cellular kinases; activity similar to that of ZDV	Hypersensitivity reaction, lactic acidosis and severe hepatosplenomegaly with steatosis
Emtricitabine (FTC)	Emtriva, Gilead	Thio-5-fluorocytosine analogue	Converted to triphosphate analogue of dCTP by cellular kinases; activity similar to that of ZDV	Minimal toxicity, lactic acidosis and severe hepatosplenomegaly with steatosis, posttreatment exacerbation of hepatitis B coinfection
Tenofovir (TDF)	Viread, Gilead	Acyclic diester prodrug tenofovir disoproxil fumarate; acyclic analogue of adenosine monophosphate	Diester hydrolysis required for conversion to tenofovir; monophosphate analogue requires diphosphorylation by cellular kinases; activity similar to that of ZDV	Asthenia, headache, GI symptoms, lactic acidosis and severe hepatosplenomegaly with steatosis, posttreatment exacerbation of hepatitis B coinfection
Zidovidine-lamivudine	Combivir, GSK	Combination of ZDV and 3TC	See individual descriptions above	See individual descriptions above
Abacavir-lamivudine	Epzicom, GSK	Combination of ABC and 3TC	See individual descriptions above	See individual descriptions above
Abacavir- zidovudine-lamivudine	Trizivir, GSK	Combination of ABC, ZDV, and 3TC	See individual descriptions above	See individual descriptions above
Tenofovir-emtricitabine	Truvada, Gilead	Combination of TDF and FTC	See individual descriptions above	See individual descriptions above
NNTRIs				
Delavirdine (DLV)	Rescriptor, Pfizer	Nonnucleoside inhibitor of HIV-1 RT	Noncompetitive inhibitor binds to HIV-1 RT; blocks RNA-dependent and DNA-dependent DNA polymerase activity by disruption of catalytic site	Skin rash (Stevens-Johnson syndrome), headache, elevated transaminase levels

Efavirenz (EFV)	Sustiva, BMS	Similar to DLV but structurally unrelated	Activity similar to that of DLV	Skin rash (Stevens-Johnson syndrome), psychiatric symptoms, nervous system symptoms, elevated transaminase levels, teratogenic effects
Nevirapine (NVP)	Viramune, BI	Similar to DLV but structurally unrelated	Activity similar to that of DLV	Severe hepatotoxicity, skin rashes (Stevens-Johnson syndrome)
Protease inhibitors				
Indinavir (IDV)	Crixivan, Merck	Peptidomimetic substrate analogue of HIV-1 and HIV-2 proteases	Binds competitively to active site of HIV protease; prevents cleavage of viral polyprotein precursors; produces immature, noninfectious viral particles	Nephrolithiasis, urolithiasis, hemolytic anemia, headache, hyperglycemia, GI symptoms, fat redistribution, hyperlipidemia, increased bleeding episodes in hemophiliacs
Saquinavir (SQV)	Fortovase, Roche	Similar to IDV; soft-gel formulation	Activity similar to that of IDV	Hyperlipidemia, fat redistribution, GI symptoms, hyperglycemia, hyperlipidemia, elevated transaminase levels, headache, increased bleeding episodes in hemophiliacs
	Invirase	Hard-gel formulation	Activity similar to that of IDV (not interchangeable with Fortovase)	Similar to those of Fortovase
Ritonavir (RTV)	Norvir, Abbott	Similar to IDV	Activity similar to that of IDV	GI symptoms, circumoral paresthesias, hyperlipidemia, hepatitis, asthenia, taste perversion, hyperglycemia, fat redistribution, increased bleeding episodes in hemophiliacs
Lopinavir (LPV)-ritonavir	Kaletra, Abbott	Coformulation of LPV and RTV, 4:1 ratio; similar to IDV	Activity similar to that of IDV	GI symptoms, asthenia, hyperlipidemia, elevated transaminase levels, hyperglycemia, fat redistribution, increased bleeding episodes in hemophiliacs
Amprenavir (APV)	Agenerase, GSK	N,N-Disubstituted hydroxyethylamino sulfonamide	Activity similar to that of IDV	GI symptoms, rash, oral paresthesias, hyperlipidemia, elevated transaminase levels, hyperglycemia, fat redistribution, increased bleeding episodes in hemophiliacs
Fosamprenavir (f-APV)	Lexiva, GSK	Phosphorylated prodrug of APV	Converted to amprenavir by cellular phosphatases; activity similar to that of IDV	Skin rash; GI symptoms, headache, hyperlipidemia, fat redistribution, elevated transaminase levels, hyperglycemia, increased bleeding episodes in hemophiliacs
Atazanavir (ATV)	Reyataz, BMS	Azapeptide aspartyl substrate analogue similar to IDV	Activity similar to that of IDV	Indirect hyperbilirubinemia, prolonged PR interval, hyperglycemia, fat redistribution, increased bleeding episodes in hemophiliacs
Nelfinavir (NFV)	Viracept, Pfizer	Monomethane sulfonate salt of protease substrate analogue similar to IDV	Activity similar to that of IDV	Diarrhea, hyperlipidemia, hyperglycemia, fat redistribution, elevated transaminase levels, increased bleeding episodes in hemophiliacs

(Continued on next page)

TABLE 1 Antiviral agents for HIV (*Continued*)

Antiviral agent (abbreviation)	Trade name, pharmaceutical company[a]	Structure	Mechanism of action	Major adverse effects
Tipranavir (TPV)	Aptivus, BI	Nonpeptidic protease inhibitor; coadministered with RTV	Activity similar to that of IDV	Hepatotoxicity, hyperglycemia, sulfa allergy, rash, hyperlipidemia, fat redistribution, increased bleeding episodes in hemophiliacs
Entry inhibitors				
Enfuvirtide (T20)	Fuzeon, Roche	36-Amino-acid synthetic peptide (residues 127–162 of gp41); N terminus acetylated; C-terminus carboxamide	Binds to first heptad repeat in gp41; prevents conformational changes required for fusion of viral and cellular membranes; administered by injection	Local injection site reactions, pneumonia, hypersensitivity reactions

[a] Pharmaceutical companies: Abbott Laboratories, North Chicago, Ill.; BI, Boehringer Ingelheim Pharmaceuticals, Ridgefield, Conn.; BMS, Bristol-Myers Squibb, Princeton, N.J.; Gilead Sciences, Foster City, Calif.; GSK, GlaxoSmithKline, Research Triangle Park, N.C.; Merck & Co., Whitehouse Station, N.J.; Pfizer, New York, N.Y.; Roche Pharmaceuticals, Nutley, N.J.
[b] GI symptoms include nausea, vomiting, and diarrhea.

Zidovudine

Pharmacology and Therapeutic Regimens

ZDV was the first agent used in antiretroviral therapy (41). The oral bioavailability is 60%, the half-life in serum is 1.1 h, and the intracellular half-life is 7 h. ZDV can be administered without regard to meals. It is metabolized to the glucuronide, which is excreted by the kidneys.

ZDV crosses the blood-brain barrier to achieve effective concentrations in the central nervous system (CNS) and also crosses the placenta (107). It can be given as standard monotherapy that is administered intravenously (i.v.) to pregnant women during labor to prevent maternal-fetal transmission and can be administered orally to the child at birth (24, 107). As alternative intrapartum and postpartum therapy, ZDV can be given orally in combination with lamivudine (3TC) to both mother and neonate. For adults and adolescents, ZDV is recommended as one of the two NRTIs or NtRTIs in preferred or alternative NNRTI-based and PI-based regimens (33).

Drug Interactions

ZDV inhibits the phosphorylation of stavudine (d4T) by thymidine kinase (TK) (56). Ribavirin inhibits the phosphorylation of ZDV (104). Ganciclovir (GCV) and alpha interferon (IFN-α) may enhance the hematologic toxicity associated with ZDV (19, 23).

Didanosine

Pharmacology and Therapeutic Regimens

The oral bioavailability of didanosine (ddI) is 30 to 40%. The half-life in serum is 1.5 h, and the intracellular half-life is >20 h. ddI should be administered without food. However, ddI is unstable at acidic pHs and therefore requires an enteric formulation, antacids, or buffering agents. Half of the drug is excreted by the kidneys. There is limited penetration of the CNS, but ddI has been shown to cross the human placenta (107). ddI is recommended only as alternative therapy with another NRTI or NtRTI in either NNRTI-based or PI-based drug regimens (33).

Drug Interactions

The combination of ddI with either d4T or zalcitabine (ddC) can increase the rates and severities of toxicities associated with each individual drug. Tenofovir (TDF), ganciclovir (GCV), and valganciclovir (val-GCV) increase ddI exposure, leading to increased ddI toxicity (106, 110). Ribavirin may increase the active metabolite of ddI, producing increased toxicity (33).

Zalcitabine

Pharmacology and Therapeutic Regimens

The oral bioavailability of ddC is 85%. The half-life in serum is 1.2 h, and the intracellular half-life is undetermined. ddC can be administered with or without food; however, there is a reduced absorption rate when the drug is taken with meals. Seventy percent of the drug is eliminated unmetabolized by renal excretion. There is limited penetration of the CNS. In animal studies, ddC has been shown to cross the placenta (107). It is recommended as alternative therapy with another NRTI or NtRTI in NNRTI- or PI-based combination therapy (33).

Drug Interactions

The combination of ddC with either ddI or d4T can increase the rates and severities of toxicities associated with each individual drug (33). There is overlapping resistance between ddC and ddI. ddC inhibits the phosphorylation of 3TC.

Stavudine

Pharmacology and Therapeutic Regimens

The oral bioavailability of d4T is 86%. The half-life in serum is 1.0 h, and the intracellular half-life is 7.5 h. d4T can be administered with or without food. Fifty percent of the drug is excreted by the kidneys. Placental passage occurs in animals, and d4T penetrates into the cerebrospinal fluid (CSF) (44, 107). d4T is recommended as an alternative drug in dual NRTI-NtRTI backbones in combination with an NNRTI or PIs (33).

Drug Interactions

d4T combined with either ddI or ddC can increase the rates and severities of toxicities associated with each individual drug. ZDV and ribavirin inhibit the phosphorylation of d4T (56, 63, 104).

Lamivudine

Pharmacology and Therapeutic Regimens

The oral bioavailability of 3TC is 86% in adolescents and adults and 66% in children. The half-life in serum is 5 to 7 h, and the intracellular half-life is 18 to 22 h. 3TC can be administered with or without food. Seventy-one percent of the drug is excreted by the kidneys by active organic cationic secretion. The drug crosses the human placenta (107). 3TC is recommended as part of a preferred therapy regimen in combination with another NRTI or NtRTI and either an NNRTI or PIs for treatment-naïve patients (33).

Drug Interactions

3TC and ddC each inhibit the phosphorylation of the other (33).

Abacavir

Pharmacology and Therapeutic Regimens

The oral bioavailability of abacavir (ABC) is 83%. The half-life in plasma is 1.5 h, and the intracellular half-life is 12 to 26 h. ABC can be administered with or without food. It is metabolized by alcohol dehydrogenase and glucuronyl transferase, and 82% of the metabolites are excreted by the kidneys. Ingestion of ethanol decreases the metabolism and elimination of the drug. Placental passage has been demonstrated in animal studies (107). ABC penetration of the CNS is adequate to inhibit HIV replication (16). It is recommended as an alternative drug in dual NRTI-NtRTI backbones in combination with an NNRTI or PIs (32, 33). Combination formulations of two and three NRTIs or NtRTIs including ABC are commercially available (Table 1).

Drug Interactions

ABC decreases the level of methadone. Alcohol increases the concentration of ABC in plasma through the common metabolic pathways of alcohol and ABC (33).

Emtricitabine

Pharmacology and Therapeutic Regimens

The oral bioavailability of emtricitabine (FTC) is 93%. The half-life in plasma is 10 h, and the intracellular half-life is >20 h. FTC can be administered with or without food. It is excreted mostly unchanged (86%) by the kidneys, and 14% is eliminated in the feces. It is not known whether FTC crosses the placenta (107) or if there is penetration of cells of the CNS (88). FTC is recommended as a preferred drug in combination with another NRTI or NtRTI in either NNRTI-based or PI-based regimens for treatment-naïve patients (33).

Drug Interactions

No significant interactions with other antiretroviral agents have been reported (33, 88).

Tenofovir

Pharmacology and Therapeutic Regimens

The oral bioavailability of tenofovir (TDF) from the prodrug tenofovir disoproxil fumarate is 25% in fasting patients and 39% in patients taking it with a high-fat meal, although the drug is administered without regard to meals. The half-life in plasma is 17 h, and the intracellular half-life is >60 h. The drug is excreted mostly unchanged (70 to 80%) by the kidneys. TDF has been shown to cross the placenta in animal studies (107). It is less likely than other NRTIs and NtRTIs to be associated with mitochondrial toxicity (48). TDF is recommended as a preferred NRTI or NtRTI in dual combination backbones with the NNRTI efavirenz (EFV) (33).

Drug Interactions

TDF increases the concentration of ddI in plasma, leading to increased toxicity (106, 110). There may be increased toxicity associated with the coadministration of GCV, val-GCV, acyclovir (ACV), or cidofovir (CDV) (33, 48).

NRTI and NtRTI Combination Formulations

There are four fixed-dose double or triple combinations available as commercial formulations for convenience of administration: ABC-3TC-ZDV (Trizivir), ABC-3TC (Epzicom), FTC-TDF (Truvada), and 3TC-ZDV (Combivir). The triple combination ABC-3TC-ZDV is recommended only when preferred or alternative NNRTI- or PI-based regimens cannot be used for treatment-naïve patients because of potential drug interactions. Clinical trials have shown the ABC-3TC-ZDV combination to be equivalent to PI-based regimens but inferior to NNRTI-based regimens in reducing HIV RNA loads in plasma below detectable levels (33, 51, 121). The dual combinations are used as NRTI-NtRTI backbones in combination with an NNRTI or PIs in triple or quadruple drug therapy.

Nonnucleoside Reverse Transcriptase Inhibitors

Drugs in the NNRTI class do not require intracellular anabolism for activation. There is no common structure; however, they bind noncompetitively to a catalytic site on the HIV-1 RT but not the HIV-2 RT. The disruption of DNA polymerization activity leads to premature DNA chain termination (27, 31). There are currently three FDA-approved NNRTIs: NVP, delavirdine (DLV), and EFV. All are metabolized by the cytochrome P450 (CYP450) system, which also metabolizes the PIs (see below) and other drugs used to

treat conditions associated with HIV infection. Drug-drug interactions can lead to either induction or inhibition of the metabolism of other antiretroviral agents and medications for associated symptoms. NNRTIs are used as part of triple therapy regimens with two NRTIs or NtRTIs to preserve the effectiveness and delay exposure to the adverse effects of PIs. A disadvantage of the NNRTIs is the relatively low number of mutations required to confer cross-resistance to all drugs in this class (33).

Delavirdine

Pharmacology and Therapeutic Regimens

The bioavailability of DLV is 85%, and the half-life in plasma is 5.8 h. DLV can be administered with or without food. The level of plasma protein binding is 98%, and there is little CNS penetration. DLV is metabolized primarily in the liver by the CYP450 system and is an inhibitor of CYP3A4. The metabolites are excreted in the urine (51%) and eliminated in the feces (44%). Placental passage of DLV has not been determined (107). DLV is not recommended for use in combination regimens for treatment-naïve patients, because it has lower antiviral activity and requires more frequent dosing than the other NNRTIs (33).

Drug Interactions

Dose modifications or cautious use is indicated for antifungal agents ketoconazole and voriconazole, oral contraceptives, methadone, and erectile dysfunction drugs. Coadministration of the following drugs with DLV is contraindicated: lipid-lowering agents simvastatin and lovastatin; the gastrointestinal (GI) motility agent cisapride; histamine$_2$ (H2) blockers; proton pump inhibitors; anticonvulsants phenobarbitol and phenytoin; sedatives and hypnotics; ergot derivatives; antimycobacterial drugs rifampin, rifapentin, and rifabutin; the herb St. John's wort; and the PIs amprenavir (APV) and fosamprenavir (f-APV) (33, 104).

Efavirenz

Pharmacology and Therapeutic Regimens

The oral bioavailability of EFV is undetermined. The half-life in serum is 5.8 h. The levels in plasma are increased when the drug is administered with a high-fat or high-calorie meal. EFV is 99.5% protein bound in the plasma, mainly to albumin. CNS penetration is low, but EFV has been shown to cross the placenta in animals (107). EFV is metabolized by CYP3A4 and CYP2B6 and is an inducer and inhibitor of CYP3A4. Glucoronidated metabolites are excreted in the urine (14 to 34%) and eliminated in the feces (16 to 61%). EFV is the preferred NNRTI in triple regimens with two NRTIs or NtRTIs, except for pregnant women, because teratogenic effects have been observed in cynomologus monkeys during the first trimester of pregnancy (33).

Drug Interactions

Dose modifications may be necessary for potential drug interactions between EFV and the following: indinavir (IDV), lopinavir (LPV), APV, ritonavir (RTV), nelfinavir (NFV), clarithromycin, methadone, and oral contraceptives (33, 104). Contraindicated drugs are rifapentine, cisapride, sedatives and hypnotics, ergot derivatives, St. John's wort, voriconazole, and saquinavir (SQV) as the sole PI.

Nevirapine

Pharmacology and Therapeutic Regimens

The oral bioavailability of NVP is >90%, and the half-life in serum is 25 to 30 h. NVP is 60% protein bound, and the concentration in the CSF is 45% of the concentration in plasma. NVP can be administered with or without food. It is metabolized in the liver by CYP450 isoenzymes and is a CYP3A4 inducer. Glucuronidated metabolites are excreted in the urine (80%) and feces (10%). NVP is known to cross the human placenta (107). It has been used in resource-limited regions as a single agent, which can be administered orally, in an intrapartum and newborn prophylaxis regimen to prevent maternal-fetal transmission (107). NVP is recommended only as an alternative to EFV in NNRTI-based combination regimens with NRTIs and NtRTIs for adult females with $CD4^+$ T-cell counts of $\leq$250 cells/mm^3 or males with counts of $\leq$400 cells/mm^3 (33).

Drug Interactions

NVP reduces the concentrations of IDV, SQV, oral contraceptives, ketoconazole, clarithromycin, and methadone in plasma (104). Coadministration of rifampin, rifapentine, or St. John's wort with NVP is contraindicated (33).

Protease Inhibitors

The introduction of PIs in 1996 significantly improved HIV antiviral therapy by inhibiting HIV disease progression and greatly improving prognosis. This class of drugs, like the NNRTIs, requires no intracellular anabolism for antiviral activity. The target is the protease encoded by HIV-1 and HIV-2, which is required for posttranslational processing of the precursor Gag and Gag-Pol polyproteins (27, 39, 45). Competitive inhibition of the protease by PIs results in production of immature noninfectious viral particles.

PIs are commonly used in HAART regimens in combination with NRTIs and NtRTIs to achieve maximum antiretroviral activity and to minimize the development of resistance. PI-based regimens introduced initially led to treatment failure related to the limited bioavailability, frequent dosing, and toxicity. There are several characteristics of PIs that affect their bioavailability and toxicity. They are highly bound to plasma protein, mainly alpha-1 acid glycoprotein (AAG) (42, 71). The low concentration of unbound drug is responsible for the therapeutic activity as well as the toxicity. PIs are substrates for P-glycoprotein and multidrug resistance-associated protein (136). These are efflux transporters, which enhance the elimination of the drugs from cells in the intestine, liver, and kidneys (138) and reduce intracellular drug concentrations. All of the PIs are metabolized in the intestine and liver by enzymes of the CYP450 system (12), mainly CYP3A, CYP2C9, and CYP2C19. An individual PI can induce and/or inhibit specific CYP450 isoenzymes, which can enhance or reduce the PI's own metabolism or that of other PIs. As noted above, the CYP450 system metabolizes the NNRTIs and numerous other drugs that may be used for conditions associated with HIV infection. Thus, the choice of treatment regimens is complicated by multiple potential drug-drug interactions which may enhance toxicity and/or require modifications of doses of coadministered drugs (33).

Although most PIs are inhibitors of CYP3A4, RTV is the most inhibitory. For this reason, RTV is used in boosting regimens to improve the pharmacokinetic profile of a second PI (109, 138). Subtherapeutic concentrations of RTV increase the systemic exposure of a second PI by reducing the rate

of metabolism and increasing the half-life (12), which lowers dosing requirements and lessens food effects for the second drug. An example is LPV, which alone has very little bioavailability and a very short half-life but in combination with RTV is used therapeutically both for treatment-naïve patients and for salvage therapy (25). The effect of RTV on the pharmacokinetics of other PIs varies as a result of differences in interactions with components of the CYP450 system that determine bioavailability (138). Specific recommendations are described below for each drug.

Indinavir

Pharmacology and Therapeutic Regimens

The bioavailability of IDV is 65%, and the half-life in serum is 1.5 to 2.0 h. IDV should be administered with low-calorie, low-fat food. It is 60% plasma protein bound, mainly to AAG (71). IDV is an inhibitor of CYP3A4, although it is less inhibitory than RTV (see below). The majority of the drug (83%) is eliminated as metabolites in the feces. There is minimal passage of IDV across the placenta (107), but it penetrates into the CSF (64, 139).

IDV can be used in RTV-boosted regimens with two NRTIs or NtRTIs for treatment-naïve patients (33). Unboosted IDV is not recommended in PI-based regimens because of its multiple dosing and fasting requirements.

Drug Interactions

Coadministered drugs that require dose modifications or cautious use include antifungal agents, anticonvulsants, the lipid-lowering agent atorvastatin, and erectile dysfunction agents. Other drug classes that are contraindicated for coadministration with IDV include nonsedating antiarrhythmics, the lipid-lowering agents simvastatin and lovastatin, antimycobacterial drugs rifampin and rifapentine, ergot derivatives, sedatives and hypnotics, the GI motility agent cisapride, the neuroleptic pimozide, and St. John's wort. IDV should not be used with atazanavir (ATV) because of additive adverse effects of hyperbilirubinemia and jaundice (33, 104).

Saquinavir

Pharmacology and Therapeutic Regimens

The oral bioavailability of Invirase, the hard-gel capsule formulation of SQV, is approximately 4%. The bioavailability of Fortovase, the soft-gel capsule formulation, is higher but not completely determined. The half-life in serum of both formulations is 1 to 2 h. SQV is metabolized by CYP450 and is a CYP3A4 inhibitor. It is 97% bound to plasma proteins and is eliminated mainly in the feces as metabolic products (33). There is minimal passage of SQV across the placenta (107).

SQV can be used in RTV-boosted regimens with two NRTIs or NtRTIs for treatment-naïve patients. Unboosted SQV is not recommended in PI-based regimens because of low oral bioavailability, multiple dosing requirements, and a high pill burden (33).

Drug Interactions

Coadministered drugs that require dose modifications or cautious use include antifungal agents, lipid-lowering agents atorvastatin and pravastatin, anticonvulsants, methadone, and erectile dysfunction agents. Drugs that are contraindicated for coadministration with SQV include simvastatin and lovastatin, rifampin and rifabutin, cisapride, pimozide, sedatives and hypnotics, ergot derivatives, St John's wort and garlic supplements, and dexamethasone (33, 104).

Ritonavir

Pharmacology and Therapeutic Regimens

The oral bioavailability of RTV is undetermined, and the half-life in serum is 3 to 5 h. RTV needs to be administered with food. It is 98% plasma protein bound and is metabolized by CYP3A. The major metabolite is isopropylthiazole, which has the same antiviral activity as the parent drug. The route of elimination of the oxidative metabolites and unaltered drug is fecal (33). Passage across the placenta is minimal (107).

The main role of RTV in current HIV therapeutics is to enhance the pharmacokinetics of a second PI (25, 138), because RTV is such a strong inhibitor of CYP450 isoenzymes. RTV-boosted PIs are recommended in combination with two NRTIs or NtRTIs in several PI-based regimens for treatment-naïve patients. RTV is a pharmacoenhancer of IDV, APV, SQV, LPV, ATV, and to a lesser degree NFV. The use of RTV alone in PI-based regimens is not recommended because of GI intolerance (33).

Drug Interactions

In general, RTV is a very strong inhibitor of CYP3A4 that has numerous potential drug interactions requiring close monitoring (33, 104). Coadministered drugs that require dose modifications or cautious use include antifungal agents, rifampin, rifabutin, clarithromycin, atorvastatin, pravastatin, anticonvulsants, methadone, erectile dysfunction agents, desipramine, and theophylline. Drugs whose effects may be decreased by RTV coadministration include anticoagulants, anticonvulsants, and antiparasitics. Drugs that are contraindicated for coadministration with RTV include the calcium channel blocker bepridil; cardiac drugs amiodarone, flecainide, propafenone, and quinidine; simvastatin and lovastatin; rifapentine; cisapride; pimozide; sedatives and hypnotics; ergot derivatives; oral contraceptives (ethinyl estradiol); and St. John's wort.

Lopinavir-Ritonavir

Pharmacology and Therapeutic Regimens

LPV is administered only in combination with low-dose RTV (r). The bioavailability of LPV/r is undetermined, and the half-life is 5 to 6 h. It should be taken with food of moderate fat content. The level of plasma protein binding is 99%. LPV/r is metabolized by the CYP450 isoenzymes and inhibits CYP3A4 and to a lesser extent CYP2D6. It is eliminated mainly in the feces and urine as metabolites. It is not known whether LPV crosses the placenta (107). LPV/r is the preferred drug combination in PI-based regimens with two NRTIs or NtRTIs for treatment-naïve patients (33).

Drug Interactions

The interactions of LPV/r are similar to those of RTV alone (33, 104) (see above).

Amprenavir

Pharmacology and Therapeutic Regimens

The bioavailability of APV is undetermined, and the half-life in serum is 7.1 to 10.6 h. APV can be administered with or without food, but high-fat-content meals should be avoided. The level of plasma protein binding is 90%. APV is metabolized in the liver by CYP3A4, of which it is an

inhibitor and inducer (33, 40). Approximately 90% is eliminated as metabolites in the feces and urine. It is not known whether APV crosses the placenta (107).

APV either boosted or unboosted with RTV is not recommended for treatment-naïve patients because of the high pill burden (33). The oral solution contains propylene glycol, which is contraindicated for pregnant women and young children.

Drug Interactions

Drugs that require dose modifications or cautious use with APV include antifungal agents, rifabutin, atorvastatin, anticonvulsants including methadone, erectile dysfunction agents, H2 receptor antagonists, and proton pump inhibitors (33, 43). Drugs that are contraindicated for coadministration with APV include the calcium channel blocker bepridil, simvastatin and lovastatin, rifampin and rifapentine, cisapride, pimozide, sedatives and hypnotics, ergot derivatives, St. John's wort, and oral contraceptives (33, 104). The oral solution of RTV contains ethanol; therefore, it should not be used to boost the oral solution of APV, because the two solutions use the same metabolic pathway for elimination (33). APV and its prodrug f-APV should not be used together, because there is no benefit and there is potential additive toxicity. DLV is also contraindicated with APV.

Fosamprenavir

Pharmacology and Therapeutic Regimens

f-APV is a prodrug with no antiviral activity but must be converted to APV by cellular phosphatases (40) (see "Amprenavir" above). It is not known whether f-APV crosses the placenta (107). RTV-boosted f-APV is recommended in alternative PI-based regimens with two NRTIs or NtRTIs for treatment-naïve patients (33).

Drug Interactions

See "Amprenavir" above.

Atazanavir

Pharmacology and Therapeutic Regimens

ATV is an azapeptide PI that differs structurally from other peptidomimetic PIs. The bioavailability is undetermined, and the half-life in serum is 7 h. The bioavailability, however, is increased by administration with food. ATV is 86% protein bound and penetrates the CNS (99). It is metabolized in the liver by CYP3A4, and it is also an inhibitor of this enzyme. The metabolites are eliminated in the feces. It is not known whether ATV crosses the placenta (107).

ATV has the advantage of once-daily dosing and a low pill burden, and the drug exposure can be increased by boosting with RTV (55). ATV is recommended for use in alternative PI-based regimens with two NRTIs or NtRTIs (33).

Drug Interactions

ATV should not be used with IDV because of additive adverse effects of hyperbilirubinemia and jaundice. Drugs that require dose modifications or cautious use include antifungal agents; rifabutin and clarithromycin; oral contraceptives; atorvastatin; anticonvulsants carbamazepine, phenobarbital, and phenytoin; erectile dysfunction agents; H2 receptor antagonists; proton pump inhibitors; and antacids and buffered medications. Drugs that are contraindicated for coadminstration with ATV include the calcium channel blocker bepridil, simvastatin and lovastatin, rifampin and rifapentine, cisapride and proton pump inhibitors, pimozide, sedatives and hypnotics, ergot derivatives, St. John's wort, and irinotecan (33, 104).

Nelfinavir

Pharmacology and Therapeutic Regimens

The bioavailability of NFV is 20 to 80%, and the half-life in serum is 3.5 to 5 h. NFV shows the greatest accumulation in cells compared to the other PIs; however, the level of protein binding is >98% (42). It is administered with food, which increases the bioavailability. NFV is metabolized by CYP450 isoenzymes and is both an inhibitor and an inducer of CYP3A4 (40). There is minimal placental passage (107). NFV is used in alternative PI-based regimens with two NRTIs or NtRTIs for treatment-naïve patients (33).

Drug Interactions

Drugs that require dose modifications or cautious use include antifungal agents, rifabutin, atorvastatin, anticonvulsants including methadone, and erectile dysfunction agents. Drugs that are contraindicated for coadministration with NFV include simvastatin and lovastatin, rifampin and rifapentine, cisapride, pimozide, sedatives and hypnotics, ergot derivatives, St. John's wort, and oral contraceptives (33, 104).

Tipranavir

Pharmacology and Therapeutic Regimens

Tipranavir (TPV), a nonpeptidic PI (136), is the most recently approved of the PI class of drugs. The bioavailability is undetermined, and the half-life in serum is 4.8 to 6 h. The drug should be taken with food. TPV is >99.9% protein bound in plasma to both albumin and AAG. Metabolism is mainly through CYP3A4. However, most of the drug is eliminated unchanged in the feces. It is not known whether TPV crosses the placenta (107). TPV requires coadministration with RTV to reach effective levels in plasma (12, 86). The current indicated use is for patients who are highly treatment-experienced or who are infected with virus strains resistant to multiple PIs.

Drug Interactions

TPV decreases ethinyl estradiol concentrations, and therefore nonhormonal contraception methods should be used, and it also reduces methadone levels. Classes of drugs that are contraindicated include antiarrhythmics, antihistamines, ergot derivatives, cisapride, pimozide, and sedatives and hypnotics. Coadministration of APV, LPV, or SQV with RTV-boosted TPV is not recommended (12).

Fusion Inhibitors

A new class of antiretroviral agents has been developed that target the entry of HIV into the host cell. Enfuvirtide (T20) is the first member of this class to be approved. It is a linear synthetic peptide of 36 amino acids that binds to the first heptad repeat in the gp41 subunit of the HIV-1 envelope glycoprotein. The sequence of the peptide was derived from that of HIV-1(LAI), a subtype B strain. The binding prevents conformational changes that are required for fusion between the virus envelope and the cell membrane (93). Entry is inhibited, thereby preventing infection of the target cell. This is a novel antiviral mechanism, because the drug is active outside rather than inside the cell.

Enfuvirtide

Pharmacology and Therapeutic Regimens

T20 is administered by subcutaneous injection. The bioavailability is 84% (93), and the half-life in serum is 3.8 h. It is assumed that the metabolism of the drug produces the constituent amino acids, which enter the amino acid pool in the body and are recycled. It is not active against HIV-2, but there are recent data that suggest that it is active against non-B subtypes and possibly group O as well (105). It is not known whether T20 crosses the placenta (107). It is presently used for the treatment of HIV-1 infection in treatment-experienced patients (33).

Drug Interactions

There is no evidence that T20 induces or inhibits any of the CYP450 isoenzymes; therefore, it is unlikely to interact with any of the drugs that are metabolized by the CYP450 system. No significant interactions with other antiretroviral drugs have been identified (93).

AGENTS ACTIVE AGAINST HEPATITIS B VIRUS

There are two major classes of drugs available to treat HBV infection: nucleoside and nucleotide analogues and interferons. Because a large percentage of patients are coinfected with HIV, these agents can also be categorized by whether they have activity against both viruses or only HBV. Of the drugs that are specifically approved for use against HBV, IFN-α2a and IFN-α2b, pegylated IFN-α2a and IFN-α2b, adefovir (ADV), and entecavir are active only against HBV, and 3TC is active against both HBV and HIV. TDF and FTC are approved only for use against HIV but have also been shown to have activity against HBV (8, 33, 50, 73, 127). The common target for antiviral drugs active against both viruses is the RT function of the HIV and HBV replication enzymes (137).

Chronic HBV infection plays an important role in the morbidity and mortality of HIV-infected patients (119). The strategy for treating coinfected patients is to determine whether infection with only one virus or that with both viruses needs to be treated. If only HIV infection needs to be treated, drugs with activity against both HIV and HBV, such as 3TC and TDF, should be withheld. If only HBV infection needs to be treated, drugs without anti-HIV activity, such as pegylated interferon and ADV, can be used. If infection with both viruses needs to be treated, HAART regimens with NRTIs or NtRTIs such as 3TC and TDF that suppress replication of both viruses should be considered (33). 3TC monotherapy rapidly selects for HBV resistance (89, 119); therefore, combination therapy with one NRTI (3TC or FTC) and one NtRTI (TDF or ADV) may be effective (8, 119). Pegylated interferon is used to treat HBV infection, but the side effects limit the length of therapy, and the response appears to be lower in HIV-HBV-coinfected patients (119). There is also some evidence that the HBV genotypes (A through H) may affect the response to therapy. Genotype A appears to be associated with the best response (1, 11, 68, 119).

Nucleoside and Nucleotide Analogues

Lamivudine

3TC (Table 1) was the first nucleoside analogue that was approved for the treatment of chronic HBV infection. Because it has activity against both HIV and HBV, it has been effective in reducing loads of both viruses in plasma as part of HAART regimens. However, HBV-specific drug resistance mutations are selected over long-term therapy at a higher rate (20% per year) in coinfected patients than in those that are HIV negative (10). Discontinuation of 3TC by HBV-infected patients can produce severe flare-ups of liver transaminase levels, which are usually self-limited but have been fatal in a few cases. Another common problem is the rebound viremia that occurs when therapy is terminated (33). This is thought to be derived from the viral covalently closed circular DNA, which is not affected by nucleoside or nucleotide therapy and remains in the infected hepatocytes (137).

Pharmacology

See the section on HIV antiviral agents above. 3TC has activity against both HIV and HBV. The selection of HBV drug resistance mutations eventually decreases efficacy for the treatment of chronic hepatitis. Recent recommendations suggest combination dual NTRI-NtRTI therapy using TDF for coinfected patients to reduce the rate of selection of HBV 3TC-resistant strains (119).

Drug Interactions

See the section on HIV antiviral agents above.

Adefovir

Structure and Mechanism of Action

The prodrug adefovir dipivoxil (ADV) is manufactured by Gilead Sciences (Foster City, Calif.) as Hepsera. It is a diester of ADV, which is an acyclic nucleotide analogue of adenosine monophosphate. It was originally developed as an antiretroviral drug; however, the high doses required were associated with nephrotoxicity (137). A much lower dosage is effective against HBV (52). It is diphosphorylated by cellular kinases into the triphosphate form, which is a substrate for the RT activity of the HBV DNA polymerase. Incorporation of ADV into the replicating viral DNA causes chain termination.

Adverse Effects

The most frequently reported adverse reactions associated with ADV therapy are asthenia, headache, and GI symptoms. Nephrotoxicity can occur in patients with underlying renal dysfunction and those taking nephrotoxic agents such as cyclosporine, tacrolimus, aminoglycosides, vancomycin, and nonsteroidal anti-inflammatory drugs. Discontinuation of therapy can produce severe acute exacerbation of hepatitis (33).

Pharmacology

The bioavailability is 59%, and the half-life in serum is 7.5 h. ADV is administered without regard to food. It is excreted by the kidneys. There are no data for placental passage of the drug. ADV is recommended for the treatment of HBV infection in patients with 3TC-resistant virus (128).

Drug Interactions

ADV does not alter the pharmacokinetics of 3TC and TDF. The bioavailability appears to be increased by ibuprofen.

Entecavir

Structure and Mechanism of Action

Entecavir is manufactured by Bristol-Myers Squibb (Princeton, N.J.) as Baraclude and received FDA approval in

March of 2005. It is a carboxylic 2′-deoxyguanosine analogue, which inhibits three stages of HBV replication: priming, reverse transcription, and plus-strand DNA synthesis (74, 115, 137). It does not appear to affect mitochondrial DNA replication (74, 119).

Adverse Effects

Severe acute exacerbations of HBV infection may occur in patients who discontinue therapy. Common adverse effects are headache, fatigue, dizziness, and GI symptoms (74).

Pharmacology

The bioavailability is approximately 100%, and the half-life in serum is 15 h (74). Entecavir should be administered without food. It is excreted mainly as unmetabolized drug by the kidneys.

Drug Interactions

Entecavir is not a substrate, inhibitor, or inducer of any of the CYP450 isoenzymes. There is no interaction with 3TC, ADV, or TDF. It is possible that drugs that reduce renal function or compete for active tubular secretion may increase the concentration of entecavir in serum and/or that of the coadministered drug.

Tenofovir Disoproxil Fumarate

TDF (Table 1) is approved for the treatment of HIV-infected patients. It has repeatedly been shown to have activity against HBV, but it is not licensed for this purpose (127, 137). It does not show cross-resistance with HBV 3TC-resistant mutants, and it appears to have a lower potential for the selection of resistance mutations. For this reason, it is being considered as a component in combination therapy for coinfected patients to reduce the possibility of selection of HBV drug-resistant mutants (8, 119). Severe flare-ups of hepatic enzyme levels have been reported in coinfected patients who have discontinued TDF therapy (33).

Emtricitabine

FTC (Table 1) is approved for therapy of HIV-infected patients. It has activity against HBV but is not licensed for anti-HBV therapy. FTC and 3TC are biochemically similar and appear to be interchangeable for potential use in the treatment of HIV-HBV-coinfected patients (119). However, they also share the same HBV resistance mutations; therefore, combination therapy with these two drugs is not recommended (137). In addition, as with 3TC, severe acute exacerbations of HBV infection can occur once therapy is discontinued (33).

Interferons

IFN-α is a type I interferon naturally secreted by hematopoietic cells. There are multiple members of the IFN-α family, with broad biological effects including antiviral, antiproliferative, and immunomodulatory activities (116). Recombinant forms of some of these molecules have been developed as antiviral agents and approved for the treatment of HBV and HCV infection (see below).

Interferon-α2a and Interferon-α2b and Pegylated Interferon-α2a and Interferon-α2b

Structure and Mechanism of Action

The IFN-α agents are produced through recombinant DNA technology from cloned human leukocyte interferon genes expressed in *Escherichia coli*. The standard IFN-α2a (RoferonA; Roche Pharmaceuticals, Nutley, N.J.) and IFN-α2b (Intron-A; Schering-Plough) are 20,000-molecular-weight proteins. The pegylated interferons (peginterferon) are covalent conjugates of standard interferon with branched (2a) or straight (2b) polyethylene glycol (PEG) chains. The molecular masses are 31,000 (2a) and 60,000 (2b) Da. Peginterferons 2a and 2b are manufactured as Pegasys (Roche) and PEG-Intron (Schering-Plough), respectively.

Interferons bind to specific cell membrane receptors. Binding to the receptors activates an intracellular signaling cascade, which leads to the expression of a multitude of interferon-stimulated genes. There are broad biological effects resulting from the expression of these genes, which include the inhibition of viral replication in infected cells as well as decreased cell proliferation, increased apoptosis, and increased phagocytosis by macrophages (116).

Pharmacology

Interferon is administered by subcutaneous injection. Peginterferon has decreased renal clearance and a greatly extended half-life compared to standard interferon. The longer half-life allows for once-weekly dosing (122). Peginterferon is recommended for the treatment of patients with HBe antigen-negative or antigen-positive chronic hepatitis B (47, 68, 75, 84). In recent studies, combination therapy with peginterferon and 3TC did not produce a better response than therapy with peginterferon alone in these patients (75, 84), although staggered therapy with peginterferon and 3TC may improve the rate of virologic response (20). There is some evidence that HBV genotypes may affect the response to treatment (1, 68, 75).

Adverse Effects

There are a number of serious conditions induced by interferon therapy. These include life-threatening or fatal neuropsychiatric reactions (suicide, depression, and drug addiction), severe bacterial infections associated with neutropenia, bone marrow toxicity with severe cytopenias, flu-like syndrome (headache, fatigue, myalgia, and abdominal pain), GI symptoms (anorexia, diarrhea, and nausea), myocardial infarction, and stroke (68, 101, 122).

Drug Interactions

The potential for interaction of peginterferon with drugs metabolized by the CYP450 isoenzymes in patients with hepatitis is not known. Peginterferon therapy is contraindicated in patients with advanced HBV-related cirrhosis (89).

AGENTS ACTIVE AGAINST HEPATITIS C VIRUS

The only antiviral agents that have shown any activity in achieving a sustained virologic response against chronic HCV infection are standard IFN-α2a and IFN-α2b, pegylated IFN-α2a and IFN-α2b, and combinations of these interferons with ribavirin. Ribavirin monotherapy is not effective against HCV infection (126). Numerous studies have led to the current standard of care, which is combination therapy with pegylated IFN-α and ribavirin (5, 46, 83, 122, 125). There is also good evidence that the HCV genotype is predictive of the response to therapy. Of the six HCV genotypes, genotypes 2 and 3 are the most susceptible to therapy, and the rates of sustained virologic responses are lower for genotypes 1, 4, 5, and 6 (77, 82, 101, 122).

Interferons

Interferon-α2a and Interferon-α2b and Pegylated Interferon-α2a and Interferon-α2b

See the section on HBV agents above.

Other Agents

Ribavirin

Structure and Mechanism of Action

Ribavirin is a synthetic nucleoside analogue with the structure 1-beta-D-ribofuranosyl-1*H*-1,2,4-triazole-3-carboxamide. It is manufactured as Rebetol by Schering-Plough and as Copegus by Roche for the treatment of HCV infection. It is not known how ribavirin combined with peginterferon inhibits HCV.

Ribavirin has in vitro activity against several other RNA viruses, including respiratory syncytial virus (RSV) and Lassa fever virus (100). In cell cultures, the inhibitory activity of ribavirin for RSV is selective. Reversal of the in vitro antiviral activity by guanosine or xanthosine suggests that ribavirin may act as an analogue of these cellular metabolites, although the mode of action is unknown. However, clinical trials with ribavirin show that the drug is not effective in the treatment of RSV infections (90).

Pharmacology

The bioavailability is increased by a high-fat meal; therefore, ribavirin should be administered with food. The multiple-dose half-life of ribavirin in plasma is 12 days, and the drug may persist in other body compartments for up to 6 months. The pathway for elimination has not been determined. Ribavirin appears not to be a substrate for the CYP450 isoenzymes.

Adverse Effects

Animal studies have demonstrated teratogenic and embryocidal effects. These observations, along with the persistence of the drug, are the reasons ribavirin is strongly contraindicated during pregnancy. Major adverse effects include hemolytic anemia, aggravation of underlying cardiac disease, hepatic failure, and pulmonary symptoms (83, 122). Ribavirin is always given in combination with interferon for the treatment of HCV infection; therefore, additional adverse effects are associated with interferon therapy (see above).

Drug Interactions

HIV-HCV coinfection can be treated; however, the pill burden and potential drug interactions may affect the regimen. Cases of hepatic failure have been reported with NRTIs, in particular ddI and ZDV. ZDV plus ribavirin is linked to higher rates of anemia (33).

AGENTS ACTIVE AGAINST HERPESVIRUSES

The majority of the antiviral compounds that are active against the herpesviruses are nucleoside or nucleotide analogues, which inhibit DNA replication. Several of these compounds require phosphorylation by a viral enzyme as well as cellular kinases for activation. In most cases the ultimate target is the activity of DNA polymerase, although other enzymatic steps in DNA synthesis also may be inhibited (31). Besides the nucleoside and nucleotide analogues, the antiherpes compounds include a pyrophosphate analogue (foscarnet [PFA]) that targets the DNA polymerase directly, an antisense oligonucleotide (fomivirsen), and an entry inhibitor (docosanol). The structure, mode of action, pharmacology, and adverse effects of each drug are summarized in Table 2.

Acyclovir and Valacyclovir

The selective activity of the acyclic guanosine analogue ACV against herpes simplex virus (HSV) was first reported in 1977 (37). ACV was one of the first effective anti-HSV compounds available clinically and has been in general use for the treatment of serious HSV infections for the past 25 years. Val-ACV, the L-valyl ester, is rapidly converted to ACV (67) after administration.

ACV is phosphorylated by the viral TK (36) and then phosphorylated into the di- and triphosphate forms by cellular enzymes. ACV triphosphate competes with dGTP for the viral DNA polymerase. Incorporation of ACV into the growing DNA chain prevents further nucleotide extension of the viral DNA.

Spectrum of Activity

ACV is active against HSV-1, HSV-2, and varicella-zoster virus (VZV) (80) and is commonly used to treat serious infections caused by these viruses. ACV is also activated by the UL97 kinase of human cytomegalovirus (HCMV) (124) and is recognized by the HCMV DNA polymerase. ACV has been used for prophylactic treatment to prevent HCMV disease in patients following transplantation (7, 60, 96, 111).

Pharmacology

The pharmacokinetics of ACV after oral administration have been evaluated in healthy volunteers and in immunocompromised patients with HSV and VZV infection. The level of protein binding in plasma is from 9 to 33%, and the average bioavailability is from 10 to 20%. The elimination half-life in plasma is 2.5 to 3.3 h. ACV may be administered with or without food.

ACV is excreted by the kidneys in an amount accounting for 62 to 91% of the dose administered. The only known urinary metabolite is 9-[(carboxymethoxy)methyl] guanine. The half-life and total body clearance of ACV are dependent on renal function. A dosage adjustment is recommended for patients with reduced renal function. The half-life and total body clearance of ACV in pediatric patients over 1 year of age are similar to those in adults with normal renal function.

The oral formulation of ACV continues to be used widely. In addition, an oral prodrug of ACV, val-ACV, is available and has improved bioavailability compared to the parent compound (120).

Drug Interactions

No clinically significant interactions have been identified for ACV.

Penciclovir and Famciclovir

Penciclovir (PCV) is structurally related to ACV and is also a selective inhibitor of HSV. Famciclovir (FCV), the oral prodrug, is the diacetyl 6-deoxy analogue of PCV (129) and undergoes rapid transformation into the active compound PCV. There are differences in the anabolism of PCV compared to that of ACV. PCV has a higher affinity for the HSV TK than ACV, resulting in higher levels of PCV triphosphate in HSV-infected cells. PCV triphosphate is more stable in HSV-infected cells, resulting in a 10- to 20-fold longer half-life than that of ACV triphosphate. The HSV DNA polymerase, however, has a higher affinity for ACV

TABLE 2 Antiviral agents for herpesviruses

Antiviral agent (abbreviation)	Trade name, pharmaceutical company[a]	Structure	Mode of action and route of administration	Major adverse effects
Acyclovir (ACV)	Zovirax, GSK	2-Amino-1,9-dihydro-9-[(2-hydroxyethoxy)methyl]-6*H*-purine-6-one	Converted to monophosphate by viral TK; converted to triphosphate by cellular kinases; DNA chain terminator; available as oral or i.v. formulations	Minimal toxicity, nausea, vomiting, headache, diarrhea, precipitation in renal tubules if maximum solubility exceeded
Valacyclovir (val-ACV)	Valtrex, GSK	L-Valyl ester of acyclovir	Mode of action same as that of ACV; oral formulation results in increased bioavailability	Nausea, headache, vomiting, dizziness, abdominal pain
Penciclovir (PCV)	Denavir, Novartis	9-[4-Hydroxy-3-(hydroxymethyl)butyl] guanine	Mode of action same as that of ACV; topical formulation for treatment of herpes labialis	Headache and application site reaction no different from those with placebo
Famciclovir (FCV)	Famvir, Novartis	Diacetyl 6-deoxy analogue of PCV	Mode of action same as that of ACV; oral form of penciclovir	Headache, nausea, diarrhea
Ganciclovir (GCV)	Cytovene, Roche	9-[2-Hydroxy-1-(hydroxymethyl) ethoxy] methyl guanine	Converted to monophosphate by HCMV UL97 kinase or HSV or VZV TK; converted to triphosphate by cellular kinases; DNA chain terminator; oral and i.v. formulations	Fever, neutropenia, anemia, thrombocytopenia, impaired renal function, diarrhea
Valganciclovir (val-GCV)	Valcyte, GSK	L-Valyl ester of GCV	Mode of action same as that of GCV; oral form of ganciclovir that results in increased bioavailability	Diarrhea, neutropenia, nausea, headache, anemia
Foscarnet (PFA)	Foscovir, AstraZeneca	Trisodium phosphonoformate	Pyrophosphate analogue; interferes with pyrophosphate binding site of DNA polymerase; i.v. administration	Renal impairment, fever, nausea, anemia, diarrhea, vomiting, headache, seizures, altered electrolytes in serum
Cidofovir (CDV)	Vistide, Gilead	1-[(*S*)-3-Hydroxy-2-(phosphonomethoxy)propyl] cytosine; formerly called HPMPC	Converted to di- and triphosphate by cellular kinases; DNA chain terminator; i.v. administration with probenecid	CDV: renal toxicity, decreased intraocular pressure, neutropenia, fever; probenecid: nausea, vomiting, headache, rash
Fomivirsen	Vitravene, Novartis	Oligonucleotide complementary to IE2 transcript of HCMV	Binding of the oligonucleotide to mRNA blocks translation of IE2 gene product and virus replication; administered by ophthalmic injection	Uveitis, iritis, vitritis
Vidarabine (Ara-A)	Generic	9*H*-Purin-6-amino-9-β-D-arabinofuranosyl-monohydrate	Mode of action not well understood; appears to inhibit viral DNA synthesis; ophthalmic ointment for topical use	Lacrimation, conjunctival pain, keratitis, photophobia
Trifluridine	Viroptic, Monarch	α,α,α-Trifluorothymidine	Mode of action not well understood; appears to inhibit viral DNA synthesis; ophthalmic aqueous solution for topical use	Burning on instillation and palpebral edema, punctuate keratopathy, hypersensitivity reaction, stromal edema, keratitis sicca, hyperemia, increased ocular pressure
Docosanol	Abreva, GSK	*n*-Docosanol	Prevents HSV entry into cells by inhibition of fusion between HSV envelope and cell membrane; over-the-counter topical cream preparation	Headache, skin rash

[a] Pharmaceutical companies: AstraZeneca, Wilmington, Del.; Gilead Sciences, Foster City, Calif.; GSK, GlaxoSmithKline, Research Triangle Park, N.C.; Monarch Pharmaceutical, Bristol, Tenn.; Novartis, East Hanover, N.J.; Roche Pharmaceuticals, Nutley, N.J.

triphosphate than for PCV triphosphate. ACV is an obligate DNA chain terminator, but PCV allows limited chain elongation. The overall effect of the differences between ACV and PCV is that the two compounds have similar anti-HSV potencies (6).

Spectrum of Activity

PCV and FCV are active against HSV and VZV (35). Neither of these compounds is active against other human herpesviruses.

Pharmacology

PCV is available only as a cream for the topical treatment of herpes labialis (113). FCV was developed to improve the bioavailability of the parent compound PCV (79). Following oral administration, FCV is deacetylated and oxidized to form PCV; therefore, little or no FCV is detected in plasma or urine. The absolute bioavailability of FCV is 77% ± 8%.

FCV is eliminated as PCV in urine (108). Elimination half-life, urinary recovery, and renal clearance of PCV do not change with increasing dose. PCV displays linear pharmacokinetics in the anticipated therapeutic dose range of FCV.

Drug Interactions

No clinically significant interactions have been identified for PCV.

Ganciclovir and Valganciclovir

GCV was the first effective anti-HCMV drug developed for clinical use and in older literature is referred to as DHPG (85). GCV is an acyclic nucleoside analogue of 2′-deoxyguanosine, which requires phosphorylation to become active (123). GCV triphosphate concentrations may be 100-fold greater in HCMV-infected cells than in uninfected cells, indicating preferential phosphorylation of GCV in infected cells (28). Unlike ACV, GCV is not an absolute chain terminator, and short subgenomic fragments of HCMV continue to be synthesized (53, 54). Val-GCV, the L-valyl ester of GCV, is rapidly converted to GCV (30).

Spectrum of Activity

GCV is active against HCMV as well as HSV, VZV, Epstein-Barr virus, and human herpesvirus 6 (78).

Pharmacology

A pharmacokinetic study was performed with patients with HIV who received GCV as a single i.v. infusion over 1 h, 500 mg orally every 3 h, or 1,000 mg orally twice daily. The mean peak concentrations in serum were 8.27, 1.02, and 1.18 μg/ml, respectively, for each of these regimens. The absolute bioavailabilities of the drug in the oral regimens were 8.84 and 8.53%, respectively. The extent of GCV absorption, peak concentrations, and average concentration at steady state were not statistically different between the oral regimens. Both oral regimens resulted in concentrations in the range of the concentration that inhibits 50% of most HCMV isolates (2). Oral GCV is available for the treatment and prophylaxis of HCMV infection; however, val-GCV has increased absorption that results in increased bioavailability (103, 131). Renal excretion of unchanged drug by glomerular filtration and active tubular secretion is the major route of elimination of GCV.

Drug Interactions

Coadministration of GCV and ddI results in increased levels of ddI.

Foscarnet

PFA is an organic analogue of pyrophosphate, which exerts its antiviral activity by selective inhibition at the pyrophosphate binding site on the viral DNA polymerase at concentrations that do not affect cellular DNA polymerases (97). PFA does not require intracellular activation.

Spectrum of Activity

Although PFA is active against several herpesviruses, including HSV, HCMV, VZV, and Epstein-Barr virus, it is most commonly used to treat infections with antiviral drug-resistant HSV and HCMV.

Pharmacology

Pharmacokinetic data indicate that PFA undergoes negligible metabolism, appears to be distributed widely from the circulation, and is eliminated via the renal route. The available data, however, indicate that the pharmacokinetics of the drug vary among patients and within an individual patient, particularly with regard to plasma PFA levels (78). The PFA terminal half-life determined by urinary excretion is 87.5 ± 41.8 h, possibly due to the release of PFA from bone (22). Approximately 90% of PFA is excreted as unchanged drug in urine. Systemic clearance of PFA decreases and half-life increases with diminishing renal function. Based on these observations, it is necessary to modify the dosage of PFA for patients with renal impairment (4).

Drug Interactions

Possible drug-drug interaction between PFA and intravenous pentamidine, which results in hypocalcemia, has been described. Caution is advised for patients receiving drugs known to affect serum calcium levels. Because of the tendency for renal toxicity, the use of PFA in combination with other potentially nephrotoxic drugs should be avoided (114).

Cidofovir

CDV is a nucleotide analogue of deoxycytidine monophosphate. In earlier published literature it is referred to as HPMPC (118). CDV phosphorylation does not require a viral enzyme. For HCMV, two successive CDV molecules must be incorporated in order to affect chain termination (135).

Spectrum of Activity

CDV is active against several herpesviruses, including HCMV, HSV, and VZV (70, 118). CDV also has activity against poxviruses (81), adenovirus (95), polyomaviruses (72), and human papillomavirus (117).

Pharmacology

CDV must be administered with probenecid (29, 134). Approximately 90% of the CDV dose administered is recovered unchanged in the urine within 24 h. When CDV is administered with probenecid, the renal clearance of CDV is reduced to a level consistent with creatinine clearance, suggesting that probenecid blocks active renal tubular secretion of CDV (29). In vitro, CDV is less than 6% bound to plasma or serum proteins.

Drug Interactions

No clinically significant interactions have been identified for CDV. However, the required administration of probenecid with CDV may produce drug-drug interactions resulting from the potential block of acidic drug transport in the kidneys (29).

Fomivirsen

Fomivirsen is a phosphorothioate oligonucleotide, which is complementary (antisense) to the HCMV immediate-early (IE2) transcript with the following sequence: 5′-GCG TTT GCT CTT CTT CTT GCG-3′ (98). The target region of the mRNA encodes several proteins responsible for the regulation of viral gene expression that are essential for HCMV replication. Hybridization of the oligonucleotide with the mRNA target leads to inhibition of specific protein synthesis by mechanisms including translational arrest and degradation of the mRNA by RNase H cleavage. The end result is inhibition of virus replication. This sequence appears to be unique to HCMV and is not complementary to any known mRNA sequence in humans.

Spectrum of Activity

Fomivirsen is approved only for the treatment of retinitis caused by HCMV (66).

Pharmacology

After intravitreal injection, the long residence time in the retina and the vitreous provides a pharmacokinetic basis for the treatment intervals (76). Fomivirsen is cleared from the vitreous over the course of 7 to 10 days by a combination of tissue distribution, metabolism, and vitreal egress. In the eye, fomivirsen concentrations are greatest in the retina and iris (49).

Fomivirsen is metabolized by exonucleases in a process that sequentially removes residues from the terminal ends of the oligonucleotide, yielding shortened oligonucleotides and mononucleotide metabolites. Data with related compounds indicate that mononucleotide metabolites are further catabolized, similar to endogenous nucleotides, and are excreted as low-molecular-weight metabolites.

Vidarabine

Vidarabine (Ara-A) is a purine nucleoside analogue for the topical treatment of epithelial keratitis caused by HSV (9). It appears to interfere with the early steps in viral DNA synthesis. Ara-A is phosphorylated by cellular kinases before being incorporated into the viral DNA, resulting in the inhibition of viral DNA synthesis.

Spectrum of Activity

Ara-A was the first anti-HSV compound shown to reduce the mortality of proven HSV encephalitis (130). It has activity against HSV, VZV, HCMV, and vaccinia virus. It is approved for the topical treatment of acute keratoconjunctivitis caused by HSV.

Pharmacology

Ara-A is rapidly deaminated into arabinosylhypoxanthine, the main metabolite, which has much lower antiviral activity (9), but it may act synergistically with the parent drug to inhibit DNA replication. Because of low solubility, systemic absorption of Ara-A should not be expected to occur following ocular administration. Trace amounts of the metabolite are found in the aqueous humor.

Trifluridine

Trifluridine is a fluorinated pyrimidine nucleoside used for the topical treatment of epithelial keratitis caused by HSV (21). Trifluridine interferes with DNA synthesis in cultured mammalian cells, but its antiviral mode of action is not completely known.

Spectrum of Activity

Trifluridine has in vitro and in vivo activity against HSV-1, HSV-2, and vaccinia virus (102). Some strains of adenovirus are also inhibited in vitro by trifluridine. It is approved for the topical treatment of acute keratoconjunctivitis caused by HSV.

Pharmacology

Intraocular penetration by trifluridine occurs after topical instillation into the eye. Decreased corneal integrity or stromal or uveal inflammation may enhance the penetration of trifluridine into the aqueous humor. Systemic absorption following therapeutic dosing with trifluridine appears to be negligible (17).

n-Docosanol

n-Docosanol, also known as behenyl alcohol, is a saturated 22-carbon aliphatic alcohol (69). Docosanol works by inhibiting the fusion between the plasma membrane of human cells and the envelope of HSV. Consequently, docosanol prevents viral entry into cells and subsequent viral replication.

Spectrum of Activity

n-Docosanol exhibits in vitro activity against several lipid-enveloped viruses, including RSV, HSV-1, and HSV-2.

Pharmacology

A topical preparation of *n*-docosanol is available as a 10% cream for the treatment of HSV infection. It is available without a prescription as an over-the-counter medication.

AGENTS ACTIVE AGAINST INFLUENZA VIRUSES

There are two classes of agents for the treatment of influenza: M2 protein inhibitors and neuraminidase inhibitors. The M2 inhibitors are active only against influenza type A, and the neuraminidase inhibitors have activity against both types A and B (91).

M2 Protein Inhibitors

The viral M2 protein facilitates the hydrogen ion-mediated dissociation of the matrix protein-ribonucleoprotein (RNP) complex within the endosome and the release of the viral RNP into the cytoplasm of the host cell (94). The inhibitors block the passage of H^+ ions through the M2 ion channel, which prevents uncoating of the virus (31, 91).

Amantadine and Rimantadine

Amantadine was the first anti-influenza agent discovered, and it was licensed in 1966 for therapy and prophylaxis of influenza A virus infections (112). Rimantadine was licensed in 1993 (92). Both drugs have similar antiviral activities, but they differ in their metabolisms and adverse effects.

Structure and Mechanism of Action

Amantadine hydrochloride is designated chemically as 1-adamantanamine hydrochloride, and rimantadine is methyl-1-adamantanemethyl amine hydrochloride. Both amantidine and rimantadine inhibit the uncoating and release of viral RNA into the host cell.

Pharmacology

Both amantadine and rimantadine are water soluble and absorbed from the GI tract. Oral administration of amantadine or rimantadine shows that the maximum nasal concentrations are similar for both drugs (58). Both compounds have nearly complete oral bioavailability and have a long half-life of >12 h in plasma. Amantadine is excreted primarily unchanged in the urine by glomerular filtration and tubular secretion, and rimandatine is metabolized by the liver (91).

When these compounds are started within 48 h of the onset of influenza symptoms, there has been an associated 1-day reduction of the duration of clinical symptoms including fever, systemic complaints, and virus shedding. Efficacy rates of up to 85% have been shown for preventing influenza illness (132).

Drug Interactions

Agents with anticholinergic properties may potentiate the anticholinergic-like side effects of amantadine. The chronic use of cimetidine may reduce the clearance of rimantadine. Coadministration with acetaminophen or aspirin may reduce the peak concentration of rimantadine.

Adverse Effects

The adverse reactions reported most frequently at the recommended dose of amantadine are nausea; vomiting; dizziness; insomnia; CNS complaints such as difficulty concentrating, jitteriness, and nervousness; and exacerbation of seizure disorders. Less frequently reported adverse reactions are depression, anxiety and irritability, hallucinations, confusion, anorexia, dry mouth, constipation, ataxia, livedo reticularis, peripheral edema, orthostatic hypotension, headache, somnolence, nervousness, dream abnormality, agitation, dry nose, diarrhea, and fatigue.

The common adverse effects associated with rimantadine include nausea, vomiting, anorexia, and CNS effects including headache, insomnia, nightmares, nervousness, lightheadedness, and difficulty concentrating. There are significantly fewer side effects associated with rimantadine than with amantidine (91).

Neuraminidase Inhibitors

The influenza virus neuraminidase is an envelope glycoprotein that cleaves the terminal sialic residues, releasing the virion from the infected cell. The viral neuraminidase allows the influenza virus to spread from cell to cell. Two neuraminidase inhibitors are approved for the treatment of infections with influenza A and B viruses: oseltamivir and zanamivir (34). Both compounds are sialic acid analogues that competitively and reversibly bind to the active site of the enzyme, which results in the inhibition of virus particle release from infected cells.

Oseltamivir

Structure

Chemically, oseltamivir phosphate is (3*R*,4*R*,5*S*)-4-acetylamino-5-amino-3(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid, ethyl ester, phosphate, which is an ethyl ester prodrug requiring conversion to the active form, oseltamivir carboxylate, by enzymes in the liver, GI tract, and blood.

Spectrum of Activity

Oseltamivir is active against both influenza A and B viruses (38).

Pharmacology

Oseltamivir is readily absorbed from the GI tract after oral administration of oseltamivir phosphate and is extensively converted predominantly by hepatic esterases to oseltamivir carboxylate (62). At least 75% of an oral dose reaches the systemic circulation as oseltamivir carboxylate. The half-life in plasma is 6 to 10 h. The level of binding of oseltamivir carboxylate to plasma protein is low. Coadministration with food has no significant effect on the peak concentration of oseltamivir carboxylate in plasma.

The efficacy of oseltamivir in preventing naturally occurring influenza illness has been demonstrated in treatment and prophylaxis studies with a nursing home population (13). In a study of postexposure prophylaxis for household contacts of index cases, oseltamivir reduced the incidence of clinical influenza without the emergence of drug-resistant virus (57).

Absorbed oseltamivir is eliminated primarily by conversion to oseltamivir carboxylate. Concentrations of oseltamivir in plasma decline in most subjects after oral administration. Oseltamivir carboxylate is not further metabolized and is eliminated in the urine. The renal clearance rate exceeds the glomerular filtration rate, indicating that tubular secretion occurs in addition to glomerular filtration.

Drug Interactions

Studies of oseltamivir suggest that clinically significant drug interactions are unlikely. In vitro studies demonstrate that neither oseltamivir nor oseltamivir carboxylate is a good substrate for P450 mixed-function oxidases or for glucuronyl transferases.

Adverse Effects

The most frequently reported adverse events in clinical studies were nausea and vomiting (87), which were generally of mild to moderate degree and usually occurred on the first two days of administration.

Zanamivir

The chemical name of zanamivir is 5-(acetylamino)-4-[(aminoiminomethyl)-amino]-2,6-anhydro-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enonic acid. For the treatment of influenza, zanamivir is administered to the respiratory tract by oral inhalation only by using a blister pack (65). The contents of each blister are inhaled using a specially designed breath-activated plastic device for the inhaling powder. A blister that contains the medication is pierced, and zanamivir is dispersed into the air stream created when the patient inhales through the mouthpiece.

Most studies of zanamivir have involved topical administration by inhalation of dry powder. This route rapidly provides high local concentrations at the sites of delivery. Zanamivir treatment has been shown to reduce the severity and duration of naturally occurring, uncomplicated influenza illness in adults (15).

Spectrum of Activity

Zanamivir is active against both influenza A and B viruses (59).

Pharmacology

The absolute oral bioavailability of zanamivir is low, averaging 2%. After intranasal or oral inhaled administration, a median of 10 to 20% of the dose is systemically absorbed, with maximum concentrations in serum generally reached within 1 to 2 h. The median half-life in serum ranges between 2.5 and 5.05 h, suggesting that the elimination rate

is limited by absorption. The low level of absorption of the drug after inhaled administration results in low concentrations in serum, and therefore there is modest systemic exposure to zanamivir after inhalation. Zanamivir is not metabolized, and the potential for clinically relevant drug-drug interactions is very low (18). After i.v. administration, 90% is excreted unchanged in the urine.

Drug Interactions

No clinically significant drug interactions are predicted based on data from in vitro studies (18).

Adverse Effects

The adverse effects in patients in treatment studies were similar to those in patients receiving placebos, although bronchospasm has been reported in patients with underlying asthma. Additional adverse reactions occurring in fewer than 1.5% of patients receiving zanamivir include malaise, fatigue, fever, abdominal pain, myalgia, arthralgia, and urticaria (59, 61; http://us.gsk.com/products/assets/us_relenza.pdf).

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Mechanisms of Resistance to Antiviral Agents

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Understanding the mechanisms of viral drug resistance is critical for clinical management of individuals receiving antiviral therapy, for development of new antiviral drugs, and for drug resistance surveillance. In this chapter, we review the mechanisms of resistance to antiviral drugs used to treat seven common viral infections: those with herpes simplex virus (HSV), cytomegalovirus (CMV), varicella-zoster virus (VZV), human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2), influenza A and B viruses, hepatitis B virus (HBV), and hepatitis C virus (HCV).

Drug-resistant viruses are identified by in vitro passage experiments in which wild-type viruses are cultured in increasing concentrations of an inhibitory drug and by ex vivo analysis of virus isolates obtained from individuals receiving antiviral therapy. Measuring drug susceptibility involves culturing a fixed inoculum of virus in serial dilutions of an inhibitory drug. The drug concentrations required to inhibit virus replication by 50 or 90% are the most commonly used measures of susceptibility. Drug susceptibility assays are not designed to determine the drug concentration required to inhibit virus replication in vivo. They are instead designed to identify viruses with reduced drug susceptibilities relative to wild-type viruses. The clinical significance of reductions in drug susceptibility is determined by studying the treatment responses of individuals harboring viruses with reduced susceptibilities.

HERPESVIRUSES

All currently licensed systemic drugs for herpesvirus infections target viral DNA polymerase. Nucleoside analogs that are selectively phosphorylated by viral enzymes are commonly used as initial therapy. To improve oral bioavailability, some are formulated as prodrugs. Acyclovir, its prodrug valacyclovir, and famciclovir (the prodrug of penciclovir) have been used successfully to treat genital HSV infections, VZV infections, and mucocutaneous HSV infections in immunocompromised hosts. Ganciclovir and its prodrug valganciclovir are used to treat CMV infection. These selective nucleoside analogs are supplemented by the drugs foscarnet (phosphonoformate) and cidofovir that inhibit viral DNA polymerase without prior activation by viral enzymes. Foscarnet is a pyrophosphate analog, and cidofovir is a phosphonomethoxycytosine nucleotide analog. These are generally used as second- and third-line drugs when there is a lack of response to initial therapy.

Herpes Simplex Virus

Both acyclovir and penciclovir are initially monophosphorylated by the HSV thymidine kinase (TK) and then triphosphorylated by cellular enzymes. In their active forms, these drugs inhibit viral DNA polymerase by competing with guanosine triphosphate for incorporation into newly synthesized DNA chains, where they cause chain termination. Because the viral TK enzyme is not essential for HSV replication, a wide variety of TK mutations may decrease the phosphorylation of nucleoside analogs, and this is by far the most common mechanism of acyclovir and penciclovir resistance in HSV (61).

Most TK mutants are classified as TK negative, usually in which frameshift or stop mutations delete important functional domains, or TK partial, in which the mutation reduces the phosphorylation of both natural nucleosides and antiviral drugs (130). TK-altered mutants, which selectively reduce the phosphorylation of antiviral nucleosides while natural nucleosides are normally phosphorylated, occur less commonly. The TK mutations selected by penciclovir often differ from those selected by acyclovir, but cross-resistance is expected (159). The effect of specific substitutions on TK activity and drug susceptibility often must be defined by site-directed mutagenesis (53). Because there are so many TK mutations, phenotypic approaches to the detection of HSV resistance have been favored over genotypic approaches (101).

TK-negative HSV mutants are attenuated in mouse models with respect to virulence, latency, and reactivation potential (24). However, animal studies and clinical case reports (66, 131, 156) show that resistant viruses cause overt disease and recurrent infection. Even isolates with frameshift mutations that are predicted to be TK negative may in fact express some TK activity that enables reactivation from latency (66). Although drug-resistant HSV TK mutants can easily be selected in cell culture, recent data indicate that the frequency of isolation of acyclovir-resistant HSV in clinical practice has remained stable at about 0.3% for immunocompetent hosts and 7% for immunocompromised hosts (170).

Drug-resistant TK mutants are expected to remain susceptible to drugs that do not depend on virally mediated phosphorylation, including foscarnet, which is currently the main alternative therapy for acyclovir-resistant HSV infection, and cidofovir. DNA polymerase *(pol)* mutations are occasionally responsible for HSV resistance to acyclovir, penciclovir, foscarnet, and cidofovir. Because the viral DNA polymerase gene is essential for replication, mutations are

more selective than those affecting TK and usually consist of single-amino-acid changes in functional domains of the enzyme. The resistance and cross-resistance properties vary depending on the mutation and are best evaluated by transfer of specific mutations to control viral strains (12). These analyses show that *pol* mutations conferring acyclovir or foscarnet resistance are distributed among several conserved regions, with some clustering of mutations in regions II, VI, and III. A few mutations confer resistance to both acyclovir and foscarnet (61). Little is known about the range of cidofovir resistance mutations in clinical isolates, but available information suggests that there is no consistent cross-resistance with acyclovir or foscarnet (12).

Varicella-Zoster Virus

Because VZV also expresses a TK, the drugs used and the mechanisms of resistance are similar to those for HSV. Acyclovir is less potent against VZV than HSV, and either intravenous acyclovir or oral prodrugs (valacyclovir or famciclovir) are needed to obtain levels of reliable antiviral activity in blood (139). VZV treatment efficacy depends more on the prompt initiation of adequately bioavailable therapy than on the loss of drug susceptibility (31). The notable exceptions are AIDS patients who may develop chronic, spreading verrucous skin lesions in which prolonged viral replication selects for resistant strains (136).

Analogous to acyclovir-resistant HSV isolates, most acyclovir-resistant VZV isolates contain mutations that result in TK deficiency or substitutions that alter substrate specificity (61). A tendency for resistance mutations to cluster at certain TK gene loci has been reported (61, 132), possibly simplifying genotypic resistance testing, which may have a greater role with this virus than with HSV because of the slower growth of VZV in cell culture.

Alternative drugs for acyclovir-resistant VZV are foscarnet and cidofovir. Foscarnet resistance maps to codons corresponding to conserved functional domains II and III of the VZV DNA polymerase (61), similar to that in foscarnet-resistant HSV and CMV mutants. Some mutants selected by foscarnet in cell culture have a slow-growth phenotype indicating the potential consequence of drug resistance mutations for DNA polymerase, an essential viral gene product (184). DNA polymerase mutations conferring resistance to these drugs and to acyclovir appear to be mostly distinct, although specific information relating to foscarnet or cidofovir resistance in clinical isolates is limited and not validated by mutagenesis studies (61).

Cytomegalovirus

CMV lacks a TK enzyme but expresses a phosphotransferase (UL97) which is essential for the production of normal amounts of infectious virus in cell culture (152). Ganciclovir, the standard initial therapy for CMV disease, is monophosphorylated by UL97 and subsequently converted into the active triphosphate form in infected cells (173). Risk factors for CMV resistance are high levels of continuing viral replication, as seen in immunocompromised hosts, coupled with continuing antiviral drug exposure, usually a few months or more (62). Since the advent of combination antiretroviral therapy, there has been a marked decrease in CMV infection in HIV-1-infected persons. During the same time, however, CMV infection has been increasingly diagnosed in transplant recipients, especially those with posttransplant primary infection and lung or pancreas transplantation (62).

More than 90% of ganciclovir-resistant CMV isolates found following treatment contain UL97 mutations (29). Because of the important function of UL97 in the viral life cycle, UL97 ganciclovir resistance mutations occur at a restricted number of positions and usually preserve the UL97 gene's reading frame. Resistance mutations have been observed at codons 460, 520, and 590 to 607, with the most common being those at codons 460, 592, 594, 595, and 603 (29). Several deletion mutations conferring resistance have been observed between codons 590 and 607. Rarely in clinical practice, a *pol* mutation may cause ganciclovir resistance, without mutation in the UL97 gene (48). Genotypic testing is attractive because of the limited number of UL97 codons involved and the technical difficulty of phenotypic testing of clinical isolates. UL97 is not known to be involved in resistance to foscarnet or cidofovir.

After continued exposure to ganciclovir, mutations in the CMV DNA polymerase gene (*pol*) may be selected and contribute to an increased level of resistance to the drug (26, 169). Recombinant phenotyping confirms the high-level ganciclovir resistance conferred by a combination of UL97 gene and *pol* mutations (28). Ganciclovir and cidofovir resistance mutations have been reported at codons 301, 408 to 413, and 501 to 545, which correspond to exonuclease, IV, and δC regions of *pol*, and at codons 978 to 988 (region V) (25).

Foscarnet resistance mutations in CMV *pol* often involve conserved regions II (e.g., codons 700 and 715), III (e.g., codons 802 and 809), and VI (e.g., codon 781) and some nonconserved loci (e.g., codon 756) (62). There appears to be limited cross-resistance between foscarnet and the other current anti-CMV drugs. Some foscarnet resistance mutations in region III also appear to confer low-grade ganciclovir or cidofovir resistance of uncertain clinical significance. This region of *pol* may have a role in recognition of the incoming nucleotide triphosphate and pyrophosphate exchange. An unusual deletion mutation (codons 981 and 982) in region V confers resistance to all three drugs: ganciclovir, cidofovir, and foscarnet (28). Some CMV *pol* mutations have been associated with a slow-growth phenotype (62).

Genotypic detection of CMV *pol* resistance mutations is feasible based on extensive knowledge of natural polymorphisms associated with interstrain variation and of well-characterized drug resistance mutations. The wide distribution of known and potential resistance mutations makes it necessary to determine the *pol* sequence from codons 300 to 1,000, and interpretation of results is more complicated than that for the UL97 gene. Moreover, there continue to be reports of sequence changes in *pol* that are indeterminate for drug resistance and that require further genetic analysis (62).

Fomivirsen is a little-used antisense topical CMV antiviral. Fomivirsen resistance has not been associated with a mutation in the cognate CMV sense sequence (135). A drug that has entered early clinical trials is maribavir, a benzimidazole L-riboside that inhibits the viral UL97 phosphotransferase (13). Maribavir-resistant laboratory CMV mutants have been isolated that contain mutations in UL97 (13) or UL27 (27). Another class of drugs targets viral DNA cleavage and processing, with resistant strains showing mutations in UL56 or UL89 (46).

HUMAN IMMUNODEFICIENCY VIRUS TYPE 1

Twenty antiretroviral drugs have been approved for treatment of HIV-1 infection, including seven nucleoside and one nucleotide reverse transcriptase (RT) inhibitors (NRTIs), eight protease inhibitors (PIs), three nonnucleoside RT inhibitors (NNRTIs), and one fusion inhibitor. In previously untreated individuals with drug-susceptible HIV-1 infection,

combinations of three or four drugs from two drug classes lead to prolonged virus suppression and, in most patients, immune reconstitution. Once complete HIV-1 suppression is achieved, it usually persists indefinitely if therapy is not interrupted (149). However, because antiretroviral therapy does not inhibit proviral HIV-1 DNA, viral eradication is not possible. Recurrent viremia and immunological decline ensue whenever therapy is discontinued, regardless of the previous duration of virological suppression.

HIV-1 genetic variability results from the high rate of RT enzyme errors, the high rate of virus replication in vivo, recombination when viruses with different sequences infect the same cell, and the accumulation of proviral variants during the course of infection (30, 116). The virus population within an individual does not consist of a single genotype but is instead an ensemble of innumerable related genotypes or quasispecies. The selection of drug-resistant variants depends on the extent to which virus replication continues during incompletely suppressive therapy, the ease of acquisition of a particular mutation (or set of mutations), and the effect of drug resistance mutations on drug susceptibility and virus replication.

The presence of drug resistance before the start of a new antiretroviral treatment regimen is an independent predictor of virological response to that regimen, and clinical trials have shown that drug resistance testing, particularly genotypic testing, provides clinical benefit in selecting treatment for infected individuals (reviewed in references 82 and 160). Although drug resistance is usually acquired during therapy, it can also be transmitted between individuals. In the United States, Europe, and Brazil, about 10 to 15% of new HIV-1 infections are with strains harboring resistance to one or more of the currently approved RT inhibitors or PIs (150).

Reverse Transcriptase

The RT enzyme is responsible for RNA-dependent DNA polymerization and DNA-dependent DNA polymerization. RT is a heterodimer consisting of p66 and p51 subunits. The p66 subunit contains the DNA binding groove and the active site; the p51 subunit displays no enzymatic activity and functions as a scaffold for the enzymatically active p66 subunit. The p66 subunit has five subdomains, including the "finger," "palm," and "thumb" subdomains, which participate in polymerization, and the "connection" and RNaseH subdomains (84, 94). Most RT inhibitor resistance mutations are in the 5' polymerase-encoding regions, particularly those corresponding to the finger and palm subdomains (158).

Nucleoside and Nucleotide RT Inhibitor Resistance

The NRTIs are prodrugs that are phosphorylated by host cellular enzymes. The occurrence of triphosphorylation (or diphosphorylation in the case of tenofovir) at different rates in different cell types complicates the in vitro assessment of both NRTI activity and phenotypic resistance testing, leading to marked discordances between in vitro and in vivo NRTI potencies (59). Differences between the highly activated lymphocytes required for susceptibility testing and the wider variety of cells that are infected in vivo also provide the most likely explanation for why the NRTIs differ in their dynamic ranges and in the levels of in vitro resistance that are clinically significant (104, 122). Clinical isolates from persons failing NRTI therapy may have several-hundred-fold reductions in susceptibility to zidovudine, lamivudine, and emtricitabine but will rarely have more than fivefold reductions in susceptibility to didanosine, stavudine, and tenofovir. However, very slight reductions in susceptibility to this second category of drugs—as low as 1.5 fold—are clinically significant (193).

There are two biochemical mechanisms of NRTI resistance. One mechanism is mediated by discriminatory mutations that reduce RT affinity for an NRTI, preventing its addition to the growing DNA chain (157). Another mechanism is mediated by primer-unblocking mutations that increase the hydrolytic removal of the chain-terminating NRTIs (5, 15, 123).

M184V, a discriminatory mutation that causes high-level in vitro resistance to lamivudine and emtricitabine, is the most commonly occurring NRTI resistance mutation. M184V also causes low-level in vitro resistance to didanosine and abacavir and increased susceptibility to zidovudine, stavudine, and tenofovir (193). However, viruses with M184V replicate less well than wild-type viruses, possibly explaining why abacavir and particularly didanosine appear to be fully active against viruses with this mutation (128).

The primer-unblocking mutations M41L, D67N, K70R, L210W, T215Y/F, and K219Q/E are selected by zidovudine and stavudine and are also called thymidine analog mutations. A subset of these mutations (M41L, L210W, and T215Y) are particularly important for causing cross-resistance to didanosine, abacavir, and tenofovir (97, 124, 128). An uncommon mutation, a double-amino-acid insertion at position 69, is responsible for high-level resistance to all NRTIs when it occurs in combination with multiple thymidine analog mutations (195).

The remaining clinically significant NRTI resistance mutations include mutations at positions 65, 74, 75, 115, and 151. K65R causes resistance to all NRTIs except zidovudine and possibly stavudine (140). L74V causes resistance to didanosine and abacavir. V75T/M/A cause resistance to stavudine and possibly other NRTIs; Y115F causes resistance to abacavir; Q151M, which is usually accompanied by accessory mutations at positions 62, 75, 77, and 116, causes resistance to each of the NRTIs (161, 165).

Nonnucleoside RT Inhibitor Resistance

The NNRTIs delavirdine, efavirenz, and nevirapine inhibit HIV-1 RT allosterically by binding to a hydrophobic pocket close to the active site. This hydrophobic NNRTI binding pocket is less well conserved than the deoxynucleoside triphosphate binding site. Indeed, HIV-1 group O and HIV-2 are intrinsically resistant to most NNRTIs (197). Moreover, wild-type HIV-1 group M isolates tend to have greater interisolate variability in susceptibilities to NNRTIs than in susceptibilities to NRTIs and PIs (141).

A single mutation in the NNRTI binding pocket may result in high-level resistance to one or more NNRTIs. Resistance emerges rapidly when NNRTIs are administered as monotherapy or in the presence of incomplete virus suppression, suggesting that NNRTI resistance can be caused by the selection of rare, preexisting populations of mutant viruses within an individual. A single dose of nevirapine used to prevent mother-to-child HIV transmission can select for NNRTI-resistant mutants that are detectable for two or more months and may interfere with the subsequent response to an NNRTI-containing treatment regimen (87, 88).

The NNRTI resistance mutations are clustered between residues 98 and 108, 179 and 190, and 225 and 238. K103N, which is the most common, confers a >20-fold increase in resistance to each of the NNRTIs and appears to be sufficient to cause virological treatment failure with each of the NNRTIs. High levels of clinical cross-resistance among the NNRTIs occur because most NNRTI resistance mutations confer resistance to multiple NNRTIs (7, 167) and because

a single NNRTI often selects for multiple different NNRTI-resistant populations within a person even if only one drug-resistant population is detected during direct PCR sequencing.

There is no cross-resistance between NRTIs and NNRTIs. Indeed, several NNRTI resistance mutations increase susceptibility to certain NRTIs (98) and combinations of certain NRTI resistance mutations increase NNRTI susceptibility (166, 192).

HIV-1 Protease

The HIV-1 protease enzyme is responsible for the posttranslational processing of the viral *gag* and *gag-pol* polyproteins to yield the structural proteins and enzymes of the virus. The enzyme is an aspartic protease composed of two noncovalently associated, structurally identical monomers 99 amino acids in length. The hydrophobic substrate cleft recognizes and cleaves nine different peptide sequences to produce the matrix, capsid, nucleocapsid, and p6 proteins from the *gag* polyprotein and the protease, RT, and integrase proteins from the *gag-pol* polyprotein. In addition to the substrate cleft, a mobile flap which sits above the substrate cleft plays a major role in substrate recognition and drug resistance. The three-dimensional structures of many wild-type and mutant proteases have been cocrystallized with either inhibitors or peptide substrates.

Protease Inhibitor Resistance

There are eight Food and Drug Administration (FDA)-approved PIs: amprenavir, atazanavir, indinavir, lopinavir, nelfinavir, ritonavir, saquinavir, and tipranavir. Fosamprenavir is a prodrug of amprenavir with improved bioavailability that has also recently been approved by the FDA. With the exception of nelfinavir, each of these PIs is usually administered with subtherapeutic doses of ritonavir because ritonavir is a potent cytochrome P450 enzyme inhibitor that increases the levels of all PIs except nelfinavir in serum by 10-fold or more. Lopinavir is the only PI that is coformulated with ritonavir. Ritonavir is rarely administered by itself because of its poor tolerability when administered at therapeutic dosages.

Mutations at more than 20 protease positions are associated with drug resistance (Table 1). Most of these mutations contribute resistance to more than one PI (154, 155). Mutations in the substrate cleft cause resistance by reducing the binding affinity between the inhibitor and the mutant protease enzyme. Mutations elsewhere in the enzyme either compensate for the decreased kinetics of enzymes with active-site mutations or cause resistance by altering enzyme catalysis, dimer stability, or inhibitor binding kinetics or by reshaping the active site through long-range structural perturbations (49). Mutations at several of the protease cleavage sites are also selected during PI treatment (35, 115). Protease cleavage site mutations improve the kinetics of protease enzymes with PI resistance mutations. By themselves, they have not been shown to reduce PI susceptibility.

The requirement for multiple mutations to overcome the activity of certain PIs has been referred to as a "genetic barrier" to drug resistance (34, 90). This genetic barrier is highest in persons receiving their first PI, particularly if it is coadministered with ritonavir. Ritonavir-boosted regimens are so potent that when administered to PI-naïve individuals, failure is almost universally caused by nonadherence rather than by drug resistance (91).

Cell Entry Inhibitor Resistance

The HIV-1 envelope consists of surface (gp120) and transmembrane (gp41) glycoproteins. gp120 binds to the CD4 receptor and to one of the chemokine coreceptors (CCR5 or CXCR4) on target cells. After gp120-CD4-coreceptor binding, gp41 undergoes a conformational change that promotes fusion of viral and cellular membranes. Two heptad repeat regions (HR1 and HR2) of gp41 form a helical bundle that contains trimers belonging to HR1 and HR2. Enfuvirtide is a highly active synthetic peptide that inhibits fusion by binding to HR1 and preventing its bundling with HR2 (92). Enfuvirtide-resistant isolates contain either single or double mutations between positions 36 and 45 of gp41 HR1 (125, 168, 188). Single mutants typically have on the order of 10-fold-decreased enfuvirtide susceptibility, whereas double mutants typically have on the order of 100-fold-decreased susceptibility.

CCR5 inhibitors bind to one of the transmembrane loops of CCR5, thereby distorting the receptor and restricting HIV-1 gp120 binding. Several CCR5 inhibitors have shown potent activity in phase I and II clinical trials in treating persons that do not harbor CXCR4-tropic HIV-1 variants (44). In vitro resistance has developed slowly and incrementally through the development of multiple gp120 mutations (119). In vivo, rapid failures have occurred in persons with preexisting minor populations of CXCR4 variants that were undetected during eligibility screening (44).

Intersubtype Variation

Naturally occurring protease and RT polymorphisms differ among subtypes, but for the most part these differences are not responsible for mutations with major effects on drug susceptibility. Indeed, nearly all published in vitro and in vivo studies suggest that all subtypes are equally susceptible to current antiretroviral drugs (142). Preliminary data suggest that the drug resistance mutations observed in subtype B isolates are the most common ones observed in non-B subtypes (89). However, subtle differences in the specific mutations observed among subtypes have been reported and will continue to be identified (16, 19, 20, 67, 68, 133, 172).

INFLUENZA VIRUS

Influenza A, B, and C viruses have segmented, encapsidated, minus-strand RNA genomes, each associated with a polymerase complex. The RNA particles are located inside a shell of Matrix 1(M1) protein that lines the viral lipid membrane. Embedded in the membranes of influenza A and B viruses are three proteins: two spiked glycoproteins (hemagglutinin [HA] and neuraminidase [NA]) and the membrane channel protein M2. Influenza C virus does not have an NA but instead has a single surface glycoprotein with both HA- and NA-like activities. There are four approved anti-influenza virus drugs belonging to two mechanistic classes: M2 channel blockers (the adamantine derivatives amantadine and rimantadine) and NA inhibitors (zanamivir and oseltamivir). Peramivir is a third NA inhibitor in clinical development. M2 channel blockers are active only against influenza A virus. NA inhibitors are active against influenza A and B viruses but not influenza C virus.

M2 Channel Blockers

M2 forms an ion channel that plays a role in virus uncoating and possibly in regulating the HA activity of newly formed viruses (64). Passage of hydrogen ions through the M2 channel into the virion following endocytosis promotes the dissociation of the M1 protein from the ribonucleoprotein complexes so that the ribonucleoprotein can enter the cell nucleus and initiate replication. By controlling the pH within trans-Golgi vesicles during virus exit, the M2 protein also prevents the exposure of the acid-sensitive HA to an adversely low pH (64, 83). Amantadine and rimantadine interfere with the penetration of hydrogen ions through the M2 channel and prevent

TABLE 1 Mechanisms and mutations associated with antiviral drug resistance

Virus	Antiviral agent(s)	Mechanism(s) of resistance	Mutations
HSV	Acyclovir, valcyclovir, penciclovir, famciclovir	TK and/or DNA polymerase mutations	TK frameshift or substitution mutations causing a TK-deficient phenotype and cross-resistance among the four drugs. Less commonly, TK-altered or DNA polymerase mutations
	Foscarnet	DNA polymerase mutations	Mutations clustering in regions II, VI, and III, (probable pyrophosphate binding regions)
	Cidofovir	DNA polymerase mutations	Mutations in regions δC and II (based on limited data from laboratory strains)
VZV	Acyclovir, valcyclovir, penciclovir, famciclovir	TK and/or DNA polymerase mutations	TK frameshift or substitution mutations causing a TK-deficient phenotype and cross-resistance among the four drugs. Less commonly, TK-altered or DNA polymerase mutations
	Foscarnet	DNA polymerase mutations	Mutations clustering in regions II, VI, and III (probable pyrophosphate binding regions)
	Cidofovir	DNA polymerase mutations	No VZV-specific data are available.
CMV	Ganciclovir	UL97 phosphotransferase mutations and/or DNA polymerase mutations	Usually UL97 mutations at residues 460, 520, 592, 594, 595, and 603 as well as mutations at codons 590–607. DNA polymerase mutations (exo, IV, δC, and V regions) sometimes confer resistance, usually with UL97 mutations.
	Foscarnet	DNA polymerase mutations	Mutations in regions II, III, and VI. Some may also decrease ganciclovir susceptibility.
	Cidofovir	DNA polymerase mutations	Mutations in regions exo, IV, δC, and V, resulting in cross-resistance to ganciclovir
HIV-1	PIs[a]: amprenavir, atazanavir, indinavir, lopinavir, nelfinavir, ritonavir, saquinavir, tipranavir	Protease mutations interfere with inhibitor binding or compensate for the decreased replication associated with other mutations.	Substrate cleft mutations: D30N, V32I, G48VM, I50VL, V82AFSTLM, I84V. Flap mutations: M46IL, I47VA, F53L, I54VMTLAS. Mutations at other conserved sites: L23I, L24I, L33F, G73SCT, L76V, N88DTS, L90M. Mutations at polymorphic sites: L10IVFR, K20RMTI, M36I, L63P, A71VTI, V77I
	NRTIs: abacavir, didanosine, emtricitabine, lamivudine, stavudine, zidovudine. Nucleotide RT inhibitor: tenofovir	RT mutations that allow the enzyme to discriminate between NRTIs and naturally occurring nucleosides or that promote ATP-dependent hydrolytic removal of chain-terminating nucleotide monophosphates	M184V confers high-level resistance to lamivudine and emtricitabine. K65R confers intermediate resistance to didanosine, abacavir, tenofovir, lamivudine, and emtricitabine. L74V confers resistance to didanosine and abacavir. Q151M occurs in association with A62V, V75I, F77L, and F116Y and confers high-level resistance to all NRTIs except possibly lamivudine and emtricitabine. M41L, D67N, K70R, L210W, T215FY, and K219QE develop in isolates from persons receiving zidovudine and stavudine but confer clinically significant cross-resistance to each of the other NRTIs except possibly lamivudine and emtricitabine.
	NNRTIs: delavirdine, nevirapine, efavirenz	Mutations in the NNRTI binding pocket of RT that interfere with inhibitor binding	A98G, L100I, K101EP, K103NS, V106AM, V108I, V179DE, Y181CIV, Y188CLH, G190ASEQ, P225H, F227L, M230L, P236L, K238T. Most mutations cause resistance to each of the NNRTIs.
	Fusion inhibitor: enfuvirtide	Mutations in the first heptad repeat region (HR1) of the gp41 transmembrane protein	Enfuvirtide is a peptide mimetic of the HR2 region of gp41. Drug resistance mutations in the HR1 region interfere with the association of the HR1 and HR2 domains necessary for virus-cell fusion. Mutations occur in a highly conserved region between gp41 residues 36 and 45.
Influenza virus	M2 channel blockers: amantadine, rimantadine	M2 transmembrane domain substitutions that interfere with hydrogen ion transfer	Mutations at residues 27, 30, 31, and 34, particularly A30V and S31N
	NA inhibitors: zanamivir, oseltamivir, peramivir[b]	NA receptor binding site mutations that decrease inhibitor affinity	R152K and R292K confer high-level resistance to each of the NA inhibitors. E119GAD confers high-level zanamivir and low-level oseltamivir resistance. E119V confers high-level oseltamivir resistance but does not affect resistance to zanamivir. H274Y confers high-level oseltamivir and low-level zanamivir resistance. D198N causes intermediate resistance to oseltamivir and zanamavir but not peramivir.

(Continued on next page)

TABLE 1 Mechanisms and mutations associated with antiviral drug resistance (*Continued*)

Virus	Antiviral agent(s)	Mechanism(s) of resistance	Mutations
		HA mutations that reduce the affinity of HA for its receptor	Mutations in many different residues in or close to the HA receptor binding site cause decreased dependence on NA activity in vitro. The clinical significance of these mutations is uncertain.
HBV	Interferon	Unknown	Unknown
	Nucleoside analogs: lamivudine, adefovir, entecavir, emtricitabine,[b] tenofovir,[b] telbivudine[b]	Polymerase substitutions that interfere with nucleotide triphosphate binding. Several may also promote primer unblocking.	M204VI, L180M, V173L: lamivudine, emtricitabine, telbivudine; variable partial cross-resistance to tenofovir, entecavir. N236T, A181V: adefovir, tenofovir. A194T: tenofovir. I169T, T184G, S202I, M250V: entecavir
HCV	Interferon	Uncertain	Genotype 1 isolates respond less well to interferon than genotype 2 or 3 isolates, but the molecular basis for this phenomenon is not known. Within genotype 1 isolates, certain sequence patterns within NS5A may be associated with interferon resistance.
	Ribavirin	Uncertain	Uncertain
	Investigational drugs: PIs, RNA-dependent RNA polymerase nucleoside and nonnucleoside RNA polymerase inhibitors	PI and nonnucleoside inhibitor resistance mutations appear to interfere with binding. Nucleoside inhibitors appear to enhance polymerase discrimination.	It is too premature to list specific mutations, as clinical experience is limited.

[a]Extensive additional information on each of the PI, NRTI, and NNRTI resistance mutations can be found at http://hivdb.stanford.edu. Several additional NRTI resistance mutations appear to act through a combination of the two mechanisms described above.
[b]Investigational drugs. Emtricitabine and tenofovir, although investigational for treating HBV infection, are approved for treating HIV-1 infection.

transport of the ribonucleoprotein complex to the nucleus (64, 77).

Cross-resistance to both drugs results from single-amino-acid substitutions at position 26, 27, 30, 31, or 34 within the M2 transmembrane domain (9, 10). Some of the mutations, such as those at positions 30 and 31, inhibit M2 channel binding, whereas others, such as those at position 27, may allow binding to occur without affecting channel function (6). M2 inhibitor resistance develops rapidly during in vitro passage experiments and during treatment in about 30% of persons receiving amantadine or rimantadine (10, 78, 79, 164). Amantadine- and rimantadine-resistant variants maintain normal infectivity and virulence in animal models and cause typical disease in humans (2, 9, 78). Patients infected with drug-resistant virus have slower responses to treatment than do patients infected with wild-type virus (79).

Large surveillance studies have shown that M2 inhibitor resistance, usually caused by the S31N mutation, increased among H3N2 isolates from 1% in the 1990s to more than 50% in China and more than 90% in the United States by 2003 and 2005 to 2006, respectively (17, 18, 205). H5N1 viruses isolated from human and avian sources since 2003 also appear to usually have the S31N mutation, making them intrinsically resistant to M2 inhibitors (21, 153). M2 inhibitor resistance among H1N1 and H1N2 viruses has not increased to the same extent as that observed in H3N2 viruses.

Neuraminidase Inhibitors

HA binds to sialic (*N*-acetylneuraminic) acid-containing cellular receptors, thus initiating viral infection. NA cleaves the alpha-ketosidic bonds linking terminal sialic acid residues to adjacent oligosaccharide moieties, preventing the formation of aggregates between HA and cell surfaces sialic acids and allowing newly formed viruses to be released from the surfaces of infected cells (33, 182). NA may also promote viral spread within mucosal secretions in the respiratory tract (33, 182). Because HA binds to cellular receptors to initiate infection, whereas NA destroys cellular receptors to allow virus release from cells, a balance between these two activities is required for efficient infection (186).

NA is a homotetramer containing 470 amino acids (33, 181, 182). A hydrophobic stalk peptide at its N terminus is responsible for membrane anchoring. A globular head contains the enzyme active site, a pocket lined by a central shell of 17 conserved, predominantly charged active-site residues and 16 neighboring conserved residues (181, 182). Zanamivir and oseltamivir are sialic acid analogs that competitively inhibit NA (185).

Zanamivir and oseltamivir are active against a wide range of strains including each of the nine identified avian NA subtypes (70) as well as avian H5N1 variants isolated in 1997 and 2004 (73, 102, 103, 134). Although these drugs appear to be less active against influenza B virus in vitro, the clinical significance of this finding is uncertain (70).

NA inhibitor activity can be measured by using enzymatic assays, cell culture, and animal models (mice and ferrets). However, NA inhibitor activity in cell culture is variable because different clinical isolates have different degrees of dependence on HA activity and because the cells used for in vitro assays have different concentrations of glycoconjugate receptors (202). Indeed, there are currently no suitable cell lines that bear receptors comparable to those in the human respiratory tract (120).

The extent to which the mechanisms of resistance that emerge in vitro are clinically relevant is not always certain. For example, during in vitro passage experiments, NA inhibitor

resistance can be conferred by mutations in HA and/or NA. Drug resistance HA mutations that decrease HA affinity to its cellular receptor allow the virus to exit infected cells without the need for significant NA activity (69, 121). However, in animal models as well as in humans, viruses with HA mutations alone remain susceptible to NA inhibitors (1, 14, 71, 81).

In contrast to HA resistance mutations, NA resistance mutations emerge both in vitro and in vivo and are clinically significant (71). NA resistance mutations emerge at residues within the catalytic site (residues 152 and 292) or the surrounding structural framework (residues 119, 198, and 274). These residues are highly conserved in untreated influenza virus strains (120), and NA inhibitor-resistant variants with mutations at these residues have reduced enzymatic activity and virulence in animal models (32, 74, 80, 81, 85, 200).

Mutations in the catalytic site usually confer resistance to each of the available NA inhibitors. For example, the uncommon mutation R152K and the common mutation R292K confer resistance to zanamivir, oseltamivir, and peramivir in both influenza A and B viruses (69, 86). The framework mutations E119G/A/D cause high-level zanamivir resistance and low-level oseltamivir resistance, whereas E119V causes high-level oseltamivir resistance but does not affect zanamavir or peramivir susceptibility. H274Y reduces oseltamivir but not zanamivir susceptibility (69, 120, 121). The effect of H274Y may also be subtype dependent, as the mutation has been reported to cause markedly reduced susceptibility in several H1N1 isolates but not in one H3N2 isolate (126, 187).

Resistance to NA inhibitors develops at a much lower rate in vitro and in vivo than does resistance to M2 blockers, presumably as a consequence of the negative impact NA inhibitor resistance mutations have on NA function and virus replication. In addition, the spread of NA inhibitor-resistant virus between individuals appears so far to be rare. Oseltamivir resistance in viruses from 10 of 182 and 9 of 50 children treated with oseltamivir has been reported previously (93, 194). Oseltamivir resistance caused by the H274Y mutation in viruses from three Vietnamese children with fatal H5N1 infections has also been reported (40, 100). Oseltamivir resistance in viruses from adults is uncommon, being reported in 1 to 4% of those from treated adults (71). There has been only one case report of zanamivir resistance (72). The higher rate of oseltamivir resistance may be due to greater selective pressure resulting from oral administration or may be a function of the structure of oseltamivir, which is more dissimilar to the natural sialic acid substrate than zanamivir (70).

HEPATITIS B VIRUS

HBV is a partially double-stranded DNA virus of about 3.2 kb. Following infection, its genome localizes to the nucleus, where it is converted into a covalently closed circular DNA (cccDNA) form that serves as the template for transcription of mRNA and genomic RNA. Genomic RNA is reverse transcribed into viral DNA, and the resulting viral cores either bud into the endoplasmic reticulum and are exported from the cell or return to the nucleus for conversion back into cccDNA (58). HBV cccDNA is highly stable and can be eliminated by cell turnover but not by drug therapy (52, 189, 206). Therefore, complete cure or eradication of infection is highly unusual with current antivirals (58).

HBV replicates at a high rate, producing an estimated 10^{11} virions per day (137). In the absence of therapy, HBV DNA levels are often as high as 10^8 to 10^{10} copies/ml (8 to 10 log-copies/ml). HBV polymerase, because it has RT activity, is functionally and structurally similar to HIV-1 RT and has an error rate higher than that of most DNA virus polymerases. However, the overlapping arrangement of open reading frames in the HBV genome limits the viability of many spontaneous mutants (56). For example, the nucleotides encoding the HBV polymerase also encode the HBV envelope in a different reading frame. Therefore, the rate at which mutations become fixed is estimated to be considerably lower than that for HIV-1 (63).

Anti-HBV Drug Therapy

There are four FDA-approved drugs for the treatment of chronic HBV infection. Alpha interferon was approved in 1992, followed by three nucleoside analogs: lamivudine (1998), adefovir (2002), and entecavir (2005). Emtricitabine and tenofovir, antiretroviral drugs that are similar to lamivudine and adefovir, respectively, are also active against HBV in vitro and in vivo. Telbivudine is a drug in advanced clinical development (96).

Alpha interferon, particularly pegylated interferon, is an important treatment option, particularly in patients with HBV e antigen-negative infections in whom it is more effective than lamivudine at causing sustained decreases in virus and hepatic enzyme levels and occasional loss of HBsAg following treatment discontinuation (118).

Because of the difficulty in eradicating HBV, the goals of therapy are to substantially reduce HBV DNA levels, clear HB e antigen when it is present, and decrease hepatic inflammation. Serum HBV DNA levels are the best marker of HBV activity and the risk of clinical progression (111, 129). Much of the literature is based on assays with various sensitivities and dynamic ranges, but there has been a trend towards the use of assays with greater sensitivities. Whereas the initial assays had sensitivities of about 10^4 to 10^5 copies/ml, more recent assays have sensitivities of about 10^2 copies/ml (111).

Clinical HBV resistance is usually defined as the failure to achieve a 1.0 $\log_{10}$ decrease in the level of HBV DNA in serum by 3 months (primary failure) or as an increase in HBV DNA levels of 1.0 $\log_{10}$ compared with the viral load nadir (secondary failure). HBV susceptibility testing is difficult because of the absence of cell culture systems that allow efficient virus infection in vitro. In research settings, assessment of the phenotypic impact of genotypic changes has relied on the PCR-mediated transfer of HBV genome cassettes and on site-directed mutagenesis within replication-competent laboratory strains (45, 199). In clinical settings, the cause of resistance is assessed genotypically by directly sequencing the HBV polymerase gene or by using a point mutation assay for known drug resistance mutations (111, 112).

HBV Polymerase and Nucleoside Analog Resistance

The three-dimensional structure of HBV polymerase has not been solved because of difficulty in obtaining sufficient amounts of highly purified active protein. However, homology modeling using other polymerases, including HIV-1 RT, has provided insight into the roles of many of its residues (8, 38). These models have shown that HBV polymerase shares regions similar to the finger, palm, and thumb configuration in HIV-1, as well as seven domains that are conserved among many published polymerase enzyme sequences. In 2001, a standardized numbering system for polymerase mutations was established for the RT part of the HBV *pol* gene (171).

High-level (increased by >1,000 fold) lamivudine resistance is caused by mutations M204V/I, which are in the YMDD motif, characteristic of all RTs and analogous to the site of HIV-1 lamivudine resistance (3, 60, 110, 171, 177).

Although M204I can be detected in isolation, M204V is always accompanied by the compensatory mutations L180M and/or V173L (3, 41, 110, 138). M204V/I and V173L, but not L180M, result in HBsAg coding changes of unknown significance.

M204V/I and their accompanying mutations cause high-level cross-resistance to emtricitabine and the investigational inhibitor telbivudine but do not appear to reduce susceptibility to the acyclic nucleotides, adefovir and tenofovir (198), and both drugs retain activity in treating persons with lamivudine-resistant viruses (11, 43, 144, 146, 148, 180, 191). M204V and L180V together cause partially decreased (≥30 fold) susceptibility to entecavir (198). Nonetheless, entecavir retains significant in vitro and clinical activity against lamivudine-resistant viruses (22, 105, 163, 176). In treating lamivudine-resistant virus infections, entecavir reduces HBV levels by about 4 to 5 logs, often enough to completely suppress virus replication but less than the usual 6- to 7-log reduction observed in previously untreated persons receiving entecavir.

M204V/I occur in viruses in 15 to 30% of patients treated with lamivudine for 1 year, 40 to 50% of those treated for 3 years, and 70% of those treated for 5 years (56, 95, 96, 118). The risk of drug resistance and subsequent virological breakthrough is associated with baseline HBV DNA levels, baseline hepatic enzyme elevations, high body mass index, and duration of detectable viremia (95, 201). The risk of resistance may also depend on the number of available uninfected hepatocytes and may therefore increase post-liver transplantation (111, 145, 147). Although viruses with M204V/I replicate less well than wild-type viruses, nearly all studies suggest that this mutation is clinically significant and that a new anti-HBV drug should be substituted for lamivudine in persons with elevated hepatic enzymes or in persons who are immunocompromised (23, 42, 56, 106, 111).

Adefovir resistance emerges more slowly than lamivudine resistance, occurring in viruses in 5 to 20% of persons after 2 years of therapy (55, 75, 76, 117, 190). Two mutations, N236T (4, 75, 183) and A181V (75), both near the polymerase active site, reduce adefovir susceptibility. Although the levels of susceptibility reduction associated with these mutations, 3- to 10-fold, are much lower than the levels associated with M204V/I and lamivudine resistance, these reductions are associated with virological breakthrough (4, 54, 183, 198). The N236T mutation confers cross-resistance to tenofovir but not to lamivudine, emtricitabine, entecavir, and telbivudine (199). N236T reduces viral replication levels in vitro (199). The effect of A181V on other drugs has not been described. N236T and A181V do not cause substitutions in the envelope protein. A194T has been reported in viruses from two patients developing virological rebound after receiving tenofovir for the treatment of lamivudine-resistant HBV infections, but the effect of this mutation on tenofovir susceptibility is not well defined (162).

Entecavir resistance has been reported in viruses from about 7% of persons receiving entecavir for 48 weeks for the treatment of infections with lamivudine-resistant isolates (163, 176); it has not yet been reported in viruses from persons receiving entecavir for initial therapy. In viruses from two well-described patients, clinical entecavir resistance was associated with the development of the mutations I169T-M250V and I169T-T184G-S202I in combination with the preexisting lamivudine-associated mutations (L180M-M204V/I) (176). Site-directed mutagenesis experiments confirmed the roles of these entecavir-selected mutations in causing high-level (>1,000-fold-reduced susceptibility) entecavir resistance. T184G and S202I (but not M250V) cause HBsAg coding changes of unknown significance.

Few studies of combination therapy have been published, but preliminary data suggest that combination therapy will prolong virological suppression and decrease the rate of drug resistance (174). Although no clinical benefit of combined therapy with interferon and lamivudine has been observed, the risk of lamivudine resistance has been shown to be markedly reduced in persons receiving combined therapy (118). In patients who are already infected with lamivudine-resistant isolates, it is not yet known with certainty whether it is more advantageous to substitute adefovir for lamivudine (148) or to add adefovir.

HEPATITIS C VIRUS

HCV is a positive-sense, single-stranded enveloped virus with a genome of about 9.5 kb. The six major HCV genotypes differ from one another by up to 35% of their sequences (203). Like HIV-1, HCV exists in vivo as a complex quasispecies consisting of an ensemble of viral genomes differing from one another by 1 to >5%. During the past 2 years, major advances have been made in developing robust methods for in vitro HCV replication (109, 204) and small-molecule HCV inhibitors (39).

Interferon and Ribavirin

Pegylated alpha interferon plus ribavirin for 6 to 12 months, the standard treatment for HCV, leads to sustained virological responses in about one-half of treated persons. Alpha interferon does not directly inhibit HCV; rather, it acts by inducing interferon-stimulated genes and by upregulating antiviral T-cell responses, which create an intracellular environment that is hostile to the replication of many viruses including HCV (51). A dose-response relationship exists between ribavirin and the likelihood of virus suppression (108), but the mechanism by which ribavirin acts is not known. The leading hypotheses are that ribavirin, a nucleoside analog, either interferes with deoxynucleoside triphosphate metabolism (99), directly inhibits HCV RNA polymerase (114), or increases HCV mutagenesis (36, 37), making the HCV quasispecies within an individual less able to respond to the alpha interferon challenge.

Two findings suggest that viral factors influence the response to therapy. First, HCV genotypes 2 and 3 are more likely to respond to therapy than genotype 1. Second, therapy is much more successful in persons with acute (and probably less complex quasispecies) rather than chronic infection. Several authors have proposed that certain changes in a 40-amino-acid region of HCV NS5A (sometimes referred to as an interferon sensitivity-determining region) or in other HCV proteins (e.g., the envelope 2 protein) interfere with interferon responsiveness (47, 50, 57, 65, 143, 151, 175, 196). However, no mechanism of antiviral resistance to either interferon or ribavirin has been identified with certainty.

Investigational Targeted Inhibitors

Small-molecule inhibitors of the NS3 serine protease and the NS5B RNA-dependent RNA polymerase with both in vitro and in vivo activities have been developed and are in early clinical trials (39). These inhibitors belong to three mechanistic classes: PIs, nucleoside polymerase inhibitors, and allosteric nonnucleoside polymerase inhibitors. In vitro experiments have already shown that resistance to first-generation HCV PIs emerges rapidly (107, 113, 178, 179).

Multiple nucleoside and nonnucleoside analogs have been shown to inhibit HCV polymerase in vitro (39). Nonnucleoside inhibitors that bind to three different polymerase locations have been identified (39, 127). Mutations selected for by both types of polymerase inhibitors have already been selected in vitro (127). The number of anti-HCV small-molecule inhibitors entering clinical trials as well as the number of drug resistance mutations associated with these inhibitors is expected to increase during the next several years.

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Susceptibility Test Methods: Viruses

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During the past decade and a half, safe and effective antiviral therapy has been developed for treatment of a number of viral infections (7, 13, 28, 31, 35, 36, 37, 50, 61). Great strides have been made, most notably in the development of antiviral agents for treatment of human immunodeficiency virus (HIV). While the overwhelming majority of clinical virus isolates from drug-naive patients are susceptible to antiviral agents, widespread use of some antiviral agents has led to the emergence of drug-resistant strains, particularly in immunocompromised hosts (7, 8, 10, 11, 12, 14, 44). Diagnostic virology laboratories are increasingly asked to perform in vitro testing of antiviral agents when patients fail to clinically respond to antiviral therapy. This chapter discusses the clinical situations where antiviral resistance has emerged, thus necessitating in vitro susceptibility testing, and provides an overview of the phenotypic and genotypic susceptibility testing methods that have been employed to detect resistance. The major antivirals in use are discussed in chapter 112. Chapter 113 details the mechanisms of resistance of antiviral agents.

CLINICAL INDICATIONS FOR ANTIVIRAL SUSCEPTIBILITY TESTING

Antiviral susceptibility testing is essential for defining mechanisms of antiviral resistance, for determining the frequency with which drug-resistant viral mutants emerge in clinical practice, to test for cross-resistance to alternative agents, and when evaluating new antiviral agents. With the exception of antiviral susceptibility testing for herpes simplex virus (HSV), results of in vitro phenotypic assays are usually not available in a time frame relevant for patient management. Nevertheless, clinical deterioration of patients undergoing antiviral therapy can be associated with resistant virus. Results of antiviral susceptibility testing may be helpful in certain clinical situations. Persistent or worsening HSV or varicella zoster virus (VZV) infection while on acyclovir may indicate drug resistance. Alternative therapy agents such as foscarnet and cidofovir are available. Furthermore, HSV or VZV causing recurrent infection is often thymidine kinase (TK) competent and therefore susceptible to acyclovir, which has minimal toxicity compared to foscarnet (7). Persistent or worsening human cytomegalovirus (HCMV) retinitis, pneumonitis, or colitis unresponsive to ganciclovir may also indicate drug-resistant virus. Cidofovir and foscarnet can be used as alternative agents. Influenza A isolates resistant to amantadine and rimantadine readily emerged during clinical trials of these drugs; therefore, continuous shedding or transmission of influenza A virus in a population undergoing prophylaxis or treatment with these agents may be due to drug resistance. Prolonged influenza virus shedding during therapy with a neuraminidase inhibitor (NI) may be due to the emergence of NI-resistant virus. Monitoring for HIV type 1 (HIV-1) antiretroviral resistance is essential for assessing the failure of a particular regimen to suppress HIV replication and to test for cross-resistance to alternative antiretroviral drugs to aid in the selection of appropriate salvage therapy. Guidelines developed by the U.S. Department of Health and Human Services Panel on Clinical Practices for Treatment of HIV Infection (http://aidsinfo.nih.gov/guidelines/) and by a European panel (130) have recommended that resistance testing be performed in cases of suboptimal viral load reduction after institution or modification of therapy and virologic breakthrough in ongoing therapy and also suggest that resistance testing of acute-phase or recently infected individuals at baseline prior to initiation of therapy will likely optimize the virologic response. Resistance testing at baseline in chronically infected patients may be reasonable if infection with a drug-resistant virus is suspected (http://aidsinfo.nih.gov/guidelines/) or if the prevalence in the population exceeds 10% (130) as it does in some areas of the United States (83).

DEFINITION OF ANTIVIRAL RESISTANCE

Antiviral resistance is a decrease in susceptibility to an antiviral drug that can be clearly established by in vitro testing and can be confirmed by genetic analysis of the virus and biochemical study of the altered enzymes. In vitro drug resistance must be distinguished from clinical resistance, in which the viral infection fails to respond to therapy. Clinical failures may or may not be due to the presence of a drug-resistant virus. Failure to achieve clinical response also hinges on other factors such as the patient's immunologic status and the pharmacokinetics of the drug for that individual patient. For example, limited penetration of the drug into the central nervous system may allow escape of HIV-1 despite suppression of the virus at other sites. Poor oral absorption and binding to plasma proteins may limit the bioavailability of certain drugs. Furthermore, administration of certain antiretroviral drugs in combination may interfere

with absorption or stimulate elimination of one or more of the coadministered drugs. Patient-specific factors such as nonadherence, intolerance to an antiretroviral drug, or an intercurrent infection can also lead to increases in HIV-1 plasma viremia despite in vitro susceptibility (21).

VARIABLES OF ANTIVIRAL SUSCEPTIBILITY TESTING

To date, few standards for antiviral susceptibility testing exist. The Clinical and Laboratory Standards Institute (CLSI; formerly National Committee for Clinical Laboratory Standards or NCCLS) recently published an approved standard for susceptibility testing of HSV as a first step in developing consensus protocols for antiviral susceptibility testing (29). The major obstacle to standardization of antiviral susceptibility testing is that many variables influence the final result. These include (i) cell line, (ii) viral inoculum titer, (iii) incubation time, (iv) concentration range of the antiviral agent tested, (v) reference strains, (vi) assay method, (vii) endpoint criteria, (viii) calculation of the endpoint, and (ix) interpretation of the endpoint. Testing of a single virus isolate may lead to greatly different endpoints, depending on the type of cell culture used (34, 60, 79). For example, the activity of acyclovir appeared greater than that of penciclovir when a plaque reduction assay (PRA) was performed with Vero cells; the converse was true when the assay was performed with WI-38 VA-13 and WISH cell lines. The two drugs had comparable activity when tested in A549 cells (79). The titer of the virus inoculum is also critical; too large an inoculum can make a susceptible isolate appear resistant; too small an inoculum can make all isolates appear susceptible (60). The length of time that the virus incubates in the presence of the drug must be sufficient to allow detection of small plaques, in the case of a PRA, or to allow growth of a subpopulation of resistant virus which may replicate at a slower rate than that of wild-type virus (5). The prolonged incubation times of peripheral blood mononuclear cell (PBMC)-based assays for HIV-1 susceptibility testing have been shown to select for subpopulations of HIV-1 variants not present in the starting inoculum (76). Moreover, different assay methods can produce different results. For example, the dye uptake (DU) assay for HSV susceptibility testing produces 50% inhibitory concentrations (IC_{50}s) higher than those produced by PRA (64). The concentration range of the drug tested affects the quality of the dose-response curve and therefore the validity of endpoint calculations. Susceptibility results are usually expressed as IC_{50}s because of the greater mathematical precision of the 50% endpoint than of a 90 or 99% endpoint. (Synonyms for IC_{50} are 50% inhibitory concentration and 50% effective concentration.) However, debate continues concerning the appropriate endpoint, i.e., 50% versus 90 or 99% inhibition. IC_{50}s are more precise and reproducible, but IC_{90}s may correlate better with clinical response and are better at detecting subpopulations of drug-resistant strains among sensitive ones (7). Moreover, few studies have correlated in vitro results with clinical response (8, 48, 70, 80, 103, 119).

Another critical variable is the heterogeneity within the population of a virus "isolate." A single clinical isolate actually represents a mixture of drug-susceptible and drug-resistant phenotypes (6, 65, 104, 120). A virus population that has never encountered an antiviral agent is predominantly drug susceptible; resistant virus may be present at low levels. The presence of low levels of resistant virus in a population that is predominantly drug susceptible might not be reflected in the IC_{50} but would manifest its presence in higher IC_{90}s or IC_{99}s. At this time it is unknown whether a small fraction of drug-resistant virus is important to the behavior of the virus in vivo or how such a fraction might affect the response of the infection to therapy in an otherwise healthy host. However, in immunocompromised patients, under the continued selective pressure of antiviral therapy, resistant virus can emerge, and its presence can often be correlated with progressive viral disease (8, 23, 42, 44, 48, 70, 97, 105, 106, 107, 116).

The genetic locus at which a mutation occurs also affects susceptibility testing endpoints. For example, DNA polymerase mutations of HSV, VZV, and HCMV usually confer relatively smaller increases in in vitro resistance than do TK mutations, which could go undetected in a mixed population of wild-type and mutant virus. In HIV-1, the level of in vitro resistance to protease (PR) inhibitors increased as the number of PR gene mutations increased (63). A good definition of such mixtures can be obtained only by testing appropriate concentrations of drug and a sufficiently large fraction of the population to detect resistant strains.

TESTING METHODS

Phenotypic versus Genotypic Assays

Phenotypic assays are in vitro susceptibility assays that measure the inhibitory effect of antiviral agents on the entire virus population in a patient isolate. A variety of endpoint measurements have been utilized, including a reduction in the number of plaques; inhibition of viral DNA synthesis; reduction in the yield of a viral structural protein, e.g., hemagglutinin of influenza or p24 antigen of HIV; or reduction in the enzymatic activity of a functional protein, e.g., HIV-1 reverse transcriptase (RT) or influenza virus neuraminidase. Phenotypic assays in use include PRA, DU, DNA hybridization, enzyme immunoassay (EIA), neuraminidase inhibition, and yield reduction assays for herpesvirus group and influenza A viruses; and PBMC cocultivation and recombinant virus assays for HIV-1. Genotypic assays analyze viral nucleic acid to detect specific mutations that cause antiviral drug resistance. Genotyping has been applied primarily to HCMV and HIV-1. Genotypic assays include DNA sequencing by automated sequencers, PCR amplification and restriction enzyme digestion of the products, and hybridization to microarrays of oligonucleotide probes. Phenotypic and genotypic assays have unique features that complement each other. Phenotypic assays are better suited to assess the combined effect of multiple resistance mutations on drug susceptibility. This is especially important for viruses such as HCMV and HIV-1, which acquire resistance-associated mutations in multiple genes that may be manifested as new patterns of resistance, cross-resistance, multidrug resistance, or even reversal of resistance (63, 69, 117, 120). However, most phenotypic assays are labor-intensive and expensive and have a long turnaround time. Genotypic assays are relatively inexpensive and have shorter turnaround times but cannot detect mutations outside the selected target. Interpretation of genotypic assays is problematic due to the complex interactions of resistance mutations which result in a particular drug resistance phenotype. The major phenotypic and genotypic assays in use are discussed below.

Control Strains

Simultaneous testing of control strains is crucial when antiviral susceptibility testing is being done. Reference strains should include genetically and phenotypically well-characterized drug-susceptible and drug-resistant isolates.

Drug-resistant strains chosen for reference should include those with drug resistance phenotypes relevant to the mode of action of the drug to be tested. For example, for testing nucleoside analogs, which require phosphorylation by viral TK, e.g., acyclovir versus HSV and VZV, TK-negative or -deficient strains should be included. Susceptibility testing of HCMV should include both UL97 and UL 54 mutants. For HIV-1 testing, mutants resistant to both nucleoside-analog and non-nucleoside analog inhibitors of RT and mutants resistant to PR inhibitors should be tested. The National Institute of Allergy and Infectious Diseases (NIAID) AIDS Research and Reference Reagent Program (Bethesda, Md.) provides upon request a number of reference strains of HSV, VZV, and HCMV, including the drug-resistant strains mentioned above and laboratory control strains HCMV AD169 and VZV Oka. For susceptibility testing of HIV-1, the NIAID AIDS Research and Reference Reagent Program has a repository of strains with various resistance phenotypes. Pharmaceutical companies and the American Type Culture Collection are also sources of control strains.

PRA for CMV, HSV, and VZV

The PRA has classically been the "standard" method of antiviral susceptibility testing to which new methods are compared (15, 17, 62, 64, 92). Because many variations of the PRA have been reported, the National Committee for Clinical Laboratory Standards (whose name was recently changed to Clinical and Laboratory Standards Institute [CLSI]) developed a standard for PRA testing of HSV (29). Likewise, the HCMV Resistance Working Group of the AIDS Clinical Trials Groups has formulated a standardized PRA for HCMV susceptibility testing (77). The challenges associated with HCMV susceptibility testing by PRA became apparent when, despite adherence to a consensus PRA protocol, some collaborating laboratories had difficulty distinguishing drug-susceptible and drug-resistant HCMV isolates.

The principle of the PRA is the inhibition of viral plaque formation in the presence of antiviral agent. The concentration of antiviral agent inhibiting plaque formation by 50% is considered the IC_{50}. Although the PRA is tedious and consumes more reagents than other methods, it is appropriate for small-scale testing of isolates. Prior to performing the antiviral susceptibility assay per se, titers of HSV isolates must be determined to ensure an inoculum appropriate for the surface area of the assay wells or plates (i.e., approximately 100 PFU/60-mm-wide tissue culture plate). Because clinical strains of HCMV and VZV are cell associated and because low-titer cell-free stocks are less stable during storage, infected cell suspensions (obtained by trypsin treatment of the infected monolayer) can be conveniently used for these viruses. Two to three passages of clinical strains of HCMV and VZV in cell culture are usually necessary to obtain a sufficient titer of virus. Low-passage isolates should be used because they are more likely to be representative of the original mixed population of the clinical isolate than a higher-passage stock would be. Well-characterized drug-susceptible and drug-resistant strains of HSV, HCMV AD169, and VZV Oka or Ellen serve as reference strains. Stepwise instructions for performance of the PRA for HSV, VZV, and HCMV have been published elsewhere (29, 64, 123, 124).

A modified PRA has been described that utilizes Vero or CV-1 cells that have been stably transformed with the *Escherichia coli lacZ* gene under the control of an HSV-1 early promoter and which express beta-galactosidase only after infection with HSV. Plaques were visualized after histochemical staining for beta-galactosidase (126, 127). Proposed susceptibility breakpoints determined by the PRA are listed in Table 1.

DU Assay

The DU assay has been used for many years for susceptibility testing of HSV (64, 92, 127). This assay is based on the preferential uptake of a vital dye (neutral red) by viable cells but not by nonviable cells. The extent of viral lytic activity is determined by the relative amount of dye bound to viable cells after infection with HSV compared with the amount bound to uninfected cells. The dye bound by viable cells is eluted by ethanol and measured colorimetrically. The drug concentration inhibiting viral lytic activity by 50% is considered the IC_{50}.

The DU assay consistently gives IC_{50}s of acyclovir that are three to five times greater than those given by PRA. This difference is most likely due to the higher inoculum used in the DU assay (500 PFU/ml) and to the use of a liquid overlay which allows drug-resistant virus to "amplify," thus resulting in a more sensitive detection of small amounts of drug-resistant virus. Therefore, the DU assay uses a cutoff IC_{50} of >3 μg/ml to denote acyclovir resistance (Table 1).

Advantages of the DU method include its ability to be semiautomated, allowing for efficient testing of large numbers of isolates, and its ability to detect smaller amounts of resistant virus than the PRA can detect. Disadvantages are the relatively high cost of automated equipment and the technical problems caused by overseeding of cells into the culture wells and precipitation of neutral red onto the monolayer. The stepwise procedure for the DU assay has been previously published (64, 136).

DNA Hybridization

DNA hybridization assays have been used to measure the effects of different antiviral compounds on DNA synthesis. These methods semiquantitatively measure the amounts of viral DNA produced in the absence and presence of antiviral drug, and IC_{50}s are calculated from these data. Good correlation between the PRA and a dot blot hybridization assay has been demonstrated (16, 54). The stepwise procedure has been detailed previously (124). DNA-DNA hybridization test kits previously commercially available (Hybriwix Probe Systems, Athens, Ohio) have been used successfully for susceptibility testing of HSV, HCMV, and VZV (24, 25, 26, 33, 44, 70, 105, 106, 107, 117, 125). The Hybriwix assays are no longer commercially available.

EIAs

EIAs have been developed for susceptibility testing of HSV, VZV, and influenza A virus (10, 12, 102, 108). EIA permits quantitative measurement of viral activity by spectrophotometric analysis; IC_{50}s are calculated as the concentrations of antiviral agent that reduce the absorbance to 50% of that of the virus control. EIA is more suited than the PRA and DU assay to the routine diagnostic laboratory. Susceptibility results for HSV and VZV determined by this method have correlated well with those obtained by PRA.

Susceptibility testing of influenza A virus by PRA is tedious and labor-intensive. EIA is technically easier and is more suitable for the testing of multiple isolates (10). The EIA utilizes antibodies to influenza A virus hemagglutinins (H1 or H3); viral hemagglutinin expression correlates with viral growth. Amantadine and rimantadine activities are measured by inhibition of hemagglutinin expression. Amantadine- or rimantadine-susceptible and -resistant isolates, whose M2 gene sequences are known, serve as controls and must be tested in

TABLE 1 Proposed guidelines for antiviral susceptibility results of herpesvirus group and influenza A viruses

Virus	Antiviral agent	Method	IC_{50} denoting resistance	Reference(s)
HSV	Acyclovir	PRA	≥2 μg/ml	92
		DNA hybridization	≥2 μg/ml	44, 125
		DU	≥3 μg/ml	64, 92
	Famciclovir (active metabolite = penciclovir)	PRA and DNA hybridization	Definitive breakpoints cannot be established	79, 121
	Foscarnet	PRA	>100 μg/ml	105
	Vidarabine	PRA	≥2-fold increase of IC_{50} compared to control or pretherapy isolate	107
VZV	Acyclovir	PRA and DNA hybridization	≥3–4-fold increase of IC_{50} compared to pretherapy isolate or to control strain	15, 70, 106
	Famciclovir	PRA and DNA hybridization	Definitive breakpoints cannot be established	121
	Foscarnet	Late antigen reduction assay	300 μM	106
HCMV	Cidofovir	PRA and DNA hybridization	>2 μM	26, 49, 117
	Foscarnet	PRA and DNA hybridization	>400 μM	26, 49
			>324 μM	117
	Ganciclovir	PRA and DNA hybridization	≥3–4-fold increase of IC_{50} compared to pretherapy isolate or control strain (~3 μg/ml)	42, 47, 99
			>6 μM	26, 49
			>8 μM	117
Influenza A	Amantadine, rimantadine	EIA	>0.1 μg/ml	10, 11
Influenza A and B viruses	Oseltamivir and zanamivir	NI assay	>1,000-fold decrease in NA activity	56, 57

parallel with patient isolates. A protocol for the EIA for susceptibility testing of influenza A virus has been published (124). There are no commercial EIA assays available.

Flow Cytometry

Flow cytometry has been applied to susceptibility testing of HSV and HCMV (82, 93, 98). Whereas the IC_{50}s measured by flow cytometry were numerically different from those determined by plaque reduction, drug-susceptible isolates could be readily distinguished from drug-resistant isolates. Advantages of antiviral susceptibility testing by flow cytometry include the potential for automation, the objectivity of the assay, and a shorter turnaround time than that of the PRA.

NI Assay

Recently, two inhibitors of influenza virus neuraminidase (NA), oseltamivir and zanamivir, have been approved for treatment of influenza A and B virus infections (22). Because there are limited data concerning the emergence of resistant virus in vivo, surveillance is important to determine the frequency with which resistance to NIs emerges during active influenza virus infection. Susceptibility studies to date have indicated that assay of NA activity was the best predictor of in vivo response to NIs (56, 94). NA activity is assayed using solubilized supernatant from viral culture as the source of viral NA. After incubation of the viral NA with varying concentrations of NI, a fluorogenic substrate is added. Fluorescence is quantitated by a fluorimeter, and the IC_{50} is calculated relative to the activity of viral NA in the control reaction (no NI). A zanamivir-resistant influenza B isolate from an immunocompromised child and oseltamivir-resistant A (H1N1) isolates from experimentally infected volunteers have been recovered (56, 57).

Yield Reduction Assay

The yield reduction assay reflects the ability of an antiviral agent to inhibit the production of infectious virus, rather than the formation of a plaque. Cell monolayers are infected with virus, incubated in the presence of antiviral compound, and then lysed. Cell-free virus titers are subsequently determined

by plaque assay. The endpoint is defined as the concentration of antiviral which reduces virus yield by 50% in comparison with that of untreated control cultures. When used for susceptibility testing of HSV against penciclovir and acyclovir, the IC_{50}s for penciclovir were equivalent to or lower than those for acyclovir (19, 79). The greater activity of penciclovir is postulated to be the result of the extended half-life of penciclovir triphosphate (19).

Measurement of HSV and VZV TK Activity by Plaque Autoradiography

Functional viral TK is required for initial phosphorylation of acyclovir. To determine whether resistance to acyclovir is due to diminished or altered viral TK, two plaque autoradiograph methods are used (89). Incorporation of [^{125}I]iododeoxycytidine (IdC), a pyrimidine analog selectively phosphorylated by the VZV- or HSV-specific TK, correlates well with the acyclovir-phosphorylating potential of HSV and VZV isolates. Incorporation of [^{14}C]thymidine (dT) specifically assesses the thymidine-phosphorylating activity of these isolates and is useful for analyzing resistance to pyrimidine nucleoside analogs (acyclovir is a purine nucleoside analog). Most acyclovir-resistant HSV and VZV strains fail to incorporate both substrates due to diminished TK activity (TK^-); occasionally, strains with altered substrate (TK^a) activity are seen which fail to incorporate IdC but are able to incorporate dT. For IdC incorporation, Vero cells (HSV) and MRC-5 cells (VZV) are used. For dT incorporation, $LMTK^-$ TK^- mouse LM cells (Roswell Park Memorial Institute, Buffalo, N.Y.) are used for HSV; the TK^- cell line 143B is used for VZV dT incorporation. These assays provide both quantitative and qualitative evaluations of the TK status of a mixed population of TK^+ and TK^-. The IdC and dT plaque autoradiograph methods have been detailed elsewhere (89, 124).

Assays for HIV

A number of phenotypic assays are in use for susceptibility testing of HIV-1 isolates to nucleoside analog RT inhibitors (63, 72, 74, 78, 91, 101, 115, 120, 131, 135). A serious limitation of some of these procedures is that not all clinical isolates grow in the cell culture lines used in these assays. The AIDS Clinical Trials Group developed an assay performed with PBMCs which allowed growth of almost all clinical isolates of HIV-1 (72). Viral activity is quantitated by measurement of p24 antigen of HIV-1. The PBMC assay, however, is labor-intensive, costly, and difficult to control because of the many variables of the assay and has a long turnaround time (weeks). Moreover, this assay requires cocultivation of infected PBMCs with uninfected donor PBMCs to produce a stock of the clinical isolate being tested, which has been shown to select for subpopulations of HIV-1 not present in the original isolate (76).

A new generation of phenotypic assays, recombinant virus assays (RVA), have been developed to circumvent these problems (63, 74, 91, 101, 115). RVA involves PCR amplification of complete RT and PR gene coding sequences directly from the patient's plasma. The amplified RT and PR gene sequences from the patient strain are ligated into a viral vector containing a luciferase gene and then cotransfected along with a plasmid that expresses murine leukemia virus envelope proteins into a suitable receptive cell line. These cells then contain the patient's RT and PR gene-coding sequences in a background of an HIV-1 strain from which the original RT and PR sequences had been deleted. The susceptibilities of the chimeric pseudotyped viruses to all clinically available RT and PR inhibitors are subsequently determined in a single assay in which the ability of virus particles to replicate in the presence of various levels of antiretroviral drug is measured by detection of luciferase activity in the target cells. Recombinant virus assays thus allow determination of the phenotypic resistance patterns of circulating virus in vivo and circumvent the problem of selection of nonrepresentative variants during cultivation. The RVA can be completed in approximately 10 days from the time of cotransfection (63, 74, 91, 101, 115). Two RVAs are commercially available, the Antivirogram assay (developed by Virco, Mechelen, Belgium, and available in the United States at Laboratory Corporation of America [63]) and the PhenoSense assay (ViroLogic, Inc., South San Francisco, Calif. [102]). A recent comparison by Zhang et al. (137) of these two phenotype assays concluded that the PhenoSense (ViroLogic) results are more precise (i.e., show lower variability) and the PhenoSense assay was more likely to detect resistance to abacavir, didanosine, and stavudine. Limitations of RVAs are the necessity for a minimum of 500 to 1,000 copies of HIV-1 RNA per ml of plasma, a lack of consensus as to the appropriate increase in IC_{50} for each drug that correlates with clinical resistance, and uncertainty about the proportion of the total population of virus that a subpopulation of resistant virus must achieve to be detectable by these assays (58, 63).

GENOTYPIC ASSAYS

The genetic basis for antiviral resistance has been extensively studied for HCMV and HIV-1. Although not all resistance-associated mutations are known, the majority have been elucidated, allowing the application of molecular diagnostic methods. The major advantage of genotypic assays is the relatively rapid turnaround time compared to phenotypic assays.

HCMV

Genotypic assays have been used to screen HCMV isolates for mutations associated with ganciclovir resistance. Both UL97 (phosphotransferase) and UL54 (DNA polymerase) mutations can be detected; UL97 mutations are responsible for most ganciclovir resistance found to date (117). A number of approaches have been successfully applied to genotyping HCMV.

PCR amplification of short fragments of the UL97 gene followed by restriction endonuclease digestion has been used to detect mutations at positions 460, 520, 594, and 595 (24, 25, 46, 47). In one study, this assay detected 78% of ganciclovir-resistant UL97 mutants (25). PCR amplification followed by restriction endonuclease digestion could recognize mutant virus when present at 10% of the total virus population (117). The major advantage of this PCR-restriction endonuclease method is the speed with which UL97 mutations can be identified, since HCMV sequences can be directly amplified from many clinical samples. However, the absence of mutations at these key codons in UL97 does not necessarily exclude ganciclovir resistance as it has been shown that resistance can be attributed to other mutations in UL97 or to mutations in UL54 alone (49).

PCR amplification and sequencing of nearly the entire UL54 gene and the fragment of the UL97 gene spanning the conserved domains of the phosphotransferase should theoretically detect all the mutations currently known to confer resistance to ganciclovir (5, 25, 49, 117). Wolf et al. (136) identified a complete set of overlapping primers to be used for sequencing the full UL97 gene. UL97-associated ganciclovir resistance mutations have also been detected directly in patient blood and cerebrospinal fluid (18, 118, 136).

Sequencing of the UL54 gene, for genotypic detection of resistance to ganciclovir, foscarnet, and cidofovir is a large task not generally undertaken by the routine clinical virology laboratory. The UL54 codons that confer resistance to all three anti-HCMV drugs are between codons 408 and 841, inclusive (46, 86). Thus, one must amplify a fragment of about 1,500 bp and use three or four sequencing primers in each direction to obtain a reliable sequence. In a study by Smith et al. (117), UL97 mutations were detected in 89% of ganciclovir-resistant isolates whereas UL54 mutations were present in all high-level ganciclovir-resistant isolates. A caveat that applies to genotypic analysis is that not every mutation is a cause of antiviral resistance. Marker transfer experiments must be performed to definitively determine that a particular mutation is associated with drug resistance. For this purpose, PCR-amplified UL97 and UL54 fragments containing resistance-associated mutations were cotransfected with HCMV strain AD169 (drug susceptible). The resulting recombinant plaques were assayed for antiviral susceptibility by PRA. To further verify transfer of the mutations in question, sequencing was performed across the transfected fragment (5, 26).

The field of HCMV genotyping for detection of drug resistance is still evolving. A standardized protocol for sequencing of the UL97 gene was developed by members of the AIDS Clinical Trials Group HCMV Laboratories and published several years ago (87). Nevertheless, the discovery of new mutations that confer drug resistance is ongoing, as evidenced by the recent description of a deletion at UL97 codon 601 that was responsible for the early development of ganciclovir resistance in a renal transplant recipient (59). A recent review has provided an updated synopsis of the current state of knowledge of drugs and resistance in HCMV (55).

HIV

The development of antiretroviral drug resistance is currently a significant cause of treatment failures in HIV-infected patients (66, 67). Genotyping for detection of mutations that confer resistance has become a routine component of the management of HIV-infected individuals. Because of the lack of proofreading activity in the RT enzyme that copies the genome of HIV, the virus exists in infected individuals as a population of variants or quasispecies (40). This results in the random appearance and subsequent selection of resistant mutants in the presence of a selection pressure such as an inhibitory drug. Initially, a single mutation may occur, confer a low level of resistance, and then grow to predominance in the population. With ongoing replication, additional mutations that confer high-level resistance appear and the population as a whole becomes highly resistant (90). For some drugs, such as lamivudine and nevirapine, a single mutation confers high-level resistance (112).

Genotypic methods that have been used for detection of mutations in HIV include sequencing, selective PCR, oligonucleotide-specific hybridization, microarray hybridization (GeneChip; Affymetrix, Inc., Santa Clara, Calif.) and reverse hybridization (Line Probe Assay; Bayer Diagnostics NAD, Norwood, Mass.). The line probe assay for detection of mutations in HIV is currently not commercially available. Sequencing methods have been developed by several noncommercial laboratories and by two commercial companies, Abbott Diagnostics (Abbott Park, Ill.; formerly available from Applied Biosystems, Inc.) and Bayer Diagnostics (Tarrytown, N.Y.; formerly available from Visible Genetics, Inc.). Shafer (112) has reviewed the various assays available for genotypic analysis of HIV-1.

Dideoxynucleotide sequencing is the most commonly used method of HIV-1 PR and RT gene sequence analysis (41, 112). Two commercially available systems have been used successfully for this purpose, the TrueGene HIV-1 Genotyping Kit and OpenGene DNA Sequencing System (Bayer Diagnostics) and the ViroSeq HIV-1 Genotyping System (Celera/Abbott Diagnostics) (27, 32, 45, 95, 129). Both systems have been approved for in vitro diagnostic use. The sequencing systems rely on initial extraction of viral RNA from patient plasma, RT-PCR of the extracted RNA to amplify about 1,500 bp of PR and RT and then a sequencing reaction with dye terminators (ViroSeq) or dye-labeled primers (TrueGene). Both companies make sequencing instruments for electrophoresis of the samples, and both provide software to assemble the segments and align the assembled patient sequence with a reference sequence to aid in the identification of mutant codons. With the primers for PCR and the sequencing primers, the ViroSeq system provides double coverage at a minimum for the entire 1.5-kb sequence of interest and quadruple coverage may be obtained for certain regions. The ViroSeq software combines the functions of several previously available software packages to trim the sequence segments, generate a contiguous consensus sequence that is generated by assemblage of the overlapping individual segments, and align the consensus sequence with a reference sequence. The operator must manually toggle through the entire sequence, edit (confirm or override) the computer base calls, and also make the final decision about polymorphic sites where a mutant base might constitute a minor proportion in the background of wild-type base. The ABI software prints a complete mutation report which identifies "reported" and "novel" mutations based on the Los Alamos National Laboratory HIV database, which is available online at http://hiv-web.lanl.gov. The ViroSeq system has been used to successfully detect RT and PR gene mutations in pediatric samples and in non-subtype B HIV-1 samples (32, 95).

The TrueGene genotyping kit may be used in conjunction with a number of extraction procedures. The purified viral RNA is amplified in a single-tube RT-PCR step, the product of which can be used directly in the subsequent sequencing reactions. It employs a proprietary methodology using four pairs of sequencing primers whereby the upstream primer of each pair is labeled with one dye and the downstream primer of each pair is labeled with another dye. Each primer pair is present in each of four dideoxy-terminator reactions, one reaction for each of the four dideoxy bases. Thus, this arrangement requires 16 different reactions and 16 lanes on the sequencing gel in order to cover the HIV PR and RT genes in both directions. The data are analyzed with the TrueGene software and interpreted to provide a resistance report. Two studies have demonstrated improved virological outcome when genotyping information is considered in patient management decisions (27, 129).

A number of studies have compared the various "home brew" and commercial sequencing methods. An interlaboratory study compared the abilities of 13 laboratories to detect RT mutations in cultured PBMC pellets. Highly concordant results were obtained overall, with some difficulty encountered with a clinical isolate that contained a mixture of wild-type and mutant codons. A mutant codon which was present at less than 50% of a mixed population of resistant and susceptible genotypes was not consistently detected (41). Two large multicenter studies compared sequencing results among participating laboratories for detection of RT mutations in either coded plasmid mixtures or spiked plasma samples. Laboratories had difficulty in detecting mutations in cases where the mutant represented 25% or less of the

total DNA population at that codon (110, 111). Even the editing process can contribute variability to the overall HIV genotype determination, as shown by Huang et al. (68). In this study, sequence concordance was high even though different editing strategies were used by different labs, but 12% of the resistance mutations present in the 10 electronic files that were distributed and analyzed were not identified in some labs.

The GeneChip method (Affymetrix) utilizes miniaturized, high-density arrays of oligonucleotide probes on a silicon-glass chip that contains oligonucleotide probes complementary to the first 1,200 nucleotides of HIV RT and the entire sequence of PR genes. The probes are positioned in a grid pattern on the array and are available for hybridization with target nucleic acid labeled with a fluorescent reporter group. The labeled target is incubated with the array, and based on patterns of hybridization of the target nucleic acid to the probes, the target sequence can be deduced (75). Studies comparing the performance of the GeneChip to dideoxynucleotide sequencing have found the latter to be more reliable (58, 112). GeneChip is not currently commercially available.

The line probe assay (LiPA) was manufactured by Innogenetics but marketed by Bayer Diagnostics NAD as VERSANT HIV-1 RT Resistance Assay and VERSANT HIV-1 Protease Resistance Assay. It has been withdrawn from the market.

The VircoGen VirtualPhenotype assay (Virco) utilizes a proprietary algorithm to compare a virus genotype to a large database of known genotypes and phenotypes to predict a phenotype based on sequence data (58, 112). Two RT and PR gene sequence analysis programs are available online from Stanford University at http://hivdb.stanford.edu/. One program compares an HIV-1 sequence submitted by the investigator to a consensus reference sequence ultimately linking RT and PR gene sequence variations to the antiretroviral therapy history of the patient from whom the sequences were obtained (113). In the second sequence analysis program, Drug Resistance Interpretation, PR and RT gene sequences are entered by the user and the program produces a phenotypic interpretation based on correlations of genotype and accumulated information on clinical outcome (112).

A concern with each of the genotypic methods is that mutant variants present at low frequency may not be detectable and that mixtures of HIV-1 strains with minor sequence variations may not be distinguishable (58, 112). Genotypic assays do allow more rapid and efficient detection of resistance than phenotypic assays and may allow earlier detection of emerging resistance than phenotypic assays. Also, because of the complex interactions among different combinations of resistance mutations, predictions of phenotype based on genotype alone may not be accurate (20, 112). Moreover, genotypic assays can detect only known resistance-associated mutations. The complexity of these tests makes them impractical for many diagnostic virology laboratories. Measurement of plasma HIV-1 RNA levels (viral load) reflects the extent of virus replication in an infected individual and remains the strongest predictor of clinical outcome. Declining HIV-1 RNA levels during treatment indicate a positive response to therapy, whereas a significant rise in RNA levels indicates treatment failure (21, 88).

None of these genotypic methods is currently able to detect resistance to the newest antiretroviral agent, T-20 or enfuvirtide. This is a synthetic peptide inhibitor that interferes with viral entry by blocking formation of a hairpin structure that is necessary for fusion of the viral membrane with the cell membrane. Resistance to T-20 is conferred rather quickly (within weeks) and is the result of a single mutation, G36D, within the *env* HR1 domain or a combination of mutations that may or may not include the G36D mutation (133). Although the drug is FDA approved, the commercially available genotype methods have not yet included PCR or sequencing primers for this region.

Of recent concern with respect to the now greater availability of antiretrovirals in countries with limited resources is the question of whether the genotyping methods that have been developed for use in Western countries mainly on HIV group M subtype B virus will work on non-B subtypes. This concern is significant because subtype B accounts for only about 12% of the global AIDS pandemic. In a set of 35 HIV isolates from group M subtypes A through J, full-length sequences were created with the ViroSeq reagents in 84% of the specimens tested and with the TrueGene in 53% of the specimens tested (9). Both methods amplified RNA from plasma levels of about 100 to 1,000 copies/ml. Eshleman et al. (51) employed the ViroSeq system to genotype 126 samples (114 of which were non-B subtypes) and successfully sequenced 124 of these. Genotypes performed in a second lab on the same 126 samples were 98 to 100% identical to the primary lab results. Thus, it appears that the two commercially available genotyping systems work tolerably well on subtypes other than B and should be generally usable in geographic regions where non-B subtypes predominate.

Aside from the mechanics of performing HIV genotypes on non-B subtypes is the question of whether these subtypes exhibit drug-resistant mutation patterns in the same way and at the same locations as the subtype B strains. In an authoritative recent paper on this topic, Kantor et al. (73) looked at the correlation of antiretroviral treatment on the distribution of mutations in HIV sequences from 3,686 individuals infected with non-B subtypes and compared this data with mutations in HIV sequences from 4,769 patients with subtype B. All of the known subtype B resistance mutations occurred in the non-B subtypes, and 80% were correlated with antiretroviral treatment of the patients with the non-B subtypes. The authors concluded that it is reasonable for global monitoring of resistance to continue to focus on the known subtype B resistance mutations as there are apparently no unique or previously unrecognized mutations in non-B subtypes.

HBV

Treatment of chronic hepatitis B virus (HBV) infections with antiviral drugs and immune modulators is becoming more widely employed as recent clinical trials have demonstrated efficacy. Three nucleoside analogs, lamivudine (3TC), adefovir dipivoxil (ADV), and entecavir, a carbocyclic deoxyguanosine analog (114), are approved for treatment of chronic HBV and act at the level of the HBV DNA polymerase. Pegylated alpha interferon is approved for use in treatment and has both antiviral and immunomodulating activity.

Three regions of the HBV genome are relevant to treatment, resistance to the various drugs, and type/subtype determination: (i) the active site of the viral DNA polymerase gene, (ii) mutations in the precore region of the HBV genome, and (iii) mutations in the core (C-gene) and the surface (S-gene) proteins.

Lamivudine (3TC) was the first clinically useful drug for treatment of HBV and is still widely used today, although resistance is virtually ensured (20% of patients taking the drug develop resistance each year) if the drug is used long enough in any given patient (85). The active site of the HBV

DNA polymerase enzyme is very similar to that of HIV (they both have the same complement of enzymatic activities), and thus it seemed reasonable that many of the nucleoside analogs that inhibit HIV RT would also inhibit HBV polymerase. In fact, many have been tested in clinical trials but mainly lamivudine and adefovir dipivoxil (an analog of adenosine monophosphate) are currently being used in HBV therapy. Sequencing of the active site of the HBV polymerase is necessary for detection of the mutations now known to confer resistance to these drugs, although other research (134) or a commercial method is available (1, 122). Famciclovir (now rarely used) resistance mutations are in polymerase domain B, lamivudine resistance mutations are in domain C (the "M" of the YMDD motif is at codon 552 [or codon 204 by some counting methods], which corresponds to codon 184 in the HIV RT gene), and the only demonstrated adefovir dipivoxil resistance mutation is in domain D (codon 236) (3, 4, 85). Recent evidence indicates that ongoing lamivudine therapy in the presence of lamivudine resistance mutations is of no clinical value (2, 81), thus possibly accentuating the need for lamivudine resistance testing in the future. Adefovir dipivoxil is active against lamivudine-resistant mutants of HBV and is quite slow to cause the acquisition of resistance mutations during initial therapy.

The U.S. FDA approved in March 2005 a third drug, entecavir, for treatment of chronic HBV. This development is particularly exciting because entecavir is active against lamivudine- and adefovir dipivoxil-resistant HBV; it is easily absorbed, well tolerated, and relatively nontoxic. Entecavir is a carbocyclic deoxyguanisine analog that acts directly on the HBV DNA polymerase to cause delayed or nonobligate chain termination and also blocks priming of the reverse transcription reaction (114). Resistance to entecavir is apparently rare, having occurred in only two patients of the more than 500 enrolled in phase II and III clinical trials (128). Interestingly, resistance to entecavir alone was not observed; the two patients with resistance to entecavir had preexisting mutations to lamivudine which, in combination with new entecavir-induced mutations, resulted in high-level resistance to both drugs. Thus, patients with lamivudine resistance due to mutations at codons 528 and 552 (180 and 204 by the other counting method) when exposed to entecavir may develop mutations at codons 532, 550, and/or 598 (184, 202, and/or 250) and be highly resistant to both drugs (128).

Secondly, the precore (preC) region of the HBV genome has recently gained significance because there are indications that it may be associated with resistance to interferon. There is long-standing evidence (52) that mutations in the preC region have a negative effect on interferon treatment, and more-recent in vitro experiments have supported this contention (132). The development of a G1896A (codon 28) mutation results in a termination codon which interrupts translation of the HBeAg precursor and thus initiates an e-antigen-negative chronic hepatitis. Sequencing of this region is a reasonable method for detection of these mutations. Alternatively, Innogenetics has a LiPA that is capable of detecting this mutation as well as a preC promoter mutation that may also be present (codon 1762).

Thirdly, classification of HBV into types A to H based on the genomic sequence has yielded unexpected correlations with the clinical course of disease. It has long been known that some types, for example, type B, are associated with less active liver disease, slower progression, and more likely spontaneous HBeAg seroconversion than other types, for example, type C (96). Recent studies with pegylated interferon (71) have yielded the clinically relevant information that the response was type dependent. In this study, patients with type A had a 47% response (i.e., loss of HBeAg), patients with type B had a 44% response, type C patients had a 28% response, and type D patients had a 25% response. Thus, there is increasing evidence that determination of the HBV type is an important predictive factor in treatment with interferon and the overall course of disease (53). The genotype can be determined by sequencing the entire genome, the core region, or the surface protein region. Innogenetics has a commercially available LiPA that identifies the HBV type via reverse hybridization with an amplicon from the DNA polymerase gene.

Current recommendations for monitoring of patients on therapy for HBV include baseline quantitative HBV DNA levels and additional levels periodically to help determine the endpoint of treatment for patients with chronic hepatitis B (American Association for the Study of Liver Diseases [85]). Genotyping for detection of resistance should be performed if resistance is suspected because of rising DNA levels or appearance of symptoms (84).

INTERPRETATION OF ANTIVIRAL SUSCEPTIBILITY RESULTS

Table 1 lists breakpoint IC_{50}s proposed by various investigators for herpes group viruses and influenza viruses. The concentration of antiviral by which the virus is considered susceptible to the antiviral agent has generally been based on median susceptibilities of large numbers of clinical isolates from patients prior to, during, and after antiviral therapy. Because of the variables that affect antiviral susceptibility results, the absolute IC_{50} value can vary from assay to assay and from laboratory to laboratory. Moreover, in vitro results indicating susceptibility or resistance may not correlate with response of the infection to therapy in vivo. The clinical response of the patient depends on a number of other factors such as immunological status and pharmacokinetics of the drug in that particular patient (the dose or route of administration could be inappropriate). A poor clinical response may occur even though the antiviral susceptibility testing denotes in vitro susceptibility (39). Patients with HSV infections who are immunocompromised may fail to respond to therapy despite in vitro IC_{50}s indicating susceptibility to vidarabine or acyclovir (44, 105, 107). Conversely, HSV isolates for which IC_{50}s of acyclovir are >2 μg/ml can occasionally be recovered from otherwise healthy hosts who have responded to acyclovir therapy (80). Thus, a high IC_{50} derived by in vitro susceptibility testing is not sufficient to designate a viral strain as resistant. Neither can in vitro susceptibility to a drug a priori predict successful clinical outcome. Whenever possible, evidence of genetic alteration of the virus should be considered as well.

Interpretation of antiviral susceptibility results is further complicated by the variability in endpoint due to testing methodologies (30, 64, 92, 99). Because endpoints are dependent on the test method, each new method and antiviral agent must be correlated with a historic standard that has been used to test large numbers of isolates. Also, the absolute IC_{50} may vary from assay to assay and from laboratory to laboratory. Moreover, because small subpopulations of resistant virus may not be reflected in IC_{50}s, IC_{90} values may be more predictive of clinical response. One approach to interpreting susceptibility endpoints is to compare the IC_{50}s of an isolate obtained prior to therapy (or of a well-characterized reference control strain) with that of an isolate obtained during therapy; a significant increase in the ratio of

such IC_{50}s denotes resistance. However, pretherapy isolates are often unavailable, and the IC_{50} ratio considered to be clinically significant is unclear. Large-scale collaborative comparisons of methods with the same viral isolates are necessary to standardize antiviral susceptibility testing and to establish definitive interpretive guidelines. Only when a standardized assay is adopted can prospective studies be performed to correlate in vivo response with in vitro susceptibility. Such studies are essential before definitive interpretive breakpoints are established.

Susceptibility testing of penciclovir illustrates the effect that cell line and testing method have on the endpoint. When acyclovir and penciclovir, which is structurally similar to acyclovir, were tested with HSV isolates in Vero cells by PRA, penciclovir appeared to be less active than acyclovir and HSV-2 isolates appeared to be resistant to penciclovir. In contrast, penciclovir appeared to be more active than acyclovir against some HSV isolates when SCC25 cells were used. The two drugs appeared to have comparable activities when tested in MRC-5 and A549 cells (79). When clinical isolates of VZV were tested by plaque reduction and DNA hybridization, IC_{50}s for DNA hybridization were significantly lower than those by PRA (121). Variability in endpoint was also seen with VZV depending on the composition of the inoculum (cell free versus cell associated). Therefore, breakpoints for susceptibility testing of penciclovir with HSV and VZV cannot be established at this time (79, 121).

A limitation of phenotypic testing for HIV-1 is the lack of consensus as to the absolute IC_{50}s denoting resistance of HIV-1 to antiretroviral drugs. In vitro susceptibility of HIV-1 results are usually expressed as the "fold increase" in the IC_{50} of an isolate obtained on therapy compared to a pretreatment isolate or a drug-susceptible isolate (63, 109). The increase in the IC_{50} considered clinically significant is likely to vary by drug or drug class (58). The Antivirogram assay (Virco) has established cutoffs denoting reduced susceptibility at 2 standard deviations above the mean value for 1,000 isolates from untreated HIV-positive individuals and for several thousand isolates of genetically wild-type virus. Both Virco and ViroLogic are aware of the importance of clinical cutoff values and are making diligent efforts to ascribe accurate values for use by physicians.

The significance and clinical utility of phenotypic and genotypic analysis of HIV-1 are increasingly being recognized. A report that reanalyzed data from previous studies demonstrated that baseline genotypic and phenotypic drug resistance predicted virological failure (38). Genotypic analysis of the PR gene in patients who are on antiretroviral therapy but PR-inhibitor naive has also been shown to be predictive of virological and immune response to therapy with PR inhibitors (100). Prospective studies have demonstrated improved virological outcome when genotyping information is considered in patient management decisions (27, 43, 129). Drug resistance testing is now recommended to help guide the selection of salvage therapy after treatment failure and for guiding therapy for pregnant women (66). However, limitations of genotypic testing include the difficulty in interpretation of resistance mutations and the inability to detect minority variants.

It is important to remember that antiretroviral therapy may fail for reasons other than the emergence of drug-resistant virus such as drug antagonism; nonadherence; increased clearance of one antiretroviral drug when coadministered with another drug; inadequate penetration of drug into a sequestered site, e.g., the central nervous system; or malabsorption of drug from the gastrointestinal tract (58, 63).

FUTURE DIRECTIONS

Consensus protocols for antiviral susceptibility testing are under development. A protocol for HSV plaque assays is now published (29), and a standardized procedure for sequencing of the HCMV UL97 gene (87) has been published. Standardization is hampered by the many variables that affect susceptibility testing results. No single assay method, cell line, or inoculum composition (cell free versus cell associated) appears sufficient for testing all viruses. A major problem with culture-based susceptibility testing of viruses other than HSV is that assays may require weeks to complete, a fact that limits their utility in management of acute cases. Genotypic assays for UL97 and UL54 mutations of HCMV may replace culture-based assays as more resistance-associated mutations are identified. Data attesting to the clinical significance of susceptibility testing of HIV-1 isolates are accumulating. Additional prospective studies are required to delineate the utility of antiretroviral susceptibility testing.

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DAVID W. WARNOCK

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There are at least 100,000 named species of fungi. However, it has been estimated that the number of undiscovered species ranges from 1 million to more than 10 million, and it has been calculated that about 1,000 to 1,500 new species are described each year (6). Of the named species of fungi, fewer than 500 have commonly been associated with human or animal disease, and no more than 50 are capable of causing infection in otherwise normal individuals. On the other hand, an increasing number of ubiquitous environmental molds are now being implicated as opportunistic pathogens, capable of producing serious or lethal disease in hosts that are immunocompromised or debilitated. These molds are organisms whose natural habitat is in the soil or on plants, wood, compost heaps, or decomposing food. Many are familiar to mycologists, plant pathologists, and food microbiologists, but they present problems for the clinical microbiologist who often has had no formal training in the identification of fungi. Fungal identification can be challenging and sometimes frustrating because of the importance placed on the morphological characteristics of the organisms and the need to become familiar with a range of different structures and terms. Indeed, it is fair to state that obscure mycological terms are one of the major factors that discourage many microbiologists from mastering fungal identification. However, investing time to learn the basic structures and principles of taxonomy, classification, and nomenclature can result in the ability to recognize and identify correctly many medically important fungi.

MORPHOLOGICAL CHARACTERISTICS OF THE FUNGI

Fungi form a separate group of eukaryotic organisms which differ from other groups, such as the plants and animals, in several major respects. Fungal cells are encased within a rigid cell wall, mostly composed of chitin, glucan, chitosan, mannan, and glycoproteins in various combinations. These features contrast with the animals, which have no cell walls, and the plants, which have cellulose as the major cell wall component. As in other eukaryotic organisms, fungal cells have a true nucleus with a surrounding membrane, and cell division is accompanied by meiosis or mitosis.

Fungi are heterotrophic, that is, lacking in chlorophyll, and therefore require preformed organic carbon compounds for their nutrition. Fungi live embedded in a food source or medium and obtain their nourishment by secreting enzymes into the external substrate and by absorbing the released nutrients through their cell wall. Fungi are found throughout nature, performing an essential service in returning to the soil nutrients removed by plants.

Fungi can be multicellular or unicellular. In multicellular organisms, the basic structural unit is a chain of multinucleate, tubular, filament-like cells (termed a hypha). In most multicellular fungi the vegetative stage consists of a mass of branching hyphae, termed a mycelium or thallus. Each individual hypha has a rigid cell wall and increases in length as a result of apical growth with mitotic cell division. In the more primitive fungi, the hyphae remain aseptate (without cross-walls). In the more advanced groups, however, the hyphae are divided into compartments or cells by the development of more or less frequent cross-walls, termed septa. Such hyphae are referred to as being septate. Fungi that exist in the form of microscopic multicellular mycelium are commonly called molds.

Many fungi that exist in the form of independent single cells propagate by budding out similar cells from their surface. The bud may become detached from the parent cell, or it may remain attached and itself produce another bud. In this way, a chain of cells may be produced. Fungi that do not produce hyphae but just consist of a loose arrangement of budding cells are called yeasts. Under certain conditions, continued elongation of the parent cell before it buds results in a chain of elongated cells, termed a pseudohypha. Unlike a true hypha, the connection between adjacent pseudohyphal cells shows a marked constriction. Yeasts are neither a natural nor a formal taxonomic group, but are a growth form shown in a wide range of unrelated fungi.

Many medically important fungi change their growth form as part of the process of tissue invasion. These so-called dimorphic pathogens usually change from a multicellular mold form in the natural environment to a budding, single-celled yeast form in tissue. *Histoplasma capsulatum, Blastomyces dermatitidis, Paracoccidioides brasiliensis,* and *Sporothrix schenckii* are the best-known examples of this dimorphic change, but many other fungal pathogens show subtle morphological differences between forms found in tissue and in culture.

* This chapter contains information presented in chapter 110 by Robert A. Fromtling, Judith C. Rhodes, and Dennis M. Dixon in the eighth edition of this Manual.

Fungi reproduce by means of microscopic propagules called spores. These may result from an asexual process (involving mitosis only) or a sexual process (involving meiosis, preceded by fusion of the nuclei of two cells). Except for the occasional mutation, asexual spores are identical to the parent. They are generally short-lived propagules that are produced in enormous numbers to ensure dispersion to new habitats. Many fungi are also capable of sexual reproduction. Some species are self-fertile (homothallic) and able to form sexual structures between different cells within an individual thallus. Most, however, are heterothallic and do not form their sexual structures unless two different isolates come into contact. Once two compatible haploid nuclei have fused, meiosis can occur, and this leads to the production of the sexual spores. In some species, the haploid sexual spores are borne singly on specialized generative cells and the whole structure is microscopic in size. In other cases, however, the spores are produced in millions in macroscopic "fruiting bodies" such as mushrooms. Sexual reproduction and its accompanying structures form the main basis for the classification of the fungi.

NOMENCLATURE OF FUNGI

The scientific names of fungi are subject to the International Code of Botanical Nomenclature (ICBN) (http://www.bgbm.org/iapt/nomenclature/code/default.htm), a convention that dates from the time when biologists regarded these organisms as "lower plants." The rules of the ICBN must be followed when proposing the name for a new fungal species; otherwise, the name being proposed can be rejected as invalid. The main requirements for valid publication are that the name must be in Latin binomial form, the publication must contain a concise description of the essential and differential characteristics of the fungus in Latin, and a living culture of the specimen on which the author based his original description of the species must be preserved.

Names of fungi may have to be changed for a number of reasons. Many common and widely distributed species of fungi have been described as new many times and thus have come to have more than one name. In general, the correct name for any species is the earliest name published in line with the requirements of the code of nomenclature. The later names are termed synonyms. To avoid confusion, however, the ICBN permits certain exceptions. The most significant of these is when an earlier generic name has been overlooked, a later name is in common use, and a reversion to the earlier name would cause problems.

Another reason for changing the name of a fungus is when new research necessitates the transfer of a species from one genus to another or establishes it as the type of a new genus. Such changes are quite in order, but with the provision that the specific epithet should remain unchanged, except for inflection according to the rules of Latin grammar. However often a species is transferred to a new genus, the correct species epithet is always the first one that was applied to that particular organism. As an example, when *Phialophora parasitica* was moved to a new genus, it became *Phaeoacremonium parasiticum*.

If there is one complication of fungal nomenclature that is confusing to many microbiologists, it is the fact that a large number of fungi appear to bear more than one name. This is an apparent departure from the basic principle of biological classification, in which an organism can have only one correct name. As described in the previous section of this chapter, many fungi have an asexual stage (or anamorph), characterized by the production of asexual spores (e.g., conidia), and a sexual stage (or teleomorph) characterized by sexual spores (e.g., ascospores or basidiospores). Many fungi propagate asexually, and the teleomorph is unknown or only rarely encountered. Because of this, mycologists have often given separate names to the asexual and sexual stages. Often this is because the anamorph and teleomorph were described and named at different times without recognition of the connection between them. The code of nomenclature permits this practice, and while the name of the teleomorph takes precedence and covers both stages, the name given to an anamorph may be used as appropriate. Thus, it is permissible to refer to a fungus by its asexual designation if this is the stage that is usually obtained in culture. For example, *Blastomyces dermatitidis* is the anamorph of the ascomycete *Ajellomyces dermatitidis*. The anamorph is the stage that is ordinarily encountered in culture, and only under certain special conditions is the sexual stage formed. Although the correct name typified by the teleomorph is *A. dermatitidis, B. dermatitidis* remains the name that is most widely recognized and used.

The taxonomy and nomenclature of fungi that have both asexual and sexual stages are challenging. Some teleomorphic fungi can produce more than one asexual form of propagation, the term synanamorph being used to describe each of these different anamorphs. These may bear separate names. As an example, the teleomorphic species *Pseudallescheria boydii* can also exist in two different synanamorphic forms, named *Graphium fructicola* and *Scedosporium apiospermum*. These three names all refer to one and the same organism (see chapter 125).

TAXONOMY AND CLASSIFICATION OF THE FUNGI

The kingdom Fungi is one of the six kingdoms of life (4). It is organized in a hierarchical manner, each rank being named with, and recognizable by, a particular ending: Phylum (or Division), -mycota; Class, -mycetes; Order, -ales; Family: -aceae (7). Each family is composed of a number of genera, and these are divided into species. The kingdom Fungi is currently divided into four phyla or divisions: the Chytridiomycota, Zygomycota, Ascomycota, and Basidiomycota. One of these phyla, the Chytridiomycota, does not contain any species that are pathogenic to humans. In addition to the true fungi, there are a number of organisms, classified in the kingdoms Chromista and Protozoa, that while not fungi sensu stricto, are "fungus-mimics" or "pseudofungi," i.e., protists that have become similar to fungi in one or more respects.

The classification of fungi is largely based on their appearance, rather than on the physiological and biochemical differences that are of such importance in bacterial classification. The sexual spores and their mode of production form the main basis for classification of fungi into the Zygomycota, Ascomycota, and Basidiomycota. In some fungi, however, the asexual stage or anamorph has proved so successful as a means of rapid dispersal to new habitats that the sexual stage or teleomorph has disappeared or at least has not been discovered. Even in the absence of the teleomorph it is now often possible to assign these fungi to the Ascomycota or Basidiomycota on the basis of DNA sequences of the anamorphs (3). In the past, these asexual fungi were classified in an artificial group, the "Fungi Imperfecti" (also termed the "form-division Deuteromycota") and were divided into artificial form-classes according to the morphological characteristics of their asexual reproductive structures. There is no longer any separate formal grouping for those fungi that appear to be strictly anamorphic or for which no teleomorph has been

TABLE 1 Simplified taxonomic scheme illustrating major groups of the Kingdom Fungi in which medically important fungi are classified

Taxonomic designation	Representative genera
Phylum: Zygomycota	
Class: Zygomycetes	
Order: Mucorales	*Absidia, Rhizopus*
Order: Entomophthorales	*Basidiobolus, Conidiobolus*
Phylum: Basidiomycota	
Class: Basidiomycetes	
Order: Filobasidiales	*Filobasidium*, *Filobasidiella* (teleomorphs of *Cryptococcus* species)
Order: Agaricales	*Schizophyllum*
Phylum: Ascomycota	
Class: Archiascomycetes	
Order: Pneumocystidales	*Pneumocystis*
Class: Hemiascomycetes	
Order: Saccharomycetales	*Clavispora, Debaryomyces, Issatchenkia, Kluyveromyces* (teleomorphs of *Candida* species); *Saccharomyces*
Class: Euascomycetes	
Order: Onygenales	*Arthroderma* (teleomorphs of *Microsporum* and *Trichophyton* species), *Ajellomyces* (teleomorphs of *Blastomyces* and *Histoplasma* species)
Order: Eurotiales	*Emericella, Eurotium, Neosartorya* (teleomorphs of *Aspergillus* species)
Order: Hypocreales	*Gibberella, Nectria* (teleomorphs of *Fusarium* species)
Order: Microascales	*Pseudallescheria* (teleomorph of *Scedosporium* species)

discovered. Nonetheless, mycologists continue to employ the asexual reproductive characteristics of molds, at least for routine identification purposes.

A simplified taxonomic scheme illustrating the major groups of medically important fungi is presented in Table 1. These groups are described in the following sections. A simplified key to their identification is provided in Table 2.

Kingdom Fungi

Phylum Zygomycota

In this group of lower fungi, the thallus is aseptate and consists of wide, hyaline (colorless) branched hyphal elements. The asexual spores (termed sporangiospores) are nonmotile and are produced inside a closed sac, termed a sporangium, the wall of which ruptures to release them. Sexual reproduction leads to the formation of a single large zygospore with a thickened wall. Most of the medically important species are heterothallic and do not form their sexual structures unless two compatible isolates come into contact.

The phylum Zygomycota is divided into two classes, the Zygomycetes and the Trichomycetes. The former class contains two orders of medical importance, the Entomophthorales and the Mucorales. The order Entomophthorales includes the genera *Basidiobolus* and *Conidiobolus,* agents of subcutaneous infections. The order Mucorales is the most clinically important and includes the genera *Absidia, Mucor, Rhizomucor,* and *Rhizopus*.

TABLE 2 Simplified key to the main groups of medically important fungi

1a. Fungus not culturable on routine media, occurs as cyst-like cells in tissue	Archiascomycetes
1b. Fungus culturable	2
2a. Colonies consist of budding cells at 30°C	3
2b. Colonies consist of hyphae at 30°C	5
3a. Colonies black	Black yeasts
3b. Colonies white, cream, pink, or red	4
4a. Urease test positive	Basidiomycetous yeasts
4b. Urease test negative	Ascomycetous yeasts
5a. Hyphae septate	6
5b. Hyphae nearly aseptate	9
6a. Clamp connections present	Filamentous basidiomycetes
6b. Clamp connections absent	7
7a. Fruiting bodies absent	Hyphomycetes
7b. Fruiting bodies present	8
8a. Fruiting bodies containing ascospores in asci	Euascomycetes
8b. Fruiting bodies containing conidia	Coelomycetes
9a. Sporulation abundant	Zygomycota
9b. Sporulation absent; zoospores formed in water cultures	Oomycota

Phylum Basidiomycota

Most members of this group of higher fungi have a septate, filamentous thallus, but some are typical yeasts. Sexual reproduction leads to the formation of haploid basidiospores on the outside of a generative cell, termed a basidium. Three classes are recognized (Basidiomycetes, Urediniomycetes, and Ustilaginomycetes), but only a few members of this large phylum are of medical importance. The most prominent are the basidiomycetous yeasts with anamorphic stages belonging to the genera *Cryptococcus, Malassezia,* and *Trichosporon*. The genus *Cryptococcus,* which contains more than 30 species, has teleomorphs that have been assigned to the genera *Filobasidium* and *Filobasidiella* (2).

In culture, filamentous basidiomycetes often produce fast-growing, nonsporulating (sterile) white colonies with clamp connections. These are hyphal outgrowths which, at cell division, make a connection between the two cells, forming a bypass around the septum to allow the migration of a nucleus. The basidia are often produced in macroscopic structures, termed basidiomata or basidiocarps, and the basidiospores are often forcibly discharged. Asexual reproduction is variable, with some species producing spores like those of the Ascomycota (see below), but many others are not known to produce spores at all. Most filamentous basidiomycetes are wood-rotting fungi or obligate plant pathogens. The most frequently reported clinically important filamentous basidiomycete is *Schizophyllum commune.*

Phylum Ascomycota

This large phylum contains almost 50% of all named fungal species and accounts for around 80% of fungi of medical importance (6). Sexual reproduction leads to the development of haploid spores, termed ascospores, which are produced in a sac-like structure, termed an ascus. The Ascomycota show a gradual transition from primitive forms that produce single asci to species that produce large structures, termed ascocarps or ascomata, containing numerous asci. Variations in ascus structure are of major importance in the classification of these fungi. Asexual reproduction consists of the production of spores, termed conidia (singular, conidium), from a generative or conidiogenous cell. In some species the conidiogenous cell cannot be distinguished from the rest of the mycelium. In others, a special structure is produced, which bears one or more conidiogenous cells.

The phylum Ascomycota includes three classes of medical importance: the Archiascomycetes, the Hemiascomycetes, and the Euascomycetes. The class Archiascomycetes contains a single genus, *Pneumocystis,* formerly classified as a member of the kingdom Protozoa, but now reassigned to the fungi on the basis of small-subunit rRNA and other gene sequence comparisons (1). The class Hemiascomycetes contains the ascomycetous yeasts, while the class Euascomycetes contains the filamentous ascomycetes.

The order Saccharomycetales, which belongs to the class Hemiascomycetes, is characterized by vegetative yeast cells which proliferate by budding or fission. These fungi do not produce ascomata, the ascus being formed by direct transformation of a budding vegetative cell, by "mother-bud" conjugation, or by conjugation between two independent single cells. Many members of this order have an anamorphic stage belonging to the genus *Candida*. This genus, which consists of around 200 anamorphic species, has teleomorphs in more than 10 different genera, including *Clavispora, Debaryomyces, Issatchenkia, Kluyveromyces,* and *Pichia* (8).

In the class Euascomycetes sexual reproduction leads to the formation of ascomata containing asci with ascospores. This class has 12 orders that include species pathogenic to humans. Among the more important are the order Onygenales, which contains the teleomorphs of the dermatophytes and a number of dimorphic systemic pathogens (including *Histoplasma capsulatum* and *Blastomyces dermatitidis*); the order Eurotiales, which includes the teleomorphs of the anamorphic genera *Aspergillus* and *Penicillium;* and the order Hypocreales, which contains the teleomorphs of the anamorphic genus *Fusarium*. In addition, the teleomorphs of numerous melanized fungi of medical importance belong to a number of orders in the class Euascomycetes. These include the Chaetothyriales, Dothidiales, Microascales, Ophiostomatales, Pleosporales, and Sordariales.

Although most of the septate molds that are isolated in clinical laboratories belong to the class Euascomycetes, it is unusual to encounter their sexual reproductive structures in routine cultures. The few species which do produce ascomata in relative abundance include *Pseudallescheria boydii* (anamorph, *Scedosporium apiospermum*) and *Emericella nidulans* (anamorph, *Aspergillus nidulans*). For the most part, however, routine identification of these molds is based on the form and arrangement of the asexual spore-bearing structures and the manner in which the spores are produced (see below).

Kingdom Protozoa

Of the different phyla within the kingdom Protozoa, the Mycetozoa (or Myxomycota) consist of free-living, unicellular or plasmodial slime molds that have acquired aerial spores for dispersion. None of these pseudofungi are human pathogens. The phylum Microspora consists of obligate intracellular parasites. In humans, microsporidia are considered to be etiologic agents of opportunistic infections, mostly in persons with AIDS. Phylogenetic analysis has indicated that these organisms are related to conventional Fungi, rather than to the Protozoa (9). However, parasitologists have been reluctant to part with this group while mycologists have been just as reluctant to accept it. For the time being at least, the microsporidia have been retained alongside the Protozoa, while acknowledging that these organisms are, in fact, fungi (see chapter 143).

Kingdom Chromista

This kingdom contains three "fungal" phyla. In the phylum Oomycota, the thallus is unicellular to mycelial and mainly aseptate. The asexual spores, or zoospores, are motile. Sexual reproduction leads to the development of nonmotile, thick-walled oospores. Members of the order Pythiales occur mostly in the soil or on plants; one species, *Pythium insidiosum,* is of clinical significance (see chapter 128).

IDENTIFICATION OF YEASTS

Yeasts are neither a natural nor a formal taxonomic group but are a growth form found in a wide range of unrelated ascomycetous and basidiomycetous fungi. Their identification relies on a combination of morphological, physiological, and biochemical characteristics. Useful morphological characteristics include the color of the colonies, the size and shape of the cells, the presence of a capsule around the cells, the production of hyphae or pseudohyphae, and the production of chlamydospores. Useful biochemical tests include the assimilation and fermentation of sugars and the assimilation of nitrate. Most yeasts of medical importance can be identified using one of the commercial test systems that are based on sugar assimilation of isolates. However, it is important to

remember that microscopic examination of cultures on cornmeal agar is essential to avoid confusion between organisms with identical biochemical profiles (see chapter 119).

The so-called black yeasts are not a formal taxonomic group, but the description is applied to a wide range of unrelated ascomycetous and basidiomycetous fungi that are able to produce melanized budding cells at some stage in their life cycle (5). Because most of these fungi are also able to produce a true mycelium, their routine identification is largely based on the morphological characteristics of the asexual spore-bearing structures and the manner in which the spores are produced. Most belong to the form-class Hyphomycetes (see below).

CLASSIFICATION AND IDENTIFICATION OF ANAMORPHIC MOLDS

Most of the septate molds that are isolated in clinical laboratories do not produce their sexual reproductive structures in routine cultures, and their identification is based on the manner in which the asexual spores are produced. Two artificial form-classes of anamorphic or "mitosporic" molds are currently recognized, based on the mode of conidium formation. The Hyphomycetes produce their conidia directly on the hyphae or on specialized conidiophores, while the Coelomycetes have more elaborate reproductive structures, termed conidiomata.

Form-Class Coelomycetes

Three artificial orders are recognized: the Sphaeropsidales, Melanconiales, and Pycnothyriales. In the Sphaeropsidales, the conidia are produced in conidiomata that are either spherical with an apical opening and with conidiogenous cells lining the inner cavity wall (termed pycnidia) or open and cup-shaped, in which case the conidiogenous cells cover the conidiomatal surface (termed acervuli). Few members of this class are common pathogens of humans. The most frequently encountered species is *Nattrassia mangiferae,* a plant pathogen which can also cause infections of the skin and nails. Until recently, this species was known by the synanamorph name of *Hendersonula toruloidea*. Another synanamorph of this species is *Scytalidium dimidiatum*.

Form-Class Hyphomycetes

The Hyphomycetes contain a large number of septate anamorphic molds of medical importance, including the genera *Aspergillus, Blastomyces, Cladophialophora, Fusarium, Histoplasma, Microsporum, Penicillium, Phialophora, Scedosporium,* and *Trichophyton*. In addition, numerous Hyphomycetes have been reported as occasional opportunistic pathogens of humans. For this reason, it is important for clinical microbiologists to be able to recognize and correctly identify this group of fungi.

As mentioned earlier, the process of conidiogenesis is of major importance in the identification of these molds. Two basic methods of conidiogenesis can be distinguished: thallic conidiogenesis, in which an existing hyphal cell is converted into one or more conidia; and blastic conidiogenesis, in which conidia are produced as a result of some form of budding process (for a detailed discussion of this topic, see reference 5).

Thallic Conidiogenesis

In this form of conidiogenesis the conidia are produced from an existing hyphal cell. Arthroconidia, which are derived from the fragmentation of an existing hypha, represent the simplest form of thallic conidiogenesis and have evolved many different groups of fungi. The first step in the examination of cultures of these molds should be to ascertain whether another spore form is present. If so, identification should be based upon that form. The few molds of medical importance which produce arthroconidia as their sole means of conidiogenesis include *Coccidioides* species.

Aleurioconidia are formed from the side or tip of an existing hypha, and during the initial stage before a septum is laid down, can resemble short hyphal branches. This form of thallic conidiogenesis is characteristic of the dermatophytes (*Epidermophyton, Microsporum,* and *Trichophyton* species), as well as a number of dimorphic systemic pathogens, including *Blastomyces dermatitidis, Histoplasma capsulatum,* and *Paracoccidioides brasiliensis*.

Blastic Conidiogenesis

Many fungi have evolved some form of repeated budding that allows them to produce large numbers of asexual spores from a single conidiogenous cell. There are two basic forms of blastic conidiogenesis: holoblastic development, in which all layers of the wall of the conidiogenous cell swell out to form the conidium, and enteroblastic development, in which the conidium is produced from within the conidiogenous cell, the outer layers of the cell wall breaking open and an inner layer extending through the opening to become the new spore wall. These two forms of blastic conidiogenesis can be further subdivided according to the details of spore development.

Almost all the molds that produce holoblastic conidia have melanized cell walls and thus are similar in colonial appearance. The morphological characteristics of their conidia and the manner in which the spores are produced serve as the main distinguishing features. Holoblastic conidia range in size from minute unicellular spores to large, thick-walled, multicelled spores. In some species, the first-formed conidium buds to produce a second, and the second produces a third, and so on until a chain of conidia is produced with the youngest at its tip. As each spore can produce more than one bud, a branching chain becomes possible (e.g., *Cladophialophora* species). In another group, the conidiogenous cell that produced the first spore then grows past it to produce a second. If this process is repeated, it will result in an elongated conidiogenous cell with numerous lateral single spores along its sides. This is termed sympodial development and is typical of species of *Bipolaris* and *Exserohilum*.

In molds that produce enteroblastic spores, the wall of the conidium is derived from the inner layer of the wall of the conidiogenous cell. This permits a succession of spores to be produced from the same point. There are two main forms of enteroblastic conidiogenesis: phialidic, in which the specialized conidiogenous cell from which the conidia are produced is termed a phialide, and annellidic, in which the conidiogenous cell is termed an annellide.

In phialidic conidiogenesis, the first blown-out cell breaks open at its tip and remains as a collarette, from the inside of which conidia are produced in succession. In some species, the collarette is distinct (e.g., *Phialophora* species), but in others it is almost invisible at the tip of the phialide. In some phialidic molds, such as species of *Fusarium* and *Acremonium,* the spores are not firmly attached to each other and often move aside to accumulate in a wet mass around the phialide. In other phialidic molds, such as species of *Aspergillus* and *Penicillium,* continuous replenishment of the inner wall of the tip of the phialide results in the formation of an unbranched chain of connected conidia, with the youngest at the base.

Annellides, like phialides, are conidiogenous cells which produce conidia at their tips in unbranched chains (e.g., *Scopulariopsis* species) or in wet masses (e.g., *Scedosporium* species). In annellidic conidiogenesis, the first blown-out cell becomes a spore and subsequent conidia are formed by blowing out through the scar of the previous one. Unlike phialides, annellides increase in length each time a new spore is produced. An old annellide that has produced many conidia has a number of apical scars or annellations at its tip.

IDENTIFICATION OF MOLDS

Most molds can be identified after growth in culture, but the criteria for recognition often differ from the fundamental characteristics that are used as a basis for classification. Macroscopic characteristics, such as colonial form, surface color, pigmentation, and growth rate, are often helpful in mold identification. Although the culture medium, incubation temperature, age of the culture, and amount of inoculum can influence colonial appearance and growth rate, these characteristics remain sufficiently constant to be useful in the process of identification. Molds that fail to sporulate in culture are often impossible to identify to the species level, and it is therefore important to select culture conditions which favor sporulation. Although molds often grow best on rich media, such as Sabouraud's dextrose agar, overproduction of mycelium often results in loss of sporulation.

COMMON MYCOLOGICAL TERMS

Acervulus (pl. -i): an open or cup-shaped structure on which conidia are formed.

Acropetal: describes a chain of conidia, in which new spores are formed at the tip of the chain.

Aleurioconidium (pl. -ia): a thallic conidium that is formed from the end of an undifferentiated hypha, or from a short side-branch.

Aleuriospore: see aleurioconidium.

Anamorph: the asexual form of a fungus.

Annellide: a specialized conidiogenous cell from which a succession of spores is produced, and which has a column of apical scars at its tip.

Annelloconidium (pl. -ia): a blastic conidium that is formed from an annellide.

Annellospore: see annelloconidium.

Apophysis: the enlargement of a sporangium below the columella.

Arthroconidium (pl. -ia): a thallic conidium produced as the result of fragmentation of an existing hypha into separate cells.

Arthrospore: see arthroconidium.

Ascocarp: a structure that contains asci.

Ascoma (pl. -ata): see ascocarp.

Ascospore: a haploid spore produced within an ascus following meiosis.

Ascus (pl. -ci): a thin-walled sac, characteristic of the Ascomycota.

Aseptate: without cross-walls or septa.

Ballistoconidium: a conidium that is forcibly discharged.

Ballistospore: see ballistoconidium.

Basidiocarp: a structure that produces basidia.

Basidioma (pl. -ata): see basidiocarp.

Basidiospore: a haploid spore produced on a basidium following meiosis.

Basidium: a cell upon which basidiospores are produced, characteristic of the Basidiomycota.

Basipetal: describes a chain of conidia in which new spores are formed at the base of the chain.

Blastic: one of the two basic forms of conidiogenesis in which enlargement of the conidial initial occurs before a delimiting septum is laid down.

Blastoconidium (pl. -ia): a blastic conidium produced by the enlargement of a part of a conidiogenous cell before a septum is laid down.

Blastospore: see blastoconidium.

Catenate: in chains.

Chlamydospore: a resting conidium, formed as a result of the enlargement of an existing hyphal cell.

Collarette: a cup-shaped structure at the tip of a conidiogenous cell.

Columella: swollen tip of the sporangiophore projecting into the sporangium in some Mucorales.

Conidiogenesis: the process of conidium formation.

Conidiogenous cell: any cell that produces or becomes a conidium.

Conidioma (pl. -ata): a specialized conidia-bearing structure.

Conidiophore: a specialized hypha or cell on which, or as part of which, conidia are produced.

Conidium (pl. -ia): an asexual spore.

Distoseptate: term used to describe spores in which the individual cells are each surrounded by a sac-like wall distinct from the outer wall.

Enteroblastic: a form of conidiogenesis in which conidia are produced from within a conidiogenous cell.

Heterothallic: a self-sterile fungus; sexual reproduction cannot take place unless two compatible mating strains are present.

Hilum: a scar at the base of a conidium.

Holoblastic: a form of conidiogenesis in which both the inner and outer walls of the conidiogenous cell swell out to form the conidium.

Homothallic: a self-compatible fungus; sexual reproduction can take place within an individual strain.

Hypha (pl. -ae): one of the individual filaments that make up the mycelium of a fungus.

Macroconidium (pl. -ia): the larger of two different sizes of conidia produced by a fungus in the same manner.

Microconidium (pl. -ia): the smaller of two different sizes of conidia produced by a fungus in the same manner.

Mitosporic: term used to describe an anamorphic fungus.

Mold: a filamentous fungus.

Mycelium: a mass of branching filaments which make up the vegetative growth of a fungus.

Oospore: a sexual spore produced in the Oomycota.

Phialide: a specialized conidiogenous cell from which a succession of spores is produced.

Pleoanamorphism: term used to describe a fungus that has more than one anamorph.

Pleomorphic: term used to describe a nonsporing strain of a fungus.

Pseudohypha (pl. -ae): a chain of yeast cells which have arisen as a result of budding and have elongated without becoming detached from each other, forming a hypha-like filament.

Pycnidium (pl. -ia): an enclosed structure with an apical opening inside which conidia are produced.

Rhizoid: a short branching hypha that resembles a root.

Septate: having cross-walls or septa.

Septum (pl. -ta): a cross-wall in a fungal hypha or spore.

Sporangiole: a small sporangium.

Sporangiophore: a specialized hypha upon which a sporangium develops.

Sporangiospore: asexual spore, characteristic of the Zygomycota.

Sporangium (pl. -ia): a closed sac containing asexual spores, characteristic of the Zygomycota.

Synanamorph: term applied to any one of two or more anamorphs which have the same teleomorph.

Teleomorph: the sexual form of a fungus.

Thallic: one of the two basic forms of conidiogenesis in which enlargement of the conidial initial occurs after a delimiting septum has been laid down.

Thallus: the vegetative growth of a fungus.

Yeast: a unicellular, budding fungus.

Zoospore: a motile asexual spore.

Zygospore: a thick-walled sexual spore, produced in the Zygomycota.

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Specimen Collection, Transport, and Processing: Mycology

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The collection, transport, and processing of clinical specimens encompass some of the most important considerations in determining the etiology of fungal disease. Only with the appropriate handling of specimens can the recovery of fungal organisms be clearly associated with a disease process. As inappropriate specimen collection may introduce potentially confusing indigenous flora and jeopardize culture result interpretation (48), it is critical to employ only those practices most likely to facilitate detection of etiologic agents. This chapter is thus devoted to obtaining, transporting, and processing clinical specimens in a manner consistent with this aim. Diagnostic procedures with direct specimens, including antigen detection, monoclonal antibody immunoassays, and molecular techniques, are addressed elsewhere.

SPECIMEN COLLECTION

Safety Policies Regarding Specimen Management

Specimen collection considerations include safety management policies for the protection of all health care workers involved in obtaining the specimen, collection from appropriate sites, the proper labeling of specimen containers, and completion of necessary requisitions accompanying the specimens (4, 16, 21, 26, 28, 32, 37, 45, 46, 47, 49, 60, 62, 75). As specimens are usually collected in a surgical suite, at the bedside, or in an outpatient setting, it is the laboratory's responsibility to provide these collection areas with appropriate instructions, usually in the form of a specimen collection procedure manual. All specimens for mycologic studies should be considered potentially hazardous, and universal precautions should be observed with all specimen collection procedures (49, 50, 66). Primary specimen collection containers must be leakproof and should have a separate compartment for the requisition. Syringes containing aspirates must have the needle removed and be recapped prior to transport.

Common Specimen Collection Sites and Guidelines

The more common collection sites for the diagnosis of mycotic disease are listed in Table 1. As for all disease processes, the best specimen for determining the etiologic agent is at the active infection site. Specimens peripheral to the primary locus, however, such as blood in hematogenously disseminated infections, may also provide a diagnosis. The recovery of ubiquitous fungal organisms from specimens likely to harbor contaminants, such as sputum samples, should be interpreted with caution, and collection of these specimens should be discouraged in favor of more disease-directed sites. The collection details for fungal cultures are very similar to those for bacterial cultures, although the volume of material required for recovery of fungi may exceed those necessary for bacteria. More material for primary inoculum and concentration of large volumes of fluid greatly increases the likelihood of recovery of fungal species. The use of swab cultures should be limited to the sites indicated in Table 1, as deeper specimens are preferred in most settings. Specimens to be discouraged due to questionable usefulness include superficial cultures of burns, wounds, decubiti, periodontal lesions, perirectal abscesses, varicose ulcers, and gangrenous lesions, as well as Foley catheter tips, gastric aspirates of newborns, lochia, vomitus, or colostomy discharge material. Nail specimens for culture should be thoroughly disinfected with 70% alcohol before collection.

Requisitions and Specimen Labeling

A properly constructed and completed requisition form is critical for the mycology laboratory to elicit the necessary information and to properly analyze the specimen for optimal patient care. The requisition should include the following: patient name, age, sex, room number or address; physician name and address or how he/she may be contacted; specific anatomic culture site; date and hour of specimen collection; the clinical diagnosis, special culture request, and relevant patient history; special procedures used to obtain the specimen; name of individual transcribing the orders; and any antifungal agents the patient has recently or is currently receiving. This requisition must accompany the specimen. The specimen must be identified with a firmly attached label indicating the patient's name, hospital number, room number, physician, culture site, and date and time of collection. Specimens thought to contain particularly dangerous organisms should be marked appropriately.

SPECIMEN TRANSPORT AND STORAGE

In addition to proper collection, the appropriate transport and storage of specimens is necessary for fungal elements to remain viable. Guidelines are indicated in Table 1, as well as

TABLE 1 Common specimen collection sites for recovery of fungi[a]

Specimen type[b] (reference)	Collection		Transport[c]	Comments
	Guidelines	Device and/or minimum vol	Local/distant time and temp	
Blood				
Yeasts (1, 2, 7, 8, 10, 13–15, 17, 34, 37–39, 40, 42, 52–54, 57, 59, 60, 62, 64–66, 69, 71–74, 76)	Collect as for bacterial cultures; disinfect skin with tincture of iodine. Use maximum amt of blood recommended.	Automated (BacT/Alert, BACTEC, ESP)	≤2 h, RT/ ≤24 h, RT; follow manufacturers' instructions.	bioMérieux, BD Diagnostic Systems, Accumed International
		Lysis-centrifugation (Isolator or manual)	As above; process in ≤16 h	Wampole
		Biphasic bottles (Septi-Chek)	≤2 h, RT/ ≤24 h, RT	BD Diagnostic Systems
Dimorphics/filamentous fungi (8, 9, 13, 38, 72)	As above	Lysis-centrifugation (Isolator or manual)	As above	As above
CSF (57)	Collect as for bacterial culture.	Screw-cap tube, ⩾2 ml, concentrate	≤15 min, RT or 30°C/ ≤24 h, RT	Never refrigerate; aspirate or biopsy of brain abscess may be necessary.
Sterile fluids (pericardial, peritoneal, pleural, and pynovial)	Collect as for bacterial cultures. Concentrate by centrifugation; use sediment for inoculation.	Sterile container with >10 ml; may use blood culture bottle for yeasts.	≤15 min, RT/ ≤24 h, 4°C	May also concentrate small volumes by syringe filtration
Bone marrow	Prepare site for surgical incision.	Lysis-centrifugation (Isolator or manual)	As above	Pediatric Isolator
Catheter, i.v. (41, 46)	Collect as for bacterial culture.	5-cm distal tip of catheter into a sterile tube with a few drops of sterile H_2O/saline	≤15 min, RT or ≤ 24 h, 4°C	Acceptable i.v. catheters for semiquantitative culture (Maki et al. method): central, CVP, Hickman, Broviac, Swan-Ganz, arterial, peripheral, hyperalimentation, umbilical
Ear, external	Firmly rotate swab in outer ear canal.	Swab	≤2 h, RT/ ≤24 h, 4°C	
Eye				
Corneal scrapings (3, 34)	Inoculate scrapings directly onto media and slides for staining.	Direct inoculation	≤15 min, RT/ ≤24 h, RT	Inoculate plates in "C" shape.
Vitreous fluid (3, 34)	Needle aspiration	Screw-cap tube or direct inoculation	≤15 min, RT/ ≤24 h, RT	
Hair	With forceps, collect 10–12 hairs with shaft intact.	Dry container, envelope	≤72 h, RT/ ≤72 h, RT	Also scrape active borders of lesions if present.
Medical devices	Collected surgically	Sterile container with a few drops of sterile H_2O/saline: gently scrape device with a sterile scalpel to dislodge biofilm material before direct inoculation into liquid medium.	≤15 min, RT	Valves, hardware, other devices

(Continued on next page)

TABLE 1 Common specimen collection sites for recovery of fungi[a] *(Continued)*

Specimen type[b] (reference)	Collection		Transport[c]	Comments
	Guidelines	Device and/or minimum vol	Local/distant time and temp	
Nails	Disinfect with 70% alcohol; clip affected areas; collect debris under nail.	Dry container, envelope	≤72 h, RT/ ≤72 h, RT	For *Candida,* dermatophytes and agents of dermatomycoses
Prostatic fluid (46)	Bladder emptied followed by prostatic massage	Direct inoculation onto media	≤15 min, RT/ ≤24 h, RT	Primarily for blastomycosis
Respiratory, lower BAL, brush, wash, aspirate, sputum (5)	Collect as for bacterial culture.	Sterile container, >1 ml	≤2 h, RT/ ≤24 h, 4°C	Short survival time for thermally dimorphic pathogens
Respiratory, upper				
Oral (11, 58)	Swab active lesions; saline swish for *Candida.*	Swab or sterile container	≤2 h, RT/ ≤24 h, RT	Selective media useful for yeasts
Nasal sinus	Surgical removal of sinus contents	Direct inoculation or in sterile, moist gauze	≤15 min, RT/ ≤24 h, RT	Maxillary and ethmoid sinuses most common sites
Skin (64)	Disinfect with 70% alcohol; scrape surface of skin at margin of lesion.	Clean container or between taped glass slides	≤72 h, RT/ ≤72 h, RT	For dermatophytes and agents of dermatomycoses
Subcutaneous granules	Collect from pus, exudate, biopsy specimen, draining sinus tracts.	Wash granules several times in saline containing antibiotics.	≤2 h, RT/ ≤24 h, RT	Fungal grains or granules seen in eumycotic mycetoma
Subcutaneous sites				
Abscess (63)	Aspirate abscess with sterile needle and syringe.	Anaerobic transport or direct inoculation; ≥1 ml	≤2 h, RT/ ≤24 h, RT	Sample base of lesion and abscess wall.
Open wound (63)	Aspirate or swab deeply.	Swab transport system	≤2 h, RT/ ≤24 h, RT	As above
Tissue biopsies	Surgical collection	Sterile container; add a few drops of sterile saline to keep moist.	≤15 min, RT/ ≤24 h, RT	Punch biopsies may be used for skin lesions.
Urine (12)	As for bacterial cultures; early-morning specimen preferred	Sterile, wide-mouth container, ≥1 ml	≤2 h, RT/ ≤24 h, 4°C	Use midstream collection technique.
Vagina (19)	As for bacterial cultures	Swab transport	≤2 h, RT/ ≤24 h, RT	Primarily for refractory vaginal candidiasis

[a] Abbreviations: CSF, cerebrospinal fluid; RT, room temperature; i.v., intravascular; BAL, bronchoalveolar lavage fluid.
[b] Specimen collection sites are not all-inclusive. Other specimen types are generally collected as for bacterial cultures.
[c] All specimens are to be transported in leakproof plastic bags having a separate compartment for the requisition. The optimum time for arrival of most specimens from the hospital floor to the laboratory (local) is within 2 h (see exceptions) so as to maintain viability and guard against desiccation of small tissue fragments or specimens not protected by adequate transport systems. Specimens submitted from distant sites should be sent within appropriate transport devices and should generally be maintained at room temperature. Exceptions include 30°C storage of CSF specimens and 4°C extended storage for specimens likely to be bacterially contaminated.

in other areas of this Manual pertaining to specimen collection. Fungal viability may be affected by excessive heat and cold; thus, room temperature transport and storage, ideally within 2 h, is recommended. Exceptions include 30°C storage of central nervous system specimens and 4°C extended storage for specimens likely to be bacterially contaminated. A clean, dry envelope may be used for submitting skin, hair, and nail specimens.

SPECIMEN PROCESSING

Specimen Acceptability

Unacceptable specimens should not be processed. These may include specimens from inappropriate sites, specimens in leaking collection containers, and specimens/requisitions lacking necessary patient/specimen information. Physicians should be notified immediately of any rejected specimens

TABLE 2 Pretreatment of clinical specimens before plating

Specimen	Pretreatment	Comments
Blood	Lysis and centrifugation (30 min) at 3,000 × *g*, 35° fixed-angle rotor, or 2,400–3,000 × *g*, swinging bucket	Method of choice, and necessary for *H. capsulatum*
Body fluids	Filtration or centrifugation at 2,000 × *g* for 10 min	Necessary for optimal recovery; used on all specimens >2 ml
Exudates, pus, drainage	Washing, centrifugation, and crushing of granules	Recommended if granules are present
Medical devices	Gentle scraping of device with a sterile scalpel to dislodge biofilm material	
Nails	Cutting into small pieces	Necessary for optimal recovery of fungi
Respiratory secretions	Liquefication with mycolytic agent	Necessary (with centrifugation) on induced sputum specimens for *P. jiroveci.* Optional for other mycoses; centrifugation may improve yield but also increases numbers of interfering organisms, e.g., bacteria and colonizing yeasts.
Tissue	Mincing, grinding, or use of Stomacher	Necessary for optimal recovery of all fungi; specimens must be cut into small pieces if a zygomycete is suspected or ground if *H. capsulatum* is suspected.
Urine	Centrifugation at 2,000 × *g* for 10 min	Necessary for optimal recovery, especially for agents of systemic mycoses

and apprised of the compromised nature of the results if specimens are processed. If a new specimen is received, the unacceptable one should be autoclaved and discarded (46, 47). In addition to being analyzed by direct microscopy, antigen detection procedures, and/or direct molecular methods, all acceptable specimens should be cultured for recovery and identification of the fungal organisms. These results should be correlated with those of smear/tissue findings to confirm the etiologic agent.

Properly collected, identified, and transported specimens should be processed as soon as possible after reaching the laboratory. Most can be stored at 4°C, with a few exceptions identified below (39, 40). Pretreatment of selected specimens is necessary to optimize fungal recovery. These procedures are outlined in Table 2 and include centrifugation (concentration of fungal elements), blood cell lysis (releasing fungal elements from within cells), and slicing or maceration (to increase the surface area of the specimen).

The handling and culture of specimens should be done in a certified, laminar-flow biologic safety cabinet whenever possible. Both plates and tubes may be used; however, plates provide a larger surface area and are more useful for isolation of colonies. A volume of 40 ml retards dehydration, and surrounding the plate with a shrink seal or air-permeable tape prevents the inadvertent opening of the petri dish. If tubes are used, they should have a large slant area, have the screw cap loose for proper aeration, and be incubated horizontally for the first 24 h to ensure that the inoculum remains on the slant.

Specimen Pretreatment, Culture, and Processing Guidelines

Blood

Disseminated fungal infections are more prevalent than previously recognized, and blood cultures are an important diagnostic tool in the absence of focal manifestations or when foci are not easily reached (18, 22, 70). While several systems for the detection of fungemia are available, the volume of blood obtained for culture is still the most important factor in recognizing bloodstream infections, with 20 to 30 ml per culture being optimal for maximal recovery of microorganisms (70). The ratio of blood to broth is also critical, with a ratio near 1:5 enhancing detection (2, 61). If blood culture bottles are used, a biphasic system is preferred (Septi-Chek; BD Diagnostic Systems, Sparks, Md.). Several automated continuously monitoring blood culture systems are also available, with a variety of media modifications to enhance fungal recovery. These include the ESP system (Trek Diagnostics, Inc., Cleveland, Ohio), the BacT/Alert system (bioMérieux, Durham, N.C.), and the Bactec system (BD Diagnostic Systems). The salient features of each instrument are explained in chapter 15 regarding automated systems for the detection and identification of clinically significant microorganisms. All the above continuously monitoring blood culture systems provide adequate detection of bloodstream infections due to most *Candida* species and *Cryptococcus neoformans* (35, 42, 43, 55), with a higher inoculum providing earlier detection (23). Recent studies evaluating the BACTEC 9240 and the BacT/ALERT 3D systems suggest that, for *Candida glabrata,* incubation times beyond 5 days may be required, terminal subcultures may be necessary, and selective fungal media provide enhanced detection (29, 30, 44). The commercially available lysis-centrifugation system, the Isolator (Wampole Laboratories, Princeton, N.J.), or manual lysis-centrifugation (9) appears to be the more sensitive method for recovery of *Histoplasma capsulatum,* other dimorphic pathogens, and filamentous fungi (24, 25, 27, 39). Its combination with the BACTEC MYCO/F Lytic bottle may be useful as a selective fungal blood culture method (67). Isolator specimens should remain at room temperature until

processing, optimally within 16 h of collection. The sediment from lysis-centrifugation should be streaked onto a variety of enriched media not containing cycloheximide. A recent evaluation of chocolate agar suggests its use for recovery of most fungi, excluding *H. capsulatum* (56). Plates for *H. capsulatum* may require up to 3 weeks of incubation. The introduction of the BACTEC MYCO/F Lytic medium for the detection of mycobacteria has also proved to be useful for the detection of fungemia (68). While normally used with the BACTEC 9240 blood culture system, it may also be used independently and requires only 5 ml of blood. In a study to detect fungemia in less-developed countries, growth was detected by fluorescence caused by shining UV light ($\lambda = 365$ nm) onto the indicator on the bottom of the bottle, and this method appeared to be as sensitive as the Septi-Chek bacterial system or the Isolator concentrate plated onto chocolate or Inhibitory Mold Agar (1).

Bone Marrow

Heparinized bone marrow may be inoculated onto enriched media at the bedside, but preferably into pediatric Isolator tubes. At least 0.5 ml of aspirated marrow is required. Lysis-centrifugation enhances fungal recovery, particularly for *H. capsulatum* and other filamentous fungi. Blood culture bottles are not recommended for bone marrow culturing.

Intravascular Catheter Tips

Intravascular catheter tip cultures should be performed only in conjunction with concurrent blood cultures. The most common technique utilized is the semiquantitative method of Maki et al. (41), in which a 5-cm distal portion of the catheter is rolled across a blood agar plate four times and the growth of more than 15 colonies is considered significant. Acceptable intravascular catheters include the Hickman, Broviac, Swan-Ganz, arterial, peripheral, central venous, hyperalimentation, and umbilical catheters, as well as other less frequently submitted types.

Medical Devices

Various medical devices may be submitted for culture. These are collected surgically and should be transported immediately. They may include heart valves, artificial joints, intrauterine devices, and other prosthetic devices or hardware. Any obvious areas of biofilm formation or vegetation should be gently scraped with a sterile scalpel before direct inoculation into a broth medium.

Sterile Body Fluids

Body fluids that are normally sterile include pericardial, peritoneal, synovial, and cerebrospinal fluids. Small volumes (less than 2 ml) may be inoculated directly or filtered through a membrane filter. The filter is placed particle side down and is periodically moved to another area on the medium to examine for growth. Specimens greater than 2 ml require centrifugation at $2{,}000 \times g$ for 10 min, with 1 to 2 ml of the sediment being used for the inoculum. Sterile fluids may be refrigerated before processing, but cerebrospinal fluids should be maintained at room temperature.

Tissue

Most tissue requires pretreatment before processing. Tissue for *H. capsulatum* should be ground or homogenized, and the fragments/homogenates should be inoculated onto enriched media containing antibacterial agents. Media containing cycloheximide may be included if contaminating organisms are anticipated. Tissue suspected of containing zygomycetes or other fungi must be minced or finely sliced, rather than ground, as grinding is detrimental to the survival of zygomycetous fungal elements.

Exudates, Pus, and Drainage

Drainage specimens should be examined for grains or granules under a dissecting microscope. If none are present, material may be inoculated directly onto isolation media. If granules are present, they should be teased apart, crushed between two glass slides, and examined microscopically for fungal hyphae. The granules in the remainder of the specimen should be washed in sterile distilled water, crushed in a sterile manner, and inoculated onto a variety of media with and without cycloheximide. Pus and exudates may be plated directly on the media indicated above.

External Eye Specimens

Specimens from patients with presumed mycotic keratitis should be obtained by an ophthalmologist and inoculated directly onto the medium and an alcohol-cleaned, flamed slide. Corneal scrapings are transferred from the surgical instrument to a noninhibitory agar plate by making a series of C- or X-shaped cuts on the medium. Inoculated plates are maintained at room temperature if transport to the laboratory is delayed.

Upper Respiratory Tract

Oropharyngeal candidiasis is readily diagnosed by smear and culture. The newer selective, chromogenic agars for *Candida* species provide preliminary identification as well as isolation and are useful for detecting infections with more than one species of *Candida.* Nasal sinus contents obtained surgically should be plated on a variety of media containing antimicrobics but lacking cycloheximide, as several agents of fungal sinusitis are inhibited by cycloheximide.

Lower Respiratory Tract

Sputum should be collected as a first early-morning sample after the patient's teeth have been brushed and the mouth has been rinsed. Flecks containing pus, blood, or caseous material should be sought and used in culture and smears. Very viscous specimens can be liquefied with crystalline *N*-acetyl-*l*-cysteine, but sodium hydroxide (used for decontamination of cultures for mycobacteria) must not be used because it inhibits the growth of many fungi. Surgically collected specimens such as bronchoalveolar lavage fluids, washes, and aspirates should also be examined for bloody, purulent, or mucus-laden portions and plated onto antibacterial media with and without cycloheximide. Centrifugation may increase yield but also increases the number of interfering organisms, e.g., bacteria and colonizing yeasts.

Urine

Urine samples (10 to 50 ml) are centrifuged, and the sediment is inoculated onto antibacterial media with and without cycloheximide. If quantification is required, uncentrifuged urine can be streaked with a calibrated loop onto a plate of noninhibitory medium. The quantity of yeasts in a urine sample does not have the same interpretative value as that of bacteria. The persistence of yeast in several carefully collected midstream urine samples and the clinical picture of the patient in whom candiduria of any count develops are the most important factors in interpreting its significance (20).

Vaginal Secretions

Vaginal candidiasis is usually diagnosed by clinical symptoms and direct microscopic examination. As yeast cells are part of the normal vaginal flora in up to 20% of healthy women, the

recovery of *Candida* on culture without considering other factors is meaningless. Cultures are useful, however, in monitoring therapy in refractory disease. Antibacterial media with and without cycloheximide should be inoculated (19).

Hair, Skin, and Nails

Hair fragments and skin scrapings are placed directly on the agar surface. Nails yield the best culture results if they are cut into smaller pieces with a scalpel before culturing. Light pressure should be applied to submerge pieces slightly under the agar surface. Media containing both antibiotics and cycloheximide should be employed for the recovery of dermatophytes. Stored cultures should be maintained at room temperature rather than 4°C, as some dermatophytes do not tolerate refrigeration. Specimens may also be mailed utilizing a clean, dry envelope.

Media for Primary Isolation

Table 3 lists a variety of media that are suitable for primary isolation and that are all commercially available. Many other media, including homemade varieties, may perform equally well, and their choice may depend on availability, cost, and personal preference. Some basic tenets, however, apply to all media used for primary recovery. A medium that supports

TABLE 3 Media for primary isolation

Type of medium[a]	Additive	Use and comments
Without antibacterial or antifungal agents		
SDA	None	Classic pigment and colony formation for dermatophytes
BHI agar	Sheep blood optional	Cultivation of all fungi including fastidious dimorphics; blood inhibits conidiation.
Sabhi agar	Sheep blood optional	Cultivation of all fungi including fastidious dimorphics; blood inhibits conidiation.
PDA or PFA	None	Production of conidia and fruiting bodies; particularly useful for filamentous, opportunistic fungi.
Any of the above	Olive oil on surface	Cultivation of *Malassezia furfur*
With antibacterial agents		
Any of the above	Separate or in combination: chloramphenicol, ciprofloxacin, gentamicin, penicillin, streptomycin	Inhibition of bacteria; BHI agar, Sabhi, and IMA are more enriched than other media and may better support the more fastidious fungi.
IMA	Contains chloramphenicol.	
With antibacterial and antifungal agents		
Mycosel or Mycobiotic agar	SDA with chloramphenicol and cycloheximide	Inhibition of bacteria and many saprobic fungi that may overgrow more fastidious fungi; may also inhibit opportunistic etiologic agents. Useful for dermatophyte recovery
BHI agar or Sabhi	Any antibacterial agent in previous group plus cycloheximide	As above
Selective for dimorphic fungi		
Yeast extract phosphate medium	Add ammonia to surface.	Inhibition of bacteria, yeasts, and many molds and enhancement of slow-growing dimorphic fungi
For dermatophytes		
DTM	Chloramphenicol, gentamicin, cycloheximide	Both are meant to isolate dermatophytes and show a color change of the medium; some nondermatophytic fungi may also produce a color change.
DIM	Penicillin, streptomycin, cycloheximide, with bromcresol purple	
Mycosel or Mycobiotic	As above	
Selective and differential for yeasts		
CHROMagar Candida, Albicans ID	Antimicrobial agents and chromogenic substrates	Isolation of yeasts with color development in colonies allows for presumptive identification of some common species of *Candida*.

[a] SDA, Sabouraud dextrose agar; BHI, brain heart infusion agar; PDA, potato dextrose agar; PFA, potato flakes agar; IMA, inhibitory mold agar; DTM, dermatophyte test medium; DIM, dermatophyte identification medium.

fungal growth while inhibiting bacteria must be used with specimens from nonsterile sites. The most commonly used media are brain heart infusion (BHI) plus antibiotics, Sabhi agar plus antibiotics, or inhibitory mold agar (IMA). Antibiotics that have been traditionally added, alone or in combination, include chloramphenicol (<16 μg/ml), gentamicin (5 to 100 μg/ml), penicillin (20 U/ml), streptomycin (40 μg/ml), and ciprofloxacin (5 μg/ml). As antibacterial agents inhibit actinomycetes, they should not be included when these organisms are suspected.

Various media may also be made selective by incorporation of the eukaryotic protein synthesis inhibitor cycloheximide. While cycloheximide (0.5 μg/ml) suppresses many saprobic fungi, it also inhibits several clinically significant fungal pathogens, namely, *C. neoformans, Trichosporon asahii, Nattrassia mangiferae (Scytalidium dimidiatum),* some species of *Candida* and *Aspergillus,* and most zygomycetes. Cycloheximide is traditionally added to Sabouraud's dextrose agar (SDA), Emmons modification with 2% dextrose at neutral pH, or Sabhi agar, in combination with the antibacterial agent chloramphenicol. In facilities where a large portion of the patient population may be neutropenic or immunocompromised, the rapid identification of opportunistic fungi, which may quickly disseminate in these individuals, is critical. The addition of cycloheximide and/or antibiotics to potato dextrose agar (PDA) or potato flakes agar (PFA) may be more appropriate in this setting, because it facilitates identification of these organisms by providing plates for tape mounts of diagnostic structures from primary media.

A battery of enriched media is generally employed for the recovery of fastidious thermally dimorphic fungi. The most common include BHI and Sabhi augmented with antibacterial agents and 5 to 10% sheep cells. While sheep cells appear to improve the yield of organisms slightly, they also inhibit conidiation. Dimorphic organisms recovered on media containing blood should be subcultured to blood-free media for macroscopic/microscopic morphology and DNA probes. The addition of ammonium hydroxide to the medium may facilitate recovery of fastidious fungi from heavily contaminated specimens. Chromogenic media (CHROMagar Candida [CHROMagar, Paris, France], BD CHROMagar Candida [Becton Dickinson BBL, Cockeysville, Md.], and Candida ID [BioMérieux, Hazelwood, Mo.]) are useful for the recovery/identification of *Candida* species (33). The addition of fluconazole to the media facilitates determining fluconazole resistance in these species (6, 51, 54). CHROMagar may also be reliably used for direct isolation of yeast from blood cultures (31). Additional media for isolation/identification of specific organisms include media containing substrates L-dopa, caffeic acids, etc., for detection of *C. neoformans* by its phenol oxidase activity, media containing or overlaid with a long-chain fatty acids source (e.g., olive oil) for the recovery of *Malassezia furfur,* or dermatophyte test medium (DTM) or dermatophyte identification medium (DIM) specifically designed for the culture and isolation of dermatophytes. Refer to chapter 117 for more detailed information on media.

Incubation Conditions

All cultures should be incubated at ≤30°C (±1°C), as this temperature is optimal for most medically significant fungi. If 30°C is not available, they should be incubated at room temperature (near 25°C). While routine incubation has traditionally been for 4 weeks, a recent study suggests that 3 weeks may be adequate for most clinically relevant isolates, excluding those from skin, hair, and nail or from specimens for the recovery of dimorphic pathogens (36). Specimens being screened primarily for yeasts, e.g., oropharyngeal and vaginal sites, need to be incubated for only 7 days. Cultures should be examined at least every 2 or 3 days for the first 2 weeks and weekly thereafter.

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Reagents, Stains, and Media: Mycology

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The medical mycology laboratory uses a variety of stains, media, and reagents for the isolation and identification of yeasts and molds. Familiarity with the composition and characteristics of these materials is critical to the diagnostic approach when processing specimens from patients with suspected mycotic disease. The direct microscopic examination of properly stained clinical material is rapid and cost-effective and may denote the presumptive etiologic agent (4, 5, 14). Results of direct examination can also guide the laboratory in the selection of media that best support fungal growth in vitro (15). Stained preparations made from fungal cultures are essential for definitive identification.

A plethora of media are available for the primary inoculation and cultivation of fungi from clinical specimens. No one specific medium or combination of media is adequate for all specimens. Media should be carefully selected based on specimen type and suspected fungal agents. Media can be dispensed into containers such as 25- by 150-mm screw cap tubes or 100-mm-diameter petri dishes. Petri plates offer the advantage of a large surface area for isolation and dilution of inhibitory substances in the specimens, but they must be poured thick, with at least 25 ml of medium, in order to resist dehydration during incubation. Because plates are vented, they are more likely to become contaminated during incubation. Plates may be placed in gas-permeable bags or sealed with gas-permeable tape to offset this disadvantage. Plates must be opened only within a biological safety cabinet to prevent contamination of the plate and exposure of personnel to potentially dangerous fungi. Media in tubes have a smaller surface area but offer maximum safety and resistance to dehydration and contamination. If the specimen is from a contaminated site, it is important to include media that contain inhibitory substances such as chloramphenicol, gentamicin, or cycloheximide. Chloramphenicol or gentamicin inhibits most bacterial contaminants, while cycloheximide inhibits most saprobic molds. Remember that cycloheximide may also inhibit opportunistic fungi such as some species of *Aspergillus, Fusarium, Scopulariopsis, Pseudallescheria,* zygomycetes, some dematiaceous fungi, and yeasts such as *Cryptococcus neoformans* and some *Candida* species. Antibacterial agents may inhibit the growth of aerobic actinomycetes like *Nocardia* spp. It is important to use media with and without inhibitory agents. Specimens from normally sterile sites can be inoculated to media without inhibitory substances.

Each new lot of medium, whether purchased or prepared in-house, must be subjected to a quality control protocol that verifies appearance, pH, and performance (7). Media for primary isolation should be tested for optimal growth of several fungal pathogens. Selective media should be tested with strains known to be sensitive and resistant to the inhibitory agents in the media, while differential media should be evaluated with fungi that produce both positive and negative reactions.

Unless stated otherwise, the reagents and media listed in this chapter should be prepared by dissolving the components in the stated liquid with a magnetic stirring bar. The standard sterilization technique of autoclaving at 121°C at 15 lb/in^2 for 15 min should be used when needed. However, certain solutions such as those containing antibiotics or carbohydrates cannot be autoclaved because they will be denatured. These solutions are sterilized by filtration through a 0.22-μm-pore-size filter.

Storage of prepared reagents in sterile, airtight, screw-cap containers is recommended. Some reagents require storage in dark containers, and some need to be stored refrigerated (2 to 8°C) instead of at room temperature. Special storage instructions are given when appropriate. Standard safety precautions should be taken when preparing the reagents. Follow the safety guidelines for the chemicals being used, in addition to the laboratory safety protocols. For reagents that are prepared in-house, proper quality control measures must be taken with appropriate positive and negative controls.

The stains, media, and reagents listed in this chapter include those commonly used and a few specialized items. For more specific information not included here, refer to literature cited in the chapter.

REAGENTS

■ *N*-Acetyl-L-cysteine (NALC) (0.5%)

NALC (Alpha Tec Systems, Inc.) is a mucolytic agent used for digestion of sputum specimens submitted for detection of *Pneumocystis jirovecii* by microscopic examination. Sodium citrate (0.1 M) is included in the mixture to exert a stabilizing effect on the acetylcysteine.

■ Dithiothreitol (sputolysin), 0.0065 M

Dithiothreitol is a mucolytic agent that can be purchased commercially and has also been used to prepare sputum specimens for detection of *P. jirovecii.* Equal volumes of sputum and dithiothreitol are mixed and incubated at 35°C. The mixture is periodically mixed vigorously until it is *almost* completely liquefied (complete liquefaction disperses the cells of *P. jirovecii*, making microscopic detection difficult).

■ Potassium hydroxide (KOH)

Wet mounts prepared in 10% KOH are used to distinguish fungi in thick mucoid specimens or in specimens with keratinous material such as skin, hair, or nails. The proteinaceous components of the host cells are digested partially, leaving the fungal cell wall intact and more apparent. An aliquot of specimen is added to a drop of 10% KOH, which can be preserved with 0.1% thimerosal (Sigma Chemical Co.). The slide is held at room temperature for 5 to 30 min after the addition of KOH, depending on the specimen type, to allow digestion to occur. Digestive capabilities can be enhanced with gentle heating or the addition of 40% dimethyl sulfoxide.

■ Potassium hydroxide (10%) with LPCB

The wet mount with KOH and lactophenol cotton blue (LPCB) is used for the same purposes as the KOH preparation but incorporates the LPCB dye (see below). LPCB enhances the visibility of fungi because aniline blue stains the outer cell wall of fungi and lactic acid acts as an additional clearing agent. The phenol component in LPCB acts as a fungicide.

■ Sodium hydroxide (10 or 25% with added glycerin)

Solutions of sodium hydroxide may be used as alternatives to potassium hydroxide for the direct microscopic examination of hair, skin, and nails for dermatophytic infections. Visualization of fungal elements may be enhanced by the addition of glucan-binding fluorescent brighteners such as calcofluor white or Congo red.

STAINS

■ Alcian blue stain

Alcian blue and the more commonly used mucicarmine stain (see below) are mucopolysaccharide stains. These stains are very useful for visualizing *C. neoformans* in histological sections of tissue.

Basic procedure

Deparaffinized sections are stained in Alcian blue (1 g in 100 ml of acetic acid, 3% solution) for 30 min, washed in running tap water, and then rinsed in distilled water. The sections are counterstained in nuclear fast red (0.1 g in 100 ml of aluminum sulfate, 5% solution). After dehydration through 95% and absolute alcohol, the sections are cleared with xylene and mounted in Permount (Fisher Scientific). Capsular polysaccharides stain blue against a pink background.

■ Ascospore stain

Ascomycetous fungi may produce ascospores when grown on media that promote their formation. Visualization of ascospores can be accomplished with a differential staining procedure consisting of malachite green and safranin. Ascospores stain green, while the vegetative portion of the fungus stains red. The classic Kinyoun acid-fast stain (see chapter 21) may also be used for visualizing ascospores, as these structures tend to be acid-fast.

Basic procedure

A thin smear of growth is applied to a glass slide and heat fixed. The slide is flooded with malachite green (5 g in 100 ml of distilled water) for 3 min, washed with tap water, decolorized with 95% ethyl alcohol for 30 s, and counterstained with aqueous safranin (5%) for 30 s. The slide is washed with tap water, allowed to dry, and examined at ×400 to ×1,000 magnification.

■ Calcofluor white

Calcofluor white and related compounds such as Uvitex 2B and Blankophor are nonspecific fluorochromes that bind to β1,3 and β1,4 polysaccharides, specifically, cellulose and chitin of cell walls in fungi. Like the auramine-rhodamine stain, calcofluor white has become commonplace in microbiology laboratories because of the rapidity with which specimens can be observed. The fluorochrome can be mixed with KOH to clear the specimen for easier observation of fungi, including *P. jirovecii.* Fungal elements appear bluish white against a dark background when excited with UV or blue-violet radiation. Optimal fluorescence occurs with UV excitation. A barrier filter such as 510, 520, or 530 should be used for eye protection. Organisms will impart a green fluorescence (1). Typical *P. jirovecii* cysts are generally 5 to 8 μm in diameter, round, and uniform in size, and they exhibit a characteristic peripheral cyst wall staining with an intense internal "double-parenthesis-like" structure (2). Yeast cells are differentiated from *P. jirovecii* by budding and intense internal staining**.** Care must be used in interpreting the calcofluor white staining result because nonspecific reactions may be observed. Cotton fibers fluoresce strongly and must be differentiated from fungal hyphae. Additionally, tissues such as brain biopsy specimens from patients with tumors may fluoresce and resemble *Aspergillus* hyphae.

Basic procedure

KOH (10%) is mixed in equal proportion with calcofluor white solution (0.1 g of calcofluor white M2R and 0.05 g of Evans blue in 100 ml of H_2O). The specimen is covered with this mixture, a coverslip is applied, and the preparation is examined with UV light at ×100 to ×400 magnification.

■ Colloidal carbon wet mounts (India ink, nigrosin)

Colloidal carbon wet mounts are used for visualization of encapsulated microorganisms, especially *C. neoformans*. The polysaccharide capsule of organisms excludes the particles of ink, and the capsules appear as clear halos around the organism. Artifacts such as erythrocytes, leukocytes, and talc particles from gloves, bubbles, and globules after a myelogram may displace the colloidal suspension and mimic yeast. These artifacts make it necessary to perform a careful examination of the wet mount for properties consistent with the organisms (e.g., rounded forms with buds of various sizes and double-contoured cell walls). Interpretation can also be hindered if the emulsion with the colloid suspension is too thick, blocking the transmission of light.

Basic procedure
Mix equal parts of the patient's cerebrospinal fluid with either Pelikan India ink or nigrosin on a slide. Add a coverslip and examine at ×100 to ×1,000 magnification.

■ Giemsa stain

The Giemsa stain is used for the detection of intracellular yeast forms of *Histoplasma capsulatum* in bone marrow and buffy coat specimens. The fungus is usually seen as small oval yeast cells that stain blue and have a hyaline halo that represents poorly staining cell wall. The stain can also be used to visualize the trophozoite stage of *P. jiroveci.*

Basic procedure
A thin smear is prepared on a glass slide and placed in 100% methanol for 1 min. The slide is drained and then flooded with freshly prepared Giemsa stain (stock Giemsa stain diluted 1:10 with phosphate-buffered water). After 5 min the slide is rinsed with distilled water and air dried. Examine at ×100 to ×400 magnification.

■ Lactophenol cotton blue

Lactophenol cotton blue is a basic mounting media for fungi consisting of phenol, lactic acid, glycerol, and aniline (cotton) blue dye. The solution may be filtered to remove precipitated dye and stored at room temperature. It is commonly used for the microscopic examination of fungal cultures by tease or tape preparation. The addition of 10% polyvinyl alcohol (LPCB-PVA) makes an excellent permanent stain or fixative for mounting slide culture preparations (3).

Basic procedure
Concentrated phenol (20% [vol/vol]) is added to a mixture of glycerol (40%), lactic acid (20%), and water (20%), followed by the addition of aniline blue (0.05 g). The solution may be filter sterilized to remove precipitated dye. A drop is added to a glass slide, and a tease or tape mount is prepared. Add a coverslip and examine at ×100 to ×400 magnification.

■ Masson Fontana stain

The Masson Fontana stain was originally developed for demonstrating melanin granules in mammalian tissue. It has a mycological application in detecting dematiaceous fungi, and to a lesser extent C. *neoformans*, in histological sections. Fungal elements appear brown to brownish black against a reddish background.

Basic procedure
A silver solution is prepared by adding concentrated ammonium hydroxide to 10% silver nitrate until the precipitated form disappears. Deparaffinized sections of tissue are hydrated and placed in heated silver solution for 30 to 60 min. The slides are then rinsed in distilled water and toned in gold chloride (0.2 g in 100 ml of distilled water) for 10 min followed by fixation in 5% sodium thiosulfate for 5 min. The slides are washed and counterstained in nuclear fast red (0.1 g in 100 ml of distilled water) for 5 min. The sections are dehydrated through increasing concentrations of alcohol, cleared in xylene, and mounted with a coverslip.

■ Methenamine silver stains

Methenamine silver stains are perhaps the most useful stains for visualizing fungi in tissue. Fungal elements are sharply delineated in black against a pale green or yellow background. They are, however, specialized stains that are more often performed in the histology laboratory rather than the microbiology laboratory. Grocott's modification of the Gomori methenamine silver stain is commonly used for the histopathological examination of deparaffinized tissues for fungi.

Basic procedure
Stock methenamine silver nitrate solution is prepared by adding 3% methenamine (3 g in 100 ml of distilled water) to 5% silver nitrate (5 g in 100 ml of distilled water) until a white precipitate forms that clears upon shaking. This solution is then diluted 1:2 with distilled water to which 5 ml of 5% photographic-grade borax is added. Prepared slides are oxidized in a solution of chromic acid (5 g in 100 ml of distilled water), neutralized in sodium bisulfite (1 g in 100 ml of distilled water), placed in the diluted methenamine silver nitrate solution, and heated in an oven to 58 to 60°C until the material turns yellowish brown. After rinsing vigorously in distilled water, the slides are toned in gold chloride (0.1 g in 100 ml of distilled water). Unreduced silver is removed by placing the slides in a sodium thiosulfate solution (2 g in 100 ml of distilled water) and finally counterstained in 0.03% light green. Rinse, blot dry, and examine at ×100 to ×400 magnification.

■ Mucicarmine stain

The mucicarmine stain is very useful for differentiating C. *neoformans* from other fungi of similar size and shape when found in samples of tissue. The mucopolysaccharide in the capsular material of the fungus stains deep rose to red, whereas the other tissue elements stain yellow.

Basic procedure
Fixed tissue sections on glass slides are stained first with Weigert's iron hematoxylin and then placed in a solution of mucicarmine (1 g of carmine combined with 0.5 g of anhydrous aluminum chloride in 2 ml of distilled water and then diluted in 100 ml of 50% ethanol) for 30 to 60 min. The slides are rinsed in distilled water and then counterstained in mentanil yellow (0.25 g in 100 ml of distilled water). Rinse, blot dry, and examine at ×100 to ×400 magnification.

■ Periodic acid-Schiff (PAS)

The periodic acid-Schiff (PAS) stain is used to detect fungi in clinical specimens, especially yeast cells and hyphae in tissues. Fungi stain a bright pink-magenta or purple against an orange background if picric acid is used as the counterstain or against a green background if light green is used. The procedure is a multistep method combining hydrolysis and staining. The periodic acid step hydrolyzes the cell wall aldehydes, which are then able to combine with the modified Schiff reagent, coloring the cell wall carbohydrates a bright pink-magenta. The PAS stain is an excellent general stain, because most fungi in clinical material take up the stain. However, the PAS staining procedure is rather involved, requiring several different reagents and time-consuming steps, and has been replaced in many laboratories by the calcofluor white staining procedure. The PAS stain cannot be used with undigested respiratory secretions, since mucin also stains bright pink-magenta.

Basic procedure
The prepared slide is fixed on formalin-ethanol for 1 min and is then air dried. The slide is then immersed in 5%

periodic acid for 5 min, followed by 2 min in basic fuchsin (0.1 g of dye in 5 ml of 95% alcohol and 95 ml of H_2O). The slide is rinsed in water and immersed in zinc or sodium hydrosulfite solution for 10 min (1 g of zinc or sodium hydrosulfite in 0.5 g of tartaric acid and 100 ml of H_2O). Rinse in water and counterstain with saturated aqueous picric acid for 2 min or with light green stain (1 g of dye in 0.25 ml of acetic acid and 100 ml of 80% alcohol) for 5 s. Rinse, blot dry, and examine at ×100 to ×400 magnification.

■ Toluidine blue O

Toluidine blue O is used primarily for the rapid detection of *P. jirovecii* from lung biopsy specimen imprints and bronchoalveolar lavage specimens**.** Toluidine blue O stains the cysts of *P. jirovecii* reddish blue or dark purple against a light blue background. The cysts are often clumped and may be punched in, appearing crescent shaped. Trophozoites are not discernible. Although the silver stain, monoclonal antibody, and calcofluor white stains are also used, the toluidine blue O stain is easy and rapid and yields reliable results with appropriate specimens (e.g., bronchial lavage specimens) (11).

Basic procedure

After the slide is air dried, place it in the sulfation reagent (45 ml of glacial acetic acid mixed with 15 ml of concentrated sulfuric acid) for 10 min. Rinse in cold water for 5 min, drain, and place in toluidine blue O (0.3 g of dye in 60 ml of H_2O) for 3 min. Rinse in 95% ethanol, followed by absolute ethanol and then xylene. Examine at ×100 to ×1,000 magnification.

MEDIA

■ Acetate ascospore agar

Acetate ascospore agar is used for the cultivation of ascosporogenous yeasts such as *Saccharomyces cerevisiae*. A potassium acetate formulation has been shown to be a better sporulation medium than the previously used formulation with sodium acetate. Ascospores produced on this medium are visible microscopically after staining with Kinyoun carbolfuchsin acid-fast stain.

■ Antifungal susceptibility testing media

Recent advances in methods for antifungal susceptibility testing have resulted in several media that are now used for this type of testing (see chapter 131). The broth dilution method for yeasts and filamentous fungi described in the M27-A and M38-A CLSI (formerly NCCLS) documents (8, 9) uses RPMI 1640 synthetic medium without sodium bicarbonate and supplemented with L-glutamine. The medium is buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS). Although this medium supports the growth of most fungi, it is not optimal for *C. neoformans* (13), nor does it accurately discriminate amphotericin B-resistant *Candida* spp. (12). Alternatives to RPMI 1640 for broth dilution testing include yeast nitrogen base broth with 2% glucose and antibiotic medium 3 broth.

■ Aspergillus differential medium

Aspergillus differential medium is a differential medium used for the presumptive identification of *Aspergillus flavus*. Tryptone and yeast extract serve as the source of nutrients to support fungal growth. Ferric ion in the medium promotes pigment production and imparts a yellow, yellow-green, or olive color to the growth of the organism.

■ Assimilation broth media for yeasts (carbohydrate)

Assimilation broth media for yeasts are used for the detection of assimilation, i.e., carbohydrate utilization by yeasts in the presence of oxygen. The medium uses a yeast nitrogen base, distilled water, and an individual carbohydrate. The assimilation patterns of various carbohydrates help to distinguish between different species of yeast. Since all yeasts assimilate glucose, it acts as a positive control. Usually, the media are prepared as liquids in tubes, and a positive test is growth that is observed as turbidity. Commercially prepared systems such as the Uni-Yeast-Tek (Remel) and the API 20C (bioMérieux, Inc.) system are available.

■ Assimilation broth media for yeasts (nitrogen)

Carbon assimilation broth media are used to determine the ability of yeasts to utilize different carbohydrates as a sole source of carbon in a chemically defined growth medium. The growth medium, yeast nitrogen base, contains vitamins required for growth but is insufficient to support growth without supplementation with glucose or other carbohydrates.

■ Birdseed agar-niger seed agar

Birdseed agar is a selective and differential medium used for the isolation of *Cryptococcus* species and *C. neoformans. C. neoformans* is unique in that it produces the enzyme phenol oxidase. The breakdown of the substrate (*Guizotia abyssinica* seeds or niger seed) produces melanin, which is absorbed into the yeast wall and which imparts a tan to brown pigmentation on the colonies. Colonies of other yeasts are beige or cream in color. Chloramphenicol is the selective agent that inhibits bacteria and some fungi. Creatinine enhances melanization of some strains of *C. neoformans.*

■ Bismuth sulfite-glucose-glycine yeast (BiGGY) agar

Bismuth sulfite-glucose-glycine yeast agar is a selective and differential medium used for the isolation and differentiation of *Candida* spp. Peptone, glucose, and yeast extract are the nutritive bases. *Candida* species reduce the bismuth sulfite to bismuth sulfide, which results in pigmentation of the yeast colony and, with some species, the surrounding medium. The specific colonial morphologies and growth patterns of the different *Candida* species are also detected. The bismuth sulfite also acts as an inhibitor of bacterial growth, making the medium selective.

■ Blood-glucose-cysteine agar

The blood-glucose-cysteine agar medium is used to promote the mold-to-yeast conversion of *H. capsulatum, Blastomyces dermatitidis, Paracoccidioides brasiliensis,* and *Sporothrix schenckii.* The medium contains tryptose blood agar base, L-cysteine, and defribrinated sheep blood. Penicillin is added to inhibit bacterial contamination.

■ Brain heart infusion agar (fungal formulation)

Brain heart infusion agar with sheep blood is a medium used for the cultivation and isolation of all fungi including

fastidious dimorphic fungi. The nutritive base is brain heart infusion agar with 10% sheep blood for added enrichment. The antibiotics chloramphenicol and gentamicin are added to make the medium selective by inhibiting bacterial organisms and saprophytic fungi.

■ Bromcresol purple-milk solids-glucose medium

Bromcresol purple-milk solids-glucose is a differential medium used for the identification of some dermatophytes. The medium's differential capacity is based on the type of growth (profuse versus restricted) and change in the pH indicator due to the production of alkaline by-products.

■ Canavanine glycine bromthymol blue agar

Canavanine glycine bromthymol blue agar is a differential medium for distinguishing the two varieties of *C. neoformans*. The medium contains glycine, thiamine, L-canavanine sulfate, and bromthymol blue. A colony of *C. neoformans* is streaked onto the surface of the agar and incubated at 30°C for 1 to 5 days. *C. neoformans* var. *gatti* (serotypes B and C) turns the medium cobalt blue, whereas *C. neoformans* var. *neoformans* (serotypes A and D) leaves the medium greenish yellow.

■ Candida bromcresol green (BCG) agar

Candida bromcresol green agar is a selective and differential medium used for the isolation and differentiation of *Candida* spp. Peptone, dextrose, and yeast extract serve as the nutritive base. Utilization of glucose in combination with the color indicator bromcresol green yields a specific colonial morphology and color for each *Candida* spp. Neomycin makes the medium selective by inhibiting bacteria.

■ CHROMagar

CHROMagar (Hardy Diagnostics, BD Biosciences) is a commercially available differential and selective medium used for the isolation and differentiation of clinically important yeast. The nutritive base is peptone and glucose. Chloramphenicol makes the medium selective by inhibiting bacteria. The medium is available with and without fluconazole, the former providing the additional selection of fluconazole-resistant yeast such as *C. krusei*. A proprietary chromogenic mixture allows the differentiation of many yeast species. For instance, *Candida albicans* forms yellow-green to blue-green colonies. Colonial morphology as well as distinctive color patterns has been shown to make the presumptive identification of yeast species very reliable (10). The medium has been shown to be more selective than Sabouraud agar and helpful in identifying mixed cultures of yeasts and as a source medium may enhance the rapid assimilation of trehalose by *Candida glabrata* (6). The colonies on the medium should be evaluated at 48 h. *C. neoformans* and *Geotrichum* species require additional incubation for identification.

■ Converse medium

Converse medium is a liquid medium designed to induce spherule formation from the mold phase of *Coccidioides immitis*. The medium is composed of glucose, ammonium acetate, tamol, potassium phosphate, magnesium sulfate, and zinc sulfate. Incubating shaken cultures at 35 to 40°C in 10% CO_2 optimizes conversion.

■ Cornmeal agar with dextrose

Cornmeal agar with dextrose is used for the cultivation of fungi and the differentiation of *Trichophyton mentagrophytes* from *Trichophyton rubrum* on the basis of pigment production. Dextrose in place of Tween 80 differentiates *T. mentagrophytes* from *T. rubrum* on the basis of the growth of *T. rubrum* with a red pigment.

■ Cornmeal agar with Tween 80

Cornmeal agar with Tween 80 is used for the cultivation and differentiation of *Candida* species on the basis of mycelial characteristics. Tween 80 or polysorbate 80 is specifically incorporated for demonstration of chlamydospore formation by *C. albicans*. The basic nutrients for yeast growth are provided by cornmeal infusion.

■ Christensen urea agar

The ability to hydrolyze urea is an important phenotypic characteristic for identifying yeasts such as *Cryptococcus* spp., *Trichosporon* spp., and *Rhodotorula* spp. Urea hydrolysis also facilitates separation of certain dermatophytes, in particular *T. mentagrophytes* and *T. rubrum*. The medium contains 2% urea. Phenol red is the indicator.

■ Czapek-Dox agar

Czapek-Dox agar is a reference medium for the identification of *Aspergillus* species (14). Sucrose is the sole carbon source. Sodium nitrate is the sole nitrogen source. Any bacteria or fungi that can utilize sodium nitrate as a nitrogen source will grow on this medium.

■ Dermatophyte milk agar

Dermatophyte milk agar is a medium used for the cultivation and differentiation of certain dermatophytes, particularly *T. rubrum, T. mentagrophytes,* and *Microsporum persicolor*. Skim milk powder and glucose serve as the nutritive base. The indicator bromcresol purple produces a diffuse violet color in the presence of *T. mentagrophytes* due to the alkalinization of casein in the milk solids, while *T. rubrum* shows a restricted growth pattern and no pH changes in the medium. A yellow color appears when the medium is acidified from contaminating bacterial or yeast isolates.

■ Dermatophyte test medium (DTM)

Dermatophyte test medium is a selective and differential medium used for the isolation of dermatophytes *(Microsporum, Epidermophyton,* and *Trichophyton)* from cutaneous specimens. Nitrogenous and carbonaceous compounds are provided by soy peptone. Cycloheximide inhibits saprophytic molds, and chloramphenicol inhibits many bacteria. Gentamicin inhibits gram-negative bacteria. The morphology and microscopic characteristics are easily identified with this medium. Pigmentation cannot be discerned because of the presence of the phenol red indicator. The medium is yellow and turns red with growth of dermatophytes.

■ Glucose peptone agar

Glucose peptone agar is a recommended medium for demonstrating the typical morphologies of the mucoraceous fungi. The medium is made with either mycological peptone or malt extract agar.

■ Inhibitory mold agar

Inhibitory mold agar is a selective and enriched medium that is used for the general cultivation of cycloheximide-sensitive fungi (e.g., *Cryptococcus, Zygomycetes*, and *H. capsulatum*) from contaminated specimens. Casein and animal tissue provide growth nutrients. Yeast extract provides a source of vitamins. Chloramphenicol inhibits many gram-positive and gram-negative bacteria. Gentamicin is another additive that inhibits some gram-negative bacteria.

■ Kelley's medium

Kelley's medium is an enriched medium used for the isolation and cultivation of the dimorphic fungi. The nutritive base includes beef extract, peptone, sodium chloride, and starch.

■ Lactrimal agar (Borelli's medium)

Lactrimal agar (Borelli's medium) is a medium composed of whole-wheat flour, skim milk, and honey that favors the sporulation of dermatophytes, particularly *Microsporum canis*. The medium is also used for the morphological examination of dematiaceous fungi.

■ Littman oxgall agar

Littman oxgall agar is a selective general-purpose medium used for the isolation of fungi from contaminated specimens. Crystal violet and streptomycin are the selective agents and inhibit bacteria. Oxgall restricts the spreading of fungal colonies. The isolation characteristics of this medium are similar to those of Sabouraud dextrose agar with chloramphenicol and inhibitory mold agar in that it allows the growth of fungi that are sensitive to cycloheximide.

■ Malt extract (2%) agar

A medium used for the cultivation of yeasts and molds is 2% malt extract agar. A variety of formulations have been described, but it typically includes malt extract with agar and is supplemented with peptones, glucose, maltose, and dextrin and/or glycerol.

■ Mycobiotic agar (Mycosel agar)

Mycobiotic (Remel) and Mycosel agar (BD Diagnostic Systems) are trade names for a selective medium principally designed for the isolation of dermatophytes but have been used for the isolation of other pathogenic fungi from specimens contaminated with saprophytic fungi and bacteria. The medium primarily consists of peptones from a pancreatic digest of soybean meal and dextrose. The selective agents are cycloheximide and chloramphenicol. Cycloheximide inhibits the faster-growing saprophytic fungi but is also inhibitory to some clinically relevant species. These inhibited fungi include some *Candida* and *Aspergillus* species, *Zygomycetes,* and *C. neoformans*. Chloramphenicol inhibits gram-negative and gram-positive organisms.

■ Mycosel agar (see Mycobiotic agar)

■ Niger seed agar (see Birdseed agar)

■ Potato dextrose agar

Potato dextrose agar is a medium used to stimulate conidium production by fungi. The medium also stimulates pigment production in some dermatophytes. This medium is most commonly used with the slide culture technique to view morphological characteristics. Infusions from potatoes and dextrose provide nutrient factors for excellent growth. The incorporation of tartaric acid in the medium lowers the pH, thereby inhibiting bacterial growth.

■ Potato flake agar

Potato flake agar is a medium useful in the stimulation of conidium production by fungi. Its advantages over potato dextrose agar may be preparation and stability. Potato flakes and dextrose provide the nutrient factors that allow excellent growth. The pH is adjusted to 5.6 to enhance the growth of fungi and to slightly inhibit bacterial growth. The medium may be made selective by the addition of cycloheximide and chloramphenicol.

■ Rapid trehalose medium

Rapid trehalose medium is a broth medium that provides a screening method for *C. glabrata* on the basis of rapid trehalose assimilation at 42°C. *C. glabrata* is capable of utilizing trehalose more quickly than other *Candida* species. The elevated incubation temperature increases test specificity. The medium consists of a yeast nitrogen base as a source of nitrogenous compounds. Bromcresol green is the indicator, allowing visualization of an acid shift that is indicative of a positive reaction. Trehalose is incorporated at a high concentration as the carbon source.

■ Rice medium with and without Tween 80

Rice medium that simply contains rice and deionized water is used for the identification of *Microsporum audouinii* and sometimes for the identification of nonsporulating isolates of *M. canis*. The addition of Tween 80 to the medium can enhance chlamydospore formation in *C. albicans.*

■ Sabouraud-brain heart infusion agar

Sabouraud-brain heart infusion agar is a general-purpose medium used for the isolation and cultivation of all fungi. The medium is a combination of brain heart infusion agar and Sabouraud dextrose agar. The combined formula allows the recovery of most fungi including the dimorphic fungi. Sheep blood provides essential growth factors for the more fastidious fungi and enhances the growth of *H. capsulatum*. Selectivity is attained by the addition of chloramphenicol, cycloheximide, penicillin, and/or streptomycin.

■ Sabouraud dextrose agar

Sabouraud dextrose agar was formulated by Sabouraud for cultivating dermatophytes. The medium consists of pancreatic digest of casein, peptic digest of animal tissue, and dextrose at a 4% concentration and buffered to a pH of 5.6. Emmons modified the original formulation by lowering the dextrose concentration from 4 to 2% and adjusting the pH nearer to neutrality at 6.9 to 7.0. Antibiotic additives in various combinations including cycloheximide, chloramphenicol, gentamicin, ciprofloxacin, penicillin, and/or streptomycin, which inhibit some fungi and gram-positive and gram-negative bacteria, achieve selectivity for this medium. Mycobiotic agar and Mycosel agar with antibiotics are similar in performance to Sabouraud dextrose agar (Emmons modification). This medium is also available as a broth.

■ Soil extract agar

Soil extract agar is a medium composed of garden soil, yeast extract, and glucose, which is used to promote sporulation in some saprobic fungi and for mating strains of *B. dermatitidis* (14).

■ Tomato juice agar

Tomato juice agar is a medium containing tomato juice and yeast powder, which is used to promote ascospore formation (5, 14).

■ Trichophyton agars #1 to #7

Trichophyton agars are a set of seven media that allow the identification of *Trichophyton* species on the basis of their growth factor requirements. The basic ingredients in the media are listed below. Growth in all seven media is then scored on a scale of 1 to 4, and an identification is assigned.

#1: Casamino Acids, vitamin-free
#2: Casamino Acids plus inositol
#3: Casamino Acids plus inositol and thiamine
#4: Casamino Acids plus thiamine
#5: Casamino Acids plus niacin
#6: Ammonium nitrate
#7: Ammonium nitrate plus histidine

■ V9 agar

V9 agar is a medium consisting of dehydrated potato flakes and tomato juice that induces early sporulation of some environmental fungi. The naturally low pH makes the medium inhibitory to most bacteria.

■ Yeast carbon agar

Yeast carbon agar is a solid medium recommended for use in qualitative procedures for the classification of yeasts according to their ability to assimilate nitrogenous compounds. The yeast carbon base provides amino acids, vitamins, trace elements, and salts, which are necessary to support growth. The ability to assimilate nitrogen is tested by the addition of various nitrogen sources such as potassium nitrate.

■ Yeast extract phosphate agar with ammonia

Yeast extract phosphate agar with a drop of ammonium hydroxide is used for isolation of *H. capsulatum* and *B. dermatitidis* from contaminated specimens. The medium is a mix of a phosphate buffer adjusted to pH 6.0 and a yeast extract agar solution. An ammonium hydroxide solution is applied to the agar surface with a dropper and is allowed to diffuse into the medium. Ammonium hydroxide and chloramphenicol suppress bacteria and many molds and yeast, thus permitting detection of the slowly growing dimorphic fungi.

APPENDIX

Commercial Manufacturers and Suppliers of Fungal Media, Stains, and Reagents

Alpha Tec Systems, Inc.
P.O. Box 5435
Vancouver, WA 98668
1-800-221-6058
http://www.alphatec.com

BD Diagnostic Systems
7 Loveton Circle
Sparks, MD 21152
1-800-675-0908
http://www.bd.com

bioMérieux, Inc.
595 Anglum Road
Hazelwood, MO 63042-2320
1-800-638-4835
http://www.biomerieux-usa.com

Fisher Scientific
1 Liberty Lane
Hampton, NH 03842-1819
1-800-640-0640
http://www.fishersci.com

Hardy Diagnostics
1430 West McCoy Lane
Santa Maria, CA 93455
1-800-266-2222
http://www.hardydiagnostics.com

Remel
12076 Santa Fe Drive
Lenexa, KS 66215
1-800-255-6730
http://www.remel.com

Sigma-Aldrich Corp.
3050 Spruce Street
St. Louis, MO 63103
314-771-5765
http://www.sigmaaldrich.com

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Algorithms for Detection and Identification of Fungi*

YVONNE R. SHEA

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As the number of invasive fungal infections increases, the rapid and accurate diagnosis of the etiologic agent becomes even more vital for the initiation of appropriate antifungal therapy. This chapter is a review of non-culture-based detection and identification methods that can be performed directly with clinical materials in a clinical laboratory. These methods include direct microscopic examination, antigen detection, detection of fungus-specific metabolites, detection of cell wall components, detection of fungus-specific nucleic acids, and serologic diagnosis. All methods discussed should be used as an adjunct to proper culturing, as culture remains the standard for detection and identification of fungi in clinical specimens. Culture methodology for the isolation of fungi is reviewed in chapter 116.

DIRECT MICROSCOPIC EXAMINATION

Gross examination of clinical material should be performed before specimen processing occurs. Specimens should be examined for areas of caseous necrosis, microabscesses, grains and granules, and firm nodules which may indicate granulomas. Granulomas and caseous necrosis may indicate histoplasmosis, and microabscesses can be seen with hepatosplenic candidiasis, whereas grains or granules can be seen in chromoblastomycosis and mycetoma. Certain fungal organisms are more likely to be recovered from specific body sites (64) (Table 1). The pathology associated with fungal organisms may vary according to the competence of the host immune defenses.

Knowledge of the patient's residence and recent travel history may provide diagnostic clues to fungal disease because some fungal pathogens are more common in certain regions of the world (Table 2). For example, although eumycetoma has been reported worldwide, most cases come from tropical and subtropical regions around the Tropic of Cancer (25). *Madurella mycetomatis, Scedosporium apiospermum, Leptosphaeria senegalensis,* and *Madurella grisea* account for 95% of eumycetoma infections (8). Grains and granules should be examined for color, size, shape, and consistency because these characteristics can help to identify the etiologic agent (Table 2).

The ability to recognize typical staining characteristics of fungi can assist in the diagnosis, guide appropriate testing, and facilitate proper antifungal therapy. A number of stains or procedures can be used to detect fungal organisms by direct examination of clinical specimens (Table 3). Stain details can be found in chapter 117.

The value of the Gram stain should not be underestimated, as it is the front-line stain in most laboratories and the stain with which most technologists are familiar. Differentiating yeast from mold is important because yeast may represent normal colonization, while mold can indicate a deeper infectious process. Although Gram reactions can vary, yeast cells and pseudohyphae generally stain gram positive and hyphae (septate and aseptate) stain gram negative. The size and shape of budding yeast-like cells can indicate a presumptive species identification. *Cryptococcus neoformans* yeast cells are generally very round and display an amorphous orange-staining material, presumably the capsule.

Examination of smears for specific morphologic patterns can help to establish the identification of the organism. *Aspergillus* species tend to form hyphae of a consistent diameter (4 to 6 μm) and commonly demonstrate 45° branching and less commonly exhibit 90° branching. *Fusarium* and *Scedosporium* species look very similar to *Aspergillus* species in tissue (38). Sporulation in clinical material may occur. Aleurioconidia can be indicative of *Aspergillus terreus* (80), and the detection of phialides and phialoconidia can assist in the preliminary identification of *Fusarium, Paecilomyces,* and *Acremonium* species. When fungal stains show both unicellular forms and filaments that appear to be hyphae or pseudohyphae, infection with *Fusarium, Paecilomyces,* or *Acremonium* species should be considered (42). Zygomycetes exhibit aseptate (or rarely septate) hyphae of irregular diameter that tend to branch at 90 degrees. The lack of regular septations causes an absence of internal support for the hyphae, and the hyphae can become twisted and folded in a ribbon-like manner. Often the hyphae of zygomycetes are very irregular and pleomorphic. Dematiaceous molds may display multiple forms such as septate hyphae with parallel walls, rounded forms arranged in chains, or muriform bodies. The diagnosis of phaeohyphomycosis can be made only if pigmented hyphae are observed. Sometimes pigmentation can be observed by using a traditional KOH preparation. Some structures can provide definitive diagnosis of the etiologic agent (e.g., spherules of *Coccidioides* spp.). Specimens obtained from patients treated with antifungal drugs may demonstrate uncharacteristic formations such as variations

* This chapter contains information presented in chapter 112 by William G. Merz and Glenn D. Roberts in the eighth edition of this Manual.

TABLE 1 Selection of clinical specimens for recovery of opportunistic fungal pathogens[a]

Suspected pathogen	Blood	Bone marrow	Brain and CSF	Catheter/ catheter exit site	Eye	Respiratory sites	Skin, mucous membranes	Urine	Multiple systemic sites
Yeasts									
Candida spp.	X	X	X	X	X	X	X	X	X
Cryptococcus neoformans	X	X	X		X	X	X	X	X
Malassezia spp.	X			X			X		
Trichosporon spp.	X			X		X	X	X	X
Molds									
Aspergillus spp.			X		X	X	X	X	X
Dematiaceous fungi			X		X	X	X		X
Dermatophytes							X		
Fusarium spp.	X				X	X	X		X
Paecilomyces spp.			X			X	X		X
Scedosporium spp.	X		X		X	X	X		X
Zygomycetes			X		X	X	X		X
Dimorphic fungi									
Blastomyces dermatitidis			X			X	X	X	X
Coccidioides immitis	X	X	X		X	X	X	X	X
Histoplasma capsulatum	X	X	X		X	X	X	X	X
Paracoccidioides brasiliensis		X	X			X	X		X
Penicillium marneffei	X	X	X			X	X		X
Sporothrix schenckii	X		X			X	X		X
Other									
Pneumocystis jirovecii						X			X

[a] Adapted from reference 55a. The anatomic site of the fungal infection can be an indicator of what mycotic agent to suspect, but in an immunocompromised patient population, virtually any fungus can be an opportunistic pathogen.

of hypha size within the same hyphal structure, clearing of septations within the hyphae, or bulb forms within the hyphae or in the hyphal tips.

Artifacts seen in smears can hinder correct diagnosis. Lysed lymphocytes in cerebrospinal fluid (CSF) have been mistaken for *Cryptococcus neoformans,* collagen fibers and swab fibers may be mistaken for hyphae as each can fluoresce with calcofluor white, and fat droplets may be confused with budding yeast-like cells. A careful smear review in each of these situations should help avoid misinterpretation, but when in doubt, a second reader or second procedure should be employed. Correct interpretation is critical to appropriate patient care.

Detection of fungi in clinical material can be difficult because the specimen may be inadequate, relatively few fungi may be present, or similarly appearing organisms may be indistinguishable on initial examination. When specimen volume is minimal, the culture should take precedence over the smear, because direct examinations are less sensitive than culture. When possible, always perform both smear and culture, given that culture alone cannot distinguish between colonization and tissue invasion. Conversely, only nonviable organisms may be present in specimens obtained while the patient is receiving antifungal therapy and microscopy may be the only method for detecting the etiologic agent. Specimen quantity and quality may be compromised when multiple clinical laboratories process portions of the specimens. Good communication between the pathology and microbiology services in union with the clinician can greatly enhance diagnostic accuracy.

Any positive smear result must be reported promptly and clearly convey what has been seen, so that the clinician understands what type of organism has been detected. When possible, additional comments should be entered to clarify the type of structures or possible organism identification. For example, "Budding yeast-like cells seen" can indicate many different species of yeasts, while "Budding yeast-like cells resembling *Malassezia* group" offers a clue to the type and significance of the infection. Figures 1 through 26 illustrate characteristic fungal structures seen in the direct examination of clinical specimens.

ANTIGEN DETECTION

Aspergillus spp.

A diagnostic marker for invasive aspergillosis (IA) is galactomannan (GM). GM is a polysaccharide cell wall component that is released into blood by growing hyphae. The Platelia *Aspergillus* GM immunoassay (Bio-Rad, Hercules, Calif.) was cleared by the Food and Drug Administration for diagnostic use in the United States in May 2003. The Platelia *Aspergillus* GM immunoassay has been evaluated in numerous studies as a diagnostic tool for IA, but the reported sensitivities and specificities have been variable. Explanations for this variability include number and type of collected specimens (serial samples, single samples, blood, bronchoalveolar lavage [BAL] fluid), the time the specimen was obtained during the course of the infection, varying test cutoff values (47), and receipt of prophylactic or empirical antifungal therapies (46). As the use of the Platelia *Aspergillus* test has increased, causes for false positives have been revealed. False positives have been associated with dietary GM, autoantibodies, mycosis with shared antigens (86), drug therapies with amoxicillin-clavulanic acid (48) or piperacillin-tazobactam (79), the galactoxylomannan component of the *Cryptococcus neoformans* capsular polysaccharide (14), and bifidobacterial lipoglycan (52). Additionally, patients undergoing hemodialysis may have falsely high titers

TABLE 2 Characteristic fungal elements seen by direct examination of clinical specimens

Morphologic fungal element found	Organism(s)	Diam range (μm)	Characteristic features	Geographic distribution
Yeast forms	*Histoplasma capsulatum*	2–5	Small; oval to round budding cells; often found clustered within histiocytes; difficult to detect when present in small numbers; often intracellular (Fig. 23)	var. *capsulatum:* Ohio and Mississippi river valleys, Mexico, Central America var. *duboisii:* tropical areas of Africa
	Sporothrix schenckii	2–6	Small; oval to round to cigar shaped; single or multiple buds present; uncommonly seen in clinical specimens (Fig. 21)	Most often reported from Mexico, Central and South America, also common in Asia. More widespread in temperate and tropical zones
	Cryptococcus spp.	2–15	Cells vary in size; usually spherical but may be football shaped, buds usually single and "pinched off"; capsule may or may not be evident; rarely, pseudohyphal forms with or without capsule may be seen (Fig. 7, 15, and 16).	*Cryptococcus gatti:* tropics and southern hemisphere, eucalyptus trees; endemic to Vancouver Island (Douglas fir zone)
	Blastomyces dermatitidis	8–15	Cells usually large and spherical, double refractile; buds usually single, but several may remain attached to parent cells; buds connected by broad base (Fig. 22).	Southeast/South central United States, Great Lakes region, near St. Lawrence River
	Paracoccidioides brasiliensis	5–60	Cell usually large and surrounded by smaller buds around periphery (mariner's wheel appearance); smaller cells (2–5 μm) that resemble *H. capsulatum* may be present; buds have "pinched off" appearance (Fig. 25).	South and Central America
Yeast forms (fission)	*Penicillium marneffei*	3	Fission yeast, not budding, elongated, curved with septa visible (Fig. 26)	Southeast Asia and China
Cysts and trophozoites	*Pneumocystis jirovecii*	1–4 4–5	Trophozoites: small pleomorphic forms Cysts: round to cup shape with up to 8 intracystic bodies (Fig. 18)	Worldwide
Spherules	*Coccidioides immitis, Coccidioides posadasii*	10–200	Spherules vary in size; some contain endospores, others are empty and collapsed; hyphae may be seen in cavitary lesions (Fig. 24).	Southwestern United States, Mexico, Central and South America (*C. posadasii* organisms are non-Californian strains)
Yeast forms and pseudohyphae or true hyphae	*Candida* spp.	3–4 (yeast forms); 5–10 (pseudohyphae)	Cells usually exhibit single budding; pseudohyphae, when present, are constricted at ends and remain attached like links of sausage; true hyphae, when present, have parallel walls and are separated (Fig. 4, 5, and 6).	Worldwide
Yeast forms and pseudohyphae	*Malassezia* species	3–8 (yeast forms); 5–10 (pseudohyphae)	Short, curved hyphal elements may be present along with round yeast cells that are round at one end and flattened at point of conidiation (Fig. 8).	Worldwide
Wide nonseptate hyphae	*Zygomycetes*	10–30	Hyphae are large, ribbonlike, often fractured or twisted. Occasional septa may be present, branching usually at right angles (Fig. 3, 11, and 12).	*Basidiobolus ranarum:* tropical areas of Asia, Africa, South America (mostly Brazil), Mexico, Australia (children); *Conidiobolus coronatus* and *C. incongruus*: tropical and subtropical regions of Africa and Southeast Asia (adults)

(Continued on next page)

TABLE 2 Characteristic fungal elements seen by direct examination of clinical specimens *(Continued)*

Morphologic fungal element found	Organism(s)	Diam range (μm)	Characteristic features	Geographic distribution
Hyaline septate hyphae	Dermatophytes (skin and nails)	3–15	Hyaline septate hyphae commonly seen; chains of arthroconidia may be present (Fig. 19).	Also consider *Scytalidium hyalinum:* Caribbean and West Africa
	Dermatophytes (hair)	3–12	Arthroconidia on periphery of hair shaft that produce sheaths indicate ectothrix infection. Arthroconidia formed by fragmentation of hyphae within hair shaft indicate endothrix infection. Long hyphal filaments or channels within hair shaft indicate favus hair infection.	
Hyaline septate hyphae	*Aspergillus* spp.	4–6	*Aspergillus* spp. are generally septate with consistent diameter throughout, often show repeated dichotomous, 45° angle branching (Fig. 1, 9, 10, 13, and 14).	Worldwide
	Scedosporium spp. (not eumycetoma); *Fusarium* spp.; *Paecilomyces* spp.	3–12	Hyphae are septate, difficult to distinguish from other hyaline molds. May exhibit less 45° angle and more 90° angle branching.	
Dematiaceous septate hyphae	Dematiaceous fungi	1.5–6	Dematiaceous polymorphous hyphae, budding cells with single septa and chains of swollen rounded cells may be present. Occasionally, aggregates may be present when infection is caused by *Phialophora* or *Exophiala* spp. (Fig. 2).	*Phaeoannellomyces werneckii:* subtropical coastal locations; *Piedra hortae:* tropical climates of Central and South America, Southeast Asia, and the South Pacific Islands; *Ramichloridium mackenziei:* Middle East
Sclerotic bodies (muriform cells)	*Cladosporium carrionii; Fonsecaea compacta; Fonsecaea pedrosoi; Phialophora verrucosa; Rhinocladiella aquaspersa*	5–20	Brown, round to pleomorphic, thick-walled cells with transverse septa. Commonly, cells contain two fission plates that form tetrads of cells. Occasionally, branched septate hyphae may be found in addition to sclerotic bodies (Fig. 20).	Occurs worldwide, although the majority of reported cases are from tropical and subtropical regions of the Americas and Africa
Granules (white grain eumycetomas)	*Acremonium* spp. *(falciforme, kiliense, recifei)*	200–300	White, soft granule, cement-like matrix absent	Asia, North, South and Central America, Europe, Oceania
	Aspergillus nidulans	65–160	White, soft granule, cement-like matrix absent	Africa
	Fusarium spp. *(F. moniliforme, F. oxysporum, F. solani)*	80–200	White-yellowish color of the grains, edges are entire or lobed; surrounded by an eosinophilic homogeneous material. Hyphae comprising the granules are not embedded in cement.	Europe, South America, Caribbean, Africa, Asia
	Neotestudina rosatii	300–600	White, soft granule with cement-like matrix at periphery	West Africa
	Pseudallescheria boydii	200–300	White-yellow, soft granules composed of hyphae and swollen cells at periphery in cement-like matrix (Fig. 17)	Worldwide, most common in North America
Granules (black grain eumycetomas)	*Curvularia* spp. *(geniculata, lunata)*	500–1,000	Black, hard grains with cement-like matrix at periphery	Worldwide
	Exophiala jeanselmei	200–300	Black, soft granules, vacuolated, cement-like matrix absent, made of dark hyphae and swollen cells	Worldwide
	Leptosphaeria senegalensis; Leptosphaeria tompkinsii	400–600	Black, hard granules; cement-like matrix; in tissue sections, the	West Africa (specifically Senegal and Mauritania)

(Continued on next page)

TABLE 2 *(Continued)*

Morphologic fungal element found	Organism(s)	Diam range (μm)	Characteristic features	Geographic distribution
			central part consists of hyphae, and a black cement-like substance is seen at the periphery	
	Madurella grisea	300–600	Black and soft with a brown cement-like material in the periphery of the granules	India, Africa, Central and South America
	Madurella mycetomatis	200–900	Black to brown hard granules of two types: (i) rust, brown, compact, and filled with cement-like matrix and (ii) deep brown, filled with numerous vesicles, 6–14 μm in diameter, cement-like matrix in periphery and central area of light-colored hyphae	India, Africa, and South America
	Pyrenochaeta romeroi; P. mackinnonii	40–100 by 50–160	Black, soft granules composed of polygonal swollen cells at periphery, cement-like matrix	Africa, India, and South America

because hemodialysis is unable to clear *Aspergillus* antigen from serum (19). Reduced detection of GM may occur in patients with chronic granulomatous disease or Job's syndrome (78), low fungal burdens, or walled-off infections or in those with IA who are receiving antifungal therapy active against molds. Weisser et al. (82) established that GM does not precede major signs on pulmonary computerized tomographic scans suggestive of invasive aspergillosis in patients with hematological malignancies. Immunodiagnosis, metabolite detection, and molecular techniques for the diagnosis of aspergillosis are further described in chapter 121.

Blastomyces dermatitidis

Antigen detection methods are generally not used for the diagnosis of blastomycosis. A *Blastomyces dermatitidis* enzyme immunoassay (EIA) (Mira Vista Diagnostics, Indianapolis, Ind.) for the detection of antigenuria has been evaluated, demonstrating a sensitivity of 92.9%, specificity of 79.3%, and cross-reactivity with patients with histoplasmosis, paracoccidioidomycosis, penicilliosis marneffei, cryptococcosis, and aspergillosis (16). Further evaluations are needed.

Candida Species

Distinguishing colonization from disease complicates the diagnosis of candidiasis by antigen detection (62). To detect candidiasis, diagnostic markers of mannan and mannoproteins have been evaluated. Mannan, which is highly immunogenic, is one of the major cell wall components of *Candida* species and is shed into the blood during infection.

The Platelia *Candida* antigen test (Bio-Rad, Marnes-La-Coquette, France) has good specificity, but frequent testing is required because mannan is rapidly cleared from the blood (72).

The Cand-Tec latex agglutination test (Ramco Laboratories, Houston, Tex.) is designed to detect a heat-sensitive circulating antigen associated with *Candida* species. Studies using the Cand-Tec have demonstrated extreme variability in both sensitivity and specificity, and thus, it is not reliable for the diagnosis of disseminated candidiasis (1, 54, 61, 65).

Pitarch et al. (66) identified numerous novel *C. albicans* antigens that elicit a specific immune response in systemic candidiasis patients with malignant hematological disorders by using immunoproteomics. In this work, differences in antigen recognition patterns that correlated with infection progression were revealed. These data can be accessed at http:/babbage.csc.ucm.es/2d/2d.html.

Coccidioides Species

Antigen tests are not generally used for the diagnosis of *Coccidioides* infection.

Cryptococcus neoformans

The glucuronoxylomannan component of the *Cryptococcus neoformans* capsular polysaccharide is used for diagnosis of cryptococcosis. Five serotypes of *C. neoformans* are recognized: A, B, C, D, and AD. Commercially available cryptococcal antigen detection methods detect all serotypes. Most clinical isolates are serotype A (53). Latex antigen detection methods (Meridian Cryptococcal Antigen Latex Agglutination System, Meridian Diagnostics, Inc., Cincinnati, Ohio; Murex Cryptococcus Test, Murex Corp., Norcross, Ga.; Crypto-LA Test, Wampole Laboratories, Cranbury, N.J.; and Latex Antigen Detection System, Immuno-mycologics, Norman, Okla.) and the enzyme immunoassay (Premier Cryptococcal Antigen sandwich EIA; Meridian Diagnostics, Inc.) are sensitive (93 to 100%) and specific (93 to 100%) diagnostic tools (32, 37, 75). All methods are well established for the detection and quantitation of circulating capsular polysaccharide antigen from *C. neoformans* in serum and CSF. Titer determinations resulting from different latex kits should not be used interchangeably because results may vary considerably. When comparing titers between current and previous specimens, titrations should be performed using the same kit at the same time. Studies for the detection of cryptococcal antigen in BAL fluid have met with variable results (2, 39).

With both the latex and EIA methodology, false-negative as well as false-positive results have been described. False-negative results can occur with low titers of cryptococcal antigen, early infection, poorly encapsulated strains with low production of polysaccharide, and prozone phenomenon (22). False-positive results have been well documented with latex agglutination systems (5, 6, 9, 20, 27, 50, 83). Most false-positive reactions have been eliminated through procedural modifications (24, 90) and incorporation of control reagents (3).

TABLE 3 Methods and stains available for direct microscopic detection of fungi in clinical specimens[a]

Method	Use	Time required (min)	Color of fungus	Comments
Alcian blue	Detection of *C. neoformans* in CSF	2	Capsule stains blue against a pink background.	Mucopolysaccharide stain, not commonly used; like India ink, does not detect all cases.
Calcofluor white	Detection of all fungi and cysts of *Pneumocystis jirovecii*	1–2	Depending on which barrier filter is used, fungal elements appear blue-white or bright green against a dark background (Fig. 10 and 12). Cysts have "double parenthesis-like" structure in center.	Requires fluorescence microscope; collagen and swab fibers also fluoresce, fat droplets may look similar to yeast cells. Counterstain minimizes background fluorescence.
Fontana-Masson (FM)	Detection of melanin of dematiaceous fungi and *C. neoformans*	60	Cell walls black. Background pale pink.	Many nondematiaceous fungi (some *Aspergillus, Zygomycetes,* and *Trichosporon* species) can be FM positive. If hyaline in H&E, examine morphology carefully.
Giemsa stain	Primarily used for the examination of bone marrow and peripheral blood smears	15	Detects trophozoite stage of *P. jirovecii*	Detects intracellular *H. capsulatum* and fission yeast cells of *P. marneffei* (Fig. 26).
Diff-Quick	Also applied to other specimens, such as BAL fluid, CSF	2–3	Yeast cells and trophozoites appear blue-purple (Fig. 15).	Differentiate from *Leishmania*.
Wright		7		Also stains other fungi.
Gomori methanamine silver stain (GMS)	Detection of fungi and *Pneumocystis jirovecii*	5–60	Fungi and *P. jirovecii* cysts stain gray to black. Background is green (Fig. 9, 11, 13, and 21).	Often stains fungi too densely to observe structural details. Yeast cells and cysts of *Pneumocystis* may appear similar in size and shape.
Gram stain	Detection of bacteria and fungi	3	Generally yeast and pseudohyphae stain gram positive and hyphae (septate and aseptate) appear gram negative (Fig. 1–8).	*Cryptococcus* spp. stain weakly in some instances and exhibit only stippling. Often have orange amorphous material around yeast (Fig. 7). Not all fungi are detected.
H&E	General purpose histologic stain	30–60	Stains some fungal elements violet to bluish purple, tissue background is displayed as shades of red. Demonstrates natural pigment of dematiaceous fungi (Fig. 14, 17, and 24).	Permits visualization of host tissue response to the fungus. *Aspergillus* spp. and *Zygomycetes* stain well.
India ink, nigrosin	Detection of *C. neoformans* in CSF	1	Capsules around yeast cells clear halos against a black background (Fig. 16)	When positive in CSF, diagnostic of meningitis. Negative in many cases of meningitis, not reliable. Cryptococcal antigen testing recommended.
Mucicarmine	Stains mucin	60	Stains capsule of *Cryptococcus* pinkish red	May also stain cell walls of *B. dermatitidis* and *Rhinosporidium seeberi.*
Papanicolaou	Cytologic stain used primarily to detect malignant cells	30	Depending on cell type detected, background stains in subtle range of green blue, orange to pink hues. *Candida* stains	Further stains should be performed.

(Continued on next page)

TABLE 3 *(Continued)*

Method	Use	Time required (min)	Color of fungus	Comments
			gold, while other fungi may not stain at all.	
PAS	Detection of fungi	20–25	Fungi stain pink, background stains shades of purple.	PAS stain-positive artifacts can appear as yeast cells. *B. dermatitidis* appears pleomorphic.
Potassium hydroxide (KOH)	Clearing of specimen to make fungi more readily visible	5–30	Hyaline molds and yeasts appear transparent, while dematiaceous molds may display golden brown hyphae.	Addition of lactophenol cotton blue or methylene blue enhances the visibility of fungi.
Toluidine blue	Detection of *P. jirovecii* in respiratory specimens	25	Cyst walls are purple (Fig. 18).	The background and other fungi stain the same color.

[a] H&E, hemoxylin and eosin; PAS, periodic acid-Schiff.

The Premier Cryptococcal Antigen EIA yields few false-positive results, including cross-reaction with *Trichosporon* and unidentified causes (23). EIA titers are not numerically equivalent to latex titers.

Histoplasma capsulatum

The diagnosis of histoplasmosis is usually made by a combination of culture, histopathology, measurement of antibodies, or detection of antigen. The sensitivities of histopathology of tissue for the detection of *Histoplasma capsulatum* are 43, 17, and 9% for disseminated, chronic pulmonary, and self-limited histoplasmosis, respectively (91).

In active infections, antigens are released into tissues and enter body fluids adjacent to the sites of the infection. *H. capsulatum* antigen is detected in the blood, urine, CSF, or BAL fluid using an EIA. Antigen can be detected in the blood or urine in up to 92% of patients with disseminated histoplasmosis and in up to 75% of those with diffuse lung involvement during acute pulmonary histoplasmosis. The sensitivity of urinary antigen detection in disseminated histoplasmosis varies with patients with AIDS (95%), nonimmunosuppressed patients (80%), and immunosuppressed patients (82%) (87). Urine antigen is more sensitive than that in blood. Antigen is detected in the CSF of 25 to 50% of patients with *Histoplasma* meningitis and in BAL fluid of 70% of patients with diffuse pulmonary histoplasmosis. The antigen test can be used to monitor therapy and diagnose relapse. Cross-reactions occur with paracoccidioidomycosis, blastomycosis, penicilliosis, and African histoplasmosis (84).

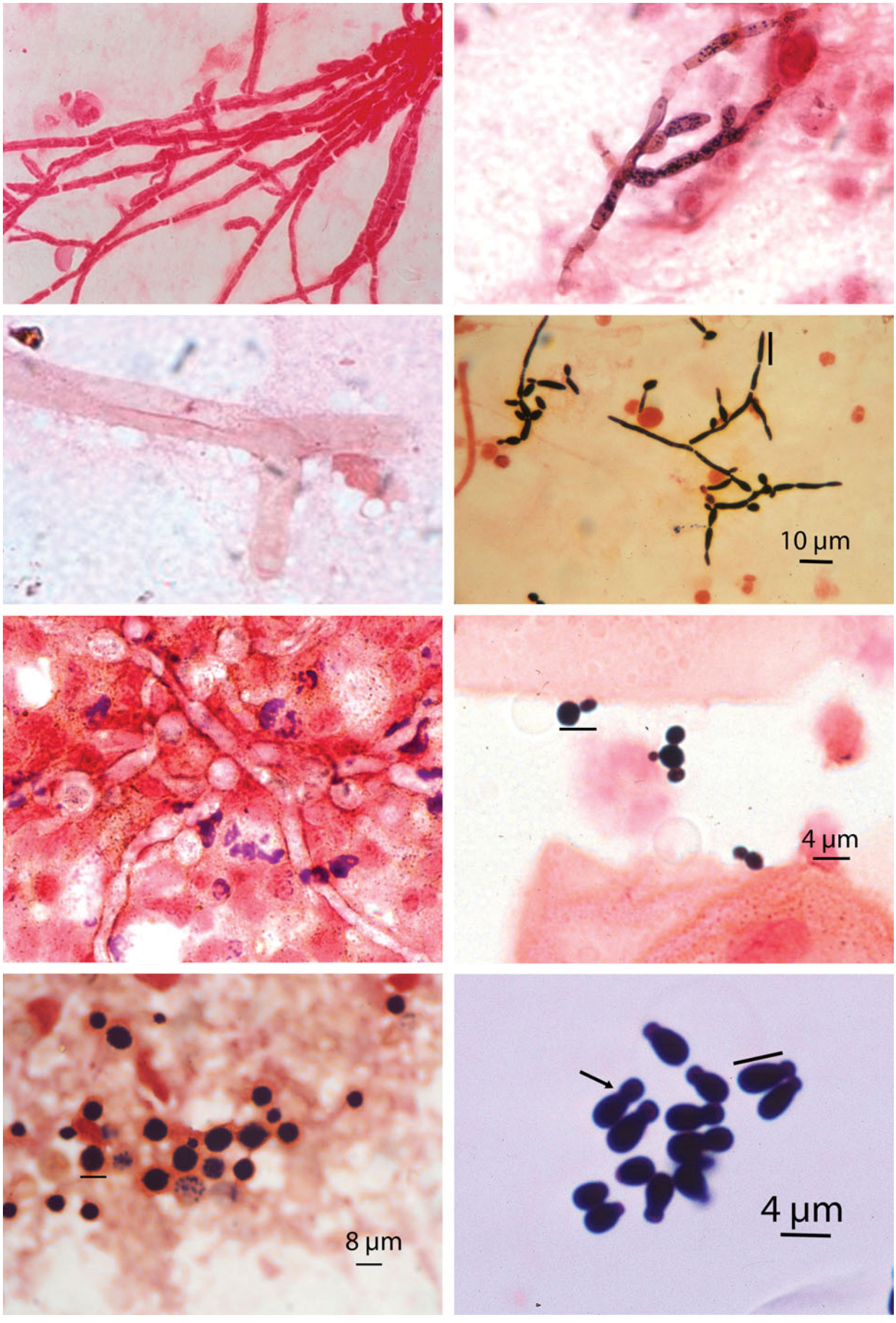
False-positive *Histoplasma* antigen results were identified for patients who received rabbit anti-thymocyte globulin (ATG) [Thymoglobulin(R)] to prevent allograft rejection. Of 107 patients receiving ATG, 17 (15.9%) demonstrated false-positive tests for *Histoplasma* antigenemia (85). It was determined that repeated administration of the rabbit immunoglobulin stimulates production of heterophile antibodies which cross-reacted with the first version of the *Histoplasma* antigen assay. In November 2004, MiraVista Diagnostics (444 Decatur Blvd., Suite 300, Indianapolis, IN) implemented its second-generation *Histoplasma* antigen EIA, which does not cross-react with patient heterophile antibodies. Currently, MiraVista Diagnostics is the only laboratory using the second-generation *Histoplasma* antigen EIA. More information about this test can be found at http://www.miravistalabs.com.

(1→3) BETA-D-GLUCAN PANFUNGAL DETECTION

The measurement of (1→3)-beta-D-glucan (BG) in blood can be used as a diagnostic marker for invasive fungal infections. BG is a cell wall polysaccharide component found in most fungi, except for the zygomycetes, which do not produce BG, and *Cryptococcus* species, which produce low levels of BG.

Although commercial assays for the detection of BG are available (FUNGITEC G TEST MK series [Seikagaku Kogo Corporation, Tokyo, Japan]; Beta-D-glucan Test Wako [Wako Pure Chemical Industries, Tokyo, Japan]; B-G Star MARUHA [Maruha Corp., Tokyo, Japan]), there is little experience in the use of these tests outside Japan (28). Comparison of the various BG tests is difficult because each utilizes different reagents and methodologies. The only commercially available test in the United States is the Fungitell (Glucatell) test (Associates of Cape Cod, Falmouth, Mass.), which was FDA approved in 2004. The Fungitell assay is based upon a modification of the Limulus Amebocyte Lysate assay (56). Briefly, BG binds to the alpha subunit of factor G found in *Limulus polyphemus* amebocyte lysates and activates Factor G, a serine protease zymogen beta subunit. Activated Factor G converts the inactive proclotting enzyme to the active clotting enzyme, which in turn cleaves the attached chromogenic peptide substrate. This chromophore is quantified spectrophotometrically at 405 nm. The recommended positive cutoff value is >80 pg/ml (Fungitell Product Insert [October 2004], Associates of Cape Cod, Inc., Falmouth, Mass.).

Studies have shown that the Fungitell serum BG detection assay is a highly sensitive and specific diagnostic tool for invasive fungal infections (including aspergillosis, fusariosis, trichosporonosis, and candidiasis) (36, 59, 60, 63). Difficulties arise when comparing the data from these studies because different cutoff values were used to interpret the results. Additionally, analysis of the patient population must be considered because test results vary with proven, probable, or possible invasive fungal infection classifications. The sensitivity and specificity results for the Fungitell BG test also vary with the frequency of sampling. Serial sampling studies tend to report a higher sensitivity than once-a-week or once-per-episode studies. Overall, the range of predicted assay accuracy is unsatisfactory (sensitivity of 60 to 100% and specificity of 64 to 99%; positive predictive values, 43 to


10 µm
4 µm
8 µm
4 µm

100%; negative predictive values, 73 to 100%). The assay appears to be less sensitive for *Candida parapsilosis*.

With all BG tests, false positives occur with patients who are on hemodialysis using cellulose membranes (35), are being treated with certain blood products (such as serum albumin and immunoglobulins) (30, 57), or have been exposed to gauze, cotton, or sponges containing glucan (58). Sera which are hemolytic or lipemic or contain bilirubin have been shown to give inconsistent results and should not be tested (70). Additionally, some mushroom extracts under investigation as antitumor drugs (including lentianan, crestin, *Sclerotinia sclerotiorum* glucan [SSG], and schizophyllan) (7) are reported to contain BG and may lead to false-positive BG assay results. All false-positive results generally show similar kinetics, displaying a sudden rise and fall in BG levels in serum. True-positive results generally show a constant rise of BG levels, which then decrease and become negative if the patient responds to antifungal therapies. Patients not responding to antifungal treatment do not show a decrease in BG levels. BG testing must always be utilized in conjunction with other diagnostic tests (e.g., GM, histopathological diagnosis, culture of samples, and radiological exams) for the conclusive diagnosis of invasive fungal infection.

FUNGUS-SPECIFIC METABOLITE DETECTION

In addition to antigen detection methods, fungus-specific metabolites have been used as markers to detect fungal infections. The fungal polyols D-arabinitol (DA) and D-mannitol (DM) and the ratio of D-arabinitol (DA)/L-arabinitol have been studied. The detection of DA in urine and serum can aid in the diagnosis of invasive candidiasis (11, 40, 68, 73, 76). DA is a 5-carbon sugar alcohol produced by most *Candida* spp., but not *Candida krusei* and perhaps *Candida glabrata*. Sensitivity differences in studies conducted for DA may relate to the prevalence of the yeast species infecting study patients (17). Human serum DA and L-arabinitol increase during renal dysfunction, so DA levels are generally reported as a ratio of DA to creatinine as serum creatinine levels are required to normalize results. Numerous techniques have been used to detect the presence of DA or determine DA/L-arabinitol ratios in human serum and urine, including gas-liquid chromatography, gas chromatography-mass spectrometry, and enzymatic methods, and are reviewed in references 12, 29, 69, 81, and 93.

DM is produced by some but not all pathogenic fungi and is not a known product of mammalian metabolism. DM has been studied primarily as a marker for *Cryptococcus neoformans* infection. DM production may contribute to *C. neoformans* pathogenesis by protecting the organism from oxidative damage (10). High concentrations of DM may cause increased intercranial pressure resulting in brain edema. No quantitative correlation can be made between the mannitol concentration and the cryptococcal antigen titer (51).

NUCLEIC ACID DETECTION

Molecular amplification techniques offer increased sensitivity over traditional staining and culture methods but may give positive results in asymptomatic individuals because of colonization or subclinical infection. Molecular methods have been applied directly to clinical materials or the contents of blood culture bottles. Numerous studies have been performed utilizing molecular methods for the direct detection and identification of fungi from specimens. (Examples can be seen in Table 4.) The process of detecting nucleic acids involves three fundamental steps. First, the nucleic acids must be extracted from the sample. Clinical materials may require further processing than fungal isolates due to the presence of PCR inhibitors found in specimens or blood culture bottles. A large number of methods for the extraction of nucleic acids from clinical materials exist (44). Cost, time, and sensitivity need to be considered when selecting an extraction method. Automated DNA extraction methods may significantly reduce contamination rates and may allow for a method of standardization between institutions performing molecular detection methods (49).

Second, an amplification target must be selected. Targets include the fungal nuclear rRNA, which consists of three genes that include the large subunit gene (26S or 28S), the small subunit gene (18S) and the 5.8S gene, separated by internal transcribed spacer (ITS) regions and housekeeping genes such as beta tubulin and actin. In general, universal primers are used to amplify the initial target, and then species-specific primers are used to identify the organism (89). The failure to amplify specific DNA may result from fungal DNA concentrations below detection limits, a focal

FIGURE 1 (row 1, left) Gram stain of BAL specimen showing gram-negative branching septate hyphae of *Aspergillus fumigatus*. Magnification, approximately ×1,000.

FIGURE 2 (row 1, right) Gram stain of skin biopsy with gram-variable branching hyphae of *Exophiala jeanselmei*. Magnification, approximately ×1,000.

FIGURE 3 (row 2, left) Gram stain of skin biopsy with gram-negative aseptate hyphae of *Rhizopus microsporus* spp. Magnification, approximately ×1,000.

FIGURE 4 (row 2, right) Gram stain of sputum with *Candida albicans* showing gram-positive budding yeast cells with pseudohyphae.

FIGURE 5 (row 3, left) Gram stain of spleen demonstrating ghost-like budding yeast-like cells with pseudohyphae. Magnification, approximately ×1,000.

FIGURE 6 (row 3, right) Gram stain of vaginal secretions with gram-positive budding yeast cells of *Candida glabrata*.

FIGURE 7 (row 4, left) Gram stain of skin abscess with *Cryptococcus neoformans* showing gram-positive and gram-variable budding yeast cells of variable size surrounded by amorphous orange halos.

FIGURE 8 (row 4, right) Gram stain of blood culture demonstrating gram-positive yeast cells of *Malassezia* spp. showing characteristic collarette at arrow.

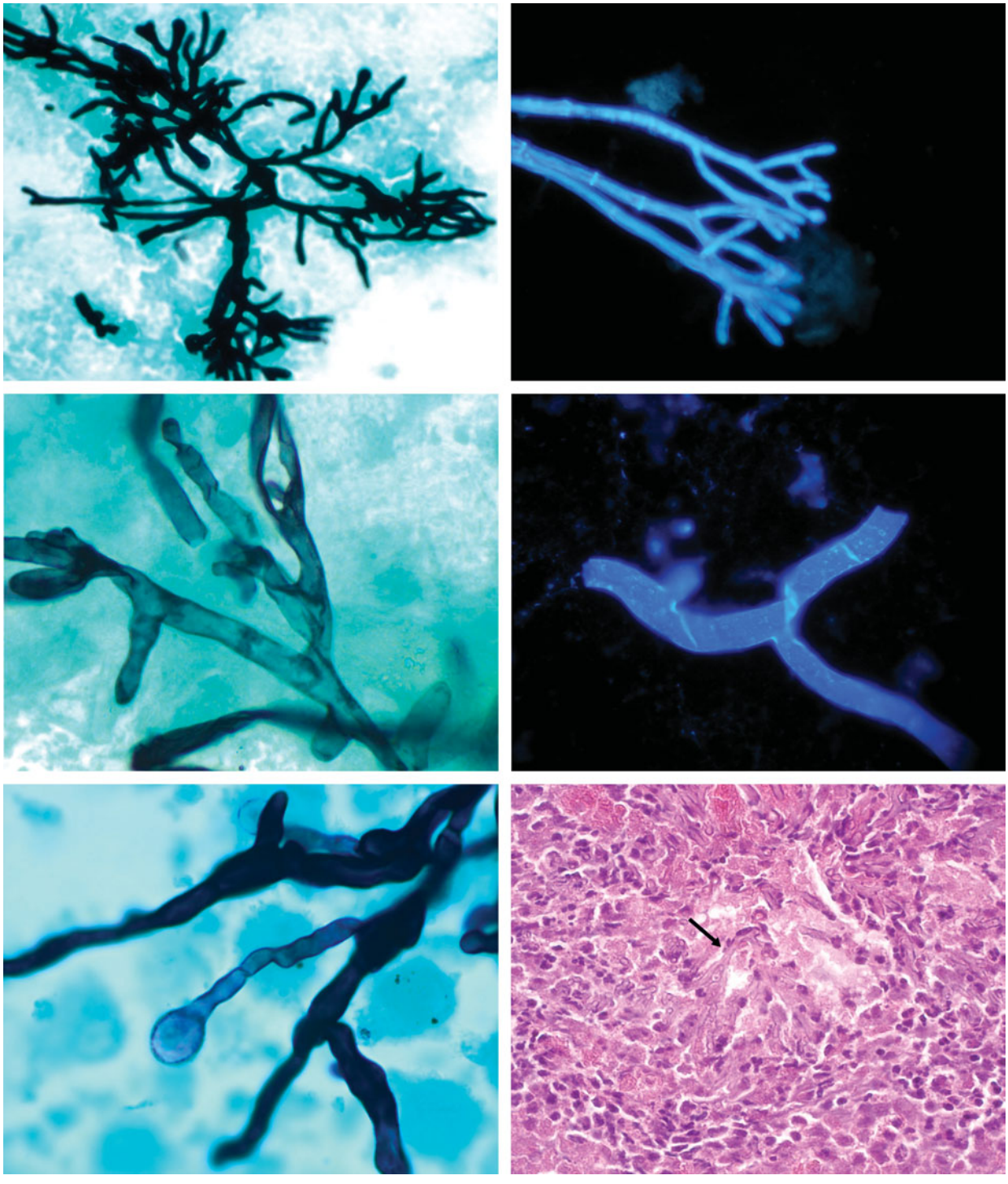


FIGURE 9 (row 1, left) Gomori methenamine silver (GMS) stain of BAL specimen showing *Aspergillus fumigatus* dichotomously branching septate hyphae. Magnification, approximately ×400.

FIGURE 10 (row 1, right) Calcofluor white stain of BAL specimen showing *Aspergillus fumigatus* dichotomously branching septate hyphae. Magnification, approximately ×500.

FIGURE 11 (row 2, left) GMS stain of skin biopsy with *Rhizopus* spp. showing ribbon-like hyphae. Magnification, approximately ×400.

FIGURE 12 (row 2, right) Calcofluor white stain of *Rhizopus* spp. showing ribbon-like hyphae. Magnification, approximately ×500.

FIGURE 13 (row 3, left) GMS stain of lung biopsy specimen showing *Aspergillus fumigatus* with bulb formation at hyphal tip. Magnification, approximately ×1,000.

FIGURE 14 (row 3, right) Hematoxylin and eosin stain of lung biopsy specimen showing *Aspergillus fumigatus* dichotomously branching septate hyphae (shown at arrow). Magnification, approximately ×400.

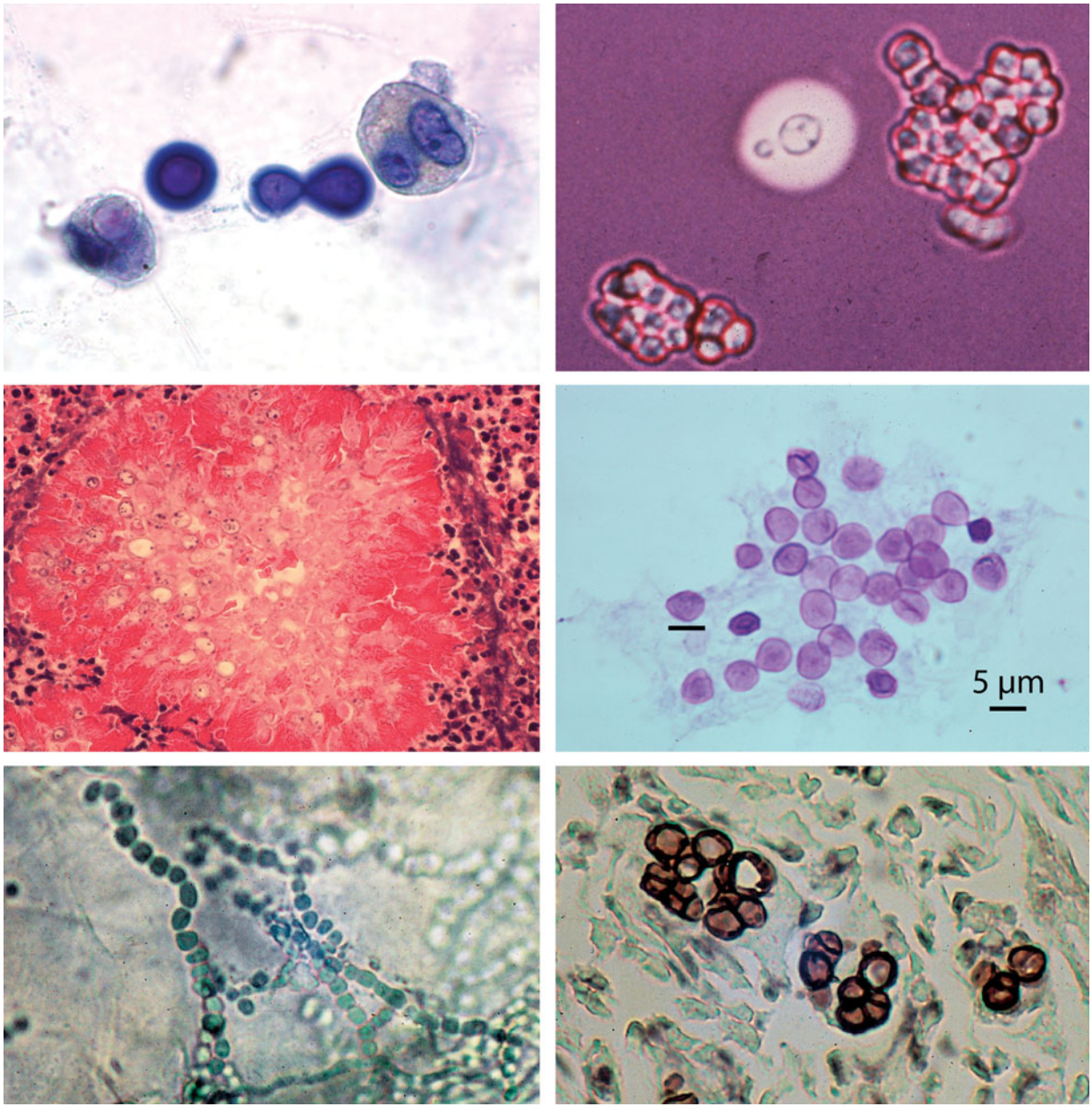


FIGURE 15 (row 1, left) Diff-Quick stain of CSF with *Cryptococcus neoformans*. Magnification, approximately ×400.

FIGURE 16 (row 1, right) India ink preparation of CSF showing encapsulated budding yeast cell of *Cryptococcus neoformans*. Magnification, approximately ×400.

FIGURE 17 (row 2, left) Hematoxylin and eosin stain of eumycotic mycetoma granule of *Scedosporium apiospermum*. Magnification, approximately ×400.

FIGURE 18 (row 2, right) Toluidine blue stain of BAL specimen showing cysts of *P. jirovecii*.

FIGURE 19 (row 3, left) Arthroconidia and hyphae of a dermatophyte. Magnification, ×200. This picture is compliments of Deanna Sutton.

FIGURE 20 (row 3, right) Sclerotic cells in tissue characteristic of the tissue form of the etiologic agents of chromoblastomycosis. Magnification, ×1,200. This picture is compliments of Deanna Sutton.

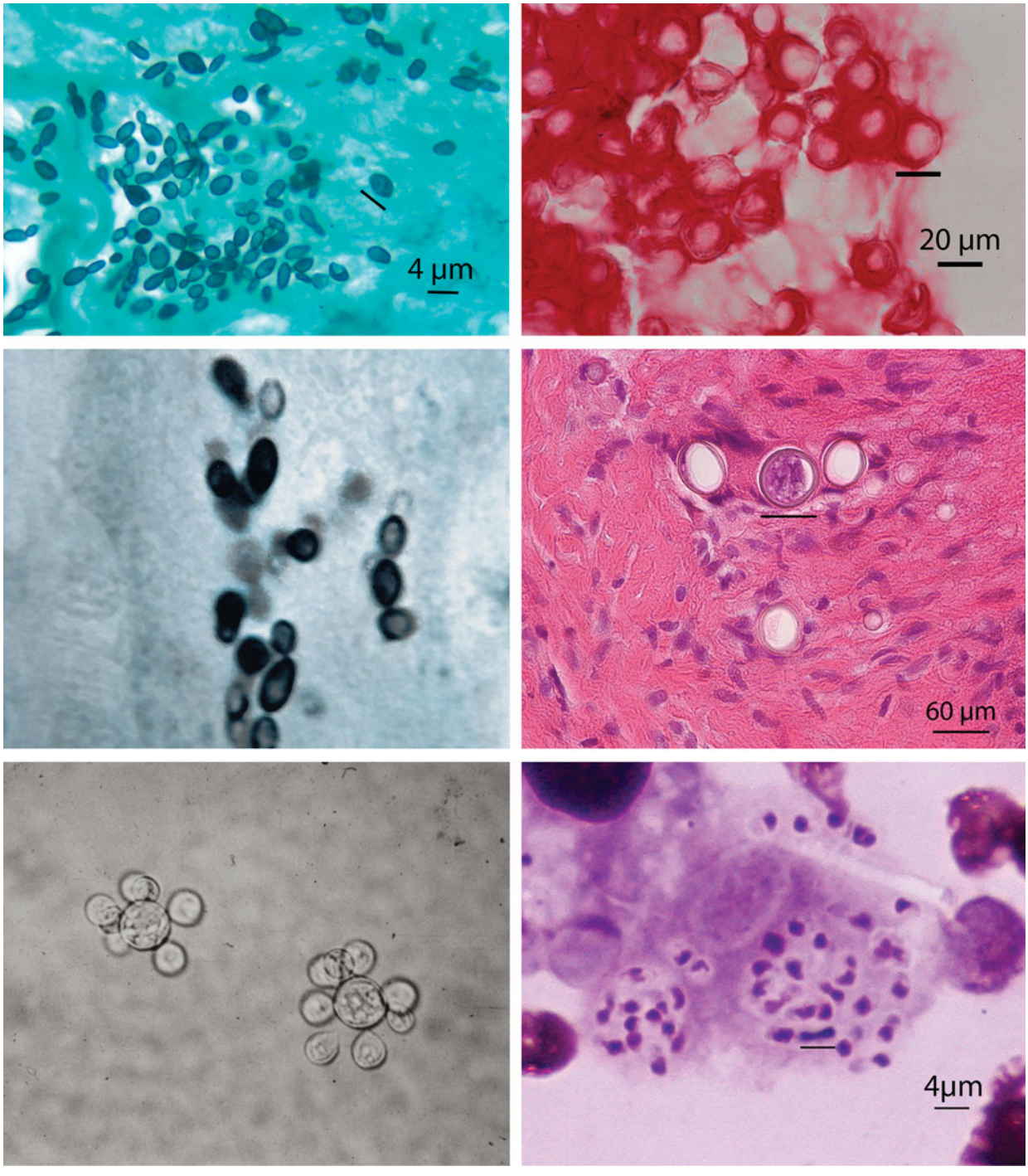


FIGURE 21 (row 1, left) GMS stain of lymph node showing characteristic cigar-shaped yeast cells of *Sporothrix schenckii*.

FIGURE 22 (row 1, right) Gram stain of abscess material showing large, broad-based, budding yeast cell with thick refractile wall characteristic of *Blastomyces dermatitidis*.

FIGURE 23 (row 2, left) GMS stain of lymph node showing blastoconidia of *H. capsulatum*. Magnification, ×625.

FIGURE 24 (row 2, right) Hematoxylin and eosin stain of *Coccidioides immitis*. Large, round, thick-walled spherules (10 to 80 µm in diameter) filled with endospores (2 to 5 µm in diameter). Young spherules have a clear center with peripheral cytoplasm and a prominent thick wall.

FIGURE 25 (row 3, left) Bright-field photomicrograph of *Paracoccidioides brasiliensis*, showing multiple budding yeast cells resembling mariner's wheels. Magnification, ×1,590.

FIGURE 26 (row 3, right) Wright-Giemsa stain of BAL specimen showing characteristic fission yeast cells of *Penicillium marneffei*.

TABLE 4 Molecular methods used for the direct detection and identification of fungi

Disease	Method	Sensitivity	Specificity	Comment
Aspergillosis	NASBA involving isothermal process of amplification and detection of *Aspergillus* spp. mRNA (94)	92.9%	62.5%	Blood samples were collected twice per week from 128 patients with hematologic diseases and neutropenic fever; 2 patients with proven IA, 14 patients with probable IA. Cutoff value, 5.24.
	Quantitative PCR targeting the 18s rRNA for the detection of *Aspergillus* spp. (55)	67%	100%	BAL specimens from 49 patients with proven or probable IA.
	PCR-ELISA for the detection of *A. fumigatus* and *A. flavus* (21)	63.6%	89.7%	1,205 serum samples from 167 patients with hematological malignancies.
	Real-time PCR to amplify the *A. fumigatus* cyp51A gene and pyrosequencing to sequence the codon for glycine 54 (77)	Not determined	Not determined	56 blood samples evaluated for mutations in the glycine 54 codon can result in reduced susceptibility to itraconazole and posaconazole.
Candidiasis	TaqMan-based PCR assay using primers for the 5.8S and 28S rRNA regions and *Candida* species-specific probes (45)	100%	97%	Prospective study 52 serum samples from 39 hematology or oncology patients with clinically proven or suspected systemic *Candida* infections.
Cryptococcosis	Nested-PCR-based assay using the ITS region for the detection of *C. neoformans* in CSF (67)	100%	100%	Detected purified DNA corresponding to as few as 10 cryptococcal cells.
	Nested PCR amplifying the *C. neoformans* URA5 gene in patients with pulmonary crytococcosis (74)	Positive for 4/5 culture-positive samples	Negative for 1 culture-positive, 10 culture-negative, and 1 undetermined	5 patients with various underlying diseases.
Zygomycosis	Evaluated two seminested PCR assays targeting the 18S rRNA of zygomycetes and the mitochondrial DNA of *Aspergillus* spp. (4)	25/40 samples with amplifiable human DNA identified fungal pathogens by both PCR assays corresponded to histopathological diagnosis; 2/8 histopathology negative detected concomitant infection with zygomycete and *A. fumigatus*	No false positives were determined	Blinded comparison of molecular identification with histopathological diagnosis; 42 paraffin wax blocks and 10 paraffin wax-embedded tissue sections from patients at risk for invasive fungal infections; all blinded.
Endemic mycoses	Detected organism by in situ hybridization and PCR assays targeting 18S and 28S rRNA (26)	*Coccidioides immitis,* 94.3%; *Blastomyces dermatitidis,* 95.0%; *Histoplasma capsulatum,* 75.0%	*Coccidioides immitis,* 100%; *Blastomyces dermatitidis,* 100%; *Histoplasma capsulatum,* 96.2%	98 formalin-fixed, paraffin-embedded tissue specimens. Each specimen had correlating culture-positive result.
Panfungal	PCR assay amplifying the complete ITS 1 and 2 regions of the rRNA complex (31)	100%	100%	Evaluated 210 ESP blood cultures; 183 were PCR/culture negative, while 27 were PCR/culture positive.
	Real-time PCR LightCycler TM technology assay amplifying 18s rRNA (33)	Sequential positive results: proven, 80%; proven/probable, 75%; proven/probable/possible, 63%	Sequential positive results: proven, 69%; proven/probable, 70%; proven/probable/possible, 72%	78 hemato-oncology patients screened twice weekly. Detected 10 CFU/ml for *C. albicans* and 20 CFU/ml for *A. fumigatus.*

infection with varying amounts of fungal elements within the tissue or destruction of DNA during formalin fixation.

Third, a method to identify the presence of the amplified target region must be determined. Numerous methodologies have been evaluated, including DNA probes, microarrays, fluorescent in situ hybridization, PCR, reverse-hybridization line probe assays, nucleic acid sequence-based amplification (NASBA), and pyrosequencing.

Real-time PCR methods, such as Taqman (Perkin-Elmer, Wellesley, Mass.) and Lightcycler (Roche Molecular Systems, Pleasanton, Calif.) techniques, enable rapid amplification, hybridization, and amplicon detection in a single reaction. Real-time technology can provide quantitative results because the point where the PCR enters the log/linear phase is detectable and reflects the amount of target DNA in the original sample.

NASBA is a technique that involves amplification and detection of mRNA. Unlike PCR, NASBA is an isothermal process, uses RNA polymerase, and amplifies mRNA. RNA is generally less stable than DNA in dead cells; therefore, its detection may indicate the viability of the organism. Because thermal denaturation is absent, there is little contaminating background of genomic DNA (43).

Pyrosequencing is a real-time, nonelectrophoretic sequencing method. This method relies on the sequential addition and incorporation of nucleotides in a primer-directed polymerase extension reaction. The pyrophosphate released on incorporation of a base-paired nucleotide is coupled to the enzymes ATP sulfurylase and luciferase to generate detectable light. The light is proportional to the number of nucleotides incorporated, allowing for the sequence to be determined in real time (15). (For detailed illustrations on pyrosequencing, go to http://www.pyrosequencing.com.)

Although molecular assays for the detection of fungi in clinical materials continue to improve, variability in PCR protocols is unavoidable due to the lack of standardization in PCR assays. Before implementation in the clinical laboratory, proper validation must be performed (13). Molecular diagnostic methods depend not only on improved methodology but also on an adequate, well-trained workforce.

SEROLOGY

Although immunocompromised patients may exhibit poor or variable antibody responses, antibody detection tests are often helpful for the diagnosis of fungal infections in immunocompetent hosts. Further information about serologic diagnosis can also be found in the appropriate organism chapters of this Manual.

Aspergillus Species

The detection of antibodies against *Aspergillus* for the diagnosis of invasive aspergillosis has produced conflicting results (34). The sensitivity and specificity of the immunodiffusion and counterimmunoelectrophoresis tests are similar. Complement fixation (CF) testing has low sensitivity and is not recommended.

Blastomyces dermatitidis

Serologic diagnosis of blastomycosis is generally performed using immunodiffusion (ID) or CF. The yeast form of *B. dermatitidis* releases two surface antigens, A antigen and W1-1. Antibodies directed against W1-1 also recognize the A antigen. The positive ID test using purified A antigen is specific and diagnostic for blastomycosis. A negative ID test does not rule out a diagnosis of blastomycosis, as this test is reported to be negative in 10% of patients with disseminated infection and over 60% of patients with localized disease. The CF test has poor sensitivity compared with the ID test (41).

Candida Species

Antibody assays generally display a low specificity for discriminating between systemic candidiasis and colonization. Ellepola et al. (18) have summarized the numerous methods developed to detect *Candida* antibodies and antigens. The Platelia *Candida*-specific antigen and Platelia *Candida* antibody (Bio-Rad Laboratories, Marnes-la Coquette, France) are EIA which can be used for the serologic diagnosis of candidiasis (71, 92). Neither test is commercially available in the United States.

Coccidioides Species

The serologic diagnosis of coccidioidomycosis is performed using ID, latex agglutination (LA), CF, or EIA methods. The ID and LA tests are useful for the initial screening of specimens and can be confirmed by other tests if positive. The ID and LA tests detect early immunoglobulin M (IgM) antibody responses to coccidioidal infection, while the CF test detects primarily IgG antibodies produced during the convalescent phase of disease or during chronic infection. Performing both the ID (or LA) and CF tests in parallel provides the highest sensitivity and specificity. The *Coccidioides* EIA (Meridian Diagnostics, Inc.) detects both anti-*Coccidioides* IgG and IgM antibodies in sera from patients with coccidioidomycosis. Although the sensitivity (97%) and specificity (94%) are high, confirmation of positive ID results is recommended (95).

Cryptococcus neoformans

Antibody detection is useful for the early diagnosis of cryptococcal disease, because antibodies are not yet neutralized by the large amount of capsular antigen released during the progression of the infection (41).

Histoplasma capsulatum

The primary serologic tests for histoplasmosis are the ID and CF tests. The ID test is an immunodiffusion test which identifies H and M precipitin bands to *Histoplasma capsulatum*. The ID test is less sensitive than the CF test. The CF test uses yeast and mycelial antigens. Serological methods are faster than culture, but false-positive results occur. The titer of specific antibodies against *Histoplasma* can remain high for months or years after the primary infection. In addition, cross-reactivity can occur in up to 40% of patients with paracoccidioidomycosis, blastomycosis, and aspergillosis; 16% of patients with coccidioidomycosis; and 8% of patients with candidiasis (88). False-negative results due to low antibody titers can be observed in immunocompromised patients with active infection.

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Candida, Cryptococcus, and Other Yeasts of Medical Importance

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TAXONOMY OF YEASTS

Yeasts of medical importance (and relevant to this chapter) belong to three classes: Hemiascomycetes (Endomycetes), Heterobasidiomycetes, and the mitosporic fungal form class Blastomycetes. Thus, the medically important yeasts present a taxonomic conundrum for the laboratorian because many of the originally classified asexual (imperfect) yeasts were subsequently shown to undergo sexual reproduction. By taxonomic convention, the yeast should no longer be classified in the Blastomycetes but rather in the phylum determined by its means of sexual reproduction. It should also be renamed with a new genus-species epithet. If this convention is followed, however, communication of the new species epithet (e.g., *Issatchenkia orientalis*) to a physician is likely to lead to confusion. A report of simply *Candida krusei* would possibly prevent the physician's inquiry. For this reason, laboratories should be mindful that most physicians will be unaware of taxonomic technicalities and should report the older, blastomycetous binomial, perhaps along with the correct binomial (e.g., *Issatchenkia orientalis* [formerly *Candida krusei*]). An exception has been made for the strictly anamorphic *Candida* species which were shown to have an ascomycetous affinity. Rather than change the names of these organisms from names that are deeply entrenched in the literature and in the minds of physicians and laboratorians, the species have retained their *Candida* genus taxon but have been placed in the family Candidaceae within the order Saccharomycetales of the Hemiascomycetes.

DESCRIPTION OF AGENTS

Yeasts are unicellular, eukaryotic, budding cells, generally round to oval or, less often, elongate or irregular in shape. They multiply principally by the production of blastoconidia (buds), such that a typical medically important yeast is composed of a progenitor cell with one or more attached progeny. When blastoconidia are produced one from the other, in a linear fashion without separating, a structure termed a pseudohypha is formed. Under certain circumstances such as growth under reduced oxygen tension, some yeasts may produce true septate hyphae.

Cultures of yeasts are moist, creamy, or glabrous to membranous in texture. Several produce a capsule which makes the colony mucoid. With rare exceptions, aerial hyphae are not produced. Colonies may be hyaline or brightly colored or may be darkly pigmented due to the presence of melanins. The latter group, referred to as phaeoid fungi and belonging to the form class Hyphomycetes, is discussed in chapter 125. Dimorphic fungal pathogens possessing a yeast phase in tissue are also discussed elsewhere.

Yeasts are generally identified (classified) by observing the macroscopic and microscopic features mentioned above. Usually, biochemical tests also are required for definitive identification to the species level. Molecular methods are currently under development and offer tremendous promise for facilitating species identification.

Genus *Blastoschizomyces*

Blastoschizomyces capitatus is a taxon derived from the combination of the obsolete taxa *Blastoschizomyces pseudotrichosporon* and *Trichosporon capitatum* (192). The teleomorph is *Dipodascus capitatus* (59). It is recognized as an emerging cause of invasive fungal disease in leukemic patients (43, 139) and endocarditis (178), is widely distributed in nature, has been recovered as normal skin flora, and has been associated with onychomycosis (55). Disseminated disease, which is usually diagnosed by blood culture, is often associated with immunosuppressive conditions (11), in particular neutropenia. Mortality from invasive disease is high in neutropenic patients, and survival is associated with neutrophil count recovery. Infections are mostly treated with amphotericin B; 5-fluorocytosine or azoles have been used, but MIC data are sparse and variable (29, 140). Macroscopically, colonies are glabrous with radiating edges, white to cream-colored, and shiny. Microscopically, isolates produce true hyphae, pseudohyphae, and annelloconidia resembling arthroconidia (Fig. 1f). Based on morphologic features alone, *B. capitatus* can be difficult to separate from *Trichosporon* spp., and physiologic tests are needed. *B. capitatus* is nonfermentative and can be separated from *Trichosporon* spp. by growth on Sabouraud glucose agar at 45°C and on cycloheximide-containing agar at room temperature and failure to hydrolyze urea.

Genus *Candida*

The heterogeneous genus *Candida* belongs to the family *Candidaceae* within the ascomycetes. The genus contains approximately 200 species. This number is not immutable. Technological advances that affect apparent taxonomic relationships will continually result in reassignments of present species and discovery of new species. These advances may also lead to the designation of new genera. Teleomorphs

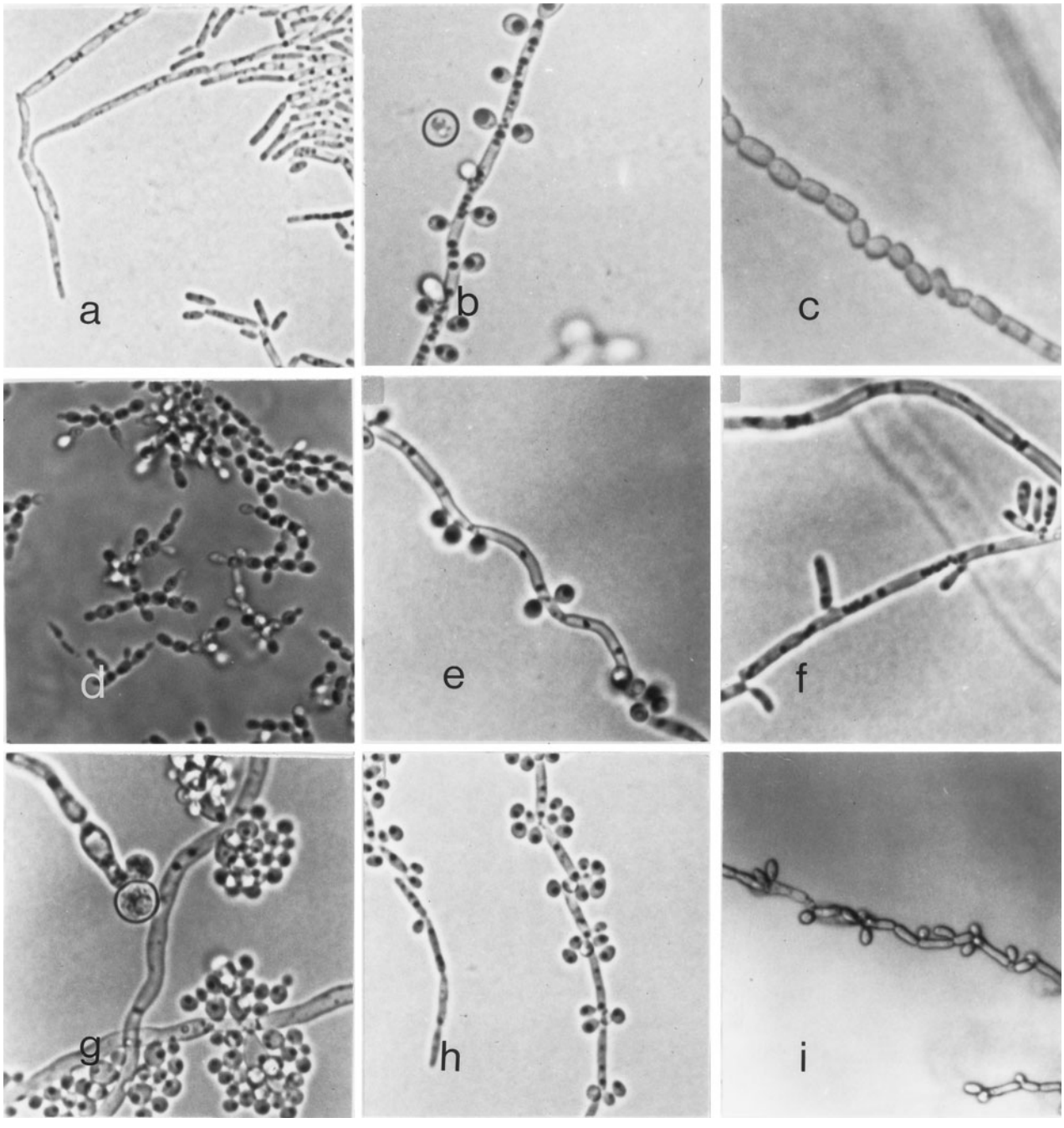


FIGURE 1 Morphologic features of some yeast and yeast-like-organisms on cornmeal agar at 24 to 48 h, ambient temperature. (a) *Candida krusei:* extremely elongated, rarely branched pseudohyphae; few blastoconidia. (b) *Candida tropicalis:* blastoconidia formed at septa and between septa. (c) *Geotrichum candidum:* arthroconidia. (d) *Candida guilliermondii:* chains of blastoconidia forming sparse pseudohyphae in a young culture. (e) *Candida lusitaniae:* short distinctly curved pseudohyphae with blastoconidia formed at, and occasionally between, septa. (f) *Blastoschizomyces capitatus:* true hyphae, annelloconidia resembling arthroconidia. (g) *Candida albicans:* blastoconidia, chlamydospores, true hyphae, and pseudohyphae. (h) *Candida parapsilosis*: elongated, delicately curved pseudohyphae with blastoconidia at septa. (i) *Trichosporon* spp.: blastoconidia formed at the corners of arthroconidia. Magnification, approximately ×339. (Courtesy of B. A. Davis.)

encompassing several genera have been demonstrated for different species of *Candida.* The teleomorphic genera include *Clavispora, Debaryomyces, Issatchenkia, Kluyveromyces, Pichia,* and *Yarrowia.* This large number of teleomorphic relationships demonstrates that the previously designated form genus *Candida* within the mitosporic fungi (formerly Deuteromycota) was a mixture of unrelated species. One reason for the wide variety of species is due to the definition

of *Candida.* The genus designation is used for any asexual yeast which does not have one of the following features: (i) acetic acid production; (ii) visually detectable red, pink, or orange pigments; (iii) arthroconidia; (iv) unipolar or bipolar budding on a broad base; (v) blastoconidia formed on sympodulae; (vi) buds formed on stalks; (vii) needle-shaped terminal conidia; (viii) triangular cells; (ix) enteroblastic-basipetal budding, usually with mucoid colonies and the ability to grow on inositol as a sole carbon source; and (x) ballistoconidia (117).

The genus *Candida* no longer contains species that are diazonium blue B positive, which is associated with yeasts having a basidiomycetous affinity (such as *Cryptococcus*). Diazonium blue B-positive organisms (e.g., *Candida humicola, Candida curvata, Candida diffluens,* and *Candida scottii*) have been reassigned to either *Cryptococcus* or *Rhodotorula* genus.

Candida claussenii and *Candida langeronii* have also been merged into *C. albicans* (235). Another change in species epithets includes the controversial fusion of *Candida stellatoidea* with *Candida albicans.* Genetic evidence supporting the fusion of *C. stellatoidea* indicates that two types (designated I and II) of *C. stellatoidea* exist (187). Type II, which does not assimilate sucrose, appears to be a sucrose-negative mutant of *C. albicans* (121). A definitive association of type I *C. stellatoidea* with *C. albicans* has not been established (22, 145), thus making the designation *C. albicans* subspecies *stellatoidea* controversial. This subspecies, unlike other *C. albicans* subspecies, is not resistant to cycloheximide. Three groups of *C. parapsilosis* have recently been described. Based on genetic and phenotypic evidence, Tavanti et al. (218) have recommended that groups II and III be designated *Candida orthopsilosis* sp. nov. and *Candida metapsilosis* sp. nov., respectively.

Candida species are ubiquitous yeasts, being found on many plants and as normal flora of the alimentary tract of mammals and mucocutaneous membranes of humans (163). Essentially all areas of the gastrointestinal tract of humans can harbor *Candida.* The most commonly isolated species (50 to 70% of yeast isolates) from the gastrointestinal tract of humans is *C. albicans,* followed by *C. tropicalis, C. parapsilosis,* and *C. glabrata.*

C. glabrata is regarded as a symbiont of humans and can be isolated routinely from the oral cavity, and genitourinary, alimentary, and respiratory tracts of most individuals. As an agent of serious infection, it has been associated with endocarditis (37), meningitis (5), and multifocal, disseminated disease (102). It is recovered often from urine specimens and has been estimated to account for as many as 21% of urinary yeast isolates (76). As it may be slow growing, routine urine cultures should be incubated for at least 2 days. A reddish colony variant of *C. glabrata,* which would otherwise have been confused with *Rhodotorula,* has been described (168). Bile-dependent strains of *C. glabrata* have also been isolated from urine (100). These isolates do not grow on routine media, or they appear as pinpoint colonies on MacConkey agar containing bile.

Candida spp. can be present in clinical specimens as a result of environmental contamination, colonization, or actual disease processes. An accurate diagnosis requires proper handling of clinical material. *Candida* spp. that are normal flora can invade tissue and produce life-threatening pathology in patients whose immune defenses have been altered by disease or iatrogenic intervention. *C. albicans* is the most common species isolated from nearly all forms of candidiasis (163). Contributing to its high association with disease is its high prevalence in the normal population, as described above. In addition, *C. albicans* appears to possess a number of virulence determinants that may promote successful parasitism. These attributes include relatively rapid germination upon seeding tissue from the bloodstream (97), protease production (122), surface integrin-like molecules for adhesion to extracellular matrix proteins (90, 220), a complement protein binding receptor (36, 81), phenotypic switching (206), and surface variation and hydrophobicity (98). These attributes have been reviewed (34, 35, 53, 159). Only *C. tropicalis* appears more virulent than *C. albicans* when present in patients with leukemia or lymphoreticular malignant disease (241). Other medically important *Candida* spp. include *C. catenulata, C. ciferrii, C. guilliermondii, C. haemulonii, C. kefyr, C. krusei, C. lipolytica, C. lusitaniae, C. norvegensis, C. parapsilosis, C. pulcherrima, C. rugosa, C. utilis, C. viswanathii,* and *C. zeylanoides* (96, 189). This list is not exclusive, as other rare agents will certainly be added in the future. The species that are emerging as opportunistic pathogens include *C. (Yarrowia) lipolytica, C. lusitaniae,* and *C. krusei.* These three species have been isolated from cases of fungemia. *C. lusitaniae*, which is generally a low-virulence organism, exhibits clinical resistance to amphotericin B (91). *C. krusei* isolates appear to be susceptible to ketoconazole but not fluconazole (242). Two other fluconazole-resistant species, *C. inconspicua* and *C. norvegensis,* are rare agents of candidiasis. *C. glabrata* is emerging as a significant pathogen with a relatively high proportion of strains exhibiting reduced susceptibility to fluconazole. *Candida* species are susceptible to caspofungin, although there is variation among the species.

An extensive review of the genus and diseases produced by *Candida* spp. has been presented by Odds (163). Regardless of the species, a single isolate of *Candida* from blood should be considered significant whether it comes from an immunocompromised or immunocompetent patient (65). Taken together with the reported rate of 10% for nosocomial bloodstream infections caused by *Candida* species (13, 24), it is evident that *Candida* fungemia represents a serious patient management problem. Ninety-seven percent of candidemias are caused by *C. albicans, C. glabrata, C. parapsilosis, C. tropicalis,* and *C. krusei* (64).

Acute pseudomembranous oral candidiasis is the presentation seen in immunocompromised patients such as those with AIDS. It is most often caused by *C. albicans* and *C. dubliniensis*. It may be present in the prodromal stages of AIDS or accompany late stages of AIDS (CD4 T-cell count of <400 cells/μl) (171, 219). Mucocutaneous forms of candidiasis are often related to defects in cell-mediated immunity, whereas systemic spread is generally associated with neutropenia (60, 74). Thus, despite the presence of oral or esophageal candidiasis, systemic spread of *Candida* spp. organisms is uncommon in AIDS patients. When it occurs, systemic spread is associated with a drop in the neutrophil count (62).

There are two serotypes, designated A and B, of *C. albicans,* and these are distributed approximately equally among immunocompetent individuals. However, serotype B is more prevalent in immunocompromised patients (31). The importance of this observation is unclear. Serotype B isolates are physiologically different from serotype A isolates in that B serotypes exhibit greater karyotype variability and are more likely to develop resistance to 5-fluorocytosine (8, 10). It is not clear how the two serotypes are distributed within the recently described geographically distributed genetic clades of *C. albicans* (207).

Blastoconidia of *Candida* spp. vary in shape, from round to oval to elongate. Occasional initial isolates, especially from patients receiving antimicrobial agents, may be highly

pleomorphic. Asexual reproduction is by multilateral budding, and true mycelium may be present. If sexual reproduction occurs, the yeasts are classified in their teleomorphic state. The appearance of pseudohyphae and attachment of blastoconidia are important characteristics to observe when identifying *Candida* spp. Figure 1 illustrates these morphologic features. Observation of germ tubes and chlamydospores is also helpful in identifying *C. albicans*. Growth on fungal media can be detected as early as 24 h; however, colonies usually are visible in 48 to 72 h and are white to cream-colored or tan. They are creamy in texture and may become more membranous and convoluted with age. Occasionally, initial isolates of *C. albicans* on Sabouraud glucose agar are wrinkled or rugose but revert to smooth colonies on subculture. In our experience, many isolates of *C. albicans* produce "colonies with feet" (i.e., colonies with short marginal extensions) on blood agar, while most other yeasts do not (note that 25% of isolates of *C. tropicalis* and *C. krusei* will also form colonies with feet [33]). These colonies should not be used to perform germ tube tests because hyphal/pseudohyphal cells will be present in the inoculum. Most *Candida* spp. grow well aerobically at 25 to 30°C, and many will grow at 37°C or above. Several species of *Candida,* most notably *C. albicans,* are diploid (190, 232).

Carbon assimilation and occasionally fermentation studies are needed to differentiate the species (Table 1), but rapid confirmation tests for particular species are becoming available (see "Rapid Identification Tests" below). Of the *Candida* spp. usually recovered from clinical specimens, *C. guilliermondii* is the only one to assimilate dulcitol (galactitol), and *C. kefyr* (previously *C. pseudotropicalis*) assimilates lactose. The assimilation of rhamnose can be helpful in separating *C. lusitaniae* from the biochemically similar but rhamnose-negative variants of *C. tropicalis* (196). For atypical isolates of *C. lusitaniae,* which can be confused with *C. tropicalis,* good growth on Trichophyton agar #1 with biotin but weak growth on vitamin-free medium indicates *C. tropicalis* (217). Certain rare strains of *C. tropicalis* may assimilate cellobiose weakly and exhibit an assimilation pattern similar to that of *C. parapsilosis*. The inclusion of arabinose is helpful, since *C. parapsilosis* readily assimilates this carbohydrate, while most strains of *C. tropicalis* do not. Urease generally is not produced, nor is KNO_3 utilized by the *Candida* species listed in Table 1. However, occasional isolates of *C. krusei* may be urease positive.

Genus *Clavispora*

Clavispora lusitaniae is the teleomorph of *Candida lusitaniae*. It is a normal component of the human gastrointestinal tract but is an emerging opportunist causing fungemia mainly of patients with malignancy. Other occasional infections include peritonitis, urinary tract infection, and meningitis (95). *C. lusitaniae* may exhibit secondary resistance to amphotericin B; therefore, clinical response to amphotericin B may be poor despite in vitro susceptibility tests indicating strains to be sensitive to the drug (95, 115). Sensitivities may be variable for 5-fluorocytosine and fluconazole. *C. lusitaniae* is sensitive to caspofungin but less so than *Candida albicans*.

C. lusitaniae cells are oval or elongated; multilateral budding occurs on a narrow base, and pseudohyphae are produced. Colonies are white to cream, smooth, and shiny or occasionally dull. The organism is heterothallic, and conjugation of opposite mating types produces asci that quickly disintegrate, releasing one or two smooth clavate ascospores. Growth occurs at 42°C. Fermentation reactions are variable, but *C. lusitaniae* will grow on a variety of sugars including sucrose, but not on mannitol or inositol. Nitrate and urease are negative.

Genus *Cryptococcus*

The genus *Cryptococcus* contains many species, six of which are given in Table 1. Of these, *Cryptococcus neoformans* is considered the only human pathogen, although *C. albidus* (105, 116) and a few others, including the recently described *C. adeliensis,* rarely have been implicated in disease in severely debilitated individuals (92, 188). *Cryptococcus* is a round to somewhat oval yeast-like fungus ranging greatly in size, from 3.5 to 8 μm or more in diameter, with single budding and a narrow neck between parent and daughter cells (Fig. 2). Unusually large yeast cells (up to 60 μm) have been observed, and this size appears to be associated with higher incubation temperatures (132). Occasionally several buds may be seen; pseudohyphae are rarely observed. The cell wall is quite fragile, and it is not unusual to find collapsed or crescent-shaped cells, especially in stained tissue sections. Cells are characterized by the presence of a polysaccharide (galactoxylomannan) capsule varying from a wide halo to a nearly undetectable, lighter zone around the cells, depending upon the strain and the medium used. Use of India ink with wet preparations, or the mucicarmine stain for polysaccharide with fixed tissue preparations, may be helpful in elucidating the capsule. Colonies typically are mucoid due to the presence of capsular material, become dry and duller with age, and exhibit a wide range of color (cream, tan, pink, and yellow). The color may darken with age. Strains possessing only a slight capsule may appear similar to colonies of *Candida.* All members of the genus produce urease, utilize various carbohydrates, and are nonfermentative.

C. neoformans was the first serious fungal pathogen of humans to be identified as a heterobasidiomycetous yeast. At least five serotypes of *C. neoformans* have been identified: A, B, C, D, and the hybrid serotype A/D. Serotypes A and D were found to produce the teleomorphic state *Filobasidiella neoformans*, and serotypes B and C produce the teleomorph originally named *Filobasidiella bacillispora* (120). The latter species was found to be identical to *C. neoformans* var. *gattii* (223). All are considered varieties of *F. neoformans,* and all are known to produce disease. Differences in disease manifestation among patients from areas where various serotypes are endemic appear to be more dependent on host immune status than on the *C. neoformans* variety (41). Serotypes A and D are opportunistic pathogens in that they attack immunocompromised individuals. Serotypes B and C attack immunocompetent individuals and are therefore primary pathogens. Standard laboratory tests do not differentiate among the serotypes. However, media have been recommended for separating serotypes A and D from serotypes B and C, but these media (glycine-cycloheximide-phenol red agar and canavanine-glycine-bromthymol blue agar) are not available commercially (123, 193). Proposals to designate the different serotypes of *C. neoformans* as separate species are under consideration, but the impact on the clinical mycology laboratory is uncertain.

The different serotypes of *C. neoformans* exhibit epidemiologic differences also (20). *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* (serotypes A and D, respectively) are the most common worldwide, with the highest prevalence of serotype A being seen in Japan and in tropical and subtropical areas such as Southeast Asia, Brazil, Australia, and Southern California. *C. neoformans* var. *gattii*

TABLE 1 Cultural and biochemical characteristics of yeasts frequently isolated from clinical specimens[a]

Species	Growth at 37°C	Pellicle in broth	Pseudo- or true hyphae	Chlamydospores	Germ tubes	Capsule, India ink	Assimilation of:												Fermentation of:						Urease	KNO_3 utilization	Phenol oxidase	Ascospores
							Glucose	Maltose	Sucrose	Lactose	Galactose	Melibiose	Cellobiose	Inositol	Xylose	Raffinose	Trehalose	Dulcitol	Glucose	Malose	Sucrose	Lactose	Galactose	Trehalose				
Candida albicans	+	−	+	+[b]	+	−	+	+	+*	−	+	−	−	−	+	−	+	−	F	F	−	−	F	F	−	−	−	−
C. catenulata	+*	−	+	−	−	−	+	+	−	−	+	−	−	−	+	−	−	−	F*	−	−	−	−	−	−	−	−	−
C. dubliniensis	+	−	+	+[b]	+	−	+	+	+	−	+	−	−	−	+*	−	+	−	F	F	−	−	F	F	−	−	−	−
C. famata	+	−	−	−	−	−	+	+	+	+*	+	+	+	−	+	+	+	+*	W	−	W	−	−	W	−	−	−	−*
C. glabrata	+	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	+	−	F	−	−	−	−	F	−	−	−	−
C. guilliermondii	+	−	+	−	−	−	+	+	+	−	+	+	+	−	+	+	+	+	F	−	F	−	F*	F	−	−	−	−*
C. kefyr	+	−	+	−	−	−	+	−	+	+	+	−	+*	−	+*	+	−*	−	F	−	F	F*	F	−	−	−	−	−
C. krusei[c]	+	+	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	F	−	−	−	−	−	+*	−	−	−*
C. lambica	+*	+	+	−	−	−	+	−	−	−	−	−	−	−	+	−	−	−	F	−	−	−	−	−	−	−	−	−*
C. lipolytica[c]	+	+	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−*
C. lusitaniae[d]	+	−	+	−	−	−	+	+	+	−	+	−	+	−	+	−	+	−	F	−	F	−	F	F	−	−	−	−*
C. parapsilosis[e]	+	−	+	−	−	−	+	+	+	−	+	−	−	−	+	−	+	−	F	−	−	−	−	−	−	−	−	−
C. pintolopesii[f]	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	F	−	−	−	−	−	−	−
C. rugosa	+	−	+	−	−	−	+	−	−	−	+	−	−	−	+*	−	−	−	−	−	−	−	−	−	−	−	−	−
C. tropicalis [d,e]	+	+	+	−[g]	−	−	+	+	+	−	+	−	+	−	+	−	+	−	F	F	F	−	F*	F*	−	−	−	−
C. zeylanoides	−	−*	+	−	−	−	+	−	−	−	−*	−	−*	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
Cryptococcus neoformans	+	−	R	−	−	+	+	+	+	−	+	−	+	+	+	+*	+	+	−	−	−	−	−	−	+	−	+	−
Cryptococcus albidus	−*	−	−	−	−	+	+	+	+	+*	+*	+	+	+	+	+	+	+*	−	−	−	−	−	−	+	+	−	−
Cryptococcus laurentii	+*	−	−	−	−	+	+	+	+	+	+	+*	+	+	+	+*	+	+	−	−	−	−	−	−	+	−	−	−
Cryptococcus luteolus	−	−	−	−	−	+	+	+	+	−	+	+	+	+	+	+	+	+	−	−	−	−	−	−	+	−	−	−
Cryptococcus terreus	−*	−	−	−	−	+	+	+*	−	+*	+*	−		+	+	+	+	−*	−	−	−	−	−	−	+	+	−	−
Cryptococcus uniguttulatus	−	−	−	−	−	+	+	+	+	−	−*	−	−*	+	+	+*	−*	−	−	−	−	−	−	−	+	−	−	−
R. glutinus	+	−	−	−	−	−*	+	+	+	−	+*	−	+	−	+	+	+	−	−	−	−	−	−	−	+	+	−	−
R. rubra	+	−	−	−	−	−*	+	+	+	−	+	−	+*	−	+	+	+	−	−	−	−	−	−	−	+	−	−	−
S. cerevisiae	+	−	−*	−	−	−	+	+	+	−	+	−	−	−	−	+	+*	−	F	F	F	−	F	F*	−	−	−	+
P. anomala	+*	−	−	−	−	−	+	+	+	−	+	−	+	−	+	−	+	−	F	F*	F	−	F	−	−	+	−	+
G. candidum[h]	−*	+	+	−	−	−	+	−	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
B. capitatus	+	+	+	−	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
P. wickerhamii[h]	+	−	−	−	−	−	+	−	−	−	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
Sporobolomyces salmonicolor	+*	+	+*	−	−	−	+	+	+	−	+*	−	+	−	W	−	+	−	−	−	−	−	−	−	+	+	−	−
Trichosporon asahi	+		+	−	−	−	+	+	+*	+	+	−	+	+*	+*	−	+*	−	−	−	−	−	−	−	+	+	−	−
Trichosporon mucoides	+		+	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	+	−	−	−
Trichosporon ovoides	+*		+	−	−	−	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+	−	−	−

[a] Modified from references 175 and 229. Symbols: +, growth greater than that of the negative control; −, negative reaction; *, some isolates may give the opposite reaction; R, rare; F, the sugar is fermented (i.e., gas is produced); W, weak reaction.

[b] *C. albicans* typically produces single and no more than two terminal chlamydospores, whereas some isolates of *C. dubliniensis* produce terminal chlamydospores in pairs, triplets, and clusters. See discussion in the text for additional methods to differentiate *C. dubliniensis* and *C. albicans*.

[c] *C. lipolytica* assimilates erythritol; *C. krusei* does not. Maximum growth temperatures: 43 to 45°C for *C. krusei* and 33 to 37°C for *C. lipolytica*.

[d] *C. lusitaniae* assimilates rhamnose; *C. tropicalis* usually does not.

[e] *C. parapsilosis* assimilates L-arabinose; *C. tropicalis* usually does not.

[f] *C. pintolopesii* is a thermophilic yeast capable of growth at 40 to 42°C.

[g] Rare strains of *C. tropicalis* produce teardrop-shaped chlamydospores.

[h] Not yeasts but may be confused with several yeast genera.

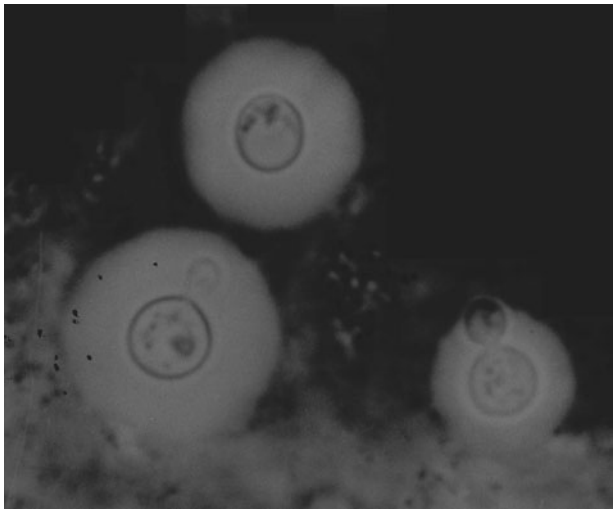


FIGURE 2 India ink preparation of *Cryptococcus neoformans*. Magnification, ×400. (Courtesy of E. S. Jacobson.)

(serotype B) is much more prevalent than serotype C strains of *C. neoformans* var. *gattii,* and the majority of serotype C has been found in Southern California (119).

C. neoformans was first detected in the environment in the late 19th century when Sanfelice recovered the yeast from peach juice. Since then, however, *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* have been most frequently associated with aged pigeon (and other bird) droppings and soils contaminated with these droppings. The yeast usually is not found in fresh droppings but is most evident in the bird excreta that have accumulated over long periods of time on window ledges, vacant buildings, and other roosting sites (119). It has only been recently that the environmental habitat of the *gattii* variety has been identified. Recovery of *C. neoformans* var. *gattii* (serotype B) from the environment coincides with the flowering of the river red gum tree (*Eucalyptus camaldulensis*). Likewise, the global distribution of cryptococcal disease due to *C. neoformans* var. *gattii* has been thought to correlate with the distribution of this tree (68). However, other sources may exist, particularly given a recent outbreak of disease in Vancouver Island, British Columbia (208, 210).

Initial cryptococcal infection begins by inhalation of the fungus into the lungs, usually followed by hematogenous spread to the brain and meninges. Involvement of the skin, bones, and joints is seen, and *C. neoformans* is often cultured from the urine of patients with disseminated infection. In patients without human immunodeficiency virus (HIV) infection, cryptococcosis, particularly cryptococcal meningitis, usually is seen in association with underlying conditions such as lupus erythematosis, sarcoidosis, leukemia, lymphomas, and Cushing's syndrome.

For nearly 45% of AIDS patients, cryptococcosis was reported as the first AIDS-defining illness. Because none of the presenting signs or symptoms of cryptococcal meningitis (such as headache, fever, or malaise) are sufficiently characteristic to distinguish it from other infections that occur in patients with AIDS, determining cryptococcal antigen titers and culturing blood and cerebrospinal fluid (CSF) are useful in making a diagnosis (45).

All species of *Cryptococcus* are nonfermentative aerobes. Separation of species is based upon assimilation of various carbohydrates and KNO_3 reduction (Table 1). *C. neoformans* may be distinguished from other species of *Cryptococcus* by these biochemical studies, as well as growth at 37°C, which may be seen with *C. albidus* and *C. laurentii*. *C. neoformans* may be differentiated from other yeasts and from other species of *Cryptococcus* by its production of brown colonies on birdseed agar, although with occasional isolates (particularly serotype C) the production of phenol oxidase may have to be induced. Differentiation from the genus *Rhodotorula,* which also occasionally forms a capsule, produces urease, and is nonfermentative, usually is accomplished by noting the utilization of inositol (Table 1) and the absence of carotenoid pigments characteristically present in *Rhodotorula. C. neoformans* can be distinguished from other non-dematiaceous yeasts in fixed tissue by the use of a Fontana-Masson stain, which detects melanin precursors in the cell wall. Mucicarmine stains stain the capsule, which helps to distinguish *Cryptococcus* spp. from yeasts with similar morphologies.

Genus *Debaryomyces*

The genus *Debaryomyces* contains at least 15 species that may be found in the environment, in the gastrointestinal tract of vertebrates, in processed foods, in dairy products, and in meat. The species of human importance is *D. hansenii*, the teleomorph of *Candida famata*. *D. hansenii* has been isolated from pigeon droppings (143) and is an infrequent cause of fungemia and endophthalmitis (183, 211).

On culture the yeast may be grayish white to yellowish, soft, shiny or dull, and smooth or with wrinkles. Budding yeasts and very occasional pseudohyphae may be seen. Conjugation between mother cell and bud occurs, and asci containing 1 to 4 round or oval warty ascospores are formed. Fermentation reactions are variable, urease is negative, nitrate is negative, inositol is negative, and the maximum growth temperature is 35°C.

Genus *Dipodascus*

Dipodascus capitatus is the teleomorph of *Blastoschizomyces capitatus*. There are two species in the genus *Dipodascus,* but only *D. capitatus* has clinical importance. *D. capitatus* is heterothallic, and asci are round to ellipsoidal and contain hyaline ellipsoidal ascospores. Only glucose, galactose, glycerol, D,L-lactate, and succinate assimilation tests are positive; hyphae, arthroconidia, and clavate conidia with truncated bases may be seen. The organism grows at 37°C.

Genus *Malassezia*

There are currently 11 species of *Malassezia: M. dermatis, M. furfur, M. globosa, M. japonica, M. nana, M. obtusa, M. pachydermatis, M. restricta, M. slooffiae, M. sympodialis,* and *M. yamatoensis* (83, 103, 213–215). These yeasts are isolated from the skin of warm-blooded animals and humans as commensals but can be agents of dermatological diseases, for example, pityriasis versicolor. The main causative agents of pityriasis versicolor are *M. globosa*, *M. sympodialis,* and occasionally *M. slooffiae* or *M. furfur* (9, 51, 89, 158). The diagnostic feature of pityriasis versicolor in skin scrapings is the presence of short, blunt-ended *Malassezia* hyphae among *Malassezia* yeasts ("spaghetti and meatballs"), observed by direct microscopic examination of the skin after digestion in a 10 to 30% KOH solution. Visualization of *Malassezia* species can be enhanced by the use of the calcofluor white stain with UV fluorescence. Other dermatological diseases, including seborrheic

dermatitis and folliculitis, have been associated with *Malassezia* yeasts often because there was a clinical response following antifungal therapy, with a reduction in yeast numbers. However, as *Malassezia* may be present on the skin in high numbers and there are no other characteristic features to provide laboratory diagnosis, the exact role of the organisms in these diseases remains unclear. Recently there have been reports of onychomycosis caused by *Malassezia* species (44, 52, 204); however, whether these yeasts are true invaders of nail or secondary colonizers remains to be decided. In addition, *M. furfur* and *M. pachydermatis* may rarely cause systemic infections, usually of neonates in intensive care units (138, 148). *M. pachydermatis* is associated with animal skin, while the other 10 species have been recovered from normal and diseased human skin. *M. sympodialis, M. slooffiae, M. globosa,* and *M. restricta* were the most frequent human colonizers (27, 28, 50, 83).

Malassezia species have a basidiomycetous affiliation within the class Ustilaginomycetes (71). Species may be distinguished genetically by karyotyping (25), large subunit rRNA gene sequencing (83), using PCR (88, 137), using PCR-restriction fragment length polymorphism (150), by amplified fragment length polymorphism and sequence analysis (87), and by phenotype, using cell morphology and a series of biochemical tests (83, 86). Ten of the 11 species require lipid for growth; only *M. pachydermatis* can grow independently of lipid supplementation of the media.

In order to isolate *Malassezia,* the growth medium has to contain a lipid supplement. Van Abbe (222) and Leeming and Notman (126) have designed media optimal for the growth and isolation of all of the *Malassezia* species. These media are not commercially available, and the alternative of overlaying Sabouraud glucose agar with a few drops of sterile olive oil has been used, but some of the more fastidious species do not survive on this medium (148). The inoculated media should be incubated aerobically at 32 to 35°C in a moist atmosphere for up to 2 weeks to permit development of the slower-growing species. Colonies are cream to beige in color and smooth to deeply folded and have a brittle texture that is frequently difficult to suspend.

Malassezia organisms are distinguished microscopically from other yeasts by the formation of a prominent monopolar bud scar, or collarette, resulting from the continued formation of daughter cells at that site (Fig. 3d). These structures are absent from *C. glabrata,* which could otherwise be confused with *Malassezia* species due to its small size and typical unipolar budding. Additional sympodial budding has been observed only in cultures of *M. sympodialis*. The species vary in shape from spherical to oval or elongated, have thick cell walls, and range in length from 1.5 to 8 μm. Identification of the individual species is not routinely attempted but can be achieved based on morphology, utilization of certain lipids including Tween 20, 40, and 80 and cremophor (castor oil), and reactions with esculin and catalase (86, 148). Organisms that are identified as *Candida lipolytica* (glucose, glycerol, and sorbitol positive) with the API 20C AUX (bioMérieux SA, Marcy l'Etoile, France) may actually be *M. pachydermatis* (138). In this case, morphology is helpful.

Genus *Pichia*

The teleomorphic genus *Pichia* encompasses several species of *Candida* (for example, *C. guilliermondii* var. *guilliermondii* and *C. norvegensis*). Generally, the anamorphic (asexual) state is the one recovered from clinical specimens, as mating studies usually are needed to produce the teleomorphic (sexual) form. *Pichia anomala* and *Pichia augusta* are the teleomorphs of the obsolete species *Hansenula anomala (Candida pelliculosa)* and *Hansenula polymorpha*, respectively, and are the two species in this genus that are most commonly associated with disease in humans. *Pichia anomala* is found mainly in the environment but is an opportunistic pathogen that can colonize human skin and has been a cause of fungemia in neonates (12, 39). It has also been associated with catheter-related infections (4, 114, 160). *Pichia augusta* has been recovered from the mediastinal lymph nodes of a child with chronic granulomatous disease (146). Variations in colony morphology may cause confusion with *Cryptococcus* and *Candida* spp. The texture may be smooth to wrinkled, and the color may be white, cream, or tan. Microscopically, multilateral budding cells are observed; yeasts and pseudohyphae may be seen. Individual species of *Pichia* are either homothallic or heterothallic. *P. anomala* is heterothallic, but the sporogenous diploid form occurs naturally and is the one usually recovered from clinical specimens. These diploid cells convert to form asci which will disintegrate to reveal one to four hat (or saturn)-shaped ascopores (Fig. 3a), a morphology which is characteristic of *Pichia* species. Carbohydrate assimilation and fermentation studies are needed to identify the individual species, although urease is negative, nitrate is positive, inositol is negative, and growth at 37°C can be variable. *Kodomaea ohmeri*, previously classified as *Pichia ohmeri*, is the teleomorph of *Candida guilliermondii* var. *membranaefaciens*. This species has been increasingly reported as an agent of disease in immunocompromised patients (reviewed in reference 94).

Genus *Rhodotorula*

Rhodotorula spp. are normal inhabitants of moist skin and can be recovered from such environmental sources as shower curtains, bathtub grout, and toothbrushes (48). In rare instances *Rhodotorula* spp. have been reported to cause septicemia (173), meningitis (181), systemic infection (107, 191), peritonitis associated with peritoneal dialysis (66), and sepsis related to complications from indwelling central venous catheters (113).

Rhodotorula spp. share many similar physiologic and morphologic properties with *Cryptococcus* spp. Both are round to oval, multilateral budding yeasts with capsules, produce urease, and fail to ferment carbohydrates. *Rhodotorula* spp. differ from cryptococci by their inability to assimilate inositol and their obvious carotenoid pigment. When a capsule is present, it is typically small, unlike *C. neoformans.*

Genus *Saccharomyces*

Saccharomyces cerevisiae is the most common species of the genus *Saccharomyces* to be recovered in the clinical laboratory. Usually thought to be nonpathogenic, it has been reported to cause thrush, vulvovaginitis, empyema, and fungemia (48, 70, 96). Person-to-person contact and exposure to commercial strains associated with health foods and baking may contribute to the ability of the organisms to colonize and infect human hosts (144).

Multilateral budding yeast cells are round to oval, and short, rudimentary (occasionally well-developed) pseudohyphae may be formed. Ascospore production can be enhanced easily by growing the yeast on Fowell's acetate agar for 2 to 5 days at room temperature. Asci contain one to four round, smooth ascospores (Fig. 3c). Other physiologic properties are listed in Table 1. Assimilation of raffinose by *S. cerevisiae* is noteworthy. Very few yeasts encountered in the clinical laboratory utilize this carbon source.

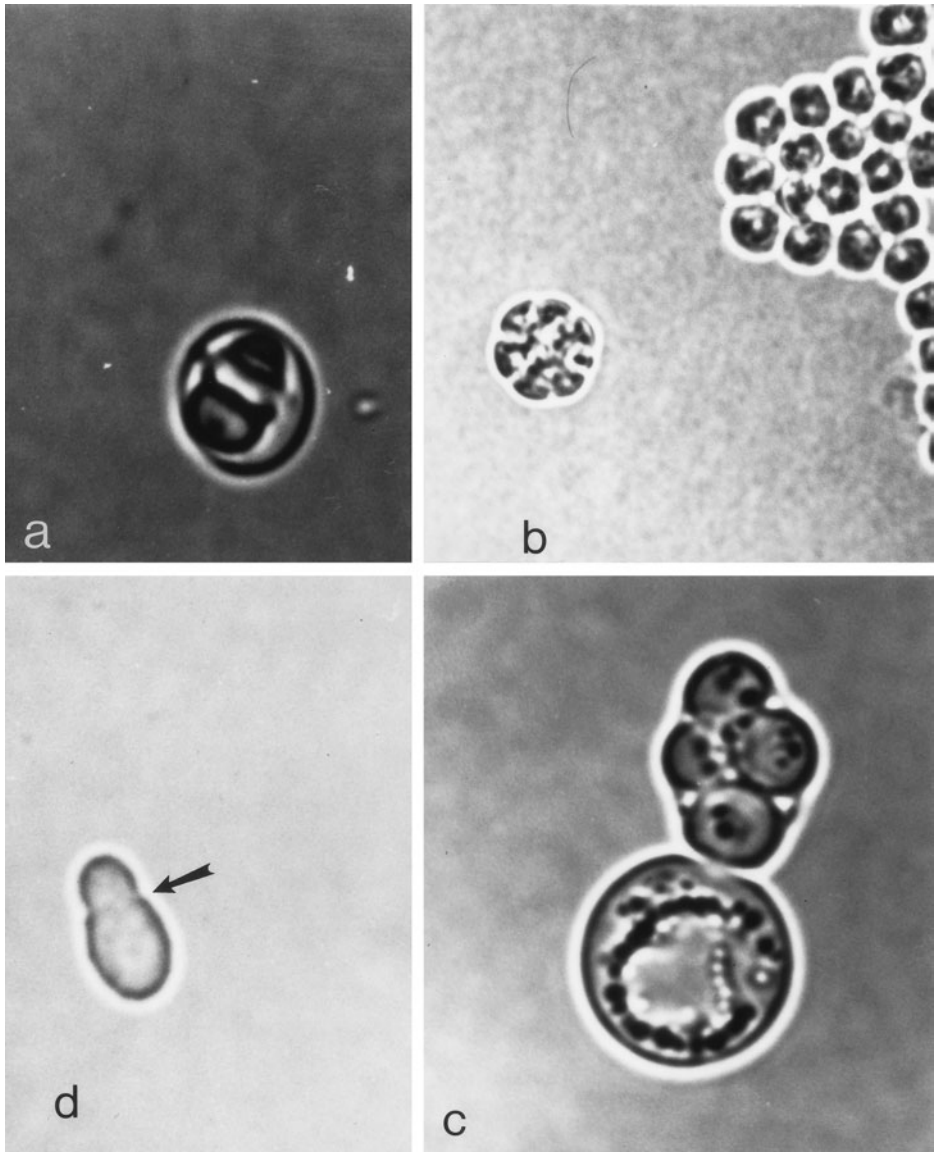


FIGURE 3 Diagnostic features of selected yeasts. (a) Ascus of *Pichia anomala* containing hat-shaped ascospores. (b) Sporangium of *Prototheca wickerhamii* containing sporangiospores. (c) *Saccharomyces cerevisiae* with vegetative cell and ascus containing four globose ascospores. (d) Bottle-shaped, budding yeast demonstrating annelloconidium and collarette (arrow) of *Malassezia furfur*. Magnification, ×910. (Courtesy of B. A. Davis.)

Genus *Sporobolomyces*

Sporobolomyces species are basidiomycetous yeasts that are environmental organisms, some of which have been linked to human disease including dermatitis (21), respiratory disease (47, 202), and AIDS (153, 177) and have been found to be a cause of endophthalmitis (203). Three species have been isolated from human infection, *S. roseus, S. holsaticus,* and *S. salmonicolor,* the last two species having the teleomorphs *Sporidiobolus johnsonii* (homothallic) and *Sporidiobolus salmonicolor* (heterothallic), respectively.

The colonies are soft and display shades of salmon pink. Budding oval to ellipsoidal yeasts, pseudohyphae, and ballistoconidia on large sterigmata may be seen. Fermentation is absent and assimilation is variable, with sucrose, urease, and nitrate being positive, inositol being negative, and growth at 37°C being variable.

Genus *Trichosporon*

Trichosporon beigelii used to be known as the major human pathogen of the genus *Trichosporon,* primarily causing the superficial hair infection white piedra, and rarely causing the systemic disease referred to as trichosporonosis. However, isolates of *T. beigelii* have been described as variable in their morphology and physiology (125). In 1992 (85), this genus underwent a major revision and 19 taxa were recognized, 6 of which could be found on humans, casting doubt on the use of *T. beigelii* as a valid species name. However, some mycologists continue to designate *T. beigelii* as the etiologic agent responsible for clinical disease caused by *Trichosporon* yeasts because differentiating between species can be problematic.

Yeasts of this genus may be isolated from soil, animals, and humans, but nearly all human infections are usually caused by one of six species: *T. asahii*, *T. asteroides*, *T. cutaneum*,

TABLE 2 Characteristics of clinically important *Trichosporon* species[a]

Characteristic	*T. asahii*	*T. asteroides*	*T. cutaneum*	*T. inkin*	*T. loubieri*	*T. mucoides*	*T. mycotoxinivorans*	*T. ovoides*
Assimilation of:								
Melibiose	−	−	+	−	+	+	+	−
Raffinose	−	−	+	−	+	+	+	V
L-Rhamnose	+	−	+	−	V	+	+	+
L-Arabinitol	+	+	+	−	V	+	W	−
Galactitol (dulcitol)	−	−	−	−	−	+	+	−
Ribitol (adonitol)	V	V	+	−	?	+	?	−
Sorbitol	−	V	+	−	V	+	+	−
Xylitol	V	+	+	−	V	+	W	V
Growth at 37°C	+	V	−	+	+	+	+	+
Growth at 42°C	−	−	−	V	+	−	−	−
Urease	+	+	+	+	+	+	+	+
0.01% cycloheximide	+	V	−	V	+	+	+	+
0.1% cycloheximide	−	V	−	−	+	+	+	−
Appressoria (on slide culture)	−	−	−	+	−	−	−	+
Fusiform giant cells	−	−	−	−	+	−	+	−

[a] Modified from references 84, 151, 166, and 229. Symbols: +, growth greater than negative control; −, negative; V, variable; W, weakly positive; ?, not reported.

T. inkin, *T. mucoides*, and *T. ovoides*. *T. loubieri* is an apparently rare cause of disseminated trichosporonosis (142, 166), and the recently described species *T. mycotoxinivorans* sp. nov. (151) has also been associated with disseminated disease (K. C. Hazen, C. C. Moore, and D. Padgett, unpublished data). White piedra is a superficial infection characterized by nodules of approximately 0.5 mm attached to the hair shafts on the head, axilla, or genital area. Infections in the genital area are usually due to *T. inkin*, and rarely encountered infections of hairs on the head are caused by *T. ovoides*. *T. asteroides* and *T. cutaneum* have been associated with superficial skin lesions, and *T. asahii*, and less often *T. mucoides*, is the cause of disseminated infection associated mainly with neutropenic patients with hematological malignancy or receiving immunosuppressive therapy (63). Others at risk for trichosporonosis include patients with AIDS, extensive burns, or intravenous catheters or patients receiving corticosteroids or who have undergone heart valve surgery (38, 49, 152). In addition, *T. asahii* and *T. mucoides* are thought to be the major causes of summer-type hypersensitivity pneumonitis in Japan, a condition that results from inhalation of *Trichosporon* arthroconidia present in the homes of the patients (161).

Patients with neutropenia tend to have a poor prognosis because achievable concentrations of antifungals in the serum are only inhibitory to growth and not sufficient to kill the yeast in vivo (49). MICs of the six human *Trichosporon* species indicate all species to have resistance to 5-fluorocytosine (6.25 to 50 μg/ml), to have higher MICs to fluconazole than most *Candida* species (0.15 to 25 μg/ml), and to be variably susceptible to amphotericin B (0.01 to >4 μg/ml), ketoconazole, and itraconazole (0.1 to 0.6 μg/ml) (89). Studies suggest that while in vitro levels of amphotericin B can be achieved in the sera of treated patients, the drug is inhibitory only to *Trichosporon* species (227). Echinocandins (caspofungin) are considered ineffective against *Trichosporon* species.

Trichosporon yeasts have no known sexual state, are basidiomycetes, and are closely related to *Cryptococcus*. They grow easily on standard mycological laboratory media, do not ferment carbohydrates, can assimilate various complex sugars, and are urease positive. Colonies form usually within a week on solid media and are cream colored and smooth. They may become dry, moist or shiny, folded, cerebriform, and elevated, with or without marginal zones with age.

They produce blastoconidia of various shapes, well-developed hyphae, pseudohyphae, and arthroconidia (Fig. 1i). *T. loubieri* also produces one- and two-celled giant cells (166), while *T. mycotoxinivorans* produces giant cells with up to eight cells depending on the growth medium (Hazen et al., unpublished). In cases where a *Trichosporon* isolate produces only few blastoconidia, differentiation from *Geotrichum* spp. may be difficult. Inoculation of malt extract broth at room temperature will encourage blastoconidia production in *Trichosporon* spp., usually within 48 to 72 h. These morphological and physiological characteristics have been used to distinguish between the species in the laboratory (89) (Table 2).

EPIDEMIOLOGY AND TRANSMISSION

Medically important yeasts are found on humans and warm-blooded animals and in the environments they inhabit (reviewed in reference 163). There are some yeasts which appear obligatory saprobes of humans and some warm-blooded animals. These include *Candida albicans, Candida glabrata,* and most likely *Candida dubliniensis.* Environmental sampling of foods, plants, potable water, and juices (pasteurized and not pasteurized) reveals an amazing array of yeast species known as opportunistic pathogens. In all cases, the presence of these yeasts could be attributed to direct and indirect contamination by warm-blooded animals. For example, Arias et al. (7) noted the presence of *C. parapsilosis, C. tropicalis, Clavispora lusitaniae, Candida zeylanoides,* and several other yeast species in single-strength orange juice which had been pasteurized but subsequently contaminated. These observations make clear that immunocompromised patients are repeatedly exposed to potential yeast pathogens other than those residing as part of their normal microbiota and care should be exercised in preparing foods and in monitoring the level of contamination associated with devices or creams which may be applied to the patient (134, 169). Parenteral nutrition fluids and devices are particularly prone to contamination with yeasts, especially *C. parapsilosis* (230). It is also noteworthy that the hospital environment can contribute to

the development of colonization with *Candida* species as well as facilitate replacement of less virulent colonizing species with more virulent species (26).

Person-to-person transmission has a negligible impact on disease development, except in nosocomial outbreaks where hands or fomites are often the source. Invasive yeast infections are associated with opportunism; thus, the yeasts causing disease must be present when the conditions are such that disease can be initiated. Several studies have shown that transmission of yeasts through sexual contact (including oral) does occur, but establishment of the transferred organisms in the recipient is affected by a variety of factors, most notably, the recipient's current normal microbiota, immunological status, and antifungal status (61, 184). Regarding the last factor, recipients with HIV and on low-dose fluconazole have been documented to develop oral colonization of a partner's fluconazole-resistant isolate through salivary exchange (61).

CLINICAL SIGNIFICANCE

As in clinical bacteriology, the significance of isolating a species known to be a member of the normal microbiota or a common contaminant depends on numerous factors, including, but not limited to, the patient's underlying disease, geographic and hospital environment, specimen site, preceding antimicrobial and device management, specimen collection and quality, and specimen processing. In general, the isolation of yeasts from sterile body sites is suggestive of infection, but the caveat to consider is the influence of the method of collection (e.g., *C. albicans* in CSF collected by lumbar puncture). The more challenging decision about significance is when the yeast is present in specimens obtained from nonsterile body sites.

Review of the literature reveals a surprising lack of evidence-based studies regarding the development of criteria for establishing significance in nonsterile body sites. Clinical microbiology laboratories will typically apply bacterial criteria to assess specimens for possible fungal infection. An example of this is to not report yeasts unless they are predominating (above bacteria). There are several problems with this approach. First, yeasts are not as abundant as bacteria in the normal microbiota, so an increase in relative abundance may reflect a yeast infection. Roughly 10 times more bacteria than yeast can occupy the same volume of tissue. Secondly, a specimen from a patient who is on antibacterial agents may contain more-abundant yeasts, the reporting of which may mislead the patient's physician about the yeast's significance. Third, when the abundance of a yeast is considered insufficient to warrant further investigation, the species may not be reported. This could lead to unfortunate consequences because certain yeasts, such as *C. tropicalis*, are considered particularly aggressive in immunocompromised patients.

The presence of yeasts in sputum has little significance (157) unless (i) no other etiology for infectious pneumonia can be determined, (ii) the yeast is consistent with an endemic dimorph, or (iii) there is evidence of minimal oropharyngeal contamination in the specimen and the yeast is relatively abundant. The presence of a few *C. neoformans* CFU in sputum does not necessarily imply etiologic significance unless there is patient information which is strongly suggestive of cryptococcosis.

The significance of yeasts in urine is also controversial due to lack of adequate studies. In one study to address levels of yeasts associated with urinary tract infection (UTI), Ahearn et al. (1) demonstrated that quantification of yeast levels provides useful information when correlated with identification of the species. Based on their study, high levels ($\geq 10^5$ CFU/ml of urine) were clearly associated with UTI, although levels of 10^4 CFU/ml should be regarded with suspicion. High levels of *C. albicans* were also seen in women during uncomplicated pregnancy but with little relationship to UTI. *C. glabrata* is also present in high numbers during UTI, especially in diabetics. However, other studies have shown that persistence of *C. glabrata* in urine regardless of level (10^3 CFU/ml or greater) is suggestive of chronic infection (100). *C. tropicalis* at levels of 10^3 to $\geq 10^5$ CFU/ml may be associated with UTI. Other species were much less often isolated from urine during the study by Ahearn et al., so correlations to disease are less clear. The presence of the same species of yeast in urine as in blood may suggest disseminated disease arising from a different site.

The importance of identifying to the species level urine isolates considered potential agents of UTI, especially in debilitated patients, is not trivial. Specific identification provides an indication of the severity of underlying disease and provides information needed for therapeutic management. Debilitated patients are more likely to have infections caused by unusual, as well as azole-resistant, species.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

No special practices for collection or transport of specimens with suspected yeast infection need to be followed. The reader is referred to chapter 116 of this Manual for standard practices for collection, transport, and storage. However, when specimens are collected and sent to the laboratory consideration should be given to the possibility that a particular fungus is highly infectious (e.g., *Coccidioides immitis*) or has unusual growth requirements (e.g., lipids for *Malassezia* species). Such information regarding the suspected etiologic agent should be provided to the laboratory in a manner that precedes manipulation of the specimen.

Transportation of specimens should be accomplished in less than 2 h. If a delay is anticipated, then normally sterile specimens should be stored at 37°C. Specimens obtained from sites potentially contaminated with normal flora should be stored at 4°C. Yeasts can withstand normal refrigeration temperatures.

DIRECT EXAMINATION OF SPECIMENS

The appropriate examination of a clinical specimen is essential prior to proper processing of the material. Additionally, it often will aid the laboratorian and the physician to obtain a preliminary identification and in either ruling in or ruling out certain pathogenic yeasts. Some methods are universal for the preliminary observation of fungi in a specimen, e.g., Gram stain, calcofluor white, and 20% KOH. However, others, such as the India ink preparation for the demonstration of a capsule, are used for yeasts only. This preparation is used with cultures and specimens of urine, cerebrospinal fluid, etc., which have been centrifuged. It generally is not useful for primary specimens such as sputum or other material which do not allow an even distribution of the ink. If 20% KOH is used on a preparation, neither India ink nor Gram stain may be subsequently added.

In performing the microscopic examination of a specimen for yeasts, structures that may aid in identification may be observed as follows (Fig. 4): (i) size and shape of the organism, (ii) morphology of the bud attachment site and number of

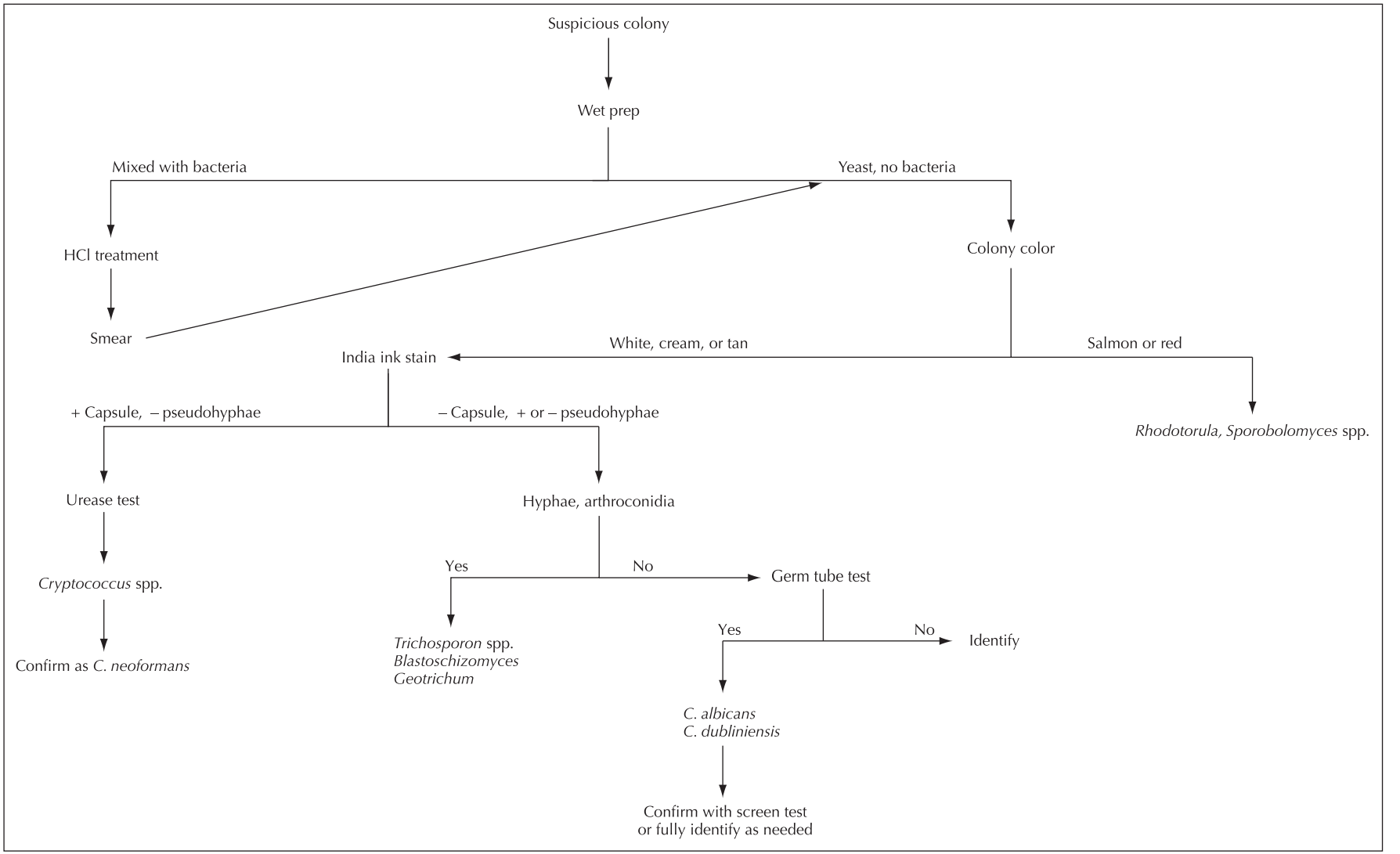


FIGURE 4 Scheme for identification of yeasts from clinical specimens.

buds, (iii) presence or absence of a capsule, (iv) thickness of the cell wall, (v) presence of pseudohyphae, and (vi) presence of arthroconidia. A great deal of information can be gleaned from careful direct microscopic examination of an appropriately prepared and stained specimen.

KOH

Wet mounts prepared in a 10 to 30% potassium hydroxide solution may be used to distinguish fungi in mucoid secretions or in skin, hair, or nail. The KOH digests the proteinaceous material, leaving the fungal elements intact. Gentle warming may speed this process, but care should be taken when using strong concentrations of KOH because the KOH rapidly crystallizes if overheated or left for prolonged periods before examination. The specimen is prepared by placing an aliquot onto a slide, adding to it a drop of KOH, and placing a coverslip on top. Ensure that enough KOH is present to completely cover the specimen. For skin and nail the specimen may be gently squashed while removing excess KOH. In these thick materials this makes visualization of the fungus easier. Hairs should always be viewed without squashing to obtain maximal information about the type of fungal infection. Dimethyl sulfoxide may be added to the KOH reagent to facilitate clearing without the need for heating or incubation, but the specimen should be examined quickly. Bubbles can be confused with yeast cells. Budding cells with internal heterogeneous material should be seen in order to enhance confidence that the object is a yeast. Round and oval objects lacking buds are common in tissue specimens and could be erroneously identified as yeasts.

The calcofluor white stain (Blankophore or Uvitex 2B) fluoresces under filtered UV light to enhance the detection of fungus as it binds to polysaccharides in chitin in the cell wall showing as bright green (4). A specimen may be prepared as above; after the coverslip is removed and a drop of calcofluor white is added, excess fluid is removed and the specimen can be viewed. Alternatively, a drop of calcofluor white can be added at the time of mounting in KOH. However, the stain may fail to penetrate thick nail samples when prepared in this way.

India Ink

India ink is used to visualize capsules which are transparent by bright-field illumination alone, as the ink is excluded from the capsule, showing a clear halo around the yeast. This stain is used to examine specimens of CSF, urine, and other body fluids for the presence of *Cryptococcus* species. Artifacts such as erythrocytes or talc from gloves appear to cause a halo effect; therefore, careful examination for the presence of yeast cells and bud formation is essential. A specimen should be prepared by placing a drop of the centrifuged sediment of a fluid specimen on a slide, adding a drop of Pelikan India Ink or Nigrasin solution, and placing a coverslip on top. While this method is relatively rapid and is generally satisfactory, a preferred method is to place a coverslip on the specimen and place of drop of ink on the side of the coverslip to allow the ink to diffuse underneath. This provides a gradient of ink which contains an optimal region to detect a capsule. India ink solution should be replaced regularly as it becomes contaminated, or alternatively a solution of 10% nigrasin in 10% formalin (using appropriate safety precautions) can be prepared.

Antigen Detection

With the exception of cryptococcal antigen tests for serum and CSF, efforts to develop other antigen detection systems have focused primarily on candidemia and disseminated candidiasis. No commercially available tests have been developed for body fluids and tissues other than blood and CSF. As described below in "Serologic Diagnosis of Yeast Infections," carbohydrate antigens have provided useful targets for several commercial kits for the detection of disseminated candidiasis. Protein antigens have also been investigated. Extracellularly secreted aspartyl proteinases appear promising (154, 244), but no commercial test is available. Another antigen, D-arabinitol, has been under investigation for over a decade and has recently been incorporated into the COBAS FARA II centrifugal autoanalyzer (Roche Diagnostic Systems) (244). Although only a limited number of laboratories have evaluated D-arabinitol as a marker for candidiasis, the antigen appears to be promising. D-Arabinitol levels appear to correlate with therapeutic success (67).

Nucleic Acid Detection

There are currently no FDA-approved nucleic acid detection systems for detection of yeasts directly from a specimen. Most efforts have been directed toward identification of yeasts which grow in blood culture bottles. Several different DNA amplification assays have been developed in research laboratories for this situation (reviewed in reference 67). A commercial peptide nucleic acid fluorescent in situ hybridization (PNA-FISH) kit has been recently approved (*C. albicans* PNA-FISH; AdvanDx, Woburn, Mass.); it specifically detects *C. albicans* in blood cultures by targeting species-specific rRNA sequences. Peptide-nucleic acid chains that mimic 26S rRNA are used to hybridize to target rRNA in *C. albicans*. The probe is also coupled to a fluorochrome which is detectable when the probe binds to its target. Several studies have shown that this assay is 100% specific (165, 186, 240). In the most comprehensive study, the test was found to have excellent sensitivity (99%), specificity (100%), positive predictive value (100%), and negative predictive value (99.3%) (240). The test can rapidly (2.5 h) indicate whether *C. albicans* is present or not and can thus indicate whether a non-*C. albicans* yeast is present (165, 186). The test does not replace subculture because blood cultures may contain mixed species. PNA-FISH-positive blood cultures should be subcultured to ensure that no other yeast species is present. *C. dubliniensis* is not detected with the PNA-FISH-probe. With this test, laboratories can report whether a positive blood culture with yeast contains *C. albicans* within a few hours after the culture becomes positive (109).

ISOLATION PROCEDURES

Processing specimens for fungal recovery is usually performed in conjunction with, and as an extension of, the processing procedure for recovery of bacteria. The primary difference is not the processing method but the selection of media onto or into which the specimen inoculum will be placed. One processing step that has unproven advantages for the isolation of yeasts from sputum specimens is the use of mucolytic agents (*N*-acetyl-L-cysteine or sputolysin).

There are, however, yeast infections for which specialized processing steps should be considered. For example, recovery of *C. neoformans* from blood or bone marrow is enhanced when lysis-centrifugation followed by inoculation onto solid media is used (80). If *C. neoformans* is suspected to be present in a respiratory specimen, setting up primary media which include a niger seed or related medium will enhance detection.

For some yeasts, a medium supplement (such as lipid) may be needed to either enhance or support growth during primary culture. A recent report showed that this approach facilitates

the detection of bile-dependent and bile-enhanced isolates of *C. glabrata*. Bile-dependent isolates have defects in the ergosterol synthesis pathway and are fluconazole resistant (14, 100). The addition of 8 μg of fluconazole per ml to primary media also helps to select for possible fluconazole-resistant isolates. This becomes a particularly powerful method when combined with a primary medium containing a chromogenic indicator used for presumptive identification of some yeasts (e.g., CHROMagar) (167).

IDENTIFICATION

Use of a sound, systematic approach to yeast identification is important. With the numerous yeast identification systems available today, most yeast species can be identified easily; however, repeat testing sometimes is necessary and morphologic characteristics on cornmeal-Tween or similar morphology agars should be mandatory.

Macroscopic Characteristics of Yeasts

Most yeasts grow well (*Malassezia* species are an exception) on common mycological and bacteriological media. Growth is usually detected in 48 to 72 h, and subcultures or lab-adapted strains may grow more rapidly. Colonies have a smooth to wrinkled, creamy appearance; some pigment may be observed initially or intensify with age. Heavily encapsulated yeasts give a very moist, mucoid appearance.

The ability of yeasts to grow at 37°C is a very important characteristic. Most pathogenic species grow readily at 25 and 37°C, whereas saprobes usually fail to grow at the higher temperature. Additionally, some yeast species, e.g., *Candida albicans*, grow in the presence of the cycloheximide (Actidione) found in Mycosel, Mycobiotic, and similar agars, whereas most other yeasts, including *Cryptococcus neoformans*, are inhibited.

Pellicle growth on the surface of liquid media such as Sabouraud glucose broth or malt extract broth has been used in the past to assist with yeast identification. More-recent evidence suggests that this characteristic can be variable; however, as an ancillary test, it may be helpful with identifying *Candida tropicalis* and *Candida krusei.*

Microscopic Characteristics of Yeasts

Upon isolation of a suspected yeast from a clinical specimen, the first examination should be a wet preparation of a colony. This provides an initial clue to the organism's identity. Observations should include the size and shape of the yeast, the method of bud attachment, and the presence or absence of pseudohyphae, true hyphae, or arthroconidia. Any round or slightly oval budding yeast with rare or no pseudohyphae seen in this preparation should be examined further for the presence of a capsule.

The same wet preparation used for the initial microscopic examination can be used for an India ink examination. The preparation then can be examined for the presence of encapsulated yeasts (Fig. 2). False-positive preparations usually are the result of contaminated ink or artifacts which can exhibit ragged edges. Capsular size cannot be used for identification purposes, as this characteristic may be influenced by culture age, media composition, and strain variation. The presence of a capsule does not automatically ensure that the yeast is *Cryptococcus neoformans* because other cryptococci, *Rhodotorula* spp., and rare *Candida* spp. produce capsule-like structures. In practice, any nonpigmented, round, encapsulated yeast recovered from CSF should be considered to be *C. neoformans* until proven otherwise.

Purity of Cultures

Before any additional physiologic tests are performed, it is essential to ensure that the culture is pure. A Gram stain of the culture can verify purity, but often bacterial contamination can be detected during the wet preparation examination. If the culture is mixed with bacteria, the isolate should be inoculated onto a blood agar plate and individual colonies should be picked or alternately, treated with hydrochloric acid (Fig. 4). The HCl procedure is performed by inoculating a colony into three tubes of Sabouraud glucose broth (each containing 5 ml). A capillary pipette is used to add 4 drops of 1 N HCl to the first tube, 2 drops to the second tube, and 1 drop to the third tube. After incubation at 25°C for 24 to 48 h, 0.1 ml of each broth is subcultured onto fresh Sabouraud glucose agar plates.

It is possible that more than one yeast species will be recovered from a clinical specimen, especially if the specimen is from a normally nonsterile site (243). In addition, multiple yeast species may be responsible for fungemia episodes. Careful attention to colonial morphology and microscopic characteristics can offer clues to a mixed population. Subculturing individual isolates to additional media can be helpful (243), and the use of a chromogenic medium may delineate the presence of more than one yeast species.

Morphology Studies

While examination of a wet preparation gives a primary indication of the yeast involved, more extensive study of morphology on cornmeal agar or other similar agars offers the opportunity to correlate morphologic characteristics with results of biochemical testing. Microscopic examination should reveal the thick-walled chlamydospores of *Candida albicans*/*Candida dubliniensis*. For features of other yeasts, see Table 3. Special attention also should be given to the size and shape of the pseudohyphae and the arrangement of blastoconidia along the pseudohyphae (Fig. 1).

Germ Tube Test

One of the most valuable and simple tests for the rapid presumptive identification of *C. albicans* is the germ tube test (Fig. 5a). The test is considered presumptive because not all isolates of *C. albicans* will be germ tube positive and false positives may be obtained, especially with *C. tropicalis,* despite well-trained staff (57). Also, *C. dubliniensis* is germ tube positive, although most isolates do not form germ tubes in a commercial synthetic germination medium (56). Microscopic observation of the preparation reveals that the short hyphal initials produced by *C. albicans* are not constricted at the junction of the blastoconidium and germ tube. Frequently, so many *C. albicans* blastoconidia produce germ tubes that the germ tubes become entwined with each other, producing clumps, especially when incubated longer than 3 h. *Candida tropicalis* also can produce hyphal initials, but the blastoconidia are larger than those of *C. albicans*, and there is a definite constricture where the hyphal initial joins a blastoconidium (Fig. 5b). In addition to using a known culture of *C. albicans* as a positive test control, negative controls using *C. tropicalis* and *C. glabrata* also should be included. Optimum conditions are obtained by using colonies grown on Sabouraud glucose agar or blood agar at 30°C for 24 to 48 h (unpublished observations). The test is performed by first lightly touching a colony with a bacteriological loop so that a thin film of organism is obtained and then transferring the inoculum into the test substrate (such as fetal bovine serum) followed by incubation at 37°C for 3 h. When *C. tropicalis* produces germ tube-like structures, less

TABLE 3 Microscopic appearance of several yeasts and yeast-like fungi on morphology agar

Organism	Pseudohyphae	True hyphae	Blastoconidia	Arthroconidia	Annelloconidia	Chlamydospores	Ascospores
B. capitatus	X	X	X		X		
C. albicans/ *C. dubliniensis*	X	X	X			X	
Other *Candida* spp.	X[a]	X[a]	X				X[a]
Cryptococcus spp.			X				
Geotrichum spp.		X		X			
Pichia spp.	X[a]		X				X
Rhodotorula spp.			X				
Saccharomyces spp.	X[a]		X				X
Trichosporon spp.	X	X	X	X			

[a] Strain variation.

than 15% of the cells do so. The preparation should not be incubated at 37°C longer than 4 h because other hypha-producing yeasts will begin to germinate after this time. In addition, a heavy inoculum of yeasts and the presence of bacteria can each lead to false-negative results.

PNA-FISH

The PNA-FISH test is described above (see "Direct Examination of Specimens"). Although the test is marketed for evaluation of positive blood cultures, the test could be used to facilitate identification of *C. albicans* growing as colonies on solid media.

Ascospore Formation

Homothallic yeasts recovered from clinical specimens may be present in their teleomorphic (sexual) state. In order to enhance production of ascospores by ascomycetous yeasts, cultures should be inoculated onto media such as Fowell's acetate agar, V-8 juice agar, or yeast extract-malt extract agar incubated at room temperature for 2 to 5 days and examined by wet preparation for the presence of ascospores within asci. Some mycologists prefer to perform special stains to detect ascospores. The Ziehl-Nielsen stain routinely employed in mycobacteriology can be used if needed; however, most ascospores can be detected easily in a drop of sterile distilled water. Ascospore production in *Saccharomyces cerevisiae* is evidenced by the presence of one to four globose spores (Fig. 3c); *Pichia anomala* produces one to four hat-shaped spores (Fig. 3a). Ascospore formation is a valuable test to aid in identification when equivocal results are obtained by other identification methods (Table 4).

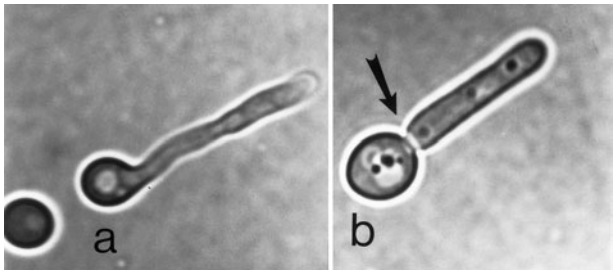


FIGURE 5 Germ tube test. (a) Germ tube formation of *Candida albicans*. (b) Blastoconidial germination with constriction (arrow) of *Candida tropicalis* not seen with true germ tubes of *C. albicans*. Magnification, ×400. (Courtesy of B. A. Davis.)

Phenol Oxidase Test

The phenol oxidase screening procedure detects the ability of *Cryptococcus neoformans* to produce phenol oxidase on substrates containing caffeic acid. The most frequently used medium is "birdseed" agar (containing niger or thistle seeds), and a number of formulations showing varying degrees of success have been described. Test performance is related to the glucose content of the medium; the more glucose in the medium, the less likely a valid test result will be obtained. *C. neoformans* colonies turn a dark brown in 2 to 5 days. Because of the wide variation in media formulations, the test must be subjected constantly to quality control measures. Some laboratories advocate the use of birdseed agar as part of the primary plating for respiratory cultures for early detection of *C. neoformans* isolates. A rapid phenol oxidase test utilizes a caffeic acid disk that is rehydrated and then inoculated with several yeast colonies and incubated at 35 to 37°C for up to 4 h. The disk should be examined every 30 min for the production of a brown pigment. If pigment is produced, it is presumptive for *C. neoformans;* if no pigment is detected but *C. neoformans* is suspected, further identification should be conducted.

Urease Test

The urease test detects a yeast's ability to produce the enzyme urease. In the presence of suitable substrates, urease splits urea, producing ammonia, which raises the pH and causes a color shift in the phenol red indicator from amber to pinkish red. The urease test aids in the identification of *Cryptococcus* spp. and *Rhodotorula* spp., which are urease positive. Most strains of *Trichosporon* spp. are positive, whereas *Geotrichum* spp. and *Blastoschizomyces capitatus* are negative. Nearly all *Candida* spp. encountered in clinical specimens are urease negative, exceptions being *Yarrowia (Candida) lipolytica* and some strains of *C. krusei*, both of which do not assimilate inositol. A rapid disk test can take from 4 to 72 h for a reaction to develop, while the more definitive urea agar slants can take from 24 to 72 h.

Rapid Trehalose Test

The rapid trehalose test can provide a presumptive identification of *C. glabrata* within 3 h. The Clinical and Laboratory Standards Institute (CLSI) has recently approved a rapid test procedure (document M35-A) for identification of *C. glabrata* (17) based primarily on rapid trehalose assimilation. False positives occur with trehalose tests (73), but these can be reduced if the results are correlated with small cell size and failure to produce germ tubes. Several modifications of

TABLE 4 Appearance of asci/ascospores of ascomycetous yeasts[a]

Anamorphic spp.	Previous synonym or obsolete name	Teleomorph (alternative spp.)	Hetero- or homothallic	Ascospores
Blastoschizomyces capitatus	*Geotrichum capitatum, Trichosporon capitatum*	*Clavispora capitatus*	Hetero	4/ascus, hyaline ellipsoidal, with slimy sheath when released
Candida cifferii		*Stephanoascus ciferrii*	Hetero	1–4/ascus, helmet or hat shaped
Candida famata	*Torulopsis candida*	*Debaryomyces hansenii*	Homo	1–2/Ascus, spherical with warts[b]
Candida guilliermondii		*Pichia guilliermondii (Yamadazyma guilliermondii)*	Hetero	Variable
Candida kefyr	*Candida pseudotropicalis, Candida macedoniensis*	*Kluyveromyces marxianus* var. *marxianus*	Homo	1–4/ascus, crescent to reniform, agglutinates on MEA[c]
Candida krusei		*Issatchenkia orientalis*	Homo	1–2/ascus, spherical[d]
Candida lipolytica		*Yarrowia lipolytica*	Hetero	1–4/ascus, spherical to hat-shaped protuberance on 1 or 2 ends
Candida lusitaniae	*Candida obtusa, Candida parapsilosis* var. *obtusa*	*Clavispora lusitaniae*	Hetero	1–4/ascus, clavate on MEA[b]
Candida norvegensis		*Pichia norvegensis*	Homo	1–4/ascus, hat shaped on acetate agar
Candida pintolopesii	*Candida slooffii*	*Arxiozyma telluris*	Homo	1–2/ascus, spherical to ovoid, rough to spiny
Candida pelliculosa		*Pichia anomala*	Hetero	1–4/ascus, hat shaped
Candida pulcherrima		*Metschnikowia pulcherrima*	Hetero	1–2/ascus, from chlamydospore, spherical with peduncle
Candida utilis		*Pichia jadinii*	Homo	1–4/ascus, hat shaped on MEA[b]
		Saccharomyces cerevisiae	Homo	1–4/ascus, spherical or short ellipsoidal (on acetate agar)

[a] Modified from reference 96.
[b] Difficult to see.
[c] MEA, malt extract agar.
[d] Difficult to induce.

the rapid trehalose assay have been introduced. The GLABRATA RTT (Fumouze Diagnostics, Levallois-Perret, France) combines hydrolysis of trehalose and maltose and includes a control (a sugar-free basal medium). *C. glabrata* hydrolyzes the trehalose rapidly, but not the maltose (which other yeasts may hydrolyze). The test provides acceptable sensitivity and specificity, but the choice of growth medium for the test inoculum influences the likelihood of obtaining false positives. Blood agar should be avoided, as both *C. albicans* and *C. tropicalis* cause high numbers of false-positive results (75, 239). The most suitable media appear to be CHROMagar and Sabouraud glucose (4%) agar. Combining colony color on chromogenic agars with the GLABRATA RTT or the rapid trehalose test could help improve specificity (156, 174). Rapid trehalose and maltose hydrolysis has also been modified into a 30-s dipstick assay. This test provides adequate specificity and sensitivity, but these parameters are affected by the medium used to grow the test inoculum (174).

Chromogenic Agars

Presumptive identification of one or two yeast species based on colony characteristics can be obtained with chromogenic agar media. CHROMagar *Candida* (CHROMagar France; Paris, France; distributed in the United States by Becton Dickinson/BBL, Sparks, Md.) allows differentiation of over 10 species and is marketed for the presumptive identification of *C. albicans, C. tropicalis*, and *C. krusei*. Colony identification is based on the differential release of chromogenic breakdown products from various substrates following exoenzyme activity. It is important to recognize that identifications are presumptive. Evidence suggests that variation in colony appearance occurs among the species (18, 170). The directions of the manufacturer must be strictly followed, as is the case in any rapid test based on exoenzyme activity. Incubation time and temperature significantly affect the colony appearance. The medium is useful for the detection of mixed yeast infections, especially in blood, and for resolving identification problems. Other chromogenic media are now commercially available. CandidaID (bioMerieux) and CandiSelect (Bio-Rad, Marnes La Coquette, France) performances are similar to that of CHROMagar. No one medium detects all instances of mixed yeast blood cultures (127, 238, 239). Approximately 2 to 10% of *C. albicans* isolates produced white colonies, rather than colored colonies, on these chromogenic media, strongly supporting the concept that these media provide only a presumptive identification. Other chromogenic media, namely Oxoid Chromogenic Candida Agar (OCCA, Basingstoke, Great Britain) and HiChrome (HiMedia Laboratories, Mumbai, India), have

been introduced recently, but published papers describing extensive comparative trials against other chromogenic media are not available.

Phenotypic Systems

A variety of manual and semiautomatic single- to multiphenotypic assay systems have been commercialized (Table 5). Most are designed to help identify the most common yeast etiologic agents. The systems vary in ease of use and accuracy. Generally the systems designed to identify multiple genera and their species are less reliable with unusual or rare pathogens (e.g., *C. inconspicua*), although sometimes rare isolates of common species such as *C. parapsilosis, C. guilliermondii,* and *C. krusei* can result in misidentification or poor species discrimination (32, 82).

Carbohydrate Assimilation Tests

The mainstay of yeast identification to the species level is the carbohydrate assimilation test (Table 1), which measures the ability of a yeast to utilize a specific carbohydrate as the sole source of carbon in the presence of oxygen. There are several reliable commercially available kits (such as API 20C AUX [bioMérieux SA], API ID32C [not available in the United States], and BBL Minitek Yeast Set [Becton Dickinson/BBL]) and automated and semiautomated systems on the market today that make the classical Wickerham and Burton method (234) unnecessary for routine clinical isolates. None of these tests are 100% concordant with the Wickerham-Burton method, and they do not uniformly agree with each other. Assimilation reactions provided in reference books, such as those by Barnett et al. (16) and Kurtzman and Fell (117) are based on the Wickerham-Burton method. Because commercial assimilation tests are only similar to the Wickerham-Burton tests, assimilation reaction profiles may differ from those provided in reference books.

Nitrate Assimilation Test

The rationale behind the nitrate assimilation test is similar to that of the carbon assimilation test. It tests the ability of a yeast to utilize nitrate as a sole nitrogen source. The test is most beneficial when trying to identify *Cryptococcus, Rhodotorula,* and *Pichia* spp.

Carbohydrate Fermentation Tests

Fermentative yeasts recovered from clinical specimens produce carbon dioxide and alcohol; therefore, production of gas is indicative of fermentation. The pH of the medium may not change. Rarely are fermentation studies needed to identify most of the commonly isolated yeasts if the mycologist is familiar with typical morphology on cornmeal agar. The test is most helpful in differentiating the various species of *Candida; Cryptococcus* and *Rhodotorula* spp. are nonfermentative.

Rapid Identification Tests

Rapid identification of yeasts impacts patient management. This is particularly true given the recognition that various yeast species (such as *C. glabrata, C. krusei, C. parapsilosis, C. lusitaniae, C. tropicalis,* and *C. neoformans*) are inherently or potentially resistant to amphotericin B, the newer azole agents, or echinocandins (78, 228).

Rapid tests are divided into those that provide definitive or presumptive identification within the same day (<24 h) of colony formation and either are specific to a single species, are limited to a few species, or apply to multiple genera (Table 5). In general, tests listed within groups 1 and 2 of Table 5 are presumptive and confirmation of the identification is needed.

Tests based on preformed enzymes may be acutely affected by incubation temperature (99) and are effectively limited to the more common yeast species isolated in the clinical laboratory. However, detection of specific exoenzymes can be useful for resolution of the confusion between two or three possible species. In particular, several rapid tests

TABLE 5 Rapid tests (<24 h) and semirapid tests (<48 h) available for presumptive or definitive identification of yeasts following colony formation[a]

Group 1 (for single species)		Group 2 (for several species)	Group 3 (for multiple genera)
Test name	Organism identified	Test name	Test name
Albicans-Sure	*C. albicans*	CandiSelect	Vitek-Yeast Biochemical Card
Albicans ID*	*C. albicans*	Candida check	Microbial Identification System (MIDI)
Albistrip*	*C. albicans*	CHROMagar	
BactiCard Candida	*C. albicans*	Fungiscreen H*	Microscan Rapid Yeast ID
Bichrolatex Albicans*	*C. albicans*		Quantum II
Fluoroplate*	*C. albicans*		IDS RapID Yeast Plus System
Germ tube	*C. albicans*		Uni-Yeast Tek
Rapidec albicans*	*C. albicans*		API 20C AUX
C. albicans screen (= Murex CA 50)	*C. albicans*		API ID32C*
			API Candida
MUAG test	*C. albicans*		Vitek2 ID-YST
PNA FISH Candida	*C. albicans*		BBL Minitek
Bichrolate krusei*	*C. krusei*		
Rapid trehalose assimilation	*C. glabrata*		
Caffeic acid disk	*Cryptococcus neoformans*		

[a]*, test not available in the United States.

are available for this purpose, particularly for detection of *Candida albicans* (for example, the *Candida albicans* screen [Carr-Scarborough Microbiologicals, Decatur, Ga.] and the Albistrip [Lab M Ltd., Bury, United Kingdom]). These tests are excellent for confirmation of a germ tube-positive organism as *C. albicans* and could be used in place of more long-term identification tests for this purpose. However, similar results are obtained for *C. dubliniensis*. To date this organism is most frequently isolated from the oropharynx of HIV-positive patients, but it may infrequently be recovered from blood, urine, vaginal, or other specimen sites, especially if the patient is immunocompromised. Using these tests for confirmation purposes can provide a financial saving to the clinical laboratory. Both tests depend on the production of two enzymes (proline aminopeptidase and β-galactosaminadase) instead of a single enzyme (β-galactosaminadase), which could otherwise lead to spurious results (209). Distinguishing *C. albicans* from *C. dubliniensis* is important, especially if the patient has been exposed to fluconazole, because the latter species can be induced to develop resistance under azole pressure. Several single tests have been suggested to help differentiate these two species from one another. These include, but are not limited to, growth at 45°C (positive for *C. albicans*), reduction of 2,3,5-triphenyltetrazolium chloride (positive for *C. dubliniensis*), production of a dark green color on CHROMagar (*C. dubliniensis*), production of β-glucosidase (*C. albicans*), and abundant chlamydoconidia on Staib agar (*C. dubliniensis*) (2, 164, 176, 198, 224). These tests are generally not rapid and are not definitive (e.g., some isolates of *C. albicans* do not grow at 45°C). Other differential agar media have been suggested (3, 112). Molecular tests and serologic tests appear to be promising rapid alternatives with higher sensitivity and specificity.

Serological identification tests, which use rabbit antisera directed against specific polysaccharides on the cell walls of *Candida* species and *Cryptococcus neoformans*, are available (Mitsubishi Kagaku Iatron, Inc., Tokyo, Japan). *C. albicans* cannot be differentiated from *C. dubliniensis* by the antisera, and *C. neoformans* serotypes B and C cannot be distinguished from each other.

Molecular Methods

At present yeasts are not identified in the routine clinical environment by use of nucleic acid technology. However, as methods are being developed for use in research, diagnostics, and epidemiology, this technology is beginning to emerge. Some reference texts (59) have included restriction maps of ribosomal operons for described fungi, Public sequence databases, most famously GenBank, are available via the Internet for laboratorians to find genetic matches for their organisms.

In order to develop a universal genetic identification system for yeasts, the most reproducible, specific, and sensitive sequence for comparison must be identified. Much of the recent research has focused on the rRNA gene regions. The large subunit, specifically the D1/D2 region, was shown to be highly discriminatory for the ascomycetous yeasts (118) and similarly for the basidiomycetous yeasts (72, 199), although differentiation of closely related species in the last study was achieved using the internal transcribed spacer (ITS) region. More recent studies have also used the ITS1 and ITS2 regions (42, 221). The potential of these methods was demonstrated by Massonet et al. (142a) when comparing 61 isolates of *Candida*, *Cryptococcus*, *Blastoschizomyces*, and *Saccharomyces* by ITS2 fragment length polymorphism with the ID32C and VITEK 2. The genetic analysis correctly identified 55 or 59 isolates depending on the method of extraction, the ID32C identified 58 isolates, and the VITEK 2 (fluorometric card) identified only 41 isolates. Hall et al. (93) used a commercially available MicroSeq D2 large-subunit ribosomal DNA sequencing kit to identify 131 clinical yeast isolates and compared the results to those obtained by conventional methods, which included the API 20C AUX system. Comparisons of 100 isolates representing 19 species of *Candida* using the MicroSeq kit matched 98% with identifications using the API 20C AUX. For the remaining isolates, representing nine species, there was 81.3% concordance between the systems. Most of the discrepancies were attributed to a lack of available data in the relevant databases. However, more research is needed before a universal genetic database can be devised because the D1/D2 and ITS regions do not appear to identify all yeasts. Using the ribosomal 5.8S-ITS sequence only 6 of 15 *Debaryomyces* species were correctly identified; the remaining species were differentiated using the sequences of the ACT1 gene (141).

For organisms that are difficult to grow or require non-commercially available materials, these methods hold great potential. For example, identification of *Malassezia* species requires both specialized growth media and phenotypic identification tests. The use of DNA technology has led to the identification of four new species in recent years (*M. yamatoensis* [213], *M. japonica* [214], *M. nana* [103], and *M. dermatis* [215]), and rapid identification methods for this genus have been developed using D1, D2, and ITS sequences (87) and by PCR-restriction fragment length polymorphism (150).

Many laboratories do not possess sequencing equipment or facilities to develop the use of these technologies for mycology; however, there are a number of other DNA-based methods available to aid in identification. Restriction enzyme analysis (REA) using the enzyme Hinf1 has been very useful in providing simple and characteristic DNA band patterns for a large number of *Candida* species, with the bonus that it is particularly good at strain differentiation for some species (77, 194). A number of species-specific probes have been developed for use with Southern blots of REA samples. These include 27A (195) and Ca3 (197) for *Candida albicans* and *Candida dubliniensis*, Ct-3 for *Candida tropicalis* (110), Cg6 and Cg12 for *Candida glabrata* (130), and Cp3–13 for *Candida parapsilosis* (69). Pulsed-field gel electrophoresis (PFGE) separates whole chromosomes (karyotyping) or large pieces of DNA that are not resolvable by conventional electrophoresis. The extraction facilitates this by taking place inside an agarose block to prevent damage and shear of the nucleic acids. Some species have characteristic karyotypes with no strain variation, for example, *Malassezia* (25), whereas other species, such as *C. albicans* (46) and *C. glabrata* (58), can be strain typed using this method.

PCR is a powerful tool and is the basis of the amplification used for sequence analysis. When sequencing is not permissible, genetic targets can be amplified and compared for size or for structure by digesting the amplicon with a suitable restriction endonuclease (237). An alternative is to use a universal sequence as a primer to generate species-specific patterns. The microsatellite sequence M13 has been found to be very useful for species identification and has even been used to identify anamorph and teleomorph pairs (124). By using a low annealing temperature for primers in PCR, a technique known as random amplification of polymorphic DNA, or RAPD, can be used for strain typing. However, problems of reproducibility prevent interlaboratory comparisons of the generated fingerprint patterns (205), confining

this to a useful method for investigation of local outbreaks or for intralaboratory studies of strain relatedness.

White et al. (233) and Ellepola and Morrison (67) have reviewed developments for rapid diagnosis of invasive candidiasis, including PCR-based methods. New techniques combining enzyme immunoassay formats for the rapid detection of species-specific amplicons and the use of real-time PCR are discussed. Standardization remains an issue before any of these tests become commonplace, but the benefits to earlier and more specific diagnosis are clear and these tests will complement those used currently in the clinical environment.

Troubleshooting Difficult Identifications

Laboratories encounter yeast isolates that do not fit easily into a specific species, particularly when commercial identification systems are used. There are key features that help separate troublesome organisms from others. For example, urease production separates basidiomycetous yeasts from ascomycetous yeasts. Maximum growth temperature, cycloheximide resistance, and the ability to assimilate cellobiose, inositol, or trehalose are helpful. For more difficult identifications, use of traditional auxanographic methods but with carbon source-impregnated disks on yeast carbon base agar plates (rather than spotting the sugar on the edge of the plate as done by Beijerinck [19]) may provide the most accurate assimilation information. Differential media such as chromogenic agars or eosin methylene blue agar (*C. glabrata* and *C. kefyr* produce distinct colonies) may sometimes help (155). Fermentation tests should also be considered, as should a second commercial system that utilizes a different endpoint (e.g., growth versus exoenzyme production). One of the molecular methods of identification could be tried for isolates that consistently fail to be identified by conventional tests.

ORGANISMS RESEMBLING YEASTS

Occasionally organisms such as molds, algae, etc., may grow on mycologic media and produce colonies that resemble those produced by yeasts. However, careful attention to morphologic characteristics can differentiate them. The following are several examples of such organisms that are recovered from clinical specimens and superficially can be confused with yeasts.

Genus *Geotrichum*

Members of the genus *Geotrichum* may be confused with yeasts because they produce a white to cream-colored, mealy, subsurface colony on fungal media. Occasionally aerial mycelium may be produced, giving a colony which superficially resembles *Coccidioides immitis*. Microscopic examination reveals septate, hyaline hyphae which break into arthroconidia upon maturation (Fig. 1c). No other structures are observed. *Geotrichum candidum* may be separated from *Trichosporon* spp. by carbohydrate assimilations and the lack of urease and blastoconidia production. *Geotrichum* spp. occasionally may be isolated from patient specimens but is rarely considered a pathogen except in extremely debilitated individuals.

Genus *Ustilago*

The genus *Ustilago* represents a heterobasidiomycetous fungus which is parasitic on seeds and flowers of many cereals and grasses. It may be inhaled and, therefore, isolated from sputum specimens. The colonies are slow growing and, when young, are moist, compact, and white. With age, short hyphae are produced and the colonies become velvety or powdery. Microscopically the cells are irregular and yeast-like or elongate to spindle-shaped and may resemble arthroconidia. With the production of short hyphae, clamp connections are formed. Physiologically, most isolates are nitrate positive and have a carbohydrate assimilation pattern similar to that of *Cryptococcus albidus*.

Black Yeasts

Occasional isolates of *Sporothrix schenckii, Aureobasidium pullulans,* and agents of phaeohyphomycosis initially produce white to tan yeast-like colonies on primary isolation media. Identification methods for these organisms are quite different from yeast identification methods and are discussed in chapter 125.

Genus *Prototheca*

Prototheca species are ubiquitous achlorophyllous algae, living on decaying organic matter, and rarely produce disease in humans and animals (111, 180). Human infection usually involves the skin and underlying tissues or olecranon bursa. Systemic protothecosis with algaemia is an extremely rare manifestation (30). *Prototheca wickerhamii* is recovered most often from human specimens, whereas *P. zopfii* usually is associated with animal infections. Colonies are white to cream-colored, yeast-like, and dull or moist to mucoid in appearance. The optimal growth temperature is 30°C, and growth is inhibited by media containing cycloheximide. Microscopically the cells are variable in size and shape and do not bud (Fig. 3b). Asexual reproduction is by release of sporangiospores from sporangia. Generally, sporangia of *P. zopfii* (14 to 16 μm) are larger than those of *P. wickerhamii* (7 to 13 μm), but this characteristic can be influenced by environmental conditions. Cells of *P. wickerhamii* are round, whereas most strains of *P. zopfii* exhibit cells which are oval to cylindrical in shape. Carbon assimilation studies can be used for identification purposes, and most commercial identification systems provide accurate identification of *P. wickerhamii*. Both species assimilate glucose and galactose; additionally, *P. wickerhamii* assimilates trehalose. *Prototheca* is a nonfermentative genus (179). *P. wickerhamii* is sensitive to voriconazole (129).

STRAIN IDENTIFICATION AND DIVERSITY

To understand the epidemiology of infection, to distinguish between endogenous and exogenous infection, and in the latter to locate the source, to examine transmission, or to monitor the spread of drug resistance, it is necessary to be able to distinguish between isolates within species.

Phenotypic typing schemes have been developed (106, 172, 182), which have the advantage of being inexpensive, but they can be prone to problems with reproducibility and the effects of storage on expression of characteristics. DNA-based schemes were developed to overcome these problems. Occasionally the results of phenotype and genotype have been combined to improve on the discrimination of either technique alone (79).

One of the oldest genetic typing techniques is REA, but the results depend on the restriction enzyme used, with EcoRI (136) and HinfI (77) being very useful for the discrimination of strains from a number of *Candida* species. However, not all species can be typed in this way; for example, isolates of C. *parapsilosis* show few differences when REA is used. Sometimes strain discrimination can be enhanced by Southern blotting the restriction digest gel and hybridizing with a DNA probe, such as the non-species-specific

poly(GT) (6, 236) or (CT)8 (216), or with species-specific probes, most notably the *Candida albicans* probe Ca3, which can demonstrate microevolution within one strain over a period of time (131).

PFGE has been used to produce electrophoretic patterns of whole chromosomes (karyotyping) for a number of species and has been used for strain typing of *C. albicans* (46), *C. glabrata* (58), *C. parapsilosis* (79), and several other *Candida* species (205). In some cases greater discrimination can be obtained by REA using a rare-cutting enzyme like SfiI or BsshII to generate large DNA fragments that are then separated using PFGE (226). However, as with ordinary REA, the discrimination of these enzymes differs between the species, with optimal results obtained using BsshII to differentiate isolates of *Candida tropicalis* (185), whereas SfiI was better for distinguishing isolates of *Clavispora lusitaniae* (115).

RAPD has been a very popular PCR-based strain typing technique (128, 245), although reproducibility can be a problem (205). Furthermore, a number of primers may have to be screened for each yeast species investigated before maximal discrimination can be established (79, 212). Successful discrimination of strains of *Cryptococcus neoformans* var. *grubii* from AIDS patients was achieved using a combination of PCR fingerprinting and RAPD analysis, with each patient having a unique strain and occasional patients being infected with more than one strain (108). Therefore, successful strain typing requires the choice of technique(s) and experimental conditions for maximum discrimination to be established for each species tested.

SEROLOGIC DIAGNOSIS OF YEAST INFECTIONS

Signs and symptoms of invasive yeast infections generally overlap those of bacterial infections, necessitating development of rapid non-culture-based methods for diagnosis. Non-culture-based tests for discriminating fungal versus bacterial infection and for detection of specific genera have been developed. Of all of these tests, the cryptococcal antigen test has proven to be the most successful, but recent progress suggests that a similarly successful *Candida* serology test may be available in the near future.

Discrimination of Fungal Infection from Other Microbial Etiologies

The unique composition of the fungal cell wall makes it particularly well suited as a focus for fungal serological tests. Many of the more common pathogenic yeasts contain β-1,3-glucan in the wall. β-1,3-Glucan is detected by two commercial kits, the Glucatell (Associates of Cape Cod) and the Fungitek G (Seikagaku Corp.). Repeat testing of patients who appear to be negative is recommended because single-test positive results provide generally good single positive test sensitivities and specificities (162) and repeatedly negative tests have a high negative predictive value. Repeat testing of patients who tested positive also improves the specificity of the test (162, 225). A positive test, however, does not provide information about the specific etiology, which is a weakness if tailored antifungal therapy is desired.

Cryptococcosis

Detection of cryptococcal antigen (capsular galactoxylomannan) in serum and CSF has been available for over 2 decades. During this time commercial tests have evolved to overcome early problems with specificity and sensitivity. The chief problem was false positives due to rheumatoid factor. Once this factor is destroyed, the sensitivity and specificity of the current generation of cryptococcal antigen tests are high. The test has been recommended as the primary evaluative tool in lieu of an India ink stain for screening CSF in suspect cases of cryptococcal meningitis. This is because the sensitivity of the India ink stain is low.

False-positive tests due to causes other than rheumatoid factor have been observed. These have been associated with trichosporonosis, *Capnocytophaga canimorsus* septicemia, malignancy, *Stomatococcus* bacteremia, some soaps and disinfectants, and hydroxyethyl starch (used in fluid resuscitation) (23, 40, 104, 135, 147, 149, 231). Interestingly, the cryptococcal galactoxylomannan contains an epitope that cross-reacts with the galactomannan antigen of *Aspergillus* spp., which is the antigen target of the Platelia *Aspergillus* assay (54).

Monitoring a decrease in antigen titer as an indication of effective anticryptococcal therapy has been suggested. However, while the titer may decrease after initiation of therapy in non-HIV patients, it may remain greater than 200 despite microbiological clearance (133). Lu et al. (133) have suggested that a better indicator of successful therapy is the return of CSF glucose, chloride, and leukocyte count to normal limits.

Candidiasis

Invasive *Candida* infections are life threatening, but detection by relatively noninvasive methods such as blood cultures is problematic. A test for the detection of antibody to *Candida* mannan is also available. Its utility is maximized when combined with the detection of antigen levels, as the antibody and antigen levels may show an inverse correlation during disease progression. The Cand-Tec test (Ramco), which detects an unspecified mannan antigen in the cell wall of *Candida* species, appeared to be relatively robust during initial studies in the 1980s, but subsequent studies during the 1990s suggested that the test provided inadequate sensitivities. A more promising test, the Platelia *Candida* Ag test (Bio-Rad), utilizes a monoclonal antibody EB-CA1, which targets an α-1,2-oligomannoside common among multiple species of *Candida*. The test has shown reasonable sensitivity and high specificities, although these vary from study to study. Sendid et al. (200) have recommended that both the antigen and the antibody tests (Platelia *Candida* Ab) be performed to maximize early diagnosis of invasive candidiasis. The specificity of the antigen test can be further enhanced by including a test for a second antigen, β-1,2-oligomannan, which is found in only a limited number of *Candida* species, *C. albicans, C. glabrata,* and *C. tropicalis* (201). *C. dubliniensis* variably expresses the antigen (101).

Specific Tests for Invasive Infections Caused by Other Yeasts

Not surprisingly, little effort has been undertaken to develop serologic tests for detection of invasive infections caused by yeasts other than *Candida* and *Cryptococcus* species. With the growing awareness of bloodstream infections caused by *Trichosporon* species, specific serologic tests for their detection may be developed. Before this occurs, a possible strategy is to use the serum cryptococcal antigen test in combination with compatible signs and symptoms and underlying disease to suggest a diagnosis of trichosporonosis.

ANTIFUNGAL SUSCEPTIBILITIES

Antifungal resistance profiles and mechanisms of yeasts and molds are described in other chapters of this Manual.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

As with bacteria, the clinical significance of an isolated yeast, especially one that may be part of the normal host microbiota, depends on a number of factors, especially the patient's signs and symptoms and underlying disease. Two specimens that are particularly problematic are sputum and urine (clean catch, indwelling catheter, and "in and out" catheter). The significance of *Candida* species in sputum regardless of quantity has been questioned (15, 157). Unfortunately, clinical microbiology laboratories often are not provided information about a patient's oropharyngeal status. Also, *Candida* species can cause pneumonia in debilitated patients. To prevent unnecessary testing but still provide useful information to physicians, Barenfanger et al. (15) recommend reporting "yeast, not *Cryptococcus*" for all respiratory secretion specimens in which a rapidly growing yeast is obtained. *Cryptococcus* species are ruled out using a rapid urease test. This approach allows physicians the opportunity to request further identification if desired. A similar approach to rapidly growing yeasts isolated from routine urine specimens (UTI is the only presentation) could also be used. However, in this situation, differentiation of *C. glabrata* from other common yeast uropathogens is the concern. As *C. albicans (C. dubliniensis)* and *C. glabrata* can be ruled out with simple rapid tests, a report of "yeast, not *C. albicans*/*C. dubliniensis*, not *C. glabrata*" could be made for those urines for which the laboratory director considers that yeast identification is indicated.

A common practice is to not identify rapidly growing yeasts present in clinically significant quantities from nonsterile body sites beyond a simple screen for *C. albicans*. This practice is potentially harmful but depends on the patient's condition. Yeast species express different antifungal profiles especially to the expanding spectrum of new antifungal agents.

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Pneumocystis

MELANIE T. CUSHION

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TAXONOMY

Organisms once known collectively by the single genus and species *"Pneumocystis carinii"* are nonfilamentous fungi that inhabit the lungs of mammals, but not birds or poikilotherms. Current evidence suggests that they can exist without consequence in mammalian hosts with intact immune systems (48, 83), but once the immune system becomes compromised, the organisms proliferate within the lung alveoli and cause a lethal pneumonia if untreated. No species of *Pneumocystis* can be cultivated outside the mammalian lung, impeding diagnostic capabilities as well as basic scientific research. Limited therapy is available with which to treat the pneumonia, since these organisms are not susceptible to standard antifungal drugs such as amphotericin B and fluconazole.

Taxonomic problems have plagued the organisms known as *"P. carinii"* since their original description in 1909 by Carlos Chagas. He included developmental forms of a cryptic *Pneumocystis* lung infection in trypanosome-infected animals as life cycle stages of the protozoan parasite *Trypanosoma cruzi* (14). In 1914, these organisms were provided an identity of their own and given the binomial epithet that reflected their predilection for the lung, *pneumo-;* the characteristic morphological form, *-cystis;* and *carinii,* to honor the Italian investigator, Antonio Carini, who provided the slides for study (29). Presumed to be a protozoan parasite at the time of identification, the potential fungal nature of *P. carinii* was raised in the 1950s (16, 58), and the controversy of the protozoan or fungal nature of these organisms continued to the late 20th century (87).

In the late 1980s, phylogenetic analyses based on the nuclear small subunit rRNA sequence alignments showed that *P. carinii* was a member of the fungal kingdom (33, 96, 97). Subsequently, additional gene sequence comparisons and cDNA and genomic sequence data emerging from the *Pneumocystis* Genome Project showed that the closest extant relative to *P. carinii* was the fission yeast *Schizosaccharomyces pombe* (18, 25). Based on genetic and morphologic criteria, *P. carinii* was placed with *S. pombe* in the Phylum Ascomycota within a newly described Class, Archiascomycetes, without designation of an order (1). In contrast, Erikkson proposed the creation of the Family *Pneumocystidaceae,* Order *Pneumocystidales,* based solely on gene sequence comparisons (35).

The morphological characteristics of *Pneumocystis* organisms are very similar regardless of the host origin. Until 1976, *"P. carinii"* was thought to represent a single zoonotic species. At that time, Frenkel described serological differences between human- and rat-derived organisms that he suggested were representative of distinct species (37), but these assertions were largely ignored by the medical community. However, it is now clear that the organism first identified as *"P. carinii"* is actually a family of related organisms that exhibit mammalian host specificity. Almost every mammal examined to date appears to harbor at least one species of *Pneumocystis* that is not found in any other mammal. Four species have been formally described (87): *P. carinii* (37, 38) and *P. wakefieldiae* (21, 26) infect rats; *P. murina* is found in the lungs of mice (53); and *P. jirovecii* is the name given to the organisms that cause infection in human beings (37, 38). The name *P. jirovecii* has not been without controversy (27, 40), and it remains to be seen if this epithet will be retained by the scientific community.

DESCRIPTION OF GENUS *PNEUMOCYSTIS*

The terminology used to describe the various life cycle stages of *Pneumocystis* bears remnants of its earlier classification as a protozoan parasite (7, 13). This discussion introduces terms more suitable for its fungal identity but retains those commonly found in the literature for continuity. Three developmental forms are generally recognized: the trophozoite (trophic stage), 1 to 5 μm (Fig. 1A); the precyst (sporocyte), 5 to 8 μm (Fig. 1B); and the cyst (spore case), about 8 μm (Fig. 1C to E). *Pneumocystis* organisms reproduce extracellularly within the mammalian lung alveoli. The trophic forms appear ameboid in structure in electron micrographs, but in freshly prepared specimens, they are ellipsoidal and often occur in clusters with other trophic forms and developmental stages. The nucleus and (often) the mitochondrion are visible in rapid Wright-Giemsa-stained specimens by light microscopy. The trophic forms do not stain with fungal stains designed to complex with the cell wall, such as methenamine silver. The sporocyte is smaller than the mature cyst and frequently oval. This stage contains a rigid cell wall lacking in the trophic forms and is visualized with fungal cell wall stains. At the sporocyte stage, the nuclei are at varying levels of nuclear division (from 2 to 8 nuclei) but have not yet been compartmentalized into separate spore structures. Aggregates of mitochondria can also be seen in this stage. The mature cyst is spherical; contains eight spores, although these may not all be visible; has a thick cell

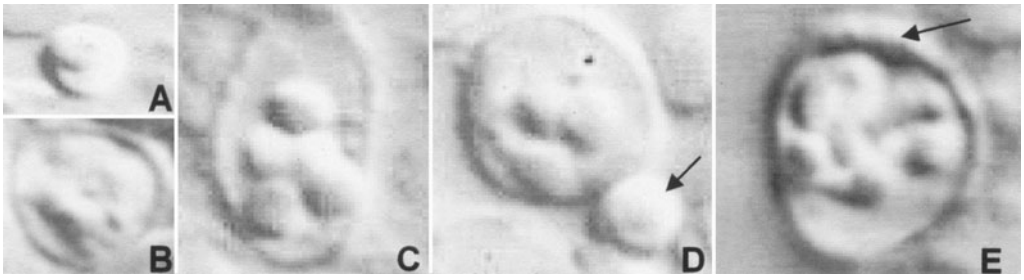


FIGURE 1 Major developmental forms of *Pneumocystis.* (A) Single trophic form; (B) sporocyte (precyst); (C) cyst with four visible spores (intracystic bodies); (D) cyst with three intracystic spores and a spore that has apparently excysted (arrow); (E) cyst with localized thickening of the cell wall (arrow) and eight visible spores. Nomarski Interference Contrast microscopy; magnification, ×1,000.

wall that excludes stains such as the rapid Wright-Giemsa stain; and stains with cell wall complexing stains such as methenamine silver. It is considered the diagnostic morphological form. All developmental forms and various poorly defined intermediate stages are often found in tightly adherent aggregates or clusters in clinical specimens, making identification of each stage difficult.

EPIDEMIOLOGY AND TRANSMISSION

In the late 1970s and early 1980s Walzer et al. (108) and Hughes (46) conducted a series of animal studies that showed that *Pneumocystis* infection was likely transmitted by an airborne route. *Pneumocystis*-free rats could acquire the infection from infected rats housed in the same room or from infected cage mates. Transmission through water, food, or ingestion of infected lungs was not observed in the same experimental studies. Although *Pneumocystis* DNA could be detected in air samples from patient rooms (5) and from an orchard after extensive sampling (104), no laboratory has been able to identify the agent of transmission.

Putative Life Cycle

Histochemical and ultrastructural studies form the basis of the current understanding of the life cycle of *Pneumocystis,* due to a historic lack of a long-term cultivation method outside the lung. Thus, any life cycle should be considered presumptive until definitive kinetic analyses are able to be performed. Human- and rat-derived *Pneumocystis* have been the most extensively studied of the organisms in this group, and discussion of the life cycle is based on these findings (6, 7, 13, 24, 64). There is no evidence for an intracellular phase, although the organisms can be frequently observed within macrophages as a result of the host response to the infection.

A schematic of a proposed life cycle can be found in Fig. 2, with a detailed description provided in the legend. Trophic forms are presumed to be the vegetative stages of the *Pneumocystis* life cycle and reproduce asexually by binary fission, not budding, as do most yeasts. It is likely that they also participate in the sexual mode of reproduction using a process similar to yeast mating type systems, although these processes are only now beginning to be defined (90, 99, 103). Several fungal meiosis-specific and mating type gene homologs have been identified in cDNA and genomic *P. carinii* databases, lending credence to the existence of these processes (17, 18, 25). Subsequent to the mating process and karyogamy (nuclear fusion), the zygotic nucleus undergoes meiosis and sporogenesis is initiated, resulting in formation of the precyst, or sporocyte. Following meiosis, an additional mitotic replication occurs, with subsequent compartmentalization of the nuclei and organelles into eight ascospores. The end product of sporogenesis is the spherical cyst, or spore case. The process of spore release has not been described but may involve a localized thickening at one pole of the spore case (cyst) (114). It should be noted that unlike other fungi, all the developmental stages of *Pneumocystis* contain a double membrane (30).

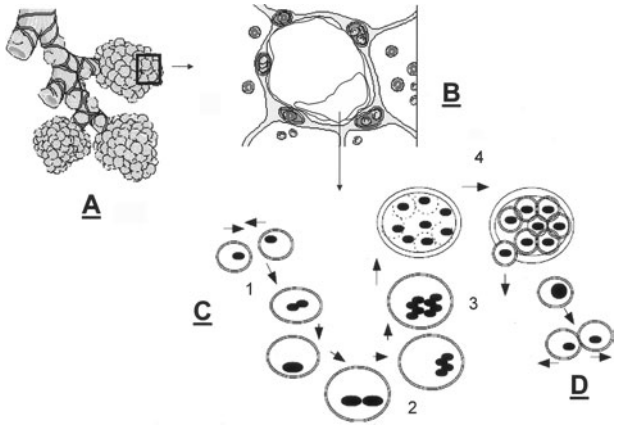


FIGURE 2 Proposed life cycle of *Pneumocystis.* (A) The primary site of *Pneumocystis* infection is the lung alveoli. Three clusters of alveoli are illustrated. An expanded schematic of an alveolus (box) is shown in panel B. (B) Single alveolus with *Pneumocystis* organisms depicted as hatched shape attached to the cells lining the alveoli in some areas (type I pneumocytes) and unattached to other alveolar cells (type II pneumocytes). (C) Putative sexual cycle of *Pneumocystis* in the lung alveoli: (1) opposite mating types fuse and undergo karyogamy, resulting in a diploid zygote; (2) the zygote then undergoes meiosis, resulting in four nuclei; (3) additional postmeiotic mitotis increases the number of nuclei to eight; (4) the nuclei and mitochondria (not shown) are compartmentalized by invagination of the inner plasma membrane, resulting in eight spores. Spores are released from the ascus (cyst) and presumably enter into the vegetative phase of the cycle. (D) Asexual replication cycle of *Pneumocystis*. Trophic forms undergo binary fission after mitotic replication of the nucleus. (Drawn with SmartDraw Suite, ed. 7.3.)

Despite numerous attempts to do so, an environmental cycle for *Pneumocystis* has not been identified. A growing body of evidence suggests that the reservoir for *Pneumocystis* is its mammalian host, a situation similar to that of other host-dependent pathogens like *Entamoeba histolytica* or *Mycobacterium tuberculosis*. Studies with humans and animal models further support a role for neonates and immunocompetent hosts as potential reservoirs, perhaps transiently or longer term.

Transmission and Epidemiology

Evidence from several different studies suggests that (i) *Pneumocystis* is transmitted by an airborne route; (ii) it is likely to be acquired early in life; (iii) it can be transmitted among immunologically intact individuals; (iv) immunosuppressed and infected hosts are able to transmit the infection to immunologically intact hosts; and (v) transmission requires a short period of exposure and low numbers of organisms.

Beard et al. reported that the expansion of *P. jirovecii* carrying a double mutation in the DHPS gene in selected members of the human immunodeficiency virus (HIV)-infected population provides strong support for transmission of *P. jirovecii* by a person-to-person route as well as a positive selective mechanism (10, 70). Previous and recent epidemiological surveys showing the clustering of *P. jirovecii*-specific genotypes with patients' place of residence are consistent with the hypothesis of person-to-person transmission of *P. jirovecii* via the airborne route (12, 68).

Serological studies performed in the 1970s through the 1980s showed that *P. jirovecii* was acquired early in life (82, 84). Most human beings become seropositive to *P. jirovecii* organisms or antigens by the ages of 2 to 4 years. Vargas et al. detected *P. jirovecii* DNA in nasopharyngeal aspirates in 24 of 72 infants (32%) suffering from mild respiratory infections (101). Seroconversion developed in 67 of 79 (85%) of the same cohort of infants by 20 months of age. In the rat model of infection, newborn rats acquired *Pneumocystis*-specific DNA in their oral cavities by 1 to 2 h after birth (49). No evidence for in utero transmission in rats was found after extensive analysis of fetal tissue by PCR, suggesting that the neonates were infected through close contact with the mother or through the air.

Experimental evidence indicates that very few organisms are required to initiate *Pneumocystis* infection and that the organism is very efficient in its method of transmission. Studies have shown that fewer than 10 *P. carinii* organisms were sufficient to establish a fulminate infection in immunosuppressed rats (22, 52) and that a 1-day period of exposure was all that was needed to transmit the infection from an infected *scid/scid* mouse to a *scid/scid* noninfected mouse (91). Likewise, transmission from infected patients to immunocompetent health care workers has been reported (102). The widespread prevalence of *Pneumocystis* in commercial colonies housing healthy rats (20, 28, 48) not only supports the very efficient dissemination of the infection throughout the members of a colony but also shows that the organism thrives in the immunocompetent host. Evidence that healthy human populations may also serve as reservoirs or sources of infection is accumulating. A recent study conducted with humans without underlying lung disease or immunosuppression found that 20% of the oropharyngeal wash samples from 50 persons were positive for *P. jirovecii* by nested PCR targeting the mitochondrial large subunit ribosomal gene (65). Further studies employing quantitative PCR should help to resolve the potential use of PCR as a diagnostic tool versus its use as a detection method.

Colonization

Emerging reports of the detection of *P. jirovecii* in populations without underlying immunosuppression as well as in those with chronic underlying diseases that have not been historically associated with its presence may suggest colonization or expansion of host range (65, 70, 71, 83). Colonization, carriage, asymptomatic infection, and subclinical infection have all been used to describe the presence of *Pneumocystis* organisms or DNA in the absence of *Pneumocystis* pneumonia (PCP). The effects of the presence of low numbers of *P. jirovecii* on the host have yet to be determined, and the role of carriage in mild respiratory infections, chronic lung disease, and progression to PCP is now being actively investigated. The most common underlying conditions in non-HIV-positive individuals associated with the presence of *P. jirovecii* include asthma and chronic lung diseases, chronic obstructive pulmonary disease, cystic fibrosis, Epstein-Barr virus infection, lupus erythematosis, rheumatoid arthritis, thyroiditis, ulcerative colitis, and pregnancy; the presence of *P. jirovecii* has also been noted in patients receiving high doses of corticosteroids (83).

CLINICAL SIGNIFICANCE

PCP remains the leading opportunistic infection associated with AIDS patients, even in the era of highly active antiretroviral therapy (51). Within the mammalian lung, the trophic forms of *Pneumocystis* adhere to the type I pneumocytes presumably through macromolecular bridges (60, 85, 113). Type I cells are responsible for the gas exchange between the alveolar capillaries and the alveolar lumen. Besides attachment to the type I cells, the various developmental stages of the parasite adhere to one another, producing large clusters that extend outwardly into the alveolar lumen. In severe, untreated infections, most of the alveoli are filled with organisms. Direct attachment to the cells responsible for gas exchange combined with the accumulation of organisms within the alveoli results in impaired gas exchange and altered lung compliance, as well as other physiologic changes associated with the pneumonia, such as hypoxia (44, 112).

Histopathologic findings can be characterized by two traits: (i) alveolar interstitial thickening and (ii) a frothy eosinophilic honeycomb-like exudate in the lumina of the lung. The interstitial thickening is a result of hyperplasia and hypertrophy of the type II pneumocyte, interstitial edema, mononuclear cell infiltration, and in some cases mild fibrosis. The exudate is apparent upon staining with hematoxylin and eosin, which does not stain the organisms. Methenamine silver or another yeast cell wall stain must be used to visualize the cyst form of the organism, considered the diagnostic stage.

Presentation in Children

PCP was first described as occurring in children and was considered a pediatric infection in its early history (39). Prominent epidemics of "interstitial plasma cell pneumonia" in undernourished children housed in suboptimal conditions after World War II were manifestations of the disease that was later identified as *P. jirovecii* pneumonia (47). The infection in these children was characterized by a plasma cell infiltrate, in contrast to the type II cell hypertrophy and scanty mononuclear infiltrate described in adults with the pneumonia (109). PCP in children can be associated with cyanosis, flaring of the nasal alae, a mild cough without fever, and retractions of intercostals in severe disease (109). Of note, whereas adults with $CD4^+$ cell counts of less than 200 per microliter are at increased risk for PCP, children less than a

year old who developed the pneumonia had $CD4^+$ counts greater than 1,000 per microliter (63).

Presentation in Adults

Adults with PCP frequently present with dyspnea, nonproductive cough, inability to breathe deeply, chest tightness, and night sweats (44). A low-grade fever (e.g., 38.5°C) and tachypnea are often present, while hemoptysis or sputum production is rare. Upon physical examination, few pronounced abnormalities are detected, but various degrees of respiratory distress, small respiratory volumes, and fine basilar rales can be observed. Patients with AIDS often have a more insidious progression to clinical disease than do those not infected with HIV but who are immunosuppressed.

The chest X ray may appear normal or reflect a disease state. Diffuse, symmetrical, interstitial infiltrates are most commonly present, while focal infiltrates, lobar consolidations, cavities, and nodules are less common. Infiltrates in early infection may be widely distributed, but consolidation increases as the disease progresses. Administration of aerosolized pentamidine as a prophylactic measure has been associated with increased frequency of apical infiltrates and pneumothoraces (92). The severity of abnormalities on the chest radiograph is considered prognostic and can be correlated with higher mortality.

The oxygenation impairment induced by pneumocystosis can be detected in most patients by a widening of the alveolar-arterial oxygen gradient [(A-a)DO_2] correlated with severity of disease and respiratory alkalosis (69). However, it should be noted that a significant number of patients can have a normal (A-a)DO_2 gradient at rest. Impaired diffusing capacity, alterations in lung compliance, total lung capacity, vital capacity, and hypoxemia are other physiologic changes that may be associated with *P. jirovecii* infection (44). Abnormalities in surfactant proteins also occur in infected individuals, with increases in surfactant protein A and D being most notable, since these proteins are considered components of innate immunity (3, 59, 86).

Extrapulmonary Pneumocystosis

The incidence of *Pneumocystis* organisms in sites other than the lung has been reported in 1 to 3% of postmortem examinations of patients with pulmonary *P. jirovecii* infections (79, 98). This is likely an underestimate due to the limited number of autopsies performed and the lack of suspicion of extrapulmonary pneumocystosis. Methenamine silver staining or immunofluorescent kits served to identify the cyst stage of *Pneumocystis* in tissue samples and fine needle aspirates in antemortem cases. The lymph nodes were the most frequent site of extrapulmonary involvement in a series of 52 patients (44%), followed by the spleen, bone marrow, and liver (33%). *P. jirovecii* has been detected in the adrenal glands, gastrointestinal tract, genitourinary tract, thyroid, ear, liver, pancreas, eyes, skin, and other sites. Infection of multiple extrapulmonary sites was associated with a rapidly fatal outcome. Pathological findings correlated with the organ where the infection was present. For example, retinal cotton wool spots were reported in infected eyes and pancytopenia was observed in patients with bone marrow lesions.

COLLECTION AND HANDLING OF SPECIMENS

P. jirovecii pneumonia should be considered in any immunocompromised patient who presents with fever, respiratory symptoms, or infiltrates on chest X ray. Although they are mostly restricted to the lung, *P. jirovecii* organisms have been found as extrapulmonary masses (e.g., pleura, intra-abdominal) in HIV-positive patients and should be included in a differential diagnosis for such patients. As no symptoms are specific for *P. jirovecii* pneumonia, a definitive diagnosis must be made by morphologic identification of the organism. An algorithm from the NIH for clinical evaluation and treatment of PCP was recently updated, and the reader is referred to this review for further details (54).

The diagnosis of PCP relies on efficient sampling. The organism can be detected in a variety of respiratory specimens including induced sputum, bronchoalveolar lavage fluid (BALF), tracheal aspirate fluid, tissue obtained by transbronchial biopsy, cellular material obtained by bronchial brush, pleural fluid, and tissue obtained by open-thorax lung biopsy. The diagnostic yield is dependent upon the underlying disease state of the patient and the expertise of the staff obtaining the sample. In some hospitals where the staff is trained to obtain sputum samples and there is a large population with AIDS, 80% of diagnoses of PCP were made from induced sputum (42). In contrast, the diagnostic yield from non-AIDS patients can be quite low and BAL or other methods may be needed to ensure an appropriate diagnosis (9, 45). It is recommended that multiple slides from non-AIDS patients be examined, especially when there is a high degree of suspicion. Hospitals serving a diverse population often rely on methods other than induced sputum. A notable drawback of using induced sputum as the primary procedure for the diagnosis of PCP is the lack of information concerning other infections or disease processes that may be present in the lung. The diagnosis of extrapulmonary pneumocystosis relies on accurate sampling of the infected organ and subsequent staining of the histological sections. For infants and young children who are unable to produce sputum or for whom this method is not warranted, tracheal aspirate fluid, open-thorax lung biopsy, or respiratory specimens must be obtained by a special pediatric bronchoscopy service (11, 101).

BAL

Fluid obtained by BAL is sufficiently liquid and does not require treatment with mucolytic agents. Twenty to 30 ml should be concentrated by centrifugation at $3,000 \times g$ for 15 min. The resultant pellet is reconstituted in 0.5 to 1.0 ml of buffer or saline, and a sterile wooden applicator is used to smear the sediment on glass slides, which are then fixed in absolute methanol, acetone, or a commercial fixative in rapid staining kits. Samples in limited quantities can be concentrated by use of a Cytospin Centrifuge (Shandon Southern, Sewickley, Pa.) or the equivalent. Addition of 1 drop of 22% bovine serum albumin to 500 μl of sample aids in adherence to the slide. The slides are air dried and then processed for staining. Morphological criteria for recognition of *P. jirovecii* stained by various procedures are discussed below and summarized in Table 1.

Induced Sputum Collection

Sputum collection is best done in a centralized facility by pulmonary function laboratory technicians, respiratory therapists, or specially trained assistants. Deep inhalation of nebulized 3% sodium chloride solution by the patient results in osmotic accumulation of fluid in and irritation of the respiratory passages with subsequent coughing and expectoration of bronchoalveolar contents. It is important to have the patient vigorously brush the teeth, tongue, and gums with a toothbrush and normal saline for 5 to 10 min prior to

TABLE 1 Comparison of stains used to detect *P. jirovecii*

Stain (reference[s])	Time to perform staining	Cyst wall	Trophic and other forms	Advantages	Disadvantages
Giemsa (2, 23)	30–60 min	Unstained. Cyst wall appears as a clear ring around spores or intracystic bodies.	Nuclei stain red-purple; cytoplasm stains light to dark blue, depending on thickness and depth of cluster.	Inexpensive. Stain simple to perform. Stains all life cycle stages of *Pneumocystis*. Stains most other pathogens (e.g., bacteria, parasites, fungi) and host cells.	Experienced reader required to distinguish *Pneumocystis* clumps from stained host cells.
Rapid Giemsa-like stains (e.g., Diff-Quik) (2, 23, 78)	<5 min				
Fluorescein-conjugated monoclonal antibody kits (direct and indirect immuno-fluorescence) (34, 41, 55, 76–78, 80)	15–30 min	Stains and fluoresces apple green. Cyst contents usually unstained (appears black) or dull. Fold in cyst wall sometimes apparent, giving a crinkled, raisin-like appearance.	Stained. Appear as small polygons or spheres outlined in apple green. Nuclei may stain. Clusters can stain with a diffuse green glow.	Recommended for less inexperienced personnel. Immunofluores-cent staining is sensitive and specific for *Pneumocystis*.	Requires fluorescent microscope. Reagents are expensive.
Methenamine silver (Gomori/Grocott) (15, 61)	30 min (microwave); 1–2 h (rapid); 6–24 h (con-ventional).	Stains brown to black. Cyst wall thickenings (double comma) and fold in the cyst wall stain dark brown to black. Does not differentiate empty cysts from those with spores.	Unstained	Easy to detect cysts. Host cells not stained.	Prolonged staining time for conventional method. Moderate costs. Strong acids used. Only the cyst form is stained. Stains other fungi.
Toluidine blue O/cresyl echt violet (15, 80)	1–6 h	Stains violet to purple. Cyst wall thickenings and folds stain darker violet to purple. Does not differentiate empty cysts and cysts with spores.	Unstained	Easy to detect cysts. Host cells not stained.	Prolonged staining time. Moderate costs. Strong acids used. Only the cyst is stained. Stains other fungi.
Calcofluor white (8, 94)	<5 min	Stains blue-white or green, depending on filter. Cyst wall and thickenings intensely fluorescent.	Unstained	Cyst fluoresces brilliantly. Simple to perform. Inexpensive.	Requires fluorescent microscope. Strong alkali used. Only cyst is stained. Stains other fungi. Some expertise is required to distinguish *Pneumocystis* cysts from other fungi.
Gram-Wiegert (4)	<5 min	Unstained wall. Intracystic bodies stain purple.	Trophic forms faintly visible.	Commonly available in cytopathology laboratories.	Faint staining. Can be overcome by experienced observer, but better stains are available.
Papanicolaou (89)	1–6 h	Unstained wall. Intracystic bodies stain purple.	Trophic forms faintly visible.	Commonly available in cytopathology laboratories.	Faint staining. Can be overcome by experienced observer, but better stains are available.

sputum induction, followed by thorough rinsing, to remove as much cellular debris of oral origin as possible. Toothpaste should not be used, as it can interfere with subsequent processing and staining of the specimen. The induced-sputum specimen is usually mucoid and translucent in appearance; only rarely is it purulent. When *P. jirovecii* clusters are present in the unstained sputum, they are typically 0.1 to 0.2 mm in diameter and cream to light tan in color. Smearing of the sputa directly onto slides and subsequent staining were shown to be a less sensitive method than treatment with a mucolytic agent followed by concentration of the specimen (62, 76).

Induced sputum is mucolyzed by the addition of an equal volume (at least 2 ml) of freshly prepared 0.0065 M dithiothreitol (Stat-Pak Sputolysin; Caldon-Biotech, Carlsbad, Calif.) or 0.5% *N*-acetyl-L-cysteine and incubation on a rotary shaker at 35°C with intermittent vigorous vortexing until the specimen is almost completely liquefied (complete liquefaction leads to dispersal of the *P. jirovecii* clusters, making microscopic detection more difficult). Some protocols require the addition of a clearing reagent for optimal results (e.g., kits for direct fluorescent-antibody [DFA] staining available from Chemicon International, Inc., Temecula, Calif.). The specimen is then concentrated by centrifugation at 3,000 × *g* for 5 min, and the sediment is smeared on glass slides, which are air dried and heat fixed by exposure to a heating block (50 to 60°C). Prolonged heat fixation is important in fixing the material to the slide (~30 min), since most of the natural cellular adhesions are removed during mucolysis. Slides containing the fixed material are then stained and examined microscopically (see below). Samples can be concentrated using a Cytospin centrifuge as described for BALF (above).

Open-Thorax Lung Biopsy

Open-thorax lung biopsy is the most invasive of the sampling procedures and is not routinely performed. This technique also suffers from a lack of sensitivity (54). However, should the laboratory receive such a specimen, the tissue should be blotted onto sterile gauze to reduce excess fluid, (which would interfere with a diagnostic imprint) and used to make touch imprints by pressing several cut surfaces onto sterile glass slides. The remainder of the tissue can be used for histological sections or for microbiological cultures after mincing or grinding. Glass slides with touch imprints should be air dried and then treated with absolute methanol or fixatives included in commercial kits of rapid Wright-Giemsa-like stains, such as Hema 3 (Fisher Scientific Inc., Cincinnati, Ohio). Infected tissue stained in this manner reveals the presence of clusters of trophic forms and cysts with reddish purple nuclei and blue cytoplasm surrounded by a halo of dye exclusion (see "Microscopic Identification" for a more detailed description). Slides should be fixed in acetone or a vendor-recommended fixative for immunofluorescent staining. Alternatively, a more concentrated sample can be achieved by maceration of the tissue with sterile scissors followed by homogenization in a Lab Blender designed to reduce the risk of aerosolization or potential contact by laboratory personnel (i.e., Stomacher Lab Blender; Tekmar Inc., Cincinnati, Ohio) for 10 to 20 min in 5 ml of saline or buffer/g of tissue. After sieving through sterile gauze to filter out large tissue particulates, the supernatant is concentrated by centrifugation (3,000 × *g* for 10 min) and then reconstituted in 1 to 5 ml of saline or buffer. Slides are prepared with 10-μl drops of the homogenate, which are then air dried, fixed, and stained as desired.

Transbronchial Biopsy

Fiberoptic bronchoscopy is the most common invasive technique for collection of tissue, but like open-thorax lung biopsy, this procedure is rarely performed today. Tissue should be handled as described above for open-thorax lung biopsy.

Nasopharyngeal Aspirates

Recent studies with humans reported the ability to detect *P. jirovecii*-specific DNA in nasopharyngeal aspirates (101) and oropharyngeal washes (71, 100) after amplification by PCR with *P. jirovecii*-specific primers. Current studies are exploring the use of oropharyngeal swabs as an additional sampling method. Although further studies addressing the correlation of the presence of *P. jirovecii* in the oral cavities and the disease state of the host are needed, handling and staining of nasopharyngeal aspirates are presented here because the use of PCR is becoming widespread for the diagnosis of infectious diseases. This method is extremely useful for sampling of pediatric populations where more invasive techniques of sampling are problematic or where the yield is low.

The aspirates are collected using a suction catheter and sterile saline. If the amount of collected specimen is small or it is highly viscous, sterile saline should be washed through the catheter to dilute the specimen or added to the final collection tube. The sample can then be treated with a mucolytic agent and subsequently stained (as described above) or prepared for PCR by DNA extraction (described below).

IDENTIFICATION AND DIAGNOSIS

Microscopic Identification

A variety of stains have been used for the identification of *P. jirovecii* organisms. One of the most common stains used by pathologists for tissue sections, hematoxylin and eosin, does not stain the organism but rather the foamy exudates within the lung alveoli, often described as "honeycombed" in appearance. Stains that illustrate the morphology of the organism by microscopic examination are those used for diagnosis in the clinical laboratory.

A common staining procedure used for the diagnosis of PCP is methenamine silver, which also stains other fungi (Table 1). Cell wall and membrane polysaccharides of fungi are oxidized to aldehydes by treatment with periodic acid, which in turn reduce the silver ion to metallic silver at alkaline pH. Addition of gold salts stabilizes the complex, and excess silver is removed by a sodium thiosulfate rinse. Variations of the methenamine silver stain (e.g., Grocott's or Gomori's) are used for both tissue sections and bodily fluids such as BALF or induced sputum (15, 61, 89). The disadvantages to use of the silver staining process have been the instability of solutions, the capricious nature of the metal impregnation, and the length of time required for staining (1 to 2 h [rapid process], or 6 to 24 h [conventional process] [Table 1]). These disadvantages have been largely overcome with kits using standardized laboratory microwave ovens for controlled processing and supplied reagents (e.g., Modified GMS Microwave staining kit; EBSciences, Agawam, Mass.). Unlike other pathogenic fungi, *P. jirovecii* does not bud, and this feature can be used to discriminate between these organisms and other fungi found in the lung that bud, e.g., *Histoplasma capsulatum*. Silver-stained cysts have a distinctive, black, cup-shaped morphology against green-colored host cell architecture. In some staining reactions, cyst wall thickenings appear as a "double comma" morphology. More

often the folds in the wall stain a dark brown to black to produce a crinkled, raisin-like appearance (Fig. 3C). Intracystic daughter forms cannot be seen with this stain, and cysts that are empty (nonviable) appear the same as those with the full contingent of eight spores.

Other stains that complex with components of the cyst wall include periodic acid-Schiff, toluidine blue, cresyl echt violet, and calcofluor white. The reactions to some of the more commonly used stains are described in Table 1. Cresyl echt violet stain produces results similar to those of the toluidine blue O (Fig. 3D) and has the same drawbacks, since a mixture of sulfuric acid and glacial acetic acid is necessary for the step prior to staining with the dye (15).

In contrast to the cyst wall stains, Giemsa and rapid Giemsa-like stains do not stain the cyst wall but instead stain the nuclei of all the various life cycle stages a reddish purple and stain the cytoplasm a light blue (Fig. 3A1–4). The cyst wall excludes the dyes and appears with a circumscribed clear zone surrounding the reddish purple nuclei of the daughter forms within (Fig. 3A1). Originally used for quantitation of rat *P. carinii* organisms (23, 78), the rapid variants of the Giemsa stain are recommended for the diagnosis of PCP by using BALF, induced sputum, or impression imprints because of the low cost, ease, and rapidity of the staining procedure. Commercial kits such as Hema-Quik (Fisher Scientific Co.) produce similar results. The staining procedure requires less than a minute to perform, and all forms of the organism are detected. Since there are approximately 10-fold more trophic forms than cysts, the sensitivity of detection is likely to be increased. This stain also permits the assessment of specimen quality of BALFs by demonstration of host alveolar macrophages, which should be present in a productive sample. In addition, the distinctive Giemsa-stained morphological appearances of other organisms likely to be encountered in the lung environment, such as *Cryptococcus neoformans* or *Toxoplasma gondii,* permit rapid diagnosis of pulmonary infections caused by these pathogens, which may not be detected with other stains. Because background host cells also stain, training and expertise in interpreting cellular elements in the Giemsa-stained preparations are necessary. Laboratories with a lower volume of *P. jirovecii* specimens may prefer to use immunofluorescent staining or one of the other stains described in Table 1.

Direct and indirect fluorescein-conjugated monoclonal anti-*P. jirovecii* antibodies used for immunofluorescent assay are targeted to a family of surface glycoproteins that contain both common and distinct epitopes, within and among *Pneumocystis* species (95). Depending on the monoclonal antibody supplied with the kit, staining may target only the cyst form or all forms of the organism. Since trophic forms are more numerous than cysts, kits using those antibodies directed to all forms of the organism (such as "2G2" and "3F6"; available from DAKO Corp., Carpinteria, Calif., or Chemicon International Inc.) are more sensitive (54). The typical fluorophore conjugated to the antibody or used in an indirect assay is fluorescein isothiocyanate, which produces a brilliant apple green color. The staining reaction shows a diffuse surface pattern distributed over the entire cluster of organisms (Fig. 3B) and often stains the matrix in which the organisms are embedded. Single cysts usually appear with a distinctive rim of fluorescence and duller interior fluorescence. It should be noted that kits using a direct staining procedure may not react with *P. jirovecii* on slides fixed in ethanol (34) and fixation in acetone or vendor recommendations for fixation should be followed.

Papanicolaou's stain, frequently used for cytopathological specimens, stains the clusters of extracellular organisms a pink color, although cysts within a macrophage appear greenish in color (Fig. 3F) (4). Organism architecture is better observed with the Giemsa-like stains. Likewise, Gram's stain produces a negative (pink) reaction with poorly defined organism morphology.

PCR Detection and Other Diagnostic Modalities

Detection of *Pneumocystis* by growth in artificial media or tissue culture is not a diagnostic option since no species of the organism can be continuously cultivated outside the mammalian lung.

Serological assays to detect anti-*P. jirovecii* antibodies are useful for epidemiological studies but not for diagnosis of PCP. Most human beings become seropositive for *P. jirovecii* antibodies early in their childhood, at 2 to 4 years of age (107) and likely come in contact with the organism many times over their lifetimes. In some cases, a rise in antibody titer can be detected in some PCP patients over time; in others, antibody titers can just as frequently drop or remain the same. Likewise, antigen capture assays for *P. jirovecii* antigens have not proven clinically useful.

A number of other laboratory tests have been used for the diagnosis of PCP but do not provide a definitive diagnosis. These include an increased arterial-alveolar gradient; an elevation of serum lactic dehydrogenase levels; and gallium and diethylenetriamine penta-acetic scans (109). The last two tests are not routinely used due to higher costs.

Amplification of *P. jirovecii* DNA is not yet used routinely for the diagnosis of PCP in the clinical laboratory setting. Although more sensitive than conventional methods relying on organism detection by microscopic methods, the presence of a specific *P. jirovecii* PCR product has not been strictly correlated with underlying disease (2, 54, 81). In contrast to conventional methods of detection, PCR-based methods can be more labor-intensive, expensive, and time-consuming. As the clinical laboratory begins to rely more on these amplification techniques or other molecular modalities in the future, it is likely that *P. jirovecii* detection will be included in the battery of infectious disease kits offered by commercial vendors. However, a PCR technique may be considered useful at this time for clinical samples that require detection of early infection or cases in which PCP is suspected but no organisms were detected. Significantly, evidence is accumulating for the emergence of drug resistance in human *P. jirovecii* and PCR can be used for identification of potentially resistant strains of the organism. Although the significance of infections with multiple genotypes of *P. jirovecii* is currently unclear, PCR-based detection systems would also be able to detect PCP caused by single or multiple genotypes.

Detection of the presence of *P. jirovecii* can be accomplished by targeting the large ribosomal subunit of the mitochondrial rRNA (mtLSU) using direct or nested PCR (31, 105). This gene is present in multiple copies per organism and provides the most sensitive target for detection. DNA can be isolated from clinical samples by using commercial kits such as the PhaseLock System (Eppendorf Scientific, Westbury, N.Y.) or a series of products designed for the clinical laboratory by vendors such as Qiagen (Valencia, Calif.). The primers targeting the mtLSU and PCR conditions are listed in Table 2. The expected amplicon size of the first product is approximately 346 bp, and that of the second is 267 bp. If genotyping of the isolates is desired, the PCR amplicon can be sequenced directly or by cloning and subsequent sequencing of the clones. Cloning permits the

assessment of the presence of infections caused by more than a single genotype. Manuals of molecular methods or vendor instructions should be consulted for these procedures. There are 4 genotypes defined by the mtLSU locus (10) and over 60 different genotypes defined using the intertranscribed spacer regions of the nucleus-encoded rRNA locus (57), but such typing is outside the scope of the clinical laboratory at this time.

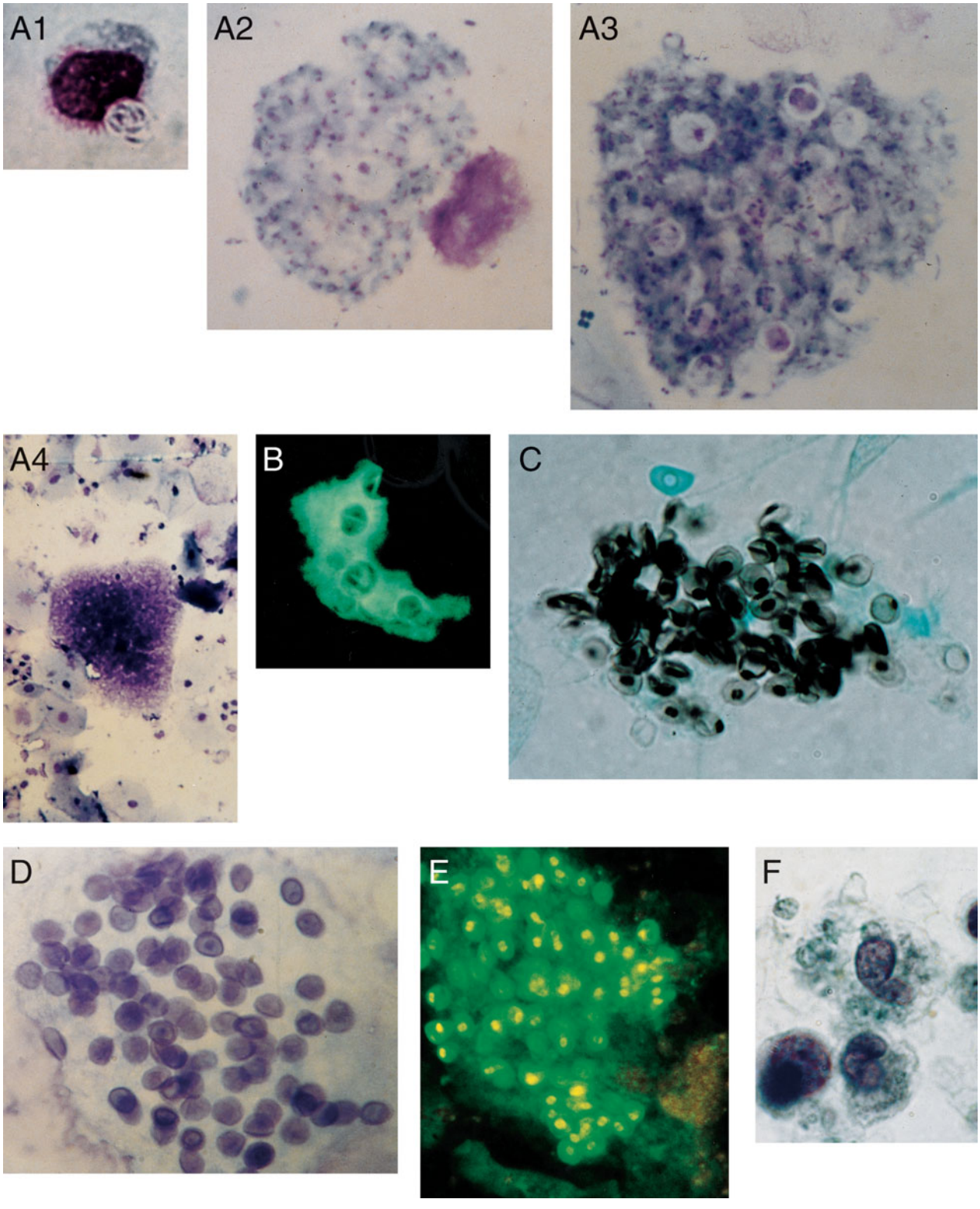


TABLE 2 Suggested primers and conditions for PCR detection of *Pneumocystis*

Gene target	Primer (5′ to 3′ sequence)	Expected product size (bp)	Conditions
MtLSU			
Primary[a]	pAZ102-E (GATGGCTGTTTCCAAGCCCA) pAZ102-H (GTGTACGTTGCAAAGTACTC)	346	94°C for 1 min, 55°C for 1 min, and 72°C for 2 min for 40 cycles; termination, 72°C for 5 min
Nested[b]	pAZ102-X (GTGAAATACAAATCGGACTAGG) pAZ102-Y (TCACTTAATATTAATTGGGGAGC)	267	94°C for 1 min, 55°C for 1 min, and 72°C for 2 min for 35 cycles; termination, 72°C for 5 min
DHPS[c]			
Round 1	F1 (CCTGGTATTAAACCAGTTTTGCC) B_{45} (CAATTTAATAAATTTCTTTCCAAATAGCATC)		94°C for 5 min, 92°C for 30 s, 52°C for 30 s, and 72°C for 1 min for 35 cycles; termination, 72°C for 5 min
Round 2	A_{HUM} (GCGCCTACACATATTATGGCCATTTTAAATC) BN (GGAACTTTCAACTTGGCAACCAC)	300	94°C for 5 min, 92°C for 30 s; 55°C for 30 s, and 72°C for 1 min for 35 cycles; termination, 72°C for 5 min

[a] From reference 105.
[b] From reference 102.
[c] Dihydropteroate synthase gene; from references 10 and 56.

ANTIMICROBIAL SUSCEPTIBILITIES

Two drugs constitute the mainstay of therapy for acute PCP, trimethoprim-sulfamethoxazole (TMP-SMX) and pentamidine isethionate (109). Secondary treatments such as atovaquone and clindamycin-primaquine have been used for milder forms of the disease, and treatment with corticosteroids has been used to improve the clinical outcome for some patients. PCP remains refractory to most common antifungal and antiprotozoal agents. TMP-SMX is a drug combination that targets the enzymes dihydrofolate reductase (trimethoprim) and dihydropteroate synthase (sulfamethoxazole), both integral steps in the folic acid pathway. Pentamidine is a cationic diamidine that was first used to treat African trypanosomiasis, "sleeping sickness," and later was found to be efficacious as PCP therapy. The mode of action of this drug is not known but may involve suppression of mitochondrial activity (19) or inhibition of topoisomerases (32). These drugs have an overall success rate of 80%, although there is a wide range of effects depending on the study population (110, 111). Neither drug is considered pneumocysticidal, and increasing cases of "breakthrough" PCP have been reported while patients were on prophylaxis with either drug (43). About 11% of those patients on TMP-SMX and 22% of those given pentamidine experienced recrudescence (88). Administration of pentamidine via an aerosolized route delivers the drug efficiently to the areas of infection, but it has been shown to be less effective than other drugs in the treatment of PCP (109). Aerosolized pentamidine administration is typically used for prophylaxis.

Mutations in the DHPS gene of *P. jirovecii* associated with sulfa resistance in other pathogens have been identified in about 50% of the isolates causing PCP in certain geographic areas (10). The presence of the mutations in the DHPS gene

FIGURE 3 Morphology of *Pneumocystis* in clinical samples stained with various stains (magnification, ×1,000 unless stated otherwise). (A) Rapid Giemsa-like (Diff-Quick) stains of induced sputum. (A1) Single mature cyst (8 μm) containing eight fusiform, intracystic bodies with purple nuclei and light blue cytoplasm. Note the clear unstained wall of the cyst at its periphery. The cyst is near an alveolar macrophage. (A2) Cluster of mostly trophic forms (2 to 3 μm) with small reddish purple nuclei and light blue cytoplasm. Boundaries of trophic forms are rarely discernible with this stain. (A3) Thick cluster of *Pneumocystis* containing various developmental forms including precysts with nuclei clustered together; mature cysts with clearly discernible nuclei; and trophic forms overlying each other to produce darker-staining blue cytoplasm. (A4) Thick cluster (magnification, ×250) of overlying *Pneumocystis* stages that appear as a mat of organisms without clearly discernible boundaries. (B) DFA stain of organism cluster from induced sputum. Note apple-green fluorescence distributed over the cluster, with accumulation on a fold in the cyst wall (upper part of cluster). Structures within the cysts are unstained and appear black. (C) Gomori's methenamine silver stain (Grocott) of organisms from BAL fluid. Cyst walls and thickenings (double comma) can be observed. Trophic forms are not stained with silver-based stains. (D) Toluidine blue O stain of *Pneumocystis* in a BAL fluid. Cyst walls are stained purple. The crinkled appearance of the cysts is illustrated with this stain. Note the lack of budding with this and other cyst wall stains. Trophic forms are not stained. (E) Calcofluor white stain of BAL fluid. Cyst walls with internal thickenings (double comma) are highly fluorescent (color varies with barrier filter used). (F) Papanicolaou's stain of pleural fluid. Pneumocystis trophic and cyst forms are phagocytized by the marcrophages. The nuclei of the organisms are dark green, and the cytoplasm is a lighter green. The macrophage nuclei stain dark purple.

of *P. jirovecii* was associated with previous TMP-SMX therapy (66, 72–74, 93), but the impact of the mutations in terms of outcome and response to therapy is not yet clear.

It may become desirable in the future to track the emergence of *P. jirovecii* strains that are resistant to TMP-SMX or to evaluate the potential for therapeutic response. In anticipation of this goal, the primers targeting the regions of the dihydropteroate gene (DHPS) associated with sulfa resistance in other pathogens (50, 56) are shown in Table 2. The nucleotide positions in which the mutations occur are at nucleotides (nt) 165 and 171. Changes at these nucleotide positions result in changes in amino acids. The following are the four genotypes for this target gene (10): genotype 1, nt 165 (A) and nt 171 (C) (resulting in Thr and Pro); genotype 2, nt 165 (G) and nt 171 (C) (resulting in Ala and Pro); genotype 3, nt 165 (A) and nt 171 (T) (resulting in Thr and Ser); and genotype 4, 165 (G) and nt 171 (T) (resulting in Ala and Ser). The last genotype, GT, represents a double mutation in the DHPS gene that has been associated with drug resistance and is emerging as the dominant genotype for *P. jirovecii* isolates in some areas (10). Although atovaquone resistance in PCP has been associated with mutations in the mitochondrial cytochrome b_1 gene (106), resistance to TMP-SMX is much more problematic and identification of mutations in the DHPS gene will likely be the more critical genetic region to evaluate in the clinical setting.

Monitoring of drug efficacy by evaluating organism burdens in clinical specimens, especially BALF, is not practical due to the problems in sampling efficiency. Reasons for the inability to assess organism burdens in response to therapy are that (i) the organisms may colonize discrete regions of the lung not accessible to sputum induction or lavage; (ii) the volume of fluid recovered from an individual patient is variable; (iii) with induced sputum collection, patients vary in their efforts to produce the sample; (iv) microscopic enumeration of trophic and cyst forms may be problematic due to the presence of large clusters that preclude accurate quantitation; and (v) it is not known to what capacity the organisms obtained by induced sputum or BALF reflect the disease state. With the advent of highly sensitive quantitation techniques such as real-time PCR which permits detection of very low template numbers, the problems of accurate quantitation can be overcome. Experimental studies are being conducted to validate the use of real-time PCR for diagnostic purposes (36, 67, 75). However, the same questions of sampling efficiency and predictive ability will remain associated with real-time PCR as well as other issues. Assays targeting genomic sequences will have to be interpreted with caution because they do not provide assessment of organism viability or whether the intact organism is present. A more responsive assay would target quantitation of an mRNA present to determine both diagnosis and viability.

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Aspergillus, Fusarium, and Other Opportunistic Moniliaceous Fungi*

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The opportunistic moniliaceous (hyaline or lightly colored) molds constitute a phylogenetically diverse group of common to rare anamorphic (asexual) and teleomorphic (sexual) fungi that typically occur as saprobes in soil, in air, or on plant litter or as facultative plant pathogens. Some of them, especially members of the genus *Aspergillus,* may be recovered from specimens without having any clinical significance. Others are isolated infrequently enough to challenge the diagnostic proficiency of the laboratory, and critical assessment is required to evaluate the significance of their recovery. While several of the genera treated in this chapter include species having either lightly colored or dark (melanized) conidia, the emphasis is on those fungi that grow in tissue in the form of hyaline or lightly colored, septate hyphal elements.

The term "aspergillosis" is used to define infections caused by species of *Aspergillus* (Table 1), but the practice of coining disease names based on the genus of fungus involved is disadvantageous for infections caused by uncommon or rare fungal pathogens. The wide variety of fungi involved makes it difficult to place the organisms into accessible groups, and problems arise when fungus names are changed. To avoid unnecessary name changes for disease names based on the genus of the fungus involved, two major disease groups have been proposed: hyalohyphomycosis and phaeohyphomycosis (4). Although the groups were defined as encompassing similar clinical spectra, they were distinguished by the presence in tissue of septate hyphal filaments without (hyalohyphomycosis) or with (phaeohyphomycosis) pigmentation or melanin in the cell wall. However, some fungi, such as *Scedosporium* species or *Nattrassia mangiferae (Scytalidium dimidiatum),* which form darkly pigmented colonies and conidia in vitro, produce hyaline or lightly pigmented hyphae in tissue. The Masson-Fontana silver stain helps to detect melanin pigmentation of fungal elements in tissue, but the results are not always decisive (see also the discussion in chapter 125). Some fungi with variable pigmentation may stain faintly or inconsistently. In practice, the terms for the disease categories have been used to designate infections caused by fungi that are either moniliaceous or dematiaceous in vitro (see chapter 125). Although it may be useful to have terms for broad categories of mycotic diseases, there are problems in categorizing fungi by color. A subcommittee of the International Society for Human and Animal Mycology has suggested that fungal diseases be named by providing a specific description of the pathology and naming the causative agent, e.g., subcutaneous cyst caused by fungus X (184, 245).

TAXONOMY AND IDENTIFICATION

The opportunistic moniliaceous molds belong to genera of the Ascomycota and Basidiomycota (see chapter 115). Many of these molds propagate asexually, and the sexual stage (teleomorph) is unknown or rarely encountered. Today the relationships between many anamorphs (asexual stages) and their sexual relatives are known through discovery of teleomorphs or are inferred by comparison of nucleic acids. This knowledge is extremely important in understanding fungal relationships and has allowed for the placement of asexual fungi next to their sexual relatives in fungal phylogenetic trees. Rapid developments in this area have led to significant changes in generic and species concepts, particularly among the significant genera *Aspergillus, Penicillium,* and *Fusarium* (74, 176, 185, 228, 255). Although teleomorphs occasionally are isolated in culture, they may be difficult to obtain using routine culture methods. In this chapter, the name of the teleomorph is used for species that are identified mainly by their sexual structures.

Most of the pathogenic molds considered in this chapter are classified in the form-class Hyphomycetes. Identification of Hyphomycetes is based on morphology of the conidia and the mechanisms by which conidia are formed (conidiogenesis; see chapter 115). Three basic tools are necessary for practical observation of these features. (i) An ocular micrometer is essential for determining sizes of conidia or sexual spores when present. Identification of molds often requires comparison with published taxonomic descriptions in which size is often a key criterion for species distinction. (ii) A dissecting microscope with magnification of up to ×60 and basal illumination is useful for the examination of colonies in plates or tubes for the presence of conidia in chains or slimy heads, specialized structures such as Hülle cells, sclerotia, conidiomata, or sexual fruiting bodies forming under the aerial mycelium or embedded in the agar. (iii) Microscopic mounts which allow observation of how a fungus forms its conidia also are necessary. Slide culture preparations are excellent

* This chapter contains information presented in chapter 116 by Lynne Sigler and Paul E. Verweij in the eighth edition of this Manual.

TABLE 1 Classification of *Aspergillus* infection[a]

I. Disease in the otherwise healthy host
  A. Toxicosis or mycotoxicosis
    1. Ingestion of mycotoxins
    2. Ingestion of other metabolites
  B. Allergic manifestations
    1. Allergic asthma
    2. Allergic rhinitis
    3. Allergic sinusitis
    4. Extrinsic allergic alveolitis
    5. Hypersensitivity pneumonitis
    6. Allergic bronchopulmonary aspergillosis
  C. Superficial or noninvasive infections
    1. Cutaneous infection
    2. Otomycosis
    3. Sinusitis
    4. Saprophytic bronchopulmonary aspergillosis
    5. Tracheobronchitis
  D. Invasive infection
    1. Single organ
    2. Multiple organs (disseminated)
II. Infection associated with tissue damage or foreign body
  A. Keratitis and endophthalmitis
  B. Burn wound infection
  C. Osteomyelitis
  D. Prosthetic valve endocarditis
  E. Vascular graft infection
  F. Aspergilloma (fungus ball)
  G. Empyema and pleural aspergillosis
  H. Peritonitis
III. Infection in the compromised host
  A. Primary cutaneous aspergillosis
  B. Sino-orbital infection
  C. Pulmonary aspergillosis
    1. Invasive tracheobronchitis
    2. Chronic necrotizing pulmonary aspergillosis
    3. Acute invasive pulmonary aspergillosis
  D. Central nervous system aspergillosis
  E. Invasive (disseminated) aspergillosis
  F. Gastrointestinal infarction (rare)

[a] Patients infected with other fungi discussed in this chapter may present with similar clinical syndromes, and the fungal structures in tissue may resemble those of *Aspergillus* species.

for many fungi (106), but conidial structures of species of *Aspergillus, Penicillium,* or *Trichoderma* may not be typical in slide culture conditions, and tease or tape mounts are preferred.

Morphologic features of importance for identification of conidial fungi include (i) conidium size, shape, and pattern of septation; (ii) color of conidia and conidiophore, whether light (hyaline or moniliaceous) or dark (dematiaceous); (iii) developmental aspects of conidiogenesis, including the nature of the conidiogenous cell; (iv) mechanism of conidium liberation or dehiscence; and (v) structure of the conidioma (if present). Differences in conidial shape and septation are useful characters for preliminary distinction and have traditionally been used for grouping conidial fungi (known as Saccardo spore groups). Conidia may be nonseptate or may have one or more septa (amerosporae, didymosporae, and phragmosporae, respectively). Some fungi produce both nonseptate and septate conidia, and these conidia are often referred to as micro- and macroconidia. Conidia also vary in shape. Some are long and narrow (scolecosporae), as in *Fusarium* species. Development of a conidium may occur by conversion of an existing cell or several cells (thallic-arthric) or may involve new wall building or blowing out of a portion of the wall (blastic). Conidiogenesis usually occurs at a particular location on a conidiogenous cell. If development occurs at a site that remains fixed and gives rise to more than one conidium, then the site is stable or determinate. If development occurs at new points on the conidiogenous cell (or axis), then the site is unstable or indeterminate. New sites may occur on an axis which lengthens (progressive) or shortens (retrogressive). The conidiogenous cell produces a single conidium or multiple conidia. Sympodial development involves the development of a single conidium at successive sites on a lengthening axis. Conidiogenous cells which are specialized to produce multiple conidia include the phialide and annellide. Conidia are produced successively (serially) and develop in slimy masses or in chains, with the youngest at the base of the chain (basipetal). Although it is sometimes difficult to differentiate between the two types of cells, the annellide elongates and sometimes narrows during the formation of each new conidium, leaving an often imperceptible series of rings or scars on the conidiogenous cell. Scrutiny of the cell by use of an oil immersion objective may be necessary to make this distinction. Conidia may form also in acropetal chains, with the youngest conidium at the tip of the chain. The distinction between acropetal and basipetal chains may be revealed by comparison of the size and wall morphologies of the top and bottom conidia of the chain. The youngest conidium is recognizable by its smaller size, lighter color if the conidia are pigmented, and differences in wall ornamentation if the conidia are roughened. Some conidiogenous cells form multiple conidia simultaneously over the surface of the swollen cell. When mature, conidia detach by fission of a double septum (schizolytic dehiscence) or by sacrifice of a supporting cell (rhexolytic dehiscence), either by fracture of a thin-walled region or by lysis of the supporting cell. Lytic dehiscence typically occurs in the dermatophytes and related fungi.

The presence of different spore states can make identification difficult. Careful examination and sometimes subculture are required to assess whether the spore types represent different states of the same fungus or whether the isolate is contaminated. Molds having more than one independent stage are called pleomorphic (occasionally polymorphic) fungi. Other conidial forms of the same fungus are called synanamorphs. These may be represented by simple yeast stages or by the formation of complex conidiomatal structures such as sporodochia (conidiophores borne crowded on a compact mass of hyphae or a hyphal stroma), synnemata (conidiophores aggregated into a compound stalk), or pycnidia (conidiogenous cells formed inside a round or oval fruiting body). Expression of a synanamorph may be influenced by the agar medium used and may be lost upon repeated subculture.

The number of moniliaceous fungal species that have been reported to cause opportunistic infection in humans and animals is increasing, and it is beyond the scope of this chapter to describe them all. Some reports identify the fungus only to the genus level, while others provide the fungus name but without salient details of its colonial and microscopic features. A continuing and vexing problem is that verification of authenticity of reports cannot be done if the fungus is inadequately described and illustrated and if case isolates are not sent for deposit in culture collections. This

chapter describes in detail the medically important species of the genus *Aspergillus* (Table 2) and provides salient colonial and microscopic characteristics of the more common species of *Fusarium* and other currently recognized moniliaceous opportunists (Tables 3 through 5). Descriptions of species are found in several reference manuals (47, 53, 106, 127, 251).

CLINICAL SIGNIFICANCE

The spectrum of disease caused by moniliaceous molds is diverse, and disease is largely determined by the local and general immunologic and physiologic state of the host and may be symptomatic or asymptomatic. In most instances, the portal of entry for fungal propagules is either through a break in the epidermis or by way of the lungs. Exceptions to this include introduction into the body by means of contaminated surgical instruments, intraocular lenses, prosthetic devices, or other contaminated materials or solutions associated with surgery or routine health care. Individuals whose resistance is lowered as a result of a severe debilitating disease or immunosuppressive therapy typically suffer from invasive pulmonary or paranasal sinus infection, but in some instances, the infecting fungus may spread to surrounding tissues or disseminate to virtually any organ. Fungemia is uncommon except in disseminated *Fusarium* infection. Noninvasive forms of infection also have been noted in debilitated individuals as well as in individuals with apparently normal defense mechanisms. In such cases, the fungus colonizes a preexisting cavity in the lungs such as an ectatic bronchus, a tuberculous cavity, or a lung cyst. Other clinical syndromes usually occurring in immunocompetent individuals include chronic sinusitis, onychomycosis, subcutaneous abscess, keratitis, otomycosis, and allergic manifestations including bronchopulmonary mycosis and sinusitis in atopic patients.

Although the majority of saprobic and plant pathogenic molds are not considered pathogenic and appear unlikely to be able to adapt to or take advantage of risk factors predisposing to opportunistic infection, those capable of growing at or near body temperature must be considered to have latent pathogenic capability. The diversity of fungi capable of colonizing or invading human tissue has increased dramatically in recent years, as reflected by new reports of proven infection. Moreover, certain fungi are isolated often enough to be suspicious for pathogenic potential. Still, there is a need for definitive evidence of infection due to a normally saprobic mold. The laboratory procedure for confirming fungal etiology includes (i) detection in the specimen of hyphal elements which are compatible with the morphology of the isolated mold, (ii) isolation of several colonies of a fungus or isolation of the same fungus from a repeat specimen, (iii) accurate identification of the isolated mold, and (iv) confirmation of the mold's ability to grow at or near body temperature. Species of *Aspergillus, Fusarium,* or *Scedosporium* isolated from all deep tissue or body fluids must be considered potential invasive pathogens. No fungal isolate should be discarded as a contaminant without thorough examination of the clinical specimen. Quality control measures to ensure that isolation media are not contaminated and the inspection of the slant or plate to evaluate where the fungus is growing relative to where the specimen was placed may also be important in evaluating whether an isolate is involved in disease. Close communication between microbiologists and physicians also is essential, especially for rare or unusual opportunists.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Clinical specimens from patients with suspected mycosis are to be collected with prudence and transported to the laboratory and processed as soon as possible by using the standard procedures described in chapter 116 of this Manual. Because of the diverse clinical manifestations, various sites may need to be examined for fungal elements. Biopsy material, transtracheal aspirates, and sputum samples collected in the early morning all may be useful specimens for the isolation and detection of hyaline molds, as are infected nails. Swabs taken from mucous membranes and skin lesions are not recommended. Although the reliability in determining whether an isolate is a possible pathogen is increased if cultures of two different specimens yield the same organism, a tissue biopsy is more informative since the significance of culture can be verified by histological examination of the tissue. Blood cultures are of limited use for the diagnosis of invasive aspergillosis (55), but *Fusarium* or *Scedosporium* species may be reliably detected (209). For optimum recovery, the specimen should be inoculated onto several types of media and incubated at 28 to 30°C. The opportunistic molds are variably sensitive to cycloheximide, so media containing this selective agent should be used cautiously. Suspicious isolates, especially of uncommon species, should be tested for their ability to grow at 35 to 37°C.

ASPERGILLUS SPECIES

Taxonomy

The genus *Aspergillus* comprises anamorphic (asexual) species with known or presumed connections to eight different teleomorph (sexual) genera classified in the family Trichocomaceae of the Ascomycota. The family Trichocomaceae also contains teleomorphs of the genera *Penicillium* and *Paecilomyces* (some species) (228). As members of the class Euascomycetes, aspergilli are distantly related to other ascomycetes such as dimorphic fungi, dermatophytes, and *Pseudallescheria* species. Because the large number of species within the genus *Aspergillus* has made identification to the species level difficult for even the experienced microbiologist, the species were placed into groups (211) and, more recently, further subdivided into the subspecies categories of subgenera and sections (53, 114). Peterson's analysis of large-subunit rRNA sequences derived from 215 taxa correlated to a large degree with many of the species originally described by Raper and Fennell (211) but demonstrated a need for taxonomic revision (198). Three main branches on the phylogenetic tree were used to define the subgenera *Aspergillus, Fumigati,* and *Nidulantes,* and sections formerly recognized were either retained or redisposed. A result of this realignment is that the subgenus *Aspergillus* includes both uniserate and biseriate species and species of different colors, whereas in prior treatments these were key characters that separated groupings of species. At this time, the genus *Aspergillus* includes seven subgenera: *Aspergillus, Fumigati, Ornati, Clavati, Nidulantes, Circumdati,* and *Stilbothamnium*. Each subgenus contains several sections, with each section composed of a number of species (115).

In practice, *Aspergillus* species are differentiated mainly on the basis of phenotypic characteristics of the anamorph, but early monographic treatments (211) also included descriptions of the teleomorph. Because this practice was inconsistent with the International Code of Botanical Nomenclature, it caused problems in the nomenclature of some well-known species.

TABLE 2 Characteristics of some medically important *Aspergillus* species grown on identification media[a]

Section[b] and species	Seriation		Colony color	Microscopic features[c]	Comment
	Uniseriate	Biseriate			
Fumigati					
A. fumigatus	+		Dark blue-green to grayish turquoise; slate gray with age; reverse variable	Conidiophore mostly up to 300 μm long and 5–8 μm wide, smooth, uncolored or greenish; vesicle dome shaped, 20–30 μm in diam, phialides on upper half only; head strongly columnar; conidia subglobose to globose, occasionally ellipsoidal, smooth to echinulate, 2–3.5 μm in diam (Fig. 3).	Distinguished by blue-green colonies, growth at 45°C and columnar heads with single layer of phialides. Sterile white fast-growing or glabrous cerebriform slow-growing variants may be confirmed by thermotolerance and DNA sequence. Cosmopolitan airborne mold often found in compost piles, soil of potted plants; most common pathogen.
Neosartorya fischeri (anamorph *A. fischerianus*)	+		White to gray-green; exudate uncolored or pale brown	Conidiophores and conidial heads and conidia as for *A. fumigatus;* cleistothecia globose mostly 150–350 μm; creamy white, ascospores rough walled with two crests, 5–8 μm long; 3 varieties distinguished by ascospore morphologies (Fig. 26).	Conidial characteristics and thermotolerance strongly similar to *A. fumigatus* but colonies usually whiter with fewer conidial heads; rarely reported as a pathogen; some clinical isolates reported as *N. fischeri* have been reidentified as *N. pseudofischeri*.
Flavi					
A. flavus	+	+	Yellow to dark yellowish green	Conidiophore mostly 400–850 μm long and 20 μm wide, roughened; uncolored, vesicle subglobose or globose, 25–45 μm in diam; loosely radiate or splitting into columns in age; conidia globose or ellipsoidal, roughened, 3–6 μm in diam.	Toxigenic[d]; brown to black sclerotia sometimes present; growth usually enhanced at 37°C; heads vary in size and seriation; colony color may be influenced by culture medium additives such as yeast extract; second most common human pathogen.
Nigri					
A. niger		+	Black with white margin and yellow surface mycelium; reverse uncolored or pale yellow.	Conidiophore 400–3,000 μm long and 15–20 μm wide, smooth, uncolored to brownish near tip; vesicle globose, 30–75 μm in diam; radiate, then splitting into columns in age; conidia globose with thick walls, brownish black, roughened, 4–5 μm in diam.	Frequent cause of otomycosis; sometimes associated with intracavitary colonization, especially in diabetics.
Versicolores					
A. versicolor		+	Green to gray-green or tan with patches of pink or yellow; reverse variable and often deep red	Conidiophore 200–400 μm long and 5 μm wide, smooth, uncolored, yellowish, or pale brown; vesicle ovate to elliptical, 9–16 μm in diam, head radiate to loosely columnar; conidia globose, echinulate, 2.5–3 μm in diam; Hülle cells globose (Fig. 27).	Toxigenic; distinguished by slow-growing, greenish-tan or variably colored colonies, and small biseriate vesicles. Conidial heads of *A. sydowii* are similar but colonies are blue-green. Reduced structures resembling penicilli sometimes present.
A. sydowii		+	Blue-green often resembling *Penicillium* spp.	Conidiophore 150–350 μm long and 3–8 μm wide, smooth, thick walled, uncolored or pale brown; vesicle globose or elliptical, 7–13 μm in diam, head radiate; conidia globose, very rough, 2.5–4 μm in diam; reduced structures resembling penicilli common.	Distinguished from *A. versicolor* by slow-growing blue-green colonies; diminutive heads resembling penicilli common.

(Continued on next page)

TABLE 2 Characteristics of some medically important *Aspergillus* species grown on identification media[a] *(Continued)*

Section[b] and species	Seriation		Colony color	Microscopic features[c]	Comment
	Uniseriate	Biseriate			
Terrei					
A. terreus		+	Tan to cinnamon brown, rarely orange-brown, reverse yellow or tan	Conidiophore 100–250 μm long and 4.5–6 μm wide, smooth, uncolored; vesicle dome shaped, 10–16 μm in diam, phialides on upper half; head columnar; conidia globose or subglobose, smooth, 2 μm in diam; solitary single-celled conidia commonly formed sessile on submerged hyphae.	Cinnamon brown colonies, columnar heads and solitary accessory conidia are highly distinctive.
Nidulantes					
Emericella nidulans (anamorph *A. nidulans*)		+	Dark green if mainly conidial; buff to purplish brown if cleistothecial; reverse deep red to purple	Conidiophore 70–150 μm long and 3–6 μm wide, smooth, brown; vesicle hemispherical, 8–12 μm in diam; phialides on upper part, columnar; conidia globose, rough, 3–4 μm in diam; cleistothecia reddish brown, globose, 100–250 μm, Hülle cells globose; ascospores lenticular with two longitudinal crests, ca. 5 μm long, reddish purple (Fig. 4).	Toxigenic; distinguished by reddish brown cleistothecia, abundant Hülle cells, reddish purple ascospores with two crests, short conidiophores and "stout" metulae.
Emericella quadrilineata		+	Olive green to grayish purple, reverse purple	Conidial heads, ascocarps and hülle cells similar to those of *E. nidulans*. Ascospores also similar but have 2 major and 2 minor equatorial crests.	Distinguished from *E. nidulans* by 4 crests on ascospores.
Flavipedes					
A. flavipes		+	White with patches of yellow or pale grayish buff, reverse yellow to golden brown	Conidiophore 150–400 μm long and 4–8 μm wide, smooth to roughened, uncolored to pale brown, vesicle subglobose, 10–20 μm in diam; radiate to loosely columnar; conidia globose, smooth, 2–3 μm in diam; cleistothecia and Hülle cells rarely produced.	Distinguished from *A. terreus* by slower-growing colonies, metulae usually formed over entire vesicle and radiate to loosely columnar heads.
Usti					
A. ustus		+	Brownish gray or olive-gray, reverse yellow, dull reddish or purplish (Fig. 6)	Conidiophore 75–400 μm long and 4–7 μm wide, smooth, becoming brown; vesicle globose or subglobose, 7–16 μm in diam, fertile over upper two-thirds; radiate to loosely columnar; conidia globose, rough, 3–4.5 μm in diam; irregular Hülle cells often present (Fig. 7).	Distinguished by dull gray-green colonies, small vesicles, brown conidiophores, and irregularly shaped Hülle cells when present.
A. deflectus		+	Slow growing, becoming mouse gray with pinkish margins or patches of yellow	Conidiophore 40–125 μm long and 2.5–3.5 μm wide, smooth, reddish brown; vesicle hemispherical, 5–7 μm in diam typically bent at right angle to stipe; phialides on upper surface, columnar; conidia globose, 3–3.5 μm in diam, smooth to roughened; Hülle cells sometimes present.	Distinguished by vesicle bent almost at right angle to stipe; rarely reported as pathogen in humans and dogs.
Aspergillus					
Eurotium spp. Anamorphs *A. glaucus* and other spp.	+		Deep green mixed with bright yellow, reverse uncolored or pale yellow	Conidiophore 200–350 μm long and 7–12 μm wide, smooth, uncolored to pale brown; vesicle globose, 15–30 μm in diam, conidial heads large, radiate; conidia subglobose, echinulate, 5 μm in diam, cleistothecia thin walled,	Formerly called *glaucus* group; osmophilic, reproduction enhanced on high-sugar media; growth poor at 37°C; difficult to distinguish by features of conidial heads, but readily identified by ascospore morphology.

			yellow, globose, 75–150 μm, ascospores smooth or roughened, with furrow and rounded or frilled crests.		*E. repens, E. nuber,* and *E. amstelodami* rarely reported as pathogens. *E. umbrosus* reported as cause of farmer's lung is another name for *E. herbariorum* (anamorph *A. glaucus*).
Restricti					
A. restrictus	+	Dull olive green to brownish green, very slow growing	Conidiophore 80–200 μm long and 4–8 μm wide, smooth or roughened, uncolored; vesicle hemispherical, 8–20 μm in diam, phialides on upper third, head columnar; conidia cylindrical to ellipsoidal, roughened, 4–7 μm long and 3–4 μm wide.		May be confused with *A. fumigatus* but differs by very slow growth on routine media, slightly enhanced growth on high-sugar media, no growth at 37°C, cylindrical conidia developing in long, adherent columns.
Candidi					
A. candidus	+	+ White to cream	Conidiophore mostly 200–500 μm long and 7–10 μm wide, smooth to roughened, uncolored; vesicle globose or subglobose, 17–35 μm in diam, fertile over entire surface; radiate; conidia globose, smooth, 3–4 μm in diam; reddish purple sclerotia sometimes present.		Distinguished from all colored aspergilli by white, slow-growing colonies; from *A. niveus* by larger vesicles, metulae covering entire surface and absence of teleomorph; predominantly biseriate, sometimes uniserate on smaller heads.

[a] Modified from previous versions of this chapter.
[b] Modern concepts have replaced group names with subgenera and sections.
[c] Refer to "*Aspergillus* Taxonomy and Identification" for description of terms.
[d] Only species producing potent toxins are noted as toxigenic, but other species may produce toxins of lesser significance.

Experts in the field have attempted to rectify outstanding problems and have compiled a list of names in current use in the family Trichocomaceae. The list currently includes 184 species of *Aspergillus* and 70 associated teleomorphs (228).

Description of Agents

Aspergillus is a large genus of common environmental fungi containing about 184 species whose members reproduce by producing conidia in dry chains on phialides, cells with a terminal opening through which enteroblastic conidia are produced repetitively. Phialides can be produced directly from the vesicle (uniserate), or from an intermediate series of cells called metulae (biserate). In general, *Aspergillus* colonies usually grow very rapidly, producing powdery white, green, yellowish, brown, or black colonies. They are found worldwide, in soil, water, foods, and other environmental niches. Their dry conidia can be easily dispersed in the air and by birds or insects. Several *Aspergillus* species also produce mycotoxins, which are harmful to humans and animals when ingested (see chapter 127).

About 40 *Aspergillus* species have been implicated in human or animal infection, but 16 of these have been recorded only once (47). Some reports have been discounted because the fungus isolated was not reliably demonstrated to be a pathogen, or because the description clearly represented another fungus or was inadequate for evaluation (255). *Aspergillus fumigatus* still accounts for most cases of aspergillosis, with *A. flavus* and *A. niger* being the next most common pathogenic species worldwide (127). *A. terreus* is increasingly being reported (94, 132, 164), and *A. lentulus* is the newest species recognized as an agent of human disease (15).

Aspergillosis is a general term used to refer to infections caused by members of the genus *Aspergillus*. Invasive pulmonary aspergillosis is the most serious form, occurring in immunocompromised individuals. A classification of *Aspergillus* infections is given in Table 1.

Epidemiology and Transmission

Invasive aspergillosis is most commonly caused by *A. fumigatus* in humans, but other species such as *A. flavus, A. nidulans,* and *A. terreus* have also been implicated in causing invasive infection. In some centers a shift toward *Aspergillus* species other than *A. fumigatus* has been reported, although significant variation between centers exists (138, 164). Conidia of *Aspergillus* species are often found in the ambient air, but large amounts of conidia are present in dust, decomposing organic matter, and soil. Inhalation of conidia may lead to a variety of disease entities ranging from asthma in atopic subjects to invasive infection in patients with severely compromised host defenses. Most invasive infections occur in the lungs, and the incidence of infection largely depends on the risk of the patient population. Typically the incidence of invasive aspergillosis is around 5% of patients with hematological malignancy, especially in acute myeloid leukemia and myelodysplastic syndrome, but may be as high as 15% in patients receiving a hematopoietic stem cell transplant (HSCT) (164).

Although invasive pulmonary aspergillosis is believed to be primarily acquired by inhalation of airborne conidia, there is some evidence that water may play a role in the transmission. *A. fumigatus* has been cultured from hospital water (271) and other sources in the hospital such as showerheads, and strains recovered from water were found to be highly related to those recovered from patients (274). Patients might be exposed to *Aspergillus* conidia during showering by inhaling droplets contaminated with conidia.

TABLE 3 Key features of some opportunistic moniliaceous fungi

Genus	Key features	Etiologic agents	Comments
Acremonium	Colonies slow growing (usually <3 cm in diam in 10 days), often white, cottony, fasciculate (spiky), glabrous or moist and pink or salmon colored. Conidiogenous cells solitary, slender (ca. 2 μm wide), mostly unbranched, awl (needle-shaped) phialides. Conidia one celled, straight or curved, in slimy masses.	*A. alabamense, A. falciforme, A. kiliense, A. potronii, A. recifei, A. curvulum, A. strictum, Acremonium* spp.	Differs from *Fusarium* by slow growth rate, narrower hyphae (mostly <2 μm in width), and more slender and needle-like phialides. Compare *Lecythophora, Phaeoacremonium, Phialemonium.*
Acrophialophora	Colonies fast growing, growth enhanced at 37°C, yellowish white, darkening to grayish brown. Conidiophores brown, seta-like. Conidiogenous cells flask-shaped phialides borne near the tip of conidiophores or on vegetative hyphae. Conidia in long chains, one celled, lemon shaped, smooth or roughened.	*A. fusispora*	Compare *Paecilomyces.* Single report of keratitis.
Aphanoascus	Colonies moderately fast growing, yellowish white, granular; cycloheximide tolerant. A homothallic ascomycete forming globose cleistothecia containing roughened ascospores; associated with a *Chrysosporium* anamorph consisting of terminal one-celled aleurioconidia and alternate arthroconidia.	*A. fulvescens*	Compare *Chrysosporium* and dermatophytes.
Arthrographis	Colonies slow growing, growth enhanced at 37°C, initially yellowish white, and yeast-like, becoming hyphal and tan, cycloheximide tolerant. Conidiophores dendritic (tree-like), bearing lateral branches. Arthroconidia formed by fragmentation of branches or of undifferentiated hyphae.	*A. kalrae*	Compare *Scytalidium hyalinum, Onychocola, Hormographiella.*
Aureobasidium	Colonies wet, pale salmon or pink, usually darkening in age to black. For microscopic features, see chapter description.	*A. pullulans*	Differs from *L. hoffmannii* and *A. strictum* by absence of distinct phialides.
Beauveria	Colonies slow to moderately fast growing, yellowish white. Conidiogenous cells solitary, in whorls or in sporodochia, basally swollen, proliferating sympodially at the tip in a zigzag (geniculate) fashion. Conidia one celled, subglobose.	*B. alba* (also called *Engyodontium album*), *B. bassiana*	Compare *Sporothrix.*
Chaetomium	Colonies fast growing, yellowish green to gray. Conidia absent or formed from phialides. Ascomata perithecia bearing coiled, straight, branched brown or indistinct setae. Ascospores lemon shaped, brown, smooth. Fig. 21.	*C. strumarium, C. atrobrunneum, C. globosum*	*C. strumarium* and *C. atrobrunneum* show enhanced growth at 42°C, but *C. globosum* fails to grow.
Chrysosporium	Colonies slow to moderately fast growing, yellowish white. Conidia single celled smooth to roughened aleurioconidia formed sessile or at the ends or on the sides of unswollen stalks. Arthroconidia sometimes present.	Unnamed species	Reports of infection by unnamed species are difficult to evaluate because the isolates are not described well enough to confirm etiology.
Cylindrocarpon	Colonies fast growing, yellowish white, tan, orange, or purple, felty or cottony. Conidiogenous cells awl-shaped phialides with a single opening, solitary, in branched structures or in sporodochia (conidiophores borne crowded on a compact mass of hyphae). Macroconidia straight or curved, with rounded ends, multicelled; microconidia 1 or 2 celled. Chlamydospores sometimes present.	*Cylindrocarpon destructans, C. cyanescens, C. lichenicola*	Distinguished from *Fusarium* by the more rounded shape of the terminal cells of the macroconidia.

(Continued on next page)

TABLE 3 *(Continued)*

Genus	Key features	Etiologic agents	Comments
Fusarium	About 100 species grouped into sections. Colonies fast growing (>7 cm in diam after 10 days); obverse and reverse colors white, cream to orange, tan, brown, carmine red, reddish brown, pink, purple, or blue to blue-green; texture felty or cottony or sparse and wet looking. Conidiogenous cells awl-shaped phialides with one opening (monophialide) or more than one opening (polyphialide). Phialides solitary, in branched structures or in sporodochia (conidiophores borne crowded on a compact mass of hyphae). Conidia of two types that are common or rare depending on the species. Macroconidia multicelled, with 2 to 5 septa, fusiform or sickle shaped, often with a distinct notched basal cell. Macroconidia produced in the aerial mycelium less typical than those produced in sporodochia. Microconidia oval, globose, ellipsoidal, clavate or reniform (kidney shaped), 1 or 2 celled, and borne in slimy heads or in short chains in the aerial mycelium. Chlamydospores sometimes present; solitary, in chains or in clusters.	*Fusarium solani, F. oxysporum, F. verticillioides (F. moniliforme)* most common (Table 4); *F. anthophilum, F. chlamydosporum, F. dimerum, F. napiforme, F. nygamai, F. proliferatum, F. roseum, F. sacchari* rarely reported.	Differs from *Acremonium* by colonies that are faster growing, broader, more robust hyphae and phialides, and usually by presence of macroconidia; many species toxigenic.
Hormographiella	Colonies fast growing, white, amber to tan, woolly, cycloheximide sensitive. Conidiophores short, bearing short fertile branches. Arthroconidia schizolytic, thin walled, single celled, often adherent around the conidiophore. Sclerotia sometimes present.	*H. aspergillata, H. verticillata*	Differs from *A. kalrae* by cycloheximide sensitivity, fast growth rate.
Lecythophora	Colonies white to salmon, moist or fasciculate (spiky), or tan darkening to black in patches. Conidiogenous cells adelophialides (short, stumpy phialides without a basal septum), as well as awl-shaped phialides. *L. mutabilis* forms brown chlamydospores, while *L. hoffmannii* does not.	*L. hoffmannii, L. mutabilis*	Differs from *Acremonium* by predominance of adelophialides. Hyphal elements in tissue are usually reported as hyaline.
Myceliophthora	Colonies fast growing, thermophilic, cinnamon brown. Conidia pale brown, roughened, single-celled aleurioconidia formed on swollen stalks.	*M. thermophila*	Differs from *Chrysosporium* by swollen stalks.
Onychocola	Colonies restricted, raised, yellowish white to grayish white, cycloheximide tolerant. Conidia 1 or 2 celled cylindrical or swollen arthroconidia forming in adherent chains, detaching by fracture of thin-walled cells. Brown knobby setae often present. Fig. 16.	*O. canadensis*	Differs from *S. hyalinum* and *Hormographiella* by adherent chains, slow growth, and cycloheximide tolerance.
Paecilomyces	About 40 species grouped into two sections. Section *Paecilomyces* includes the thermotolerant *P. variotii* and anamorphs of *Byssochlamys* and *Thermoascus* with fast-growing yellowish brown, buff, white, or orange colonies. Section *Isarioidea* includes *P. lilacinus* and species that are slower growing with white, reddish gray, violet, or brightly colored colonies. Conidiogenous cells phialides formed on verticillately branched conidiophores. Conidia single celled, in chains. Ascospores of *T. crustaceus* are orange brown, rough walled. Fig. 12, 13.	*P. lilacinus, P. variotii* (Table 5), *P. marquandii, P. javanicus, Thermoascus crustaceus*	Differs from *Penicillium* by colonies in colors other than bright green or blue-green, and by the basally swollen phialides that taper toward the neck and that are bent away from the main axis. An isolate of *P. viridis* from human infection was reidentified as *P. variotii*. Compare *Acrophialophora*.

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TABLE 3 Key features of some opportunistic moniliaceous fungi *(Continued)*

Genus	Key features	Etiologic agents	Comments
Penicillium	About 200 species divided into 4 subgenera according to conidiophore branching. Colonies moderately fast growing, powdery, green to blue-green, rarely other colors. Conidiophores erect, with 1 to 3 levels of branching. Conidiogenous cells phialides, borne on the stipes or on branches called metula. Conidia globose or ellipsoidal, smooth to roughened, in chains. Sclerotia and ascomata sometimes present. *P. marneffei* distinguished by sparse green aerial and reddish brown vegetative hyphae, red diffusible pigment; compact biverticillate or irregular penicilli on short, roughened conidiophores and growth at 37°C in a yeastlike form. Fig. 8, 9.	*P. marneffei* (true pathogen), *P. brevicompactum, P. chrysogenum, P. citrinum, P. commune, P. decumbens, P. purpurogenum, P. spinulosum,* and unidentified species	Several species may produce red diffusible pigments but fail to convert to yeast-like form. Many species toxigenic.
Phaeoacremonium	Colonies white to tan. Conidiogenous cells solitary unbranched or branched awl-shaped phialides often arising from hyphal strands. Conidia single celled, curved, rarely straight, in slimy masses.	*P. parasiticum*	Accommodates *Phialophora parasitica* and several other species intermediate between *Acremonium* and *Phialophora*.
Phialemonium	Colonies yellowish to green. Conidiogenous cells adelophialides (short, stumpy phialides without a basal septum), as well as awl-shaped phialides.	*P. curvatum, P. obovatum*	Compare *Acremonium* and *Lecythophora.*
Schizophyllum	Colonies fast growing, growth enhanced at 37°C, white, woolly or cottony, cycloheximide sensitive. Conidia absent. Hyphae bearing clamp connections and short, thin pegs or spicules; both may be absent. Clamped isolates usually develop fan-shaped, gilled mushrooms on sporulation media after 3 to 6 weeks. Fig. 24, 25, 31.	*S. commune*	Clinical picture resembles aspergillosis and clampless isolates resemble aspergilli by histopathology. *S. commune* may be the cause of sinusitis attributed to *Myriodontium keratinophilum* and *Chrysosporium* sp.
Scopulariopsis	Colonies white, buff, gray-brown to black. Conidiogenous cells annellides formed on branched conidiophores with 1 or 2 levels of branching. Conidia one celled, globose or ellipsoidal, in chains. *S. brevicaulis* distinguished by buff or tan, granular colonies, thick-walled smooth to coarsely roughened conidia that are truncate at the base and rounded or pointed at the tip and measure 5 to 8 μm wide by 5 to 7 μm long. Ascospores of *Microascus* species are orange, lens or half-moon shaped, and released in a cirrus (a long adherent column) from a short-necked ascocarp. Fig. 20.	*S. brevicaulis, S. candida, S. brumptii, Microascus cinereus, M. cirrosus*	*S. candida* differs from *S. brevicaulis* in having smooth conidia and white colonies. *S. brumptii* differs from *Scedosporium* species by the conidia produced in chains rather than in slimy masses. *Microascus* species differ from *Chaetomium* species by the orange ascospores produced from short-necked ascomata.
Scytalidium	Colonies rapid growing (>8 cm in diam by 7–10 days), yellowish white or black, dense, cottony or woolly, cycloheximide sensitive. Conidia 1- or 2-celled arthroconidia formed by schizolytic fragmentation of branched hyphae varying in width from 2- to 10-μm diam. Hyphae and arthroconidia of *S. hyalinum* are hyaline to pale yellow; those of *S. dimidiatum* are light to dark brown. Pycnidial conidia of *Nattrassia mangiferae* stage are initially hyaline and then develop median brown band.	*S. hyalinum, S. dimidiatum* (synanamorph is known as *Nattrassia mangiferae* or formerly, *Hendersonula toruloidea*)	Isolates reported as *S. lignicola* from human infection have been reidentified as *S. dimidiatum. S. lignicola* differs from *S. dimidiatum* in colonial and microscopic features and should not be considered a synonym as has been suggested in the literature.
Sporothrix	Colonies of *S. cyanescens* moderately fast growing, white to lavender, red diffusible pigment often present; cycloheximide sensitive. Conidiophores solitary, forming conidia sympodially on small denticles on sides or at tips. Primary conidia bear 1 to 3 secondary conidia; brown-pigmented sessile conidia absent.	*S. cyanescens* (also called *Cerinosterus cyanescens*); (*S. schenckii*—see chapter 120)	Affinity of *S. cyanescens* is closer to smuts than to other species of *Cerinosterus*. *S. schenckii* differs in being dimorphic and in forming brown sessile conidia.

TABLE 4 Morphological features of the three commonly isolated *Fusarium* spp.

Characteristic	*F. solani*[a]	*F. oxysporum*	*F. verticillioides* (*F. moniliforme*[b])
Colonies on potato dextrose agar after 4 days at 25°C	3–3.5 μm in diam, creamy white with cream slimy areas, developing blue-green to bluish brown colors, never orange, reverse uncolored, rarely violet	4.5–6.5 cm in diam, creamy white, often with purple or violet tints, sporodochia cream to orange, slimy, reverse uncolored to dark blue or purple	4.5–6.5 μm in diam, white, peach to purple, sporodochia orange, reverse uncolored to dark purple
Phialides	Mono, long, slender, sometimes verticillately branched	Mono, short, stout, mostly unbranched	Mono, long, branched and unbranched
Microconidia	Cylindrical to oval, nonseptate	Oval to kidney shaped, single celled, in heads	Oval to club shaped with flattened base, mostly single celled, in chains[c] or in heads (Fig. 11)
Macroconidia	Mostly three septate (one to five septate), 28–42 by 4–6 μm, slightly curved, rounded at each end or basal cell notched (Fig. 10)	Mostly three (or up to five) septate, moderately curved, 27–60 by 3–4.5 μm, pointed at each end	Present or rare, three to five (or up to seven) septate, sickle shaped to almost straight, 30–60 by 3–4 μm, basal cell foot shaped
Chlamydospores[d]	Present, formed singly and in pairs, commonly produced	Present, formed singly and in pairs, usually abundant	Absent

[a] Molecular studies reveal *F. solani* to be a complex comprising over 50 phylogenetically distinct entities.
[b] *F. verticillioides* is preferred over the name *F. moniliforme*, which has a broader concept.
[c] Best observed on carnation leaf agar or KCL medium (174).
[d] Best observed on carnation leaf agar or in sterile distilled water (176).

Genotyping will help to further understand the significance of waterborne transmission of invasive aspergillosis.

Clinical Significance

Invasive infections caused by *Aspergillus* species are associated with high rates of morbidity and mortality, especially in transplant recipients and patients receiving chemotherapy for hematologic or other malignancies. Invasive aspergillosis now is considered the second most common fungal infection requiring hospitalization in the United States. In patients with positive fungus cultures, *Aspergillus* species are the second most common isolates after *Candida* species, but positive cultures alone may not indicate a pathogenic process (195).

Recovery of *Aspergillus* species from high-risk patients is associated with invasive infection, although the probability of infection depends on the host group. Assessment of the pathologic importance of any species of *Aspergillus* isolated from clinical specimens can be difficult, even with positive radiological or (high-resolution) computed tomography findings, because aspergilli are routinely isolated from respiratory, cutaneous, and other specimens. It is imperative to determine clinical significance by (i) demonstrating hyphae in fresh clinical material, (ii) isolating heavy growth from a single specimen or the same species from multiple specimens, and (iii) demonstrating hyphae in tissue. Fungal culture of at least three serial sputum specimens is recommended whenever fungal infection is suspected. Clinical and radiological correlations help to separate true- from false-positive cultures (88). Consensus definitions have been published by the European Organization for Research and Treatment of Cancer and the Mycoses Study Group in which host factors, clinical signs and symptoms, and mycology results are used to classify patients, i.e., proven, probable, or possible invasive fungal infection (10). For invasive aspergillosis the consensus definitions recognize antigen detection and high-resolution computed tomography as diagnostic tools but do not (yet) recognize nucleic acid detection techniques. The use of the consensus definitions will help to standardize research into performance characteristics of new diagnostic tests and procedures.

Infections may be primary or secondary, and they vary in severity and clinical course (162). The clinical manifestations (Table 1) are largely determined by the local or general immunologic and physiologic state of the host, but various forms of the disease may integrate and overlap (120, 162). The major risk factors for invasive aspergillosis include hematologic malignancy, neutropenia following stem cell or solid-organ transplantation, pulmonary diseases, and glucocorticosteroid or cytotoxic drug therapy (14, 72, 193). Aspergillosis in human immunodeficiency virus (HIV)-infected people is uncommon and occurs largely among severely leukopenic individuals (84, 169). Invasive pulmonary aspergillosis and cerebral aspergillosis are life-threatening complications in patients with advanced AIDS (71, 163, 169, 278). Modification to antiretroviral therapy, combined with antifungal therapy, results in improved survival of some HIV-infected patients (163, 278), but aspergillosis collectively is associated with poor survival (84, 163, 169). Because different species of *Aspergillus* vary in their susceptibility to antifungal agents, every effort must be made to identify the fungus to the species level when isolated from an at-risk patient.

Collection, Transport, and Storage of Specimens

Methods of collection, transport, and storage of specimens are detailed in chapter 116. Invasive pulmonary aspergillosis is very difficult to diagnose and usually requires a combination of clinical, culture, radiographic, and direct antigen findings. Sputum, bronchoalveolar lavage fluid, or other nonsterile lower respiratory tract specimens can be collected for fungus culture, but it must be appreciated that aspergilli isolated from these sites may reflect contamination or colonization (195).

TABLE 5 Morphologic features of selected *Paecilomyces* species[a]

Characteristic	*P. lilacinus*	*P. marquandii*	*P. variotii*
Colonies on potato dextrose agar after 7 days at 25°C	3–3.5 cm in diam, white becoming dull red or reddish gray,[b] cottony, reverse colorless or vinaceous (Fig. 11)	2 cm in diam, violet brown[b] with white margin, cottony, often with radial folds, reverse yellow, slight yellow diffusible pigment	7–8 cm in diameter, yellowish to olive-brown, powdery (Fig. 12)
Thermotolerance	Strongly restricted at 37°C	No growth at 37°C	Reported up to 50°C, but many isolates show no growth above 40°C
Conidiophores	Rough walled, pigmented yellow or purple, metulae densely clustered, bearing whorls of 2–4 phialides (verticillate), rarely synnematous (Fig. 13)	Smooth walled, hyaline, verticillately branched, metulae with 2–4 phialides, often forming loose synnemata	Verticillately or irregularly branched, metulae bearing 2–7 phialides
Phialides	Taper abruptly to narrow neck, about 1 μm wide	Taper abruptly to narrow neck, about 1 μm wide	Phialides robust, tapering gradually to form long neck about 2 μm wide
Conidia	Smooth to rough, ellipsoidal to fusiform, hyaline to vinaceous in mass, 2.5 to 3 by 2 to 2.2 μm	Smooth to slightly rough, ellipsoidal to fusiform, hyaline to purple in mass, 3 to 3.5 by 2 to 2.2 μm	Variable in size and shape, mostly 3.2 to 5 by 2 to 4 μm or up to 15 μm long, ellipsoidal to cylindrical smooth, hyaline to yellow-brown
Solitary conidia	Absent	Present, thin walled, often in submerged mycelium	Terminal or intercalary, brown, thick walled

[a] Modified from previous versions of this chapter.
[b] Color names from reference 118a.

The most convincing evidence of aspergillosis is provided by recovery of organisms from normally sterile sites, such as lung tissue obtained from biopsy, and histopathologic demonstration of hyphal elements in tissue.

Aspergillus can be recovered from cultures of sinus tissue, other tissue biopsy specimens, skin biopsy specimens, heart valves, and appropriate ophthalmologic samples. Although *Aspergillus* endocarditis can occur in the appropriate patient, blood cultures for *Aspergillus* are usually negative. Conversely, positive blood cultures for *Aspergillus* are frequently indicative of contamination, even in a patient population at risk for aspergillosis (246).

Direct Examination

Microscopy

Hyphal elements and the details of hyphal morphology of aspergilli may be observed readily in routine KOH preparations without or with a fluorescent compound such as calcofluor white or Blankophor P or in tissue section with fungal stains such as the Gomori methenamine silver stain. In hematoxylin-and-eosin-stained tissue, viable hyphae are often basophilic to amphophilic, whereas hyphae in macerated or necrotic tissue tend to be eosinophilic (33). The appearance of *Aspergillus* hyphae may vary with the type of infection. In invasive aspergillosis, aspergilli typically are seen as hyaline, septate hyphae that are 3 to 6 μm in diameter, branch dichotomously at acute (45°) angles (Fig. 2), and have smooth parallel walls with no or slight constrictions at the septa (33). In invasive aspergillosis, hyphae proliferate extensively throughout the tissue, often in parallel or radial arrays. Aspergilli colonizing pulmonary cavitary lesions grow as tangled masses of hyphae and in chronic infection may exhibit atypical hyphal features such as swellings measuring up to 12 μm in diameter and/or absence of conspicuous septa. When hyphal elements typical of aspergilli are present in histologic sections, a presumptive diagnosis of invasive fungal infection can be made, but culture or specific immunohistochemical staining is required to identify the pathogen. In particular, hyphae of *Fusarium* or *Scedosporium* species may resemble those of aspergilli both in their propensity to grow in the lumens of blood vessels and in their formation of dichotomously branched hyphae. More promising are the molecular biological techniques that allow species identification using species-specific probes (81).

The presence of typical conidial heads or ascomata in lung cavities or in the ear canal aids in the diagnosis and may allow a presumptive identification of the fungus. In tissue, *A. terreus* displays distinctive aleurioconidia along the lateral walls of hyphae, thus permitting early clinical recognition of this species in histopathologic specimens (270). Especially if *A. niger* is the cause of infection, calcium oxalate crystals also may be found in respiratory specimens (237). Free conidia may resemble structures such as yeast cells or cysts of *Pneumocystis jirovecii* when stained with Gomori methenamine silver stain, which masks the conidium color, but conidia of the aspergilli are often roughened. Hyphae of the zygomycetes are generally broader (up to 15 μm), have nonparallel walls which often appear collapsed and twisted, and are infrequently septate (33).

Antigen Detection

In patients with invasive infection, antigen detection may be helpful in establishing an early diagnosis (134, 158). Monitoring levels of circulating antigens also may be useful for management of such patients, especially those with immunosuppression or underlying hematologic malignancies (147). Several tests for the detection of soluble antigens of *Aspergillus* species in serum, urine, or other body fluids have been developed: radioimmunoassay, enzyme-linked immunosorbent assay (ELISA), biotin-avidin-linked immunosorbent assay, latex agglutination, and immunoblotting have been the most commonly used methods (77, 102, 117, 125, 202, 259, 280). These methods may provide sensitivity sufficient to allow early diagnosis in some cases (126, 263), but few are commercially available. A latex agglutination test, Pastorex Aspergillus (BioRad, Marnes-La-Coquette, France), has been evaluated in several hospital and clinical laboratories (86, 212, 264). Results show that the test lacks sensitivity and that a low level of false-positive results (~6%) occurs for patients with no evidence of invasive disease. The sensitivity of antigen detection was significantly improved with the development of a commercially available ELISA format, Platelia Aspergillus (BioRad), which employs the same monoclonal antibody as the latex kit. Circulating antigen was detected earlier than with the latex kit (265), and several large prospective, autopsy-controlled clinical studies have confirmed the potential of the ELISA to identify patients at high risk for invasive aspergillosis at an early stage of infection (145, 147, 253). In approximately two-thirds of patients, circulating antigen was detected before diagnosis was made by other means (145). However, the main clinical evaluations have taken place in (neutropenic) patients with hematologic malignancy, and studies with other risk groups such as solid-organ transplant recipients indicate less-favorable performance, although the number of studies in solid-organ transplant recipients is limited. Regardless of the test used, success in detecting antigenemia is directly related to the frequency of monitoring of samples (147). Monitoring of antigen titers in patients with hematologic malignancy has shown that the course of the antigen titer corresponds to the response to treatment (147, 263). The antigen galactomannan can be detected in other body fluids of patients with invasive aspergillosis including cerebrospinal fluid (267), urine, and bronchoalveolar lavage fluid (116, 168). Although this application is potentially useful, the assay has thus far been validated only for serum.

In addition to the detection of *Aspergillus* galactomannan, assays that detect (1→3)-β-D-glucan, a characteristic fungal cell wall constituent common to a broad range of fungal pathogens, have been developed (181). Although (1→3)-β-D-glucan itself is not immunogenic, the detection system is based on the activation of a proteolytic coagulation cascade. Circulating (1→3)-β-D-glucan was detected in patients with systemic fungal infections including invasive aspergillosis (104, 194), but clinical experience is at present limited. The positivity of this test, particularly when used in a serial fashion, often precedes the microbiological or clinical diagnosis of invasive fungal infection.

With the increased use of surrogate markers in the management of patients, factors that hamper the use and reliability of these markers have emerged. Most notably, exposure of high-risk patients to mold-active antifungal agents significantly reduces the sensitivity of galactomannan detection (151, 152), a phenomenon that also appears to apply to the detection of circulating fungal DNA (133). In one study the sensitivity of the ELISA was only 20% for patients on antifungal prophylaxis, as opposed to 80% for those not receiving prophylaxis (151). ELISA reactivity was reported with certain beta-lactam antibacterial agents (7), and more recently reactivity of serum was described for patients treated with piperacillin-tazobactam and amoxicillin-clavulanic acid (18, 252, 269). The ELISA reactivity observed in batches of the antibiotics might be due to the use of *Penicillium* fungi for the production of the antibacterial agents. Other exogenous sources for serum reactivity might include bacteria that are present in the microflora of the intestine. The beta-1,5-linked galactofuranosyl chain of the galactomannan molecule, which is the target for the EB-A2 immunoglobulin (IgM) monoclonal antibody used in the ELISA, was found also to be present as a membrane-associated molecule in certain bacteria, most notably the *Bifidobacterium* (159, 160). Since these bacteria are present in the gut of humans and in very high numbers in neonates it was suggested that these bacteria could cause serum reactivity in those patient groups with disruption of the integrity of the intestinal barrier (159).

False-positive reactions of (1→3)-β-D-glucan are known to occur in some patients. These include subjects who had experienced renal failure and who were undergoing hemodialysis with cellulose membranes (108), subjects treated with certain immunoglobulin products (92), and specimens (or subjects) exposed to glucan-containing gauze or related materials (171). This underscores the need to combine serological (1→3)-β-D-glucan monitoring with clinical examination of the patient and other diagnostic procedures, such as high-resolution computed tomography scan, when making a diagnosis.

Nucleic Acid Detection Techniques

The sensitivity and specificity of molecular techniques make them attractive as alternative methods for the early diagnosis of invasive aspergillosis, but they are still experimental. In the absence of a commercial nucleic acid based system, conclusions with respect to the performance and diagnostic value of molecular techniques cannot be drawn since differences between in-house systems are extremely difficult to interpret. The molecular techniques appear to be highly sensitive and specific (56, 279) and can be applied to a broad spectrum of clinical specimens including respiratory samples (168), tissue biopsy specimens (81, 191), and blood (22, 56, 105). PCR-based assays appear to be amenable to the clinical laboratory. Initially, primers were designed to specifically amplify DNA from *Aspergillus* species (279), but alternative approaches have been evaluated in which a broad range of pathogenic fungi can be detected in a first step and identification to the species level can be achieved in a second step (56). The latter approach has great potential since severely immunocompromised patients are at risk for infections due to a broad range of fungal pathogens. Automated systems are increasingly used and adapted for lysis of fungal cells in clinical specimens and recovery of fungal DNA (62). Furthermore, technical advances in postamplification analysis have enabled real-time detection and quantification of the fungal DNA load in blood or tissue samples (TaqMan, LightCycler, and molecular beacons) (42, 142).

Prospective clinical evaluations of PCR-based systems with whole blood show that circulating fungal DNA can be detected early in the course of infection with a very high specificity and a variable sensitivity (56, 79, 131). The release and circulation of fungal DNA appear to be less constant than those of antigens, which results in a high number of negative samples from patients with invasive *Aspergillus* disease. In one study it was calculated that all patients with invasive aspergillosis could be diagnosed by PCR if more than 26 samples were analyzed per risk episode (22).

Isolation

In general, *Aspergillus* species can be recovered easily on routine mycological media and do not possess specific growth requirements. Most identification schemes utilize specific media and incubation conditions for the descriptions of potentially variable characteristics such as colony color and diameter (see below). *Aspergillus* species do not require specific biohazard precautions in the laboratory, although frequently culture materials are handled in a biosafety cabinet to avoid dispersal of the dry spores throughout the environment and cross-contamination of other cultures. The main disadvantages of culture are that it is relatively insensitive and that the culture process requires several days (85).

Identification

Diagnostic features of the more common medically important aspergilli are described in Table 2, and placement of species within sections follows the scheme used by Peterson (198). Species that commonly produce sexual structures (cleistothecia and ascospores; see Fig. 5) are described under their teleomorph names, *Eurotium, Emericella,* and *Neosartorya.* Figure 1 presents a diagrammatic key to several species.

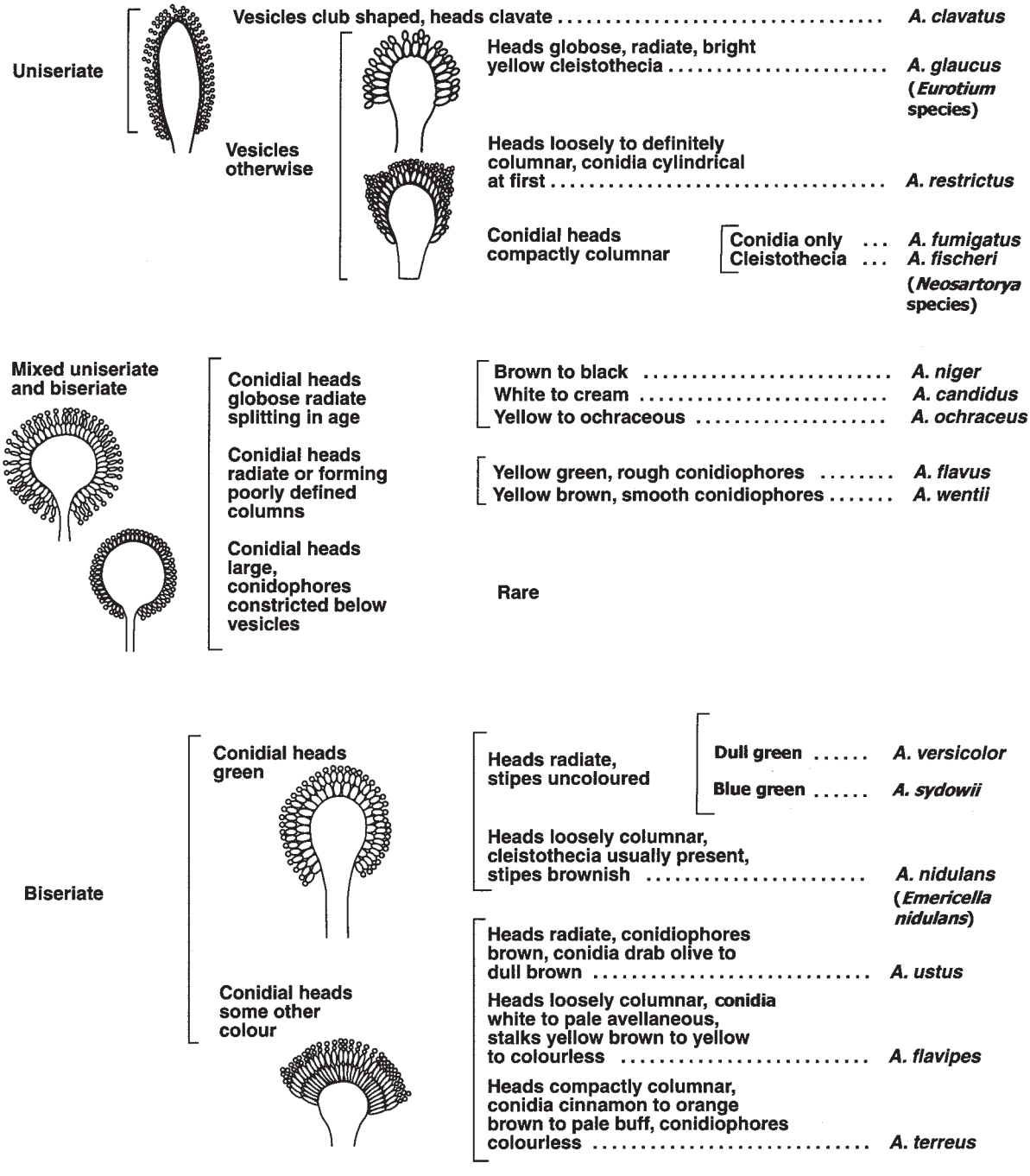


FIGURE 1 Key to representative species of *Aspergillus.* (Reprinted from reference 187 with permission of Cambridge University Press.)

Both macroscopic and microscopic characters are required for identification of *Aspergillus* species. When isolated on media such as Sabouraud dextrose agar (SDA), aspergilli tend to reproduce in the asexual form. Subculture to standard Czapek-Dox, Czapek-Dox with added glucose (20 to 30%), and 2% malt extract agar (211) or on modifications of these media (114) allows comparison of colonial and microscopic features with those given in monographs and taxonomic keys (53, 114, 115, 187, 211, 228). However, standard descriptions are often based on growth after 7 days, and it usually takes longer for development of the teleomorph. Some species are osmophilic and grow poorly on media with low concentrations of sugar. Colony diameters on standard media at 25 and 37°C, obverse and reverse colors, texture, topography, and the presence of exudate droplets or diffusible pigments are recorded after 7 days (53, 114, 187). Isolates not immediately identifiable should be retained longer for possible development of ascomata or other structures that may be helpful in the identification of an isolate.

Important microscopic features include differences in size, shape, color, and wall ornamentation of various structures; shape and size of vesicles; and arrangement of metulae and phialides. An upright stipe or conidiophore arises either directly from the vegetative hyphae or from a specialized hyphal cell called a foot cell, is usually nonseptate, and varies in color, length, and wall ornamentation in different species. The stipe terminates in a swollen cell, called a vesicle (Fig. 1, 5, and 7; see also Fig. 26 and 27), which is globose, subglobose, hemispherical, pyriform (pear shaped), or clavate (club shaped). Either the entire vesicle or the upper portion of it is covered with phialides, which form the conidia. Phialides arise simultaneously directly from the vesicle (uniseriate; Fig. 1; see also Fig. 26), from the intermediate series of cells called metulae (biseriate; Fig. 1; see also Fig. 27), or by a combination of both processes, as occurs in *A. flavus* (Fig. 1). Examination of a colony under a dissecting microscope allows observation of conidial chains to determine whether they are borne in a single column (columnar) or whether the columns are split, with some arising at right angles to the stipe (radiate). The conidia are typically ellipsoidal or globose and vary in size, color, and wall markings depending upon the species. Sclerotia are firm, fruiting-body-like structures composed of swollen hyphal cells but lacking internal spores. Hülle cells are globose or variable in shape and have thick and highly refractive walls (Fig. 5 and 7). They commonly occur immersed in the vegetative mycelium near the center of a colony, where their presence may be indicated by droplets of exudate. Hülle cells are often associated with cleistothecial ascomata such as in *Emericella* species (Fig. 5; Table 2). Cleistothecia have walls composed of enlarged hyphal cells and interiors filled randomly with asci and ascospores. Shape, color, size, and wall features of both ascomata and ascospores are important characters for differentiating teleomorphs of *Aspergillus* species. White, somewhat cottony, nonsporulating variants of aspergilli are isolated occasionally, especially from patients with chronic respiratory infections. *A. fumigatus* should be considered if a sterile white mold grows at 45°C. Variants sometimes appear highly atypical and present with glabrous cerebriform colonies not recognizable as *Aspergillus* (Fig. 3). Extended incubation on malt agar at 37°C can be used to induce sporulation in such isolates.

DNA sequencing can be particularly helpful in the identification of nonsporulating isolates or isolates with atypical morphology. The internal transcribed spacer regions (ITS1 and ITS2) of rRNA have been found to be more discriminatory than the 28S ribosomal subunit (D1-D2 region) for identification of 13 clinically important *Aspergillus* species (83). PCR-based assays utilizing these sequences have also been developed (83).

Typing Systems

Historically, genetic fingerprinting and epidemiological studies have been hampered by the lack of simple, reproducible typing methods. Better knowledge of the genetics of aspergilli and the advent of more user-friendly methods to prepare and manipulate DNA now allow for detailed analyses. Molecular techniques are useful in detection and identification systems and for epidemiological studies, although most techniques have been applied to type *A. fumigatus* strains only. Several genotypic methods can be used to genotype aspergilli including random amplified polymorphic DNA (RAPD) analysis, restriction fragment length polymorphism (RFLP) analysis, repetitive element and/or complex probes with Southern blotting (248), amplified fragment length polymorphism (AFLP) analysis, and microsatellite length polymorphism. However, a major problem with pattern-based techniques, such as RAPD, RFLP, and AFLP analyses, is the low level of concordance between laboratories (17). These drawbacks might be overcome with short tandem repeats, as was recently shown for typing of *A. fumigatus* (51a). Genetic fingerprinting of *A. fumigatus* by RFLP showed an enormous genetic variability among both clinical and environmental strains, indicating that any *A. fumigatus* strain can cause disease in humans (45, 67). Furthermore, the genetic diversity significantly reduces the potential of genotyping of *A. fumigatus* for the purpose of infection control since a considerable number of environmental isolates would have to be genotyped in order to match the patient strain with an environmental strain. For *A. fumigatus,* genotyping might be useful during clusters of infection in order to establish the presence of a common source. Nevertheless, some studies have shown genetic relatedness between environmental strains and patient strains, suggesting that aspergillosis had a nosocomial origin in some cases (45, 48, 66, 136, 137). Similar results have been obtained with RAPD typing patterns (11, 23, 127) and Southern blots probed with rRNA (249) or nonribosomal genomic sequences. Invasive aspergillosis has been linked to a nosocomial origin for *A. fumigatus* (67), *A. flavus* (136), and *A. terreus* (132). By using genotyping, specific niches within the hospital environment could be found, as was the case with *A. terreus,* where genotypes of strains recovered from potted plants in the direct environment of infected patients were identical to patient strains (132).

Serologic Tests

The detection of antibody in serum or other body fluids and reactivity in skin tests is, to some degree, dependent upon the purity, chemical nature, and uniqueness of the antigen preparation used (126). Extracts from the whole mycelium, metabolic products secreted into the medium during the growth of *Aspergillus* spp., purified antigens, and, more recently, recombinantly expressed and purified antigens have all been used in serologic tests and immunoassays. Although such antigen preparations are available commercially, standardized antigens are still not widely available. As reviewed by Kurup and Kumar (126), two of the major reasons for the lack of standardized antigens are that (i) antigen preparations are highly variable and (ii) antigens show various degrees of cross-reactivity. Work is ongoing to address these issues and to find ways to express, purify, and prepare improved antigen preparations (see, e.g., references 99, 162, and 277).

Several serologic tests with disparate sensitivities have been developed to detect circulating antibodies. As noted above, these have proven especially valuable in aiding the

diagnosis of allergic aspergillosis and aspergilloma but are less useful in the diagnosis of acute invasive disease in patients with little or no humoral response. However, antibody detection might be useful as a diagnostic tool for specific host groups such as lung transplant recipients (261) and patients with chronic granulomatous disease (225). It has also been suggested that serology could be used as a means of retrospective diagnosis for patients having undergone immunological reconstitution after an episode of neutropenia (85).

Double immunodiffusion and counterimmunoelectrophoresis remain the most widely used techniques, primarily because they are simple and easy to perform (126, 134), but they lack sensitivity and give no quantitative information on antibody concentrations. Other tests which have achieved some success for diagnosis of aspergillosis include ELISA, biotin-avidin-linked immunosorbent assay, radioimmunoassay, and indirect immunofluorescence (232). Of these, various ELISAs are the most amenable to the clinical laboratory. While in some cases these assays can be highly sensitive, reliable, and versatile, their routine use in many clinical laboratories has been limited by interlaboratory discrepancies in results and by immunological responses leading to false-positive results for some patient groups (126). The reasons for this vary but are primarily due to the ubiquitous nature of aspergilli (leading to high background antibody titers), the lack of ability to mount diagnostic humoral immune responses in immunocompromised patients, cross-reactivities between different fungi and even bacteria, and the selection of *Aspergillus* antigens used (117, 126). Using molecular biological techniques, it is now possible to produce *A. fumigatus* proteins in an expression system that yields large quantities of pure recombinant antigen. These antigens can serve as the basis for the development of ELISA methods to quantify the immune response (134). For example, the presence of antibodies directed against the recombinant protein mitogillin proved to correlate well with clinical disease, especially in patients with aspergilloma, with only 1.3% of healthy volunteers showing detectable antibodies (275). Quantitation of the immunoglobulin isotypes will be of importance to optimize the diagnostic value of the assay and to understand further the humoral response in patients with aspergillosis.

Antimicrobial Susceptibilities

The number of antifungal agents that are licensed to treat patients with invasive aspergillosis has increased. The development of lipid formulations of amphotericin B and the triazole itraconazole have broadened the therapeutic arsenal. Until recently the polyenes were considered to be the "gold standard" for treatment of invasive aspergillosis, but clinical trials have shown the triazole voriconazole to have superior efficacy, prompting many treating physicians to use voriconazole as first-line therapy (82, 192). Other antifungal drugs have been registered for treatment of invasive aspergillosis or are undergoing clinical evaluation, including the echinocandins caspofungin, micafungin, and anidulafungin and the triazole posaconazole.

A reference method for in vitro susceptibility testing of conidium-forming molds has been developed by the Clinical Laboratory Standards Institute (CLSI, formally National Committee for Clinical Laboratory Standards [NCCLS]) (172). With this method the polyenes and azoles are active in vitro. For amphotericin B there is, however, no clear correlation with clinical response because there is a narrow distribution of MICs with no clear resistant phenotype (101). The exception to this is *A. terreus,* which may have MICs as high as 16 μg of amphotericin B per ml and is considered to be resistant to the drug (270). Itraconazole, voriconazole, and posaconazole are active in vitro against *Aspergillus* species, with posaconazole typically showing the lowest MICs (199a). There is a good correlation between high MICs and treatment failure at least in experimental models of invasive aspergillosis (49, 50). Several mutations that correspond with amino acid substitutions and phenotypic resistance of *Aspergillus* against azoles have been identified (60, 157). The frequency of resistance against azoles, however, is low, and resistance appears to be found in patients who receive long-term azole prophylaxis or therapy (44, 274). Testing of the activity of echinocandins against *Aspergillus* is not reliable using the CLSI methodology and reading MIC endpoints. Exposure of *Aspergillus* to caspofungin results in morphologic changes that can be detected only by microscopic examination. Therefore, a microscopic endpoint was suggested, the minimal effective concentration, which enables in vitro testing against this class of antifungal agents (124).

Several commercial kits are available for testing the in vitro activity of antifungal drugs against molds including *Aspergillus*. The E test employs strips that contain a gradient of antifungal drug concentration and shows good correlation with the CLSI reference method (58).

Evaluation, Interpretation, and Reporting of Results

In the laboratory, *Aspergillus* isolates should be identified to the species level when they are recovered from patients at risk for aspergillus disease. Particularly important is *A. terreus,*

FIGURE 2 (row 1, left) Section of lung, stained with hematoxylin and eosin, showing dichotomously branched hyphae of *Aspergillus* in invasive aspergillosis.

FIGURE 3 (row 1, right) An atypical isolate of *Aspergillus fumigatus* that produced restricted glabrous, cerebriform colonies without sporulation. After 3 months in culture, it demonstrated growth typical of *A. fumigatus.*

FIGURE 4 (row 2, left) *Emericella (Aspergillus) nidulans* colony on potato dextrose agar.

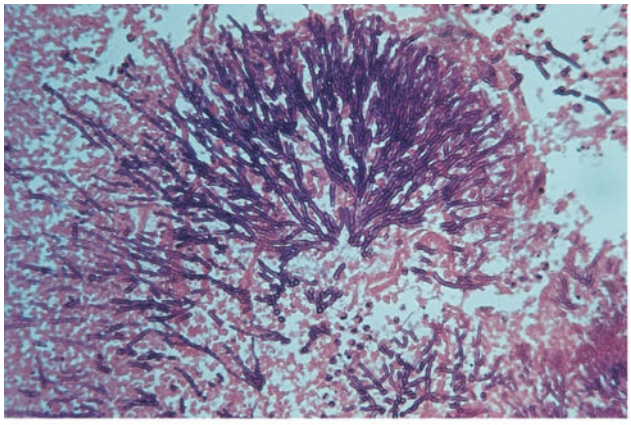
FIGURE 5 (row 2, right) *Emericella (Aspergillus) nidulans* showing *Aspergillus* heads, cleistothecia, and Hülle cells.

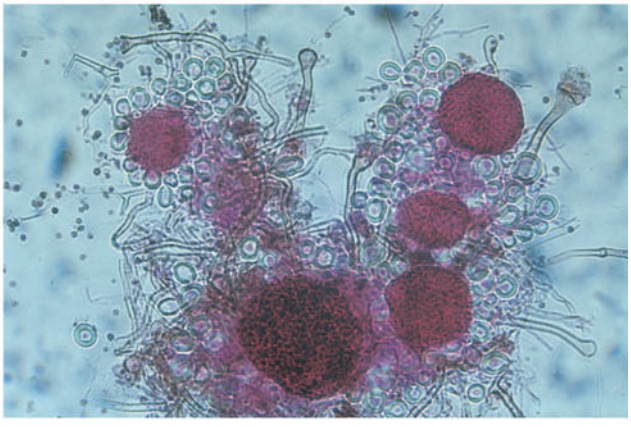
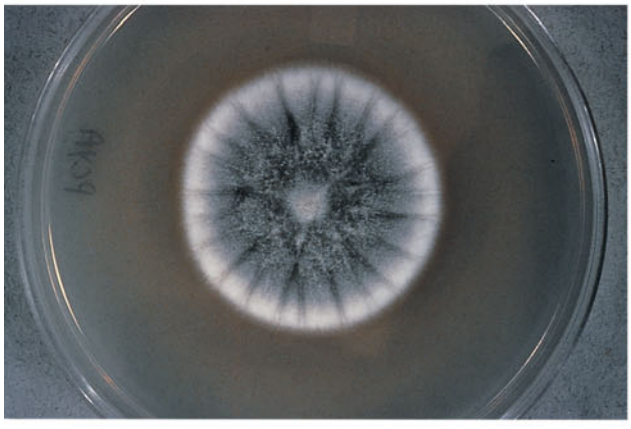
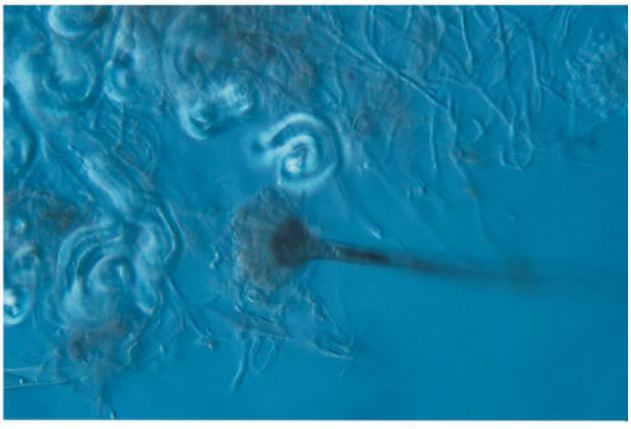
FIGURE 6 (row 3, left) *Aspergillus ustus* colony on malt extract agar.

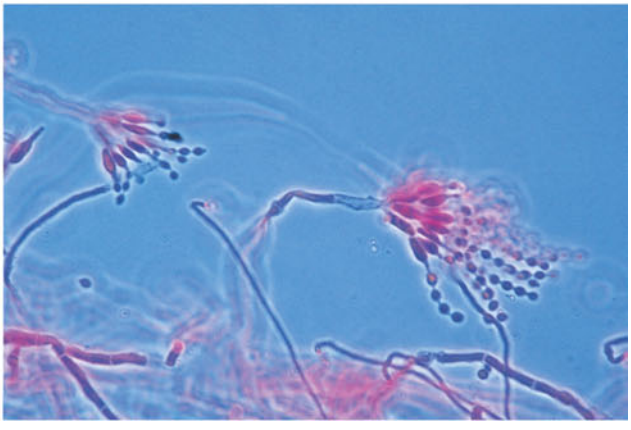
FIGURE 7 (row 3, right) *Aspergillus ustus* showing biseriate head, brown stipe, and Hülle cells.

FIGURE 8 (row 4, left) Colony of *Penicillium marneffei* showing diffusing red pigment on SDA after 7 days. Note that a few *Penicillium* species produce diffusible red pigments.

FIGURE 9 (row 4, right) Compact biverticillate penicillus of *Penicillium marneffei.*





which is resistant to amphotericin B (270). *A. nidulans* and *A. ustus* (96) have also been reported to be less susceptible to amphotericin B than *A. fumigatus* (118).

On the other hand, *Aspergillus* species recovered from nonsterile specimens in immunocompetent patients may not be causative agents of disease and may not require identification to the species level (195). Decisions regarding workup of these samples should be discussed between microbiologist and clinician.

The diagnosis of invasive aspergillosis remains very difficult, and information obtained by various diagnostic tests and procedures such as clinical symptoms and signs, chest radiograph, high-resolution computed tomography, and, if possible, culture and histology needs to be considered. The conventional approach to the management of patients at risk for invasive aspergillosis is to administer antifungal agents empirically to neutropenic patients who are persistently febrile despite treatment with broad-spectrum antibacterial agents or to those who develop pulmonary infiltrates during antibacterial therapy. The development of tests that detect circulating surrogate markers, such as fungal antigens or DNA, and the use of high-resolution computed tomography allow for alternative approaches to be evaluated. The optimal use of detection of circulating markers appears to be in prospective monitoring of high-risk patients during the period of increased risk, since circulating markers are very early indicators of infection in most patients. Once the marker is detected, patients could undergo a diagnostic workup that includes high-resolution computed tomography of the chest in order to confirm the presence of the infection. The probability of infection is very high for this group, and early antifungal treatment can then be administered selectively. The above strategy proved to be superior to the conventional approach in a decision analysis (236), and a feasibility study indicated that the preemptive treatment strategy might be an alternative to empiric therapy (146).

It should be appreciated that no MIC breakpoints are available for any molds with any antifungal drug. This prevents a straightforward correlation between MIC value and clinical outcome (214). The interpretation of MIC testing of molds remains problematic (215), with the most potential value from testing of *A. fumigatus* isolates against azoles. In general, the most useful information regarding antifungal susceptibility can be obtained from identifying the fungal agent to the species level.

FUSARIUM SPECIES

Taxonomy

Fusarium species are cosmopolitan soil saprobes and facultative plant pathogens that can cause infection or toxicosis in humans and animals (173). Teleomorphs occur in *Gibberella, Haematonectria,* and other genera in the order Hypocreales. Molecular phylogenetic studies have been used to examine species and sections within *Fusarium. F. solani* has been shown to be a complex of at least 55 phylogenetically distinct species which includes medically important isolates grouped among at least 15 different entities not yet completely defined (185, 255). The *F. solani* complex also includes *Acremonium falciforme, Cylindrocarpon lichenicola,* and *Neocosmospora vasinfecta* (47, 185, 256). Several medically important species are included within the 29 species of the *Gibberella fujikuroi* complex: *F. verticillioides, F. proliferatum, F. napiforme, F. nygamai, F. subglutinans,* and *F. sacchari* (176, 255). A synopsis of morphological features defining the *F. solani* complex (Fig. 10), *F. oxysporum,* and *F. verticillioides* (Fig. 11) is presented in Table 4. Relatives of *Fusarium* species, *Cylindrocarpon* species are also cosmopolitan soil- and plant-associated fungi which are recovered occasionally as contaminants from specimens. Rare reports concern keratitis, cutaneous and disseminated infection, and mycetoma (47, 80, 245).

Description of Agent(s)

The most frequent cause of infections in humans is *F. solani,* although other *Fusarium* species have been implicated including *F. oxysporum, F. moniliforme, F. verticillioides, F. proliferatum, F. napiforme, F. nygamai, F. subglutinans,* and *F. sacchari* (176). *F. solani* is a common cause of fungal keratitis (207).

Epidemiology and Transmission

There are numerous toxins and metabolites produced by *Fusarium* species that have been implicated to cause disease in humans, especially associated with the consumption of contaminated food products (see chapter 127). Alimentary toxic aleukia was described in individuals who ate grain contaminated with *F. sporotrichioides* or *F. poae* that produced the trichothecene mycotoxin (100). Besides orally ingested mycotoxins, systemic effects of exposure to inhaled mycotoxins have also been attributed to *Fusarium,* although these effects have been much less characterized (123).

Although invasive infection by *Fusarium* is uncommon, an increased incidence of disseminated infection has been seen in neutropenic patients with hematologic cancer, in recipients of solid-organ transplants, and allogeneic HSCT recipients (127, 154, 173, 209, 226). The incidence of fusariosis among allogeneic HSCT recipients varied between 4.21 to 5.0 cases per 1,000 in human leukocyte antigen (HLA)-matched related transplant recipients and 20.19 cases per 1,000 in HLA-mismatched transplant recipients (177). Among allogeneic HSCT recipients, a trimodal distribution was observed: a first peak before engraftment, a second peak at a median of 62 days after transplantation, and a third peak

FIGURE 10 (row 1, left) Macroconidia of the *Fusarium solani* type stained with lactofuchsin.

FIGURE 11 (row 1, right) Chains of microconidia produced by *Fusarium verticillioides (F. moniliforme)* in slide culture preparation.

FIGURE 12 (row 2, left) Colony of *Paecilomyces variotii* on potato dextrose agar after 7 days.

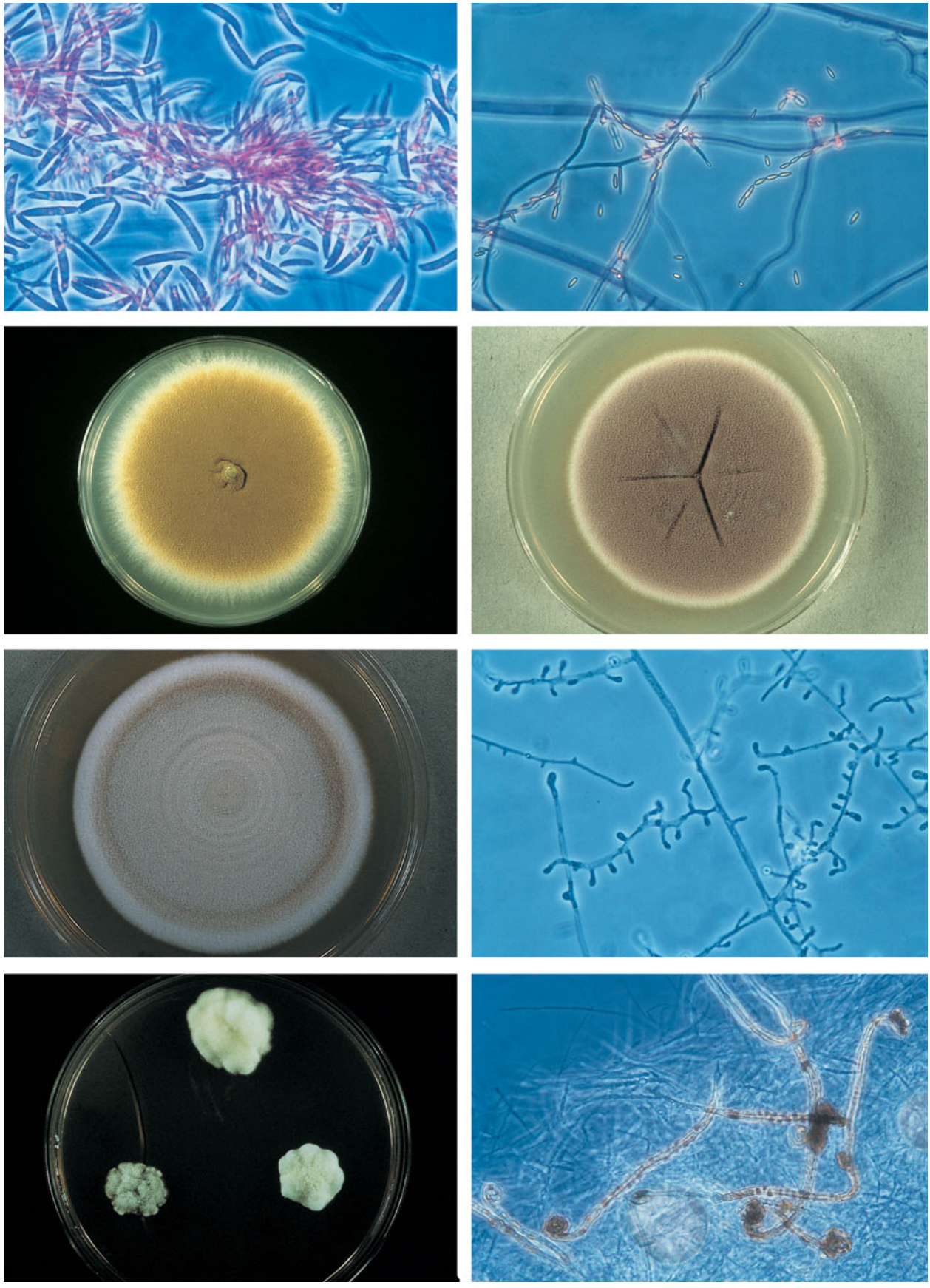
FIGURE 13 (row 2, right) Colony of *Paecilomyces lilacinus* on potato dextrose agar after 14 days.

FIGURE 14 (row 3, left) Colony of *Chrysosporium zonatum* on potato dextrose agar after 14 days at 37°C.

FIGURE 15 (row 3, right) Conidia of *Chrysosporium zonatum* formed on short curved stalks.

FIGURE 16 (row 4, left) Culture of three different isolates of *Onychocola canadensis* after 5 weeks on Mycosel agar.

FIGURE 17 (row 4, right) Setae (appendages) of *Onychocola canadensis.*



>1 year after transplantation. The actuarial survival was 13% (median, 13 days), with persistent neutropenia (hazard ratio of 5.43) and use of corticosteroids (hazard ratio of 2.18) being the most important factors associated with poor outcome (177, 178).

The portal of entry is unknown in most cases of invasive infection, although inhalation, ingestion, and traumatic inoculation have been suggested (21). In some patients disseminated infection may follow onychomycosis, often associated with cellulitis. The hospital water supply system has also been implicated as a source of infection in immunocompromised patients (6).

Clinical Significance

The spectrum of clinical presentations includes keratitis, onychomycosis, colonization of burned or necrotic skin of wounds or ulcers, sinusitis, and infection of the deeper tissues including mycetoma (see chapter 126), endophthalmitis, peritonitis, endocarditis, osteomyelitis, arthritis, and subcutaneous and brain abscess (106, 127, 173, 179). Cutaneous lesions, fungemia, rhinocerebral involvement, pneumonia, or combinations of these are common clinical findings of disseminated *Fusarium* infection (173, 205, 209, 226), and biopsy of skin lesions may be helpful in establishing a diagnosis. In contrast to the low rate of recovery of *Aspergillus* species in blood cultures in cases of invasive aspergillosis, there is a high rate of recovery (60%) from blood in disseminated *Fusarium* infection (127, 209).

Collection, Transport, and Storage of Specimens

Methods of collection, transport, and storage of specimens are detailed in chapter 116. As in invasive aspergillosis, invasive *Fusarium* infections are difficult to diagnose and usually require a combination of clinical, culture, and radiographic findings. However, unlike *Aspergillus, Fusarium* is cultured more frequently from blood, nails, and skin lesions (21, 167). The recovery from a normally sterile site and microscopic evidence of invasive growth in tissue provide the most convincing evidence of invasive fusariosis.

Direct Examination

Microscopy

In direct examination, the hyphae of *Fusarium* species resemble those of *Aspergillus, Paecilomyces,* or *Scedosporium* species in size (3 to 6 μm in width), septation, branching pattern, and predilection for vascular invasion. The hyphae are irregular in width and may show areas of collapse. Hyphae exhibit both dichotomous branching (i.e., branching at 45°), as is commonly seen in invasive aspergillosis, and branching at right angles (140). Microconidia and rarely macroconidia and budding cells, as well as phialides, may be found in blood vessel lumens or in aerated tissues (140). Immunohistologic methods may help to distinguish between infections caused by some hyaline molds, but cross-reactivity is problematic and serological tests are not in common use (110). Definitive diagnosis requires isolation and identification of the fungus, along with demonstration of fungal elements in tissue.

Antigen Detection

There is no commercial system available for detection of genus-specific or species-specific antigens released by *Fusarium* species in human infection. The diagnostic utility of polyclonal fluorescent-antibody reagents to *F. solani* in tissue sections from patients with invasive fusariosis was evaluated, but extensive cross-staining was observed with sections containing aspergilli, *Paecilomyces lilacinus,* and *Pseudallescheria boydii* (110).

There is one report that suggests that exoantigens from *F. oxysporum* cross-react with the Pastorex Aspergillus antigen latex-agglutination kit (107), but no significant cross-reactivity with *F. solani* and *F. oxysporum* was found with the Platelia Aspergillus assay despite the fact that both kits employ the same monoclonal antibody (258). There are no reports that indicate that sera from patients with invasive fusariosis show reactivity with these antigen detection kits. Antigens prepared from *F. verticillioides* were found to cause borderline cross-reactivity with the Platelia Candida antigen assay (BioRad) (218). This reactivity appeared to be specific for *F. verticillioides* since extracts prepared from *F. solani* and *F. oxysporum* showed no cross-reactivity in vitro.

Circulating (1→3)-β-D-glucan can be detected in the blood of patients with *Fusarium* fungemia and pneumonia (182, 189), although the presence of this marker is not specific for *Fusarium*. The sensitivity for detection of *Fusarium* infection was found to be 100% at a cutoff value of 60 pg/ml, among only three patients with invasive fusariosis (189). The clinical experience of (1→3)-β-D-glucan detection in patients with invasive fusariosis is limited, but the test is of value in patients with suspicion of invasive fungal infection as part of the diagnostic workup.

Nucleic Acid Detection Techniques

Given the difficulty in identification to the species level of *Fusarium* isolates by use of microscopic and macroscopic features, and the expertise required, application of molecular tools in this area seems logical. However, within the field of clinical mycology only a very limited number of studies have been published. In one study, a panfungal and species-specific *F. solani* PCR was designed based on the 18S rRNA gene sequence and applied to ocular specimens from three patients with suspected bacterial or fungal endophthalmitis. Although *F. solani* was detected in spiked specimens, no clinical samples from patients with endophthalmitis due to *Fusarium* were analyzed (98). PCR-based identification was applied to *Fusarium* isolates obtained from patients with onychomycosis (175). The *Fusarium* species identified were the same as those known to cause disseminated fusariosis in immunocompromised patients.

Panfungal PCR systems that detect a wide range of fungi including *Fusarium* have been developed (25). In one study the PCR was positive in blood samples from a patient with an invasive fusariosis and blood cultures positive for *F. solani* (25). As with other opportunistic fungi, molecular methods such as PCR appear to be a valuable tool for the detection and identification of *Fusarium* infections; however, the value of these tools in the management of patients remains to be investigated.

Isolation

In general, *Fusarium* species can be recovered easily on routine mycological media. As with *Aspergillus,* no specific growth requirements and no specific biohazard precautions are required in the laboratory.

Identification

Microscopic features of phialide shape, the number of openings on the phialides (i.e., monophialides or polyphialides), the formation of conidia in heads or chains, micro- and macroconidial shape and septation, the presence and

arrangement of chlamydospores, and colonial features including growth rates, color of colony obverse and reverse, and color of conidial masses are important characters for species identification. However, considerable proficiency is required to identify *Fusarium* species with certainty, and a reference laboratory should be consulted. Alternatively, molecular tools are being developed to help to identify clinical *Fusarium* isolates to the species level (175). Although fusaria grow well on most mycological media, the medium can profoundly influence the colonial topography, color, and conidium development. Synthetic nutrient agar, potato dextrose agar, and tap water agar supplemented with either sterilized carnation leaves or potassium chloride are widely employed (173, 174). Use of a rich medium to maintain isolates can result in cultural degeneration.

Typing Systems

Typing systems that have been applied to *Aspergillus* have also been used to differentiate strains of *Fusarium* (3, 69, 78, 231a), although most experience has been obtained in the field of agricultural research rather than in clinical mycology.

Molecular epidemiological investigations of clinical *F. oxysporum* isolates via multifocus sequence typing and AFLP analyses revealed a geographically widespread clonal lineage comprising >70% of genotyped isolates, including those obtained from different hospitals in the United States (186). These studies have identified the hospital water distribution system as a potential source of nosocomial infection (6). However, the most important mode of transmission is airborne, given the observation that the majority of patients with *Fusarium* are infected on or within 3 days of hospital admission, indicating community rather than nosocomial acquisition (208).

Serologic Tests

Conventional serological tests have been developed and used to measure exposure of specific patient populations to *Fusarium,* most commonly in the setting of occupational exposure or indoor dampness problems (97, 129, 223). In these populations IgG directed against *Fusarium* can be detected. Elevated IgE and IgG antibody levels directed against *F. vasinfectum* were found in a patient with allergic bronchopulmonary mycosis due to *F. vasinfectum* (224). Anti-*Fusarium* antibodies have not been evaluated as a diagnostic tool for patients with invasive fusariosis.

Antimicrobial Susceptibilities

In vitro studies show relatively high MICs for amphotericin B and itraconazole of *F. solani* (MIC_{90}, 4 and >8 μg/ml, respectively) and *F. oxysporum* (MIC_{90}, 1.0 and >8 μg/ml, respectively) (58). Higher MICs of these drugs were obtained with the E test, which showed an agreement of less than 80% for these species with the NCCLS M38-A method. Voriconazole and posaconazole show variable in vitro activity against clinical *Fusarium* isolates. In one series voriconazole was active against *F. solani, F. oxysporum, F. proliferatum,* and *F. moniliforme* (MIC_{90}, 2 μg/ml) but was not fungicidal against most isolates (8, 39). However, in another study both azoles showed no activity (52). The correlation between in vitro activity and clinical outcome remains suboptimal in *Fusarium,* given the clinical responses observed in patients with invasive fusariosis treated with voriconazole (196) and the efficacy of posaconazole in experimental fusariosis (144). The echinocandins show no meaningful activity against *Fusarium* in vitro (9, 200).

SCEDOSPORIUM SPECIES

Taxonomy

Based on analysis of partial sequences of the ß-tubulin and calmodulin genes and the ITS of the rRNA gene, it was demonstrated that *Pseudallescheria boydii* is a species complex (65). The three species *P. angusta, P. ellipsoidea,* and *P. fusoidea,* which are morphologically related to *P. boydii* and which were previously considered synonyms, could be differentiated genetically from *P. boydii.* Strains of *P. ellipsoidea* have caused invasive infections. The species *P. minutispora* and *Scedosporium aurantiacum* are phylogenetically separated from the other species and were proposed as new species, which is supported by morphological features. All the strains included in *S. aurantiacum* species have a clinical origin, while those included in *P. minutispora* are environmental (65).

Description of Agents

Scedosporium species are soil-, manure-, and water-inhabiting molds with virulence and clinical spectra remarkably similar to those of *Aspergillus.* The two species known to cause human and animal infection are *Scedosporium apiospermum* (the anamorph of the ascomycete *Pseudallescheria boydii*) and *S. prolificans.* The similarities between species of *Aspergillus* and *Scedosporium* in both clinical syndromes and appearance in tissue have led to their mention in this chapter, even though species of *Scedosporium* are darkly pigmented in culture.

Epidemiology and Transmission

Scedosporium is a ubiquitous saprophytic mold that is found in soil, stagnant and polluted water, sewage, plants, and decaying vegetation. Disease is caused by inhalation of spores leading to pulmonary or sinus disease or by traumatic inoculation through the skin. The fungus may colonize the respiratory tract in patients with cystic fibrosis (38), but its pathogenicity in this patient group has not been well established. *Scedosporium* is known as the causative agent of mycetoma, a chronic subcutaneous infection characterized by the production of grains (see chapter 126). Invasive infections have been increasingly observed in both immunocompetent and immunocompromised patients. The incidence of infection is typically low but may be underestimated. In organ transplant recipients *Scedosporium* accounted for approximately 25% of non-*Aspergillus* mold infections (90). The most common sites of infection are lung, sinus, skin, bone, and joints. Central nervous system involvement was present in 17 to 30% of patients, depending on the underlying disease of the patient (90). Invasive infections were found to disseminate in 46% of transplant patients, with *S. prolificans* being more likely to cause fungemia than *S. apiospermum* (91, 180).

Clinical Significance

In high-risk patients, culture of *Scedosporium* from lower respiratory tract specimens was found to have a higher positive predictive value for invasive mold infection than recovery of non-*Scedosporium* molds (139). The authors suggested treatment with antifungal agents when this mold was cultured from high-risk patients, given the significance of a positive culture and the high mortality associated with invasive disease. The survival rate of patients with invasive scedosporiosis is low, ranging from 30 to 58% in patients with infection due to *S. apiospermum* (90, 196). The survival rate in patients with *S. prolificans* infection is generally lower (19).

Patients colonized with *Scedosporium* often have risk factors that predispose them to fungal infections (e.g., leukemia, lymphoma, chronic obstructive pulmonary disease, systemic

autoimmune disease that is being treated with immunosuppressants, and AIDS). Disseminated infections with this fungus have been reported in several countries, including the United States, Canada, Australia, Germany, France, and The Netherlands. However, the infection has been reported most frequently in Spain (19), especially in the north.

Collection, Transport, and Storage of Specimens

Methods of collection, transport, and storage of specimens are detailed in chapter 116.

Direct Examination

Microscopy

In tissue sections, the hyphae of *Scedosporium* species are hyaline, septate, branched, and hematoxylinophilic and can be reliably distinguished from hyphae of the aspergilli and other hyaline molds only by direct immunofluorescence. Occasionally, conidia which are pale brown and ovoid or cylindrical are formed, generally in fungus balls or in the sinuses; their presence allows presumptive identification. Culture and serologic analysis are still necessary to establish the diagnosis.

Isolation

S. prolificans and *S. apiospermum* grow well in 2 to 4 days at 37°C in culture media that are specific for fungi, as well as in nonselective media (blood agar and chocolate agar). Their characteristic macroscopic appearance facilitates identification.

Identification

The microscopic morphology of a sporulating culture remains the cornerstone of identification of *Scedosporium*. Identification of significant strains to the species level is required given the consequences for antifungal therapy and outcome. Molecular identification using a standardized DNA sequence approach showed this to be accurate and timely when performed on pure cultures (206).

Typing Systems

Several typing systems have been used to type *Scedosporium* isolates including multilocus enzyme electrophoresis (282), RAPD (46), and inter-simple-sequence-repeat fingerprinting, a technique based on the high degree of polymorphism of the multisatellite genetic markers used (247). These studies show that *S. prolificans* is extremely diverse and that several patients were infected or colonized with more than one genotype(46). High polymorphism was also observed for *S. apiospermum,* but clustering according to geographical origin of strains was not found.

Serologic Tests

There are no systems available to detect (circulating) *Scedosporium* antigen or antibody in human infection.

Antimicrobial Susceptibilities

By using the CLSI reference method (172), *S. apiospermum* was found to be resistant to fluconazole and flucytosine, variably susceptible to amphotericin B, itraconazole, and ketoconazole, and susceptible to miconazole. Voriconazole was as active as miconazole in vitro. However, none of these presently available antifungal agents showed meaningful activity against *S. prolificans* (43). The only drug with activity against *S. prolificans* is the investigational azole albaconazole (29). In vitro MICs of voriconazole corresponded with efficacy of the drug in a guinea pig model of invasive scedosporiosis due to *S. apiospermum* (30). Although the allylamine terbinafine has no meaningful in vitro activity against *Scedosporium* sp. (64), when the drug was combined with azoles, synergistic interaction was observed (156). Several cases have been reported with survival of patients treated with a combination of voriconazole and terbinafine (89). In vitro synergistic interaction against both species was also reported for the combination of amphotericin B and micafungin (281), but there is no clinical experience with this combination.

PENICILLIUM MARNEFFEI

Taxonomy

Like its close relative *Aspergillus,* the genus *Penicillium* comprises anamorphic (asexual) species with connections to the ascomycete family Trichocomaceae (228). About 200 species have been placed into four subgenera according to conidiophore branching (53, 204), but molecular analysis has shown three of these subgenera to be polyphyletic (199). The only true pathogen is *Penicillium marneffei,* a member of the subgenus *Biverticillium* (141). The species is unique among the penicillia in being dimorphic, forming in tissue a unicellular yeastlike organelle that reproduces by planate division (division by fission). The thermally regulated growth has typical features of *Penicillium* at room temperature and as yeast cells (arthroconidium) at body temperature.

Epidemiology and Transmission

P. marneffei infection is an important emerging public health problem, especially among patients infected with HIV in the areas of endemicity in Southeast Asia, with cases being reported from Manipur State, India, Myanmar, Malaysia, southern China, Hong Kong, and Taiwan. Most cases, however, occur in Northern Thailand (41). Within these regions, *P. marneffei* infection is regarded as an AIDS-defining illness, and the severity of the disease depends on the immunological status of the infected individual.

The natural history and mode of transmission of the organism remain unclear. Exposure to soil, especially during the rainy season, has been suggested to be a critical risk factor (35, 54, 150). Even though the fungus was discovered in 1956 in the bamboo rat *(Rhizomys sinensis)* in Vietnam, decaying organic material and soil are most likely its natural habitat. Using multilocus microsatellite typing to characterize this fungus, several isolates from bamboo rats and humans were shown to share identical multilocus genotypes. These data suggest either that transmission of *P. marneffei* may occur from rodents to humans or that rodents and humans are coinfected from common environmental sources (262).

Patients are infected by inhalation of *P. marneffei* conidia. Pulmonary alveolar macrophages are the primary host defense mechanism. The fungus proliferates within phagocytic cells resulting in granulomas in immunocompetent individuals. Among AIDS patients the infection typically develops in individuals with $CD4^+$ cell counts of less than 50 cells/ml. The infection rapidly disseminates, and the yeast can be recovered from blood and bone marrow.

Clinical Significance

P. marneffei has been reported to be the fourth most common cause of disseminated opportunistic infection in patients with AIDS in areas of endemicity (35, 54, 268). Infections

are more common in immunosuppressed than immunocompetent hosts and are usually disseminated, with multiple-organ involvement including the lung, liver, and skin. The fungus can usually be isolated from characteristic skin lesions, which are present in 60% of cases, or from blood or bone marrow (54, 150). Presumptive diagnosis may be made by demonstration of the yeastlike cells of *P. marneffei* in Wright-stained smears from skin lesions or in biopsy specimens from these sites (150).

Collection, Transport, and Storage of Specimens

Methods of collection, transport, and storage of specimens are detailed in chapter 116.

Direct Examination

Microscopy

The yeastlike cells are oval or cylindrical and 3 to 6 μm long and may have a cross wall. Yeast cells of *Histoplasma capsulatum* may appear similar, especially when they are within phagocytes, but they may show evidence of budding rather than division by fission. The yeast may be stained by the methenamine silver or periodic-acid Schiff stains in tissue sections.

Isolation

P. marneffei can easily be cultured from clinical specimens. The fungus grows well on SDA. Culture of bone marrow is the most sensitive (100%) followed by culture of specimens obtained from skin biopsy (90%) and blood culture (76%). Handling and culture of specimens possibly containing *P. marneffei* should be performed with care, avoiding inhalation of conidia from sporulating mold cultures or accidental injection into the skin. Biosafety level 2 practices and facilities are recommended for propagating and manipulating cultures known to contain *P. marneffei*. The main drawback of fungal culture is the long turnaround time and the need for invasive procedures to obtain tissue.

Identification

Members of the genus *Penicillium* are the ubiquitous blue-green molds that are among the most common of all laboratory contaminants and that can be isolated easily from respiratory specimens and body surfaces. Isolates of *P. marneffei* have a distinctive morphology (Table 3; Fig. 8 and 9), with conidiophore-bearing biverticillate penicillia and each penicillus being composed of four to five metulae with smooth-walled conidia. A diffusible red pigment produced in culture is one of the first indications that an isolate may be *P. marneffei.* However, red pigment can also be produced by other *Penicillium* species that are not associated with human infection, including *P. citrinum, P. janthinellum, P. purpurogenum,* and *P. rubrum.*

Yeast-like colonies at 37°C are glabrous and beige and do not produce any red pigment.

Typing Systems

Exact typing systems have been developed to characterize genetic diversity in *P. marneffei* including multilocus microsatellite typing (61). Typing of strains showed clustering into two distinct clades corresponding with isolates obtained from patients living in the eastern parts of the *P. marneffei* range and from the western sector including Thailand and India. Potential geographic isolation of populations was also suggested by other investigators (130). Identical multilocus genotypes were found in isolates from bamboo rats and humans, but the interpretation in relation to the pathogenesis of the infection remains unclear (262).

Serologic Tests

Serologic methods have been developed and are proving to be important in the early diagnosis of infection. A number of studies have aimed at detecting fungal antibodies and/or antigens in the serum and body fluids of infected patients. Culture filtrates from *P. marneffei* cultured in liquid media and anti-*P. marneffei* rabbit sera were incorporated in an immunodiffusion test to detect antibody or antigens, respectively. Other assays included an indirect immunofluorescent-antibody test, using the yeast hyphae or the germinating conidia as antigens (110), a latex agglutination test, and an ELISA (51, 109, 150). Antigens may be detected in urine as well as serum. Specific antibodies against a recombinant cell wall-associated protein (Mp1p) were generated to produce an ELISA-based antibody test. Approximately 80% (14 of 17) of documented penicilliosis HIV^+ patients tested positive for the specific antibody. No false-positive results were found for serum samples from 90 healthy blood donors, 20 patients with typhoid fever, and 55 patients with tuberculosis, indicating a high specificity of the test. Thus, this ELISA-based test for the detection of anti-Mp1p antibody can be of significant value as a diagnostic for penicilliosis (27, 28).

Antimicrobial Susceptibilities

Amphotericin B and itraconazole are commonly used for treating patients with penicilliosis, with amphotericin B for seriously ill patients. *P. marneffei* is highly susceptible in vitro to miconazole, itraconazole, and ketoconazole but less so to fluconazole (257). Newer azoles such as voriconazole also exhibit potent activity (183). The echinocandins are active against *P. marneffei,* but in vitro the drugs are more active against the mycelial forms than the yeast-like forms (170, 183).

OTHER ASCOMYCETES

Taxonomy

Scopulariopsis

The genus *Scopulariopsis* contains both lightly colored and dark species (165), some of which have teleomorphs in the genus *Microascus* (Fig. 20) of the Microascaceae (1, 53, 165) or the genus *Kernia*.

Chaetomium

Analysis of the 18S rRNA gene confirmed the placement of *Chaetomium* in the family Chaetomiaceae under the order Sordariales (135).

Description of Agents

Scopulariopsis

Members of the genus *Scopulariopsis* are common soil fungi and agents of deterioration, especially of cellulosic substrates. Of the 30 species known, only a few are reliably reported from human infections.

Chaetomium

The perithecial ascomycetes *Achaetomium* and *Chaetomium* produce brown lemon-shaped to fusiform ascospores within ascomata that are ornamented with hairs (or setae), especially around the upper part near the opening. Anamorphs

are uncommon. The perithecial hairs of *Achaetomium* species are pale and fine and not very conspicuous, while those of *Chaetomium* species are dark, thicker, spinelike, or elaborately coiled or branched.

Gymnascella

Gymnascella species are onygenalean fungi that produce clusters of ascospores surrounded by yellow or orange filaments, but differentiated ascomata are not formed.

Clinical Significance

Scopulariopsis

Species causing onychomycosis include *S. brevicaulis* (Fig. 29), by far the most important as both pathogen and regular contaminant; *S. candida,* the next most common; and *M. cirrosus, M. cinereus,* and *S. brumptii* (106, 203, 239). Reports of onychomycosis associated with other species have not been validated; *S. koningii* is a synonym of *S. brevicaulis* (1). Like species of *Fusarium* and *Paecilomyces variotii, S. brevicaulis* may colonize necrotic tissue. *Scopulariopsis* species are rarely invasive, and *S. brevicaulis* and *Microascus cinereus* are the most common species reported. Infections include otomycosis, keratitis, prosthetic valve endocarditis, sinusitis, pneumonia, brain abscess, and (sub)cutaneous and bone invasion in immunocompetent and immunosuppressed individuals (13, 47, 122, 149, 161, 201, 231). *S. candida* and *S. acremonium* have been reported from invasive sinusitis, but few details concerning the latter species were provided in the report (57, 121). Brain abscess caused by *S. brumptii* and invasive cutaneous infection caused by *M. cirrosus* have been reported to occur in liver transplant and bone marrow recipients, respectively (122, 190).

Chaetomium

Achaetomium strumarium (reported as *Chaetomium strumarium*) (Fig. 21) and *Chaetomium atrobrunneum* may cause cerebral infection (2), but some reports concerning other *Chaetomium* species in deep infection have not been validated (239, 245). *C. globosum* (see chapter 120) occurs most commonly as a contaminant or as a rare agent of onychomycosis (2, 106, 239).

Gymnascella

Rare reports describe *Gymnascella damkaliensis* as a cause of onychomycosis (106) and *G. hyalinospora* as the cause of invasive pulmonary infection in a leukemic patient (95) (Fig. 22, 23, and 30).

Direct Examination

Microscopy

Histopathologic findings show hyphal morphology indistinguishable from that of species of *Aspergillus, Fusarium,* and *Pseudallescheria* (201).

Isolation

These species grow well on all laboratory media. *Scopulariopsis* species are strongly or moderately resistant to cycloheximide.

Identification

Darkly pigmented *Scopulariopsis* species are difficult to identify. They should be grown on a sporulation medium to promote the development of perithecia (sexual structures) of *Microascus* species. Some species form synnemata (a group of conidiophores united at the base and part of the way up their length) in culture and are classified in the closely related genus *Cephalotrichum* (also known as *Doratomyces*), but none of these has been demonstrated to be a pathogen. The genus *Ascosubramania* was described for a mold with features suggestive of a *Microascus* (74).

The *Chaetomium* species pathogenic to humans can be distinguished by the appearance of the perithecia, setae (undulate hairs), and the mature ascospores (16). Those that have been reported to cause human disease grow well at 35 to 37°C, and those with a predilection for the central nervous system often grow at up to 42 to 45°C.

Gymnascella hyalinum can be identified based on the formation of ascospores within tufts of aerial hyphae on sporulation medium (95). A case isolate and the reference isolate of this species both displayed false-positive results in the Gen-Probe test for *Blastomyces dermatitidis* (95).

Antimicrobial Susceptibilities

Scopulariopsis

Antifungals including amphotericin B and itraconazole have limited in vitro activity against *Scopulariopsis* species, and conflicting results have been reported for voriconazole and terbinafine (64, 250). In vitro, promising interaction was observed between terbinafine and fluconazole, itraconazole,

FIGURE 18 (row 1, left) Colony of *Trichoderma longibrachiatum* on potato dextrose agar after 4 days at 37°C. Note that the plate has been inoculated on one side.

FIGURE 19 (row 1, right) Microscopic appearance of *Trichoderma longibrachiatum* in tape mount preparation.

FIGURE 20 (row 2, left) Perithecium of *Microascus* species.

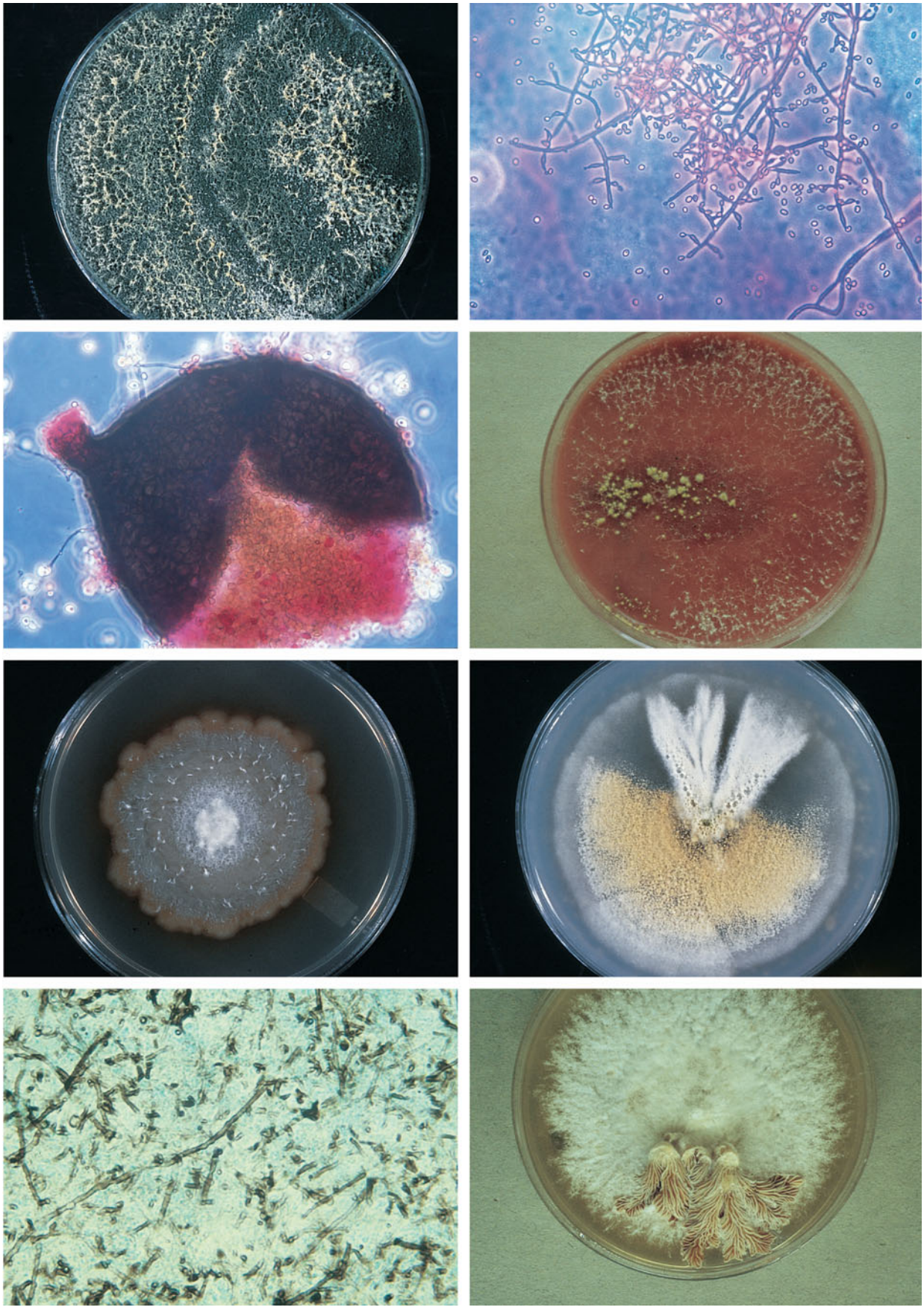
FIGURE 21 (row 2, right) Colony of *Achaetomium strumarium* showing yellowish surface mycelium and diffusing pink pigment on cornmeal agar after 5 weeks.

FIGURE 22 (row 3, left) An isolate of *Gymnascella hyalinospora* described in reference 72 was glabrous and sterile when grown on potato dextrose agar for 15 days at 30°C.

FIGURE 23 (row 3, right) The same isolate of *Gymnascella hyalinospora* as in Fig. 22 turned yellow on oatmeal agar with development of ascospores after 15 days at 30°C.

FIGURE 24 (row 4, left) Tissue section stained with Gomori methenamine silver stain showing monokaryotic (clampless) hyphae of *Schizophyllum commune* in pulmonary fungus ball.

FIGURE 25 (row 4, right) Dikaryotic culture of *Schizophyllum commune* showing development of gilled fruiting bodies on potato dextrose agar after 7 weeks in the light.



and voriconazole against isolates of *S. brevicaulis,* although clinical experience with combination therapy is very limited (222).

Chaetomium

Treatment failures of *Chaetomium* infections have been reported when patients were treated with amphotericin B alone or in combination with itraconazole. Voriconazole and the experimental triazoles ravuconazole and albaconazole showed potent activity in vitro with MICs of less than 0.5 μg/ml (235). The echinocandin micafungin was not active in vitro.

SCHIZOPHYLLUM COMMUNE AND OTHER BASIDIOMYCETES

Allergic reactions due to inhalation of mushroom spores (87) and mycetismus due to the ingestion of mushrooms have been frequently reported, but the incidence of human infections by members of the Basidiomycota is rare. *Schizophyllum commune* is recognized as a significant cause of allergy-related sinusitis and pulmonary disease including allergic bronchopulmonary mycosis and bronchial mucoid impaction, a type of hypersensitivity reaction to *S. commune* hyphae (5, 26, 40, 103, 240, 242, 244). *S. commune* has also caused infection of the brain, lungs, and buccal mucosa in both immunocompetent and immunosuppressed patients (103, 217, 220, 239, 240).

Filamentous basidiomycetes, as well as species of smuts which may appear yeastlike (Ustilaginaceae and Tilletiaceae), are commonly isolated from respiratory specimens and sometimes from blood, and their significance can be difficult to evaluate (240). Pathogenicity of members of the genus *Coprinus* (taxonomy currently under revision) has rarely been confirmed by histopathologic evidence of hyphal filaments. *C. cinereus* or its anamorph *Hormographiella aspergillata* have been reported from prosthetic valve endocarditis, fatal lung infections in leukemic patients, and cutaneous lesions (51, 128, 239, 266). Repeated isolation and the presence of filaments in sputum have implicated the thermotolerant *Sporotrichum pruinosum,* the anamorph of *Phanerochaete chrysosporium* (111), a species being investigated for its utility in biodegradation. An isolate reported as *Tilletiopsis minor* from an immunosuppressed patient appears to be *Lecythophora lignicola* (see the discussion of *Acremonium* in "Other Moniliaceous Molds" below) (210, 239).

Direct Examination

Microscopy

Direct microscopy shows septate filamentous organisms, which can be of varying widths. *Schizophyllum commune* in tissue can display histopathologic features suggestive of *Aspergillus*. Septate hyphae with clamp connections can also be demonstrated with some basidiomycete isolates (217).

Antigen Detection

Antibody and antigen tests and *Aspergillus*-specific fluorescent-antibody staining have been recommended as aids in the differential diagnosis, but they are not widely available (217).

Isolation

Filamentous basidiomycetes grow well on routine culture media. The use of the fungicide benomyl at a final concentration of 10 μg/ml in culture media can be useful to distinguish filamentous basidiomycetes, which are tolerant to benomyl, from isolates of ascomycetous origin, which may be sensitive to benomyl (254).

Identification

Hyphae in tissue are narrow (3 to 6 μm wide) or broader (up to 10 μm) and somewhat irregular. The hyphae in tissue or culture may be recognized as belonging to a basidiomycete if clamp connections are present at the septa; if characteristic pegs or spicules are present, the hyphae are typical of *S. commune* (Fig. 25 and 31). However, these structures may be absent, and the hyphae then resemble those of *Aspergillus* species or other molds (5, 240, 244) (Fig. 24). A filamentous basidiomycete can be suspected when a nonsporulating hyaline mold displays growth on benomyl agar, growth at 37°C, and sensitivity to cycloheximide (240). Identification of atypical isolates can be difficult and requires specialized techniques. Vegetative compatibility tests and sequencing of the ITS region of rRNA have been used to confirm the identification of isolates (148, 242, 244).

Antimicrobial Susceptibilities

The largest study to date evaluating the activity of antifungal agents included 44 clinical isolates of filamentous basidiomycetous fungi, including *Schizophyllum commune* ($n = 5$), *Coprinus* species ($n = 8$), *Bjerkandera adusta* ($n = 14$), and sterile, uncharacterized basidiomycetes ($n = 17$). Amphotericin B, itraconazole, voriconazole, and posaconazole showed low MICs, in contrast to fluconazole and flucytosine (70). No statistically significant differences among the genera were noted.

OTHER MONILIACEOUS MOLDS

Features of some opportunistic moniliaceous fungi are described in Table 3.

FIGURE 26 (top left) Uniseriate conidial heads of a *Neosartorya* species. Magnification, ×610.

FIGURE 27 (top right) Biseriate conidial head of *Aspergillus versicolor.* Magnification, ×770.

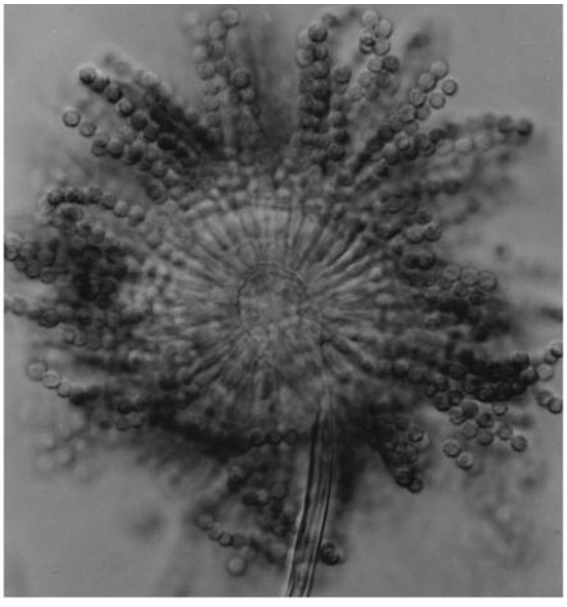
FIGURE 28 (middle left) Verticillate conidiophore of *Paecilomyces lilacinus* bearing whorls of phialides. Magnification, ×750.

FIGURE 29 (middle right) Rough-walled conidia in chains formed on annellides in *Scopulariopsis brevicaulis.* Note the branched conidiogenous apparatus. Magnification, ×580.

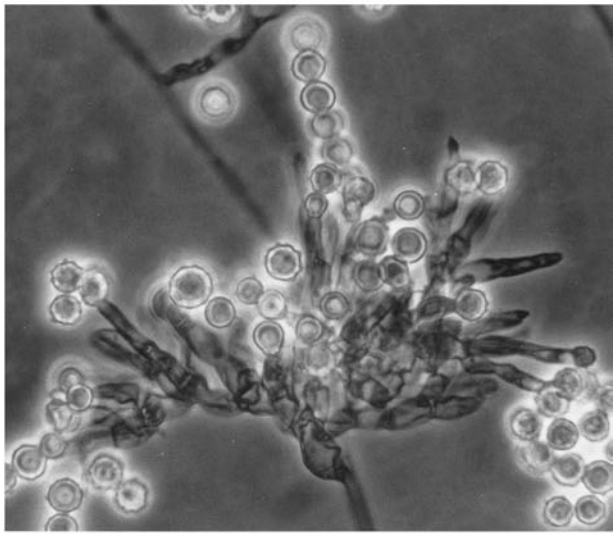
FIGURE 30 (bottom left) Ascospores of *Gymnascella hyalinospora* observed by scanning electron microscopy. Magnification, ×6,000.

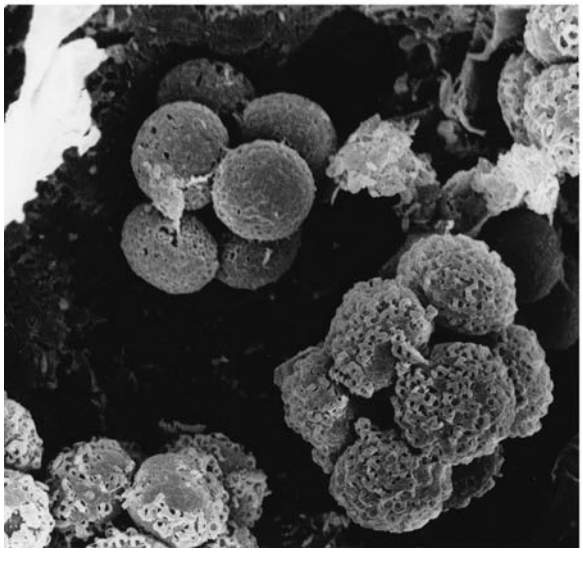
FIGURE 31 (bottom right) *Schizophyllum commune* in slide culture preparation showing clamp connections and narrow pegs or spicules (arrows). Magnification, ×580.

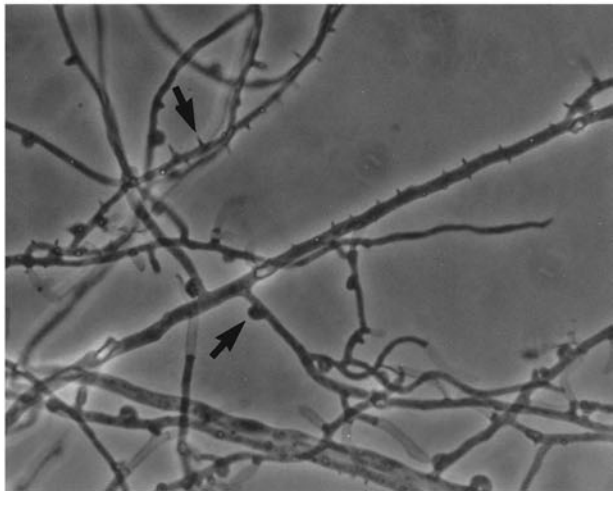












Ascomycetes: *Acremonium, Lecythophora, Phialemonium,* and *Phaeoacremonium* Species

Fungi belonging to the genera *Acremonium, Lecythophora, Phialemonium,* and *Phaeoacremonium* form single-celled conidia in slimy masses from slender phialides. The genus *Acremonium,* formerly called *Cephalosporium,* includes approximately 100 species associated with soil, insects, sewage, rhizospheres of plants, and other environmental substrates. Teleomorphs, where known, are in the genera *Nectria, Emericellopsis,* and *Thielavia,* which are placed in different ascomycete orders. Teleomorph connections and molecular data provide evidence for some restructuring of the genus along phylogenetic lines (47, 68, 73). Many reports concerning *Acremonium* species involve nail, skin, or eye infections or mycetoma (see also chapter 121) (47, 106, 127, 219). Localized and disseminated infections occur in patients following valve replacement, dialysis, or transplantation or in patients with hematologic or solid-organ malignancies (47, 73, 127, 230, 245, 255, 272). Fungemia is common. In tissue, hyphae of *Acremonium* species resemble those of *Fusarium* in branching pattern, vascular invasion, and propensity to sporulate; budding cells may rarely be seen (140, 230, 272). Recognition of *Acremonium* species in culture is not too difficult, but identification to the species level is very difficult, and many reports of infection are based on unidentified species (73, 255). Species of *Acremonium* grow well on SDA, and some species can tolerate cycloheximide. *A. kiliense,* the most common medically important species, produces cylindrical conidia and solitary hyaline chlamydospores, especially on oatmeal agar. *A. recifei* also produces chlamydospores, but its conidia are sickle shaped or slightly curved. *A. strictum,* a common soil and occasional airborne fungus, forms moist and pale-salmon-colored colonies that resemble those of *Lecythophora hoffmannii;* this fungus can be isolated from blood and can sporulate in tissue (230, 272). The genus *Phaeoacremonium,* including *P. parasiticum* and some other species associated with human infection, is distinguished from *Acremonium* by its brownish pigmented hyphae and conidiophores (see chapter 125). The genera *Lecythophora* and *Phialemonium* differ from *Acremonium* by their formation of short, stumpy phialides without basal septa (called adelophialides) in addition to the more spindle-shaped phialides (63), but these distinctions are not always readily observed. Although *L. hoffmannii* has been implicated as a cause of infection, reports may refer to *Phaeoacremonium* species. An isolate from sinus (153) was examined and determined to be *P. parasiticum* (245), and the pale brown phialides described for another isolate are more suggestive of a *Phaeoacremonium* species (219, 239). *L. mutabilis* differs from *L. hoffmannii* in forming accessory brown chlamydospores on sporulation media. Cases of endocarditis, eye infections, and peritonitis have been reported (47, 148). An isolate reported under the name *Tilletiopsis minor* (210) as causing a subcutaneous cyst may be *L. lignicola* (239). Three species of *Phialemonium* were distinguished originally by conidial shape and colony color (63), but the PCR-RFLP banding patterns showed a close relationship between isolates of *P. curvatum* and *P. dimorphosporum,* and these two species were synonymized (75). *Phialemonium* species have caused fungemia, subcutaneous and disseminated infection, peritonitis, and mycetoma.

Arthrographis, Onychocola, and *Scytalidium* Species

Fungi forming arthroconidia are placed in the genera *Arthrographis, Onychocola,* and *Scytalidium.* The thermotolerant *Arthrographis kalrae* is a rare opportunist recovered from skin, lung, corneal ulcer, and sinus (36, 197, 239, 243). Because initial growth is often yeastlike, an isolate may not be recognized as a pleomorphic mold and may be subjected to tests commonly used for yeast identification (36, 239). *Scytalidium dimidiatum* and its relative *S. hyalinum* are best known as agents of superficial infection (106), causing mainly onychomycosis of the toenails and rarely deep infection (see chapter 120). *Onychocola canadensis* is a cycloheximide-tolerant hyphomycete causing distal subungual onychomycosis or, less commonly, white superficial onychomycosis and infection of the glabrous skin (76, 106, 238, 241) (Fig. 16 and 17). Although *O. canadensis* is an uncommon cause of onychomyosis, more than 60 isolates from nails have now been recorded from New Zealand, Australia, Europe, the United Kingdom, and the United States.

Beauveria, Engyodontium, and *Sporothrix* Species

Beauveria, Engyodontium, and *Sporothrix* species have solitary conidia borne sympodially. *Beauveria bassiana* is a well-known insect pathogen with limited virulence for humans. *B. bassiana* has not been substantiated as the cause of invasive disease in humans (245) but has caused pulmonary infection in a captive alligator. *Engyodontium album* is closely related (47) and was formerly placed in the genus *Beauveria.* These species are uncommonly reported as the cause of keratitis (47, 113, 155) and endocarditis (12). *Sporothrix schenckii* is a well-known dimorphic pathogen described in chapter 125. It forms sessile pigmented conidia in addition to hyaline conidia.

Chrysosporium, Myceliophthora, and *Myriodontium* Species

Members of the genera *Chrysosporium, Myriodontium,* and *Myceliophthora* produce solitary, usually single-celled conidia which are called aleurioconidia because of their lytic method of conidium dehiscence (106). Members of these genera are related to the dermatophytes and the dimorphic pathogens, sharing with them a tolerance of cycloheximide and having teleomorphs in the ascomycete order Onygenales (106, 238).

There are few reports of infection involving *Chrysosporium* species. The thermotolerant *C. zonatum* has caused pneumonia and osteomyelitis in a patient with chronic granulomatous disease as well as noninvasive pulmonary disease in two patients (221) (Fig. 14 and 15). In other reports, the isolated organism was not identified to the species level or described well enough to confirm the etiology (reviewed in references 221, 238, and 245). *Myriodontium keratinophilum* and *Chrysosporium* species have been reported to cause sinusitis, but it seems likely that the fungi involved were misidentified *Schizophyllum commune,* an emerging agent of sinusitis (238, 245). The hyphal spicules of the latter could be confused with the stalks of the former (106). Cutaneous mycosis caused by *Aphanoascus fulvescens,* an ascomycete with a *Chrysosporium* anamorph, has been reliably documented (106, 127). Species of *Chrysosporium* and the similar *Geomyces,* as well as *Myriodontium keratinophilum,* are occasional contaminants of respiratory and cutaneous specimens (106). Members of the genus *Myceliophthora* produce aleurioconidia similar to those of onygenalean fungi, but they have affinities within the order Sordariales. The thermophilic species *M. thermophila* has been reported to cause fatal aortic vasculitis in two patients and was isolated from the brain of a patient who developed a bacterial cerebral abscess after trauma (20, 59, 260).

Metarrhizium and *Trichoderma* Species

Metarrhizium anisopliae is an insect pathogen of wide distribution that was recently documented to cause keratitis, sinusitis, and invasive infection (24, 32, 213). Conidia of *Metarrhizium* are produced from cylindrical phialides borne on verticillately

or irregularly branched conidiophores formed in sporodochia. Conidia are produced in chains that adhere together, in contrast to *Trichoderma* species, which form conidia in slimy heads. *Trichoderma* species are common soil fungi traditionally considered uncommon clinical contaminants. Many species are known, and they are difficult to identify; teleomorphs are in the genus *Hypocrea* (Hypocreales) (112, 229). Case reports attributed to species less well adapted to growth at higher temperatures, such as *T. koningii* and *T. viride,* closely related species which do not grow well above 30°C, require reevaluation (47, 112, 229, 230). *T. longibrachiatum,* which does grow well at 37°C, is probably the most important pathogenic species (166, 216, 234). Morphological criteria for recognition of this species include (i) fast-growing yellowish green colonies with a radius greater than 35 mm at 40°C after 3 days of growth on potato dextrose agar; (ii) a strong yellow diffusing pigment present at 30°C but absent at 40°C; (iii) hyphae sparingly branched and forming phialides that are mostly solitary, longer, and more gradually tapered (cylindrical); and (iv) smooth oblong to ellipsoidal conidia (229) (Fig. 18 and 19). Intercalary phialides and chlamydospores are common (229). Some reports concerning *T. pseudokoningii* may actually refer to *T. citrinoviride,* a thermotolerant species closely related to *T. longibrachiatum* (47).

Paecilomyces Species

Species of *Paecilomyces* occur worldwide as soil saprophytes, insect parasites, and agents of biodeterioration. The two most important medically significant species are *Paecilomyces lilacinus* and *P. variotii.* Their teleomorphs are unknown, but their affinity is within the ascomycete family Trichocomaceae, which also includes *Penicillium* and *Aspergillus* species; some other species have teleomorphs in the genus *Thermoascus* (Thermoascaceae). The colonial and morphologic features of these two species (Fig. 12, 13, and 28) are described in Table 5. *P. marquandii* is included for comparison with *P. lilacinus,* but this and other rarely reported species are not confirmed as agents of infection. Clinical manifestations of both species include cutaneous and subcutaneous infection, pulmonary infection, pyelonephritis, sinusitis, cellulitis, endocarditis, and fungemia in both immunocompetent and immunosuppressed patients (31, 34, 47, 245, 255, 276). Surgery, prosthetic implants, and use of contaminated solutions or lotions are risk factors predisposing to infection since the conidia of both species are relatively resistant to most sterilizing techniques (31, 53, 188), and death may occur in immunosuppressed individuals (93). Most reports of eye infection including keratitis, endophthalmitis, corneal ulcer, and orbital granuloma refer to *P. lilacinus,* but *P. variotii* may also be involved (47, 127, 233). Predisposing factors include use of extended-wear contact lenses, implantation of contaminated intraocular lenses, complications following cataract surgery, and use of topical corticosteroids before onset of clinically apparent keratitis (127, 233). Peritonitis cases caused by *Paecilomyces* species and the homothallic *Thermoascus taitungiacus* have recently been reviewed (119). Fungal elements of *Paecilomyces* in tissue are hyaline, septate, and about 2 to 4 μm wide and have parallel walls or show swellings.

Penicillium Species

Penicillium species other than *P. marneffei* have been implicated in human infection, but the validity of some reports has been questioned (255). Although *Penicillium* species grow over a wide range of temperatures from 5 to 45°C, many species are completely or strongly inhibited at 37°C and thus have low potential for causing human infection (e.g., *P. brevicompactum* and *P. commune*). Even repeated isolation of a *Penicillium* species from patient specimens does not necessarily indicate an etiologic role, unless the isolate is accompanied by typical fungal elements in tissue specimens or smears of lesional exudate. Other factors to consider when assessing the significance of repeated isolation include the isolation of any other fungal species, the patient's possible exposure to airborne conidia, and a diagnosis of bronchiectasis, since conidia may remain viable for prolonged periods without invasion or colonization (127). Nonetheless, several species have been reported to cause peritonitis, urinary tract infection, endocarditis, lung infection, fungemia, and disseminated infection (37, 47, 127, 143). The role of penicillia in allergy and hypersensitivity pneumonitis is well established (87). Identification of penicillia is a complex task, requiring growth of an isolate on several media at different temperatures and careful microscopic examination (53, 204, 227). Czapek yeast extract agar, malt extract agar, and 25% glycerol nitrate agar and incubation at 5, 25, and 37°C are used. The use of SDA alone is not recommended.

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Rhizopus, Rhizomucor, Absidia, and Other Agents of Systemic and Subcutaneous Zygomycoses*

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TAXONOMY

The term zygomycosis embraces both deep and subcutaneous mycoses caused by fungi belonging to various genera of the class Zygomycetes (Table 1), namely, mucormycosis and the entomophthoromycoses, the latter being subdivided into basidiobolomycosis and conidiobolomycosis.

MUCORMYCOSIS

The term mucormycosis is used to refer to uncommon infections due to molds belonging to the order Mucorales. These organisms can cause rhinocerebral, pulmonary, gastrointestinal, cutaneous, or disseminated infection in predisposed individuals, the different clinical forms often being associated with particular underlying conditions.

Fungi belonging to the order Mucorales are distributed into six families (Table 1), all of which can cause cutaneous and deep infections. Species belonging to the family Mucoraceae are isolated more frequently from patients with mucormycosis than those belonging to any other family. Among the Mucoraceae, *Rhizopus oryzae (Rhizopus arrhizus)* is by far the most common cause of infection. Other, less frequently isolated species of the Mucoraceae family that cause a similar spectrum of infections include *Rhizopus microsporus* var. *rhizopodiformis*, *Absidia corymbifera, Apophysomyces elegans, Mucor* species, and *Rhizomucor pusillus*. Increasingly, cases of mucormycosis due to *Cunninghamella* species (in the Cunninghamellaceae family) are being diagnosed.

ENTOMOPHTHOROMYCOSES (SUBCUTANEOUS PHYCOMYCOSIS, SUBACUTE PHYCOMYCOSIS, AND SUBACUTE ZYGOMYCOSIS)

Infection with fungi from the order Entomophthorales (Table 1), namely, *Conidiobolus coronatus, Conidiobolus incongruous,* and *Basidiobolus ranarum*, typically occurs in immunocompetent patients residing in subtropical and tropical climates. Unlike Mucorales, Entomophthorales do not cause angioinvasive disease.

Conidiobolomycosis is a chronic mycosis affecting the subcutaneous tissues. It originates in the nasal sinuses and spreads to the adjacent subcutaneous tissue of the face, causing disfigurement. Basidiobolomycosis is a chronic subcutaneous infection of the trunk and limbs.

The current taxonomy and clinical division of the Entomophthorales has been challenged recently (38). Molecular analyses during the last two decades have shown that orders within the Chytridiomycota and Zygomycota cluster among one another and therefore cannot be considered to be natural taxa (86). Members of the Entomophthorales have been found to fall within two phylogenetically distinct groups, one of which contains *Basidiobolus*, which is related closely to some chytrids, while the remaining entomophthoralean genera, including *Conidiobolus*, form another distinct group that is related closely to other zygomycetes such as the Mucorales (37). A further indication of a close link between *Basidiobolus* and the flagellated Chytridiomycota is the presence of the procentriole, the basal part of a flagellum (52). This evidence has resulted in the creation of the order Basidiobolales, containing the single genus *Basidiobolus*. At present, available data also suggest that the pathogenic agents causing mucormycosis are all members of the Mucorales (Table 1) (95).

The paraphyletic nature of the Entomophthorales, as defined previously, containing both *Basidiobolus* and *Conidiobolus*, offers a partial explanation of the highly different clinical manifestations of basidiobolomycosis and conidiobolomycosis and of the differences in the susceptibilities of the pathogens to antifungal agents. The difficulties with identification of *Conidiobolus* and *Basidiobolus* based on morphological characteristics appear to have been overcome by identification using PCR with taxon-specific primers (94). Based on these developments, one point of view is that the clinical nomenclature should reflect the modern phylogeny and that the term "entomophthoromycosis" should be restricted to mycosis caused by *Conidiobolus* spp. while mycosis caused by *Basidiobolus* spp. should be described as "basidiobolomycosis" (38).

EPIDEMIOLOGY AND TRANSMISSION

The Mucorales are opportunistic fungi capable of causing acute, rapidly developing, and often fulminant infections in the compromised host (4, 35, 68, 72, 73, 83). Major risk factors include ketoacidosis, lymphoma, leukemia, neutropenia,

*This chapter contains information presented in chapter 117 by Malcolm D. Richardson, Pirkko Koukila-Kähkölä, and Gillian S. Shankland in the eighth edition of this Manual.

TABLE 1 Pathogenic Mucorales and Entomophthorales[a]

Order	Family	Genus	Species
Mucorales	Mucoraceae	*Rhizopus*	*R. oryzae*
			R. microsporus
			R. azygosporus
			R. schipperae
		Absidia	*A. corymbifera*
		Mucor	*M. circinelloides*
			M. hiemalis
			M. racemosus
			M. ramosissimus
			M. rouxianus
		Rhizomucor	*R. pusillus*
		Apophysomyces	*A. elegans*
	Cunninghamellaceae	*Cunninghamella*	*C. bertholletiae*
	Saksenaeaceae	*Saksenaea*	*S. vasiformis*
	Syncephalastraceae	*Syncephalastrum*	*S. racemosum*
	Thamnidiaceae	*Cokeromyces*	*C. recurvatus*
	Mortierellaceae	*Mortierella*	
Entomophthorales		*Conidiobolus*	
		Basidiobolus	

[a] Table kindly provided by Eric Dannaoui.

corticosteroid or other long-term immunosuppressive therapies, and deferoxamine therapy of dialysis patients with aluminum or iron overload. To date, there have been only a few reported infections associated with AIDS, so AIDS does not appear to be a significant risk factor. Trauma to the skin including severe burns, trauma from intravenous catheters and intravenous drug abuse, and even an insect bite may also result in infection in the immunocompetent host.

The pathogenesis of mucormycosis is unclear, and although undoubtedly exogenous, possible sources of infection have only occasionally been suggested, e.g., adhesive dressings, air conditioning filter units, and food. Cutaneous trauma may be an underestimated event in the initiation of many cases of mucormycosis. However, most infections follow inhalation of spores that have been released into the air, and the lungs and nasal sinuses are common sites of infection. The most common predisposing condition for gastrointestinal infection is severe malnutrition or disruption of the gastrointestinal mucosa.

The nature of the infectious propagule responsible for the establishment of zygomycoses is unproven. Many studies have suggested that sporangiospores are responsible, and others have indicated that small hyphal fragments initiate disease. Since sporangiospores of mucoraceous fungi are both uncommon in air and readily contained by phagocytic cells normally found in pulmonary tissues, it is possible that in some cases, especially where trauma is the associated factor, hyphal fragments are the infectious propagule.

Mucormycosis

Mucormycosis is distributed worldwide. Many different organisms have been implicated, but the most common causes of human infection, listed in order of apparent incidence, are *Rhizopus oryzae* and *Rhizopus microsporus* var. *rhizopodiformis*. Other, less frequent etiological agents for which a major pathogenic role in humans has been established include *Absidia corymbifera, Apophysomyces elegans, Cunninghamella bertholletiae, Mucor* species, *Rhizomucor pusillus,* and *Saksenaea vasiformis*. These molds are ubiquitous and thermotolerant and can be isolated in large numbers from soil or decomposing organic matter, such as fruit and bread. Their spores can be found in the outside air. Nosocomial outbreaks of mucormycosis are not as common as hospital-related *Aspergillus* infections but have been reported to occur in leukemic patients. Nosocomial cutaneous infections with *Rhizopus microsporus* var. *rhizopodiformis* have been traced to contaminated dressings and wooden tongue depressors used as immobilizing splints in a neonatal intensive care unit (53). The major risk factors predisposing individuals to mucormycosis include uncontrolled diabetes mellitus, other forms of metabolic acidosis, burns, and malignant hematological disorders. Treatment is seldom of benefit unless these underlying conditions can be corrected.

Entomophthoromycosis

Conidiobolomycosis occurs mainly in the tropical rain forests of Africa, South and Central America, and Southeast Asia.

Conidiobolus coronatus (Entomophthora coronata) lives as a saprophyte in soil humus and on decomposing plant matter in moist, warm climates. It can also parasitize certain insects. The disease it causes is most common among adult males, particularly those living or working in tropical rain forests. Infection is acquired through inhalation of spores or the introduction of spores into the nasal cavities by soiled hands.

Basidiobolomycosis is encountered chiefly in the tropical regions in East and West Africa, Indonesia, and India. The most widely held view is that *Basidiobolus ranarum* is the sole agent causing the disease and that *Basidiobolus meristosporus* and *Basidiobolus haptosporus* are only synonyms of the former; however, not all authors are of this opinion.

Basidiobolus ranarum has been isolated from the guts of frogs, toads, and lizards that had apparently swallowed infected insects. It has also been isolated from ants and decaying plant matter.

It is still uncertain how the disease is acquired and what is the length of incubation. Inoculation through a thorn prick or an insect bite has been suggested, as has contamination of a wound or other abrasion. The infection is most common in children.

CLINICAL MANIFESTATIONS

Mucormycosis

In most cases, mucormycosis occurs in patients already receiving treatment because of another disease. For precisely this reason, it may not be immediately recognized. The clinical hallmark of mucormycosis is vascular invasion resulting in thrombosis and tissue infarction and necrosis. In most cases, the infection is relentlessly progressive and results in death unless treatment with a combination of surgical debridement and antifungal therapy is initiated promptly.

A number of recent reviews and commentaries underline the increasing prevalence and awareness of these infections (5, 22, 25, 35, 40, 76, 78, 84).

Mucormycosis is an opportunistic infection and is seldom seen in normal persons. Various forms are recognized, each of which is associated with particular underlying conditions. Like the etiologic agents of aspergillosis, the causal organisms of mucormycosis have a predilection for vascular invasion, causing thrombosis and infarction and necrosis of tissue. The anatomical distribution of lesions appears to correlate to a certain degree with defined predisposing conditions, e.g., craniofacial involvement in individuals with diabetic acidosis, pulmonary and disseminated infection in patients with acute leukemia, and gastrointestinal and cutaneous lesions in patients infected following local trauma. Indeed, cutaneous infections have possibly replaced craniofacial and pulmonary disease as the prevalent clinical manifestation of mucormycosis, a change mirrored by the emergence of *Rhizopus microsporus* var. *rhizopodiformis* (known as *Rhizopus rhizopodiformis* in many cases) as a significant pathogen.

Rhinocerebral Mucormycosis

The terms rhinocerebral mucormycosis and craniofacial mucormycosis are used to describe infection which begins in the paranasal sinuses and then spreads to involve the orbit, face, palate, or brain. The terms should be used only when there is documented brain involvement in addition to the involvement of the sinuses alone and in addition to the involvement of both the sinuses and the orbit, respectively. It is not clear whether the paranasal and rhino-orbital forms of mucormycosis are simply rhinocerebral mucormycosis in evolution or whether the extent to which mucormycosis progresses beyond the paranasal sinuses to involve the orbit and brain is dependent on the host response. A number of recent case studies and reviews describe a clinical variety of rhinocerebral mucormycosis termed rhino-orbital-cerebral mucormycosis (5, 27). Nasal endoscopy appears to be useful in diagnosis, tissue debridement, and follow-up with patients.

Rhinocerebral mucormycosis is most commonly seen in acidotic individuals, particularly those with uncontrolled diabetes mellitus, but it also occurs in leukemic patients and organ transplant recipients. It is the most common clinical form of mucormycosis and is often fatal within a week of onset if left untreated. It is assumed that the port of entry of the pathogen is through colonization of the nasal mucosa, allowing the fungus to spread via the paranasal sinuses into the orbit. Involvement of the brain and cavernous sinus occurs by way of the orbital apex (34). Multiple approaches to treatment are required (66, 89). Early clinical and laboratory diagnosis is crucial. If the infection spreads into the palate, a black necrotic lesion is often found. This is an important diagnostic sign, and necrotic lesions may also be found on the nasal mucosa. Nasal septum or palatal perforation is frequent. Drainage of black pus from the eye is an ominous but useful diagnostic sign. From the orbit, infection may spread into the brain, leading to frontal lobe necrosis and abscess formation. These features result from invasion of the fungus through the cribiform plate. The cerebrospinal fluid (CSF) findings are nonspecific. CSF cultures are sterile. The radiological findings are nonspecific but are useful in determining the extent of the infection (59, 79).

Pulmonary Mucormycosis

Pulmonary mucormycosis is seldom diagnosed during life. Mucormycosis may develop in the lungs as a result of aspiration of infectious material or, following inhalation, as a result of hematogenous or lymphatic spread during dissemination. Most cases occur in leukemic patients undergoing remission induction treatment. Pulmonary infiltrates develop, with infarction of focal areas of the lungs. The clinical signs are those of bronchitis, pneumonia, and thrombosis. There is necrosis of the parenchyma, leading to cavitation; the bronchi may be perforated, resulting in hemoptysis. If untreated, hematogenous dissemination to other organs, particularly the brain, will often occur. There are no characteristic symptoms or signs to distinguish mucormycosis from aspergillosis. The infection is fatal within 2 to 3 weeks.

Clinicians are aware that pulmonary mucormycosis is being seen more frequently in patients with cancer (47, 98). However, the clinical manifestation is similar to that of invasive pulmonary aspergillosis. Most cases of pulmonary mucormycosis in these patients appear to develop as breakthrough infections if treatment with antifungal agents effective against *Aspergillus* species is administered. A recent retrospective comparison of cancer patients with pulmonary mucormycosis with similar patients with invasive pulmonary aspergillosis showed that computed tomography imaging could potentially differentiate the two infections, but further validation was required (10).

Cunninghamella bertholletiae is a rare cause of pulmonary mucormycosis. A cluster of invasive pulmonary infections caused by *Cunninghamella bertholletiae* in four immunocompromised patients that occurred during a 2-year period at one center have recently been described (74).

The usefulness of fine-needle aspiration cytology (FNAC) in the diagnosis of lung lesions is well documented. Fungal lesions are among nonneoplastic lesions of the lung for which FNAC has proven to be a useful technique for both immunocompromised and immunocompetent patients. The usefulness of FNAC is highlighted in a number of case reports (2, 18). Various other approaches can be used for the diagnosis of pulmonary mucormycosis, for example, fiberoptic bronchoscopy (24).

Gastrointestinal Mucormycosis

Mucormycosis limited to the gastrointestinal tract is rare (39). It has usually been encountered in malnourished infants or children but also in patients with hematological malignancies (39). Lesions are most common in the stomach, colon, and ileum. It is seldom diagnosed during life. Frequently associated conditions include amebic colitis, pellagra, malnutrition, and kwashiorkor.

The symptoms are varied and depend on the site affected. Nonspecific abdominal pain and hematemesis are typical. Necrotic ulcers develop, and peritonitis follows if intestinal perforation occurs. Intestinal mucormycosis is a fulminant illness ending in death within several weeks due to bowel infarction, sepsis, or hemorrhagic shock.

Cutaneous Mucormycosis

Cutaneous mucormycosis is a particular problem in infected patients with burns in whom spread to underlying tissue is common. The initial signs include fever, swelling, and changes

in the appearance of the burn wound. The development of severe underlying necrosis and infarction in a burn should suggest the diagnosis.

Cutaneous infections account for 16% of all forms of mucormycosis, with an associated mortality of 16%, compared to 67% for rhinocerebral, 83% for pulmonary, and 100% for disseminated infection. The most common causative organism is *Rhizopus* spp., although others such as *Mucor* and *Absidia* are also frequently seen, whereas *Saksenaea vasiformis* and *Apophysomyces elegans* are rare pathogens (9, 45). Cutaneous mucormycosis is less likely to be associated with severe systemic illness than other forms, and local predisposing factors such as burns, trauma, surgery, needle sticks, and others play a major role. The most common sites involved in cutaneous mucormycosis are the lower and upper extremities, followed by the head and neck, and then the abdomen.

Mucormycotic gangrenous cellulitis can follow other forms of trauma to the skin. In diabetic or immunosuppressed patients, cutaneous lesions may arise at an insulin injection site or a catheter insertion site. More massive trauma, such as open fractures or crash injuries, has been present in other patients. Necrotizing cutaneous mucormycosis has occurred in patients who have had contaminated surgical dressings applied to their skin.

A number of infections have been described in a variety of individuals after traumatic injury or near-drowning (30). The most recent examples of mucormycosis in trauma patients are exemplified by survivors of the tsunami in Southeast Asia on 26 December 2004 (1).

Cutaneous lesions resembling ecthyma gangrenosum may develop following hematogenous dissemination of the fungus in immunosuppressed patients. The lesions begin as erythematous, indurated, painful cellulites and then evolve into ulcers covered with a black eschar. Isolated cases of primary cutaneous mucormycosis in a premature neonate (46) and in a patient with acute lymphocytic leukemia (54) have been described previously.

Disseminated Mucormycosis

Disseminated mucormycosis may follow any of the four forms of mucormycosis described so far, but it is usually seen in neutropenic patients with pulmonary infection. Less commonly, dissemination occurs from the gastrointestinal tract or burns or other cutaneous lesions. The most common site of spread is the brain, but metastatic necrotic lesions have also been found in the spleen, heart, and other organs. Mucormycosis can complicate peritoneal dialysis (56, 67).

Disseminated mucormycosis is usually diagnosed after the patient has died of the infection. Occasionally, metastatic cutaneous lesions permit earlier diagnosis.

Cerebral infection following hematogenous dissemination is distinct from the rhinocerebral form of mucormycosis. It results in abscess formation and infarction. Patients present with sudden onset of focal neurological deficits or coma. Investigation of the CSF is unhelpful: protein, glucose, and cell abnormalities are nonspecific, and cultures are sterile. Computed tomography and magnetic resonance imaging scans are useful in locating the lesions.

A number of other diagnostic techniques have been used, including proton magnetic resonance spectroscopy integrated with magnetic resonance imaging (79).

Mucormycosis in AIDS

Mucormycosis in human immunodeficiency virus (HIV) infection is rare. However, it can be the presenting opportunistic infection in AIDS. Predisposing factors for mucormycotic infection in HIV disease include low CD4 count, neutropenia, and active intravenous drug use. Mucormycosis can present in the basal ganglia, the skin, the gastrointestinal tract, or the respiratory tract or may be disseminated.

A total of 31 HIV-positive patients with mucormycosis have been reported (28 of these are reviewed in reference 90). It is presumed that intravenous drug injection was the portal of entry for fungal propagules. The most prevalent clinical presentations of mucormycosis in the intravenous drug-using HIV-positive patients appeared to be cerebral, cutaneoarticular, and renal (accounting for 88% of presentations in total) (26).

Mucormycosis in Hematological Malignancies

The incidence of mucormycosis in patients with hematological malignancies has increased during the last decade, probably due to the more severe and prolonged postchemotherapy neutropenia. The diagnosis is usually made at autopsy, and its incidence in autopsy studies in patients with hematologic malignancies ranges between 0.4 and 0.9%. A number of retrospective studies have been published (61, 64).

In recent years, pulmonary mucormycosis has been reported to occur in patients with leukemia and lymphoma and bone marrow transplant recipients (48, 58, 62).

Voriconazole is increasingly used as prophylaxis in patients with hematological malignancies. A number of reports have linked the prolonged use of this agent to an increase in infections caused by zygomycetes (36, 40, 50, 51, 63, 82, 91, 93).

Mucormycosis in Solid-Organ Transplant Recipients

Mucormycosis is a rare infection in renal transplant recipients; however, mortality is exceedingly high. Risk factors predisposing to this disease include prolonged neutropenia, diabetes, and immunosuppression. Mucormycosis is a rare but highly invasive infection following orthotopic liver transplantation (15, 49, 81). Reports of some 20 cases exist in the literature.

Other Forms of Mucormycosis

Isolated mucormycotic brain lesions have been reported to have occurred in a number of individuals, particularly parenteral drug abusers in whom the typical presentation is localized cerebral lesions. The infection presents as a rapid deterioration in neurological status and can be rapidly fatal (97, 99).

There is a clear association between deferoxamine use (for the treatment of iron overload) and the development of mucormycosis. Most of the reported associated cases have involved rhinocerebral disease (reviewed in reference 83). Deferoxamine is a siderophore derived from *Streptomyces pilosus*. Siderophores are high-affinity iron chelators used by microorganisms to concentrate iron for bioavailability. A number of fungi require iron for growth, and it appears that the fungi responsible for mucormycosis can utilize the iron bound to deferoxamine. The fact that deferoxamine is removed by renal clearance means that its circulation time will be prolonged in patients on dialysis, and this factor may increase the risk for these patients. Infection of bone has also been reported (33).

Differential Diagnosis

Rhinocerebral mucormycosis is a dramatic and distinctive condition, but it can be confused with cavernous sinus thrombosis, bacterial orbital cellulitis, or rhinocerebral aspergillosis or pseudallescheriasis. The clinical manifestations of pulmonary mucormycosis cannot be distinguished from those of gram-negative-bacterial pneumonia, aspergillosis, or infections caused by *Scedosporium* species.

Conidiobolomycosis

Conidiobolus infection generally begins with unilateral involvement of the nasal mucosa. The most common nasal symptom is obstruction, but frequent nosebleeding can occur and is evidence of the development of a nasal polyp in the anterior region of the inferior turbinate. Subcutaneous nodules then develop in the nasal and perinasal regions and may be associated with epidermal lesions.

The spread of the infection is slow but relentless. It is usually confined to the face, and the development of gross facial swelling involving the forehead, periorbital region, and upper lip is very distinctive. As a rule, the lesions are firmly attached to the underlying tissue, although the bone is spared. The skin remains intact. Spread to the lymph nodes has been reported. Even if, in advanced cases, the diagnosis is obvious from the clinical appearance, mycological and histological examinations are essential for its confirmation.

Basidiobolomycosis

The subcutaneous swelling that characterizes basidiobolomycosis is usually localized to the back of the shoulders and to the arms, but it may also be found on the buttocks and thighs. The initial swelling may be rapid or slow in onset, and it is hard and painless. The spread is slow but relentless, and a large mass is formed which is attached to the skin but not the underlying tissue (unlike lesions in *Conidiobolus* infection). This is a disfiguring infection, but the skin covering the lesions does not ulcerate. Lymphatic obstruction may occur and can result in massive lymphedema. There is no functional impairment so long as the joints are not blocked by the volume of the swellings. The underlying bone and joints are not affected by the disease.

Basidiobolomycosis can be diagnosed with confidence on the basis of appearance and the results of the mycological and particularly the histological examinations. Basidiobolomycosis closely resembles soft tissue sarcoma. Mycetoma has the same degree of induration as basidiobolomycosis, but the presence of draining sinuses, fixation to underlying structures, and extension to bone seen with mycetoma make the distinction relatively easy. Bacterial cellulitis is much more acute and painful than that in mucormycosis. All these conditions can be reliably distinguished from mucormycosis by biopsy and culture of the lesion. Immunodiffusion tests have been shown to determine the specific etiology of entomophthoromycosis (41).

Recently, the etiologic role of *Basidiobolus ranarum* in gastrointestinal infections has been increasingly recognized (43). Although the clinical presentation of the subcutaneous disease is quite characteristic (chronic localized subcutaneous infection of the face, trunk, or limbs) and the disease is easy to diagnose, gastrointestinal basidiobolomycosis poses diagnostic difficulties; its clinical presentation is nonspecific, there are no identifiable risk factors, and all age groups appear to be susceptible.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Mucormycosis

Because mucormycosis is such an aggressive infection, early diagnosis is essential for successful treatment. It is, however, often difficult to obtain suitable and timely specimens which have been collected correctly.

If there is any suspicion of mucormycosis, a diligent search for clues that would help to establish a specific microbiological diagnosis should be initiated without delay. The most useful clue is the presence of a black necrotic eschar either in the nasopharynx or on the palate. This eschar or material sloughing from it may be mistaken for clotted blood. Material from the eschar and underlying tissue should be obtained by biopsy or curettage and transported to the laboratory as quickly as possible for culture and microscopic examination. Other diagnostic approaches are equally effective, for example, fine-needle aspiration (18).

Entomophthoromycosis

Specimens must be taken from the subcutaneous tissue where the infection is seen as nodules which develop into massive, firm, indurated swellings which are freely movable over the underlying muscle but are attached to the skin, which is hyperpigmented but not ulcerated.

DIRECT EXAMINATION

Microscopy

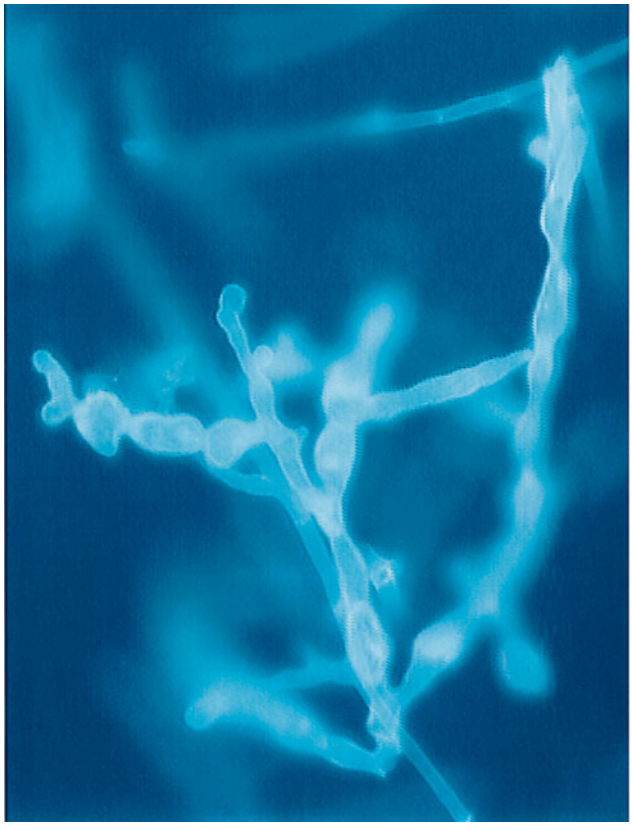
Given the limitations of imaging studies, diagnosing mucormycosis almost always requires histopathological evidence of fungal invasion of the tissues. The urgency of establishing the diagnosis is frequently underappreciated. Delayed diagnosis has been associated with a dramatically worse outcome.

A definitive diagnosis of mucormycosis is best achieved by direct histopathological examination and culture. Biopsy specimens from pulmonary, rhinocerebral, and cutaneous lesions and scrapings of necrotic mucocutaneous lesions are appropriate specimens.

The necrotic material should be gently broken apart and mounted in a few drops of 20% (wt/vol) potassium hydroxide (KOH). Typical wide, nonseptate hyphae with right-angled branching may be seen. Demonstration of mycelial elements in the correct clinical setting constitutes strong evidence for mucormycosis. If the KOH preparation is unrevealing, surgical biopsy or debridement of necrotic material should be performed. Examine any material obtained in KOH and stain with methenamine silver (Grocott's modification; GMS) and hematoxylin and eosin (H&E) (see below). If fungal elements are seen, obtain further biopsy specimens. The rapid progression seen in untreated mucormycosis allows no delays in diagnostic efforts. The sensitivity of these techniques can be enhanced by the use of optical brightners, for example, calcofluor white (Blankophor) (77) (Fig. 1 and 2).

The microscopic demonstration of the presence of Mucorales in clinical material taken from necrotic lesions is more significant than the isolation of these organisms in culture. However, different species cannot be distinguished from one another morphologically. These organisms can be distinguished from other molds, such as *Aspergillus,* due to their characteristic broad, nonseptate filaments with right-angled branching. In contrast to *Aspergillus,* zygomycetes are larger, do not have parallel walls with 45-degree-angle branching, and do not radiate from a single point in tissues. Furthermore, agents of mucormycosis stain poorly with periodic acid-Schiff stain (PAS) but stain well with H&E (Fig. 3). Negative results of microscopic examinations do not eliminate the possibility of mucormycosis even if a portion of necrotic tissue has been examined carefully.

Central nervous system involvement in rhinocerebral mucormycosis may suggest the presence of the etiologic agent in CSF. However, agents of mucormycosis have not been isolated from CSF. In cases of suspected pulmonary mucormycosis, examine biopsy material or material from fine-needle percutaneous aspiration. Sputum culture is rarely helpful.



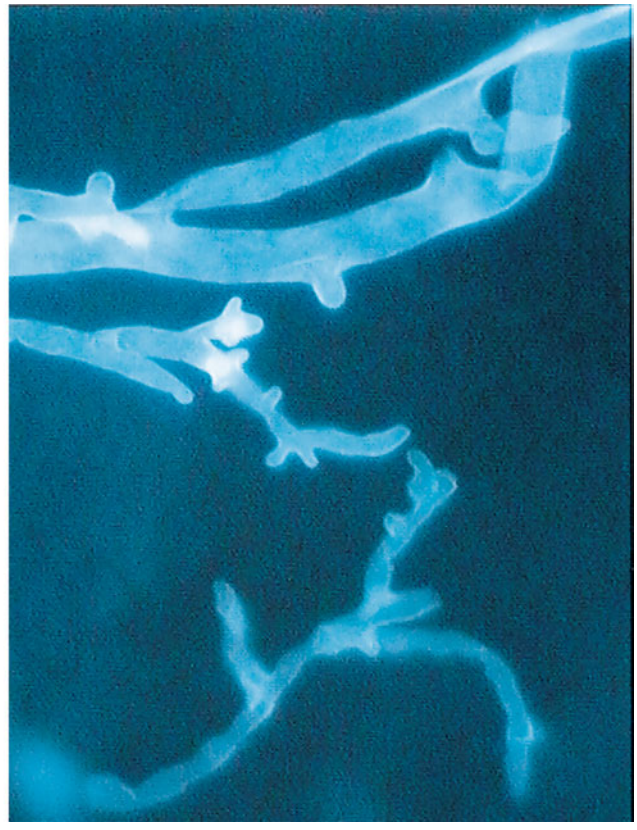


FIGURE 1 (left) Microscopic appearance of *Rhizopus* sp. hyphae in an aspirate from a mandibular gingival abscess stained with calcofluor white.

FIGURE 2 (right) Microscopic appearance of *Absidia corymbifera* hyphae in a thin section of lung tissue stained with calcofluor white.

Metastatic skin lesions, if present, may allow a diagnosis to be made without performing a lung biopsy. In isolated cases, fungal elements have been seen in bronchoalveolar lavage fluid and isolated in culture. Intestinal mucormycosis is usually a rapidly progressive disease ending in death within a few days. In almost all cases, the diagnosis is not suspected until histological examination of gangrenous tissue obtained at surgery or autopsy reveals the fungus. Premortem diagnosis is difficult. In suspected cases, the demonstration of the presence of fungi in gastric aspirates or feces may be useful.

Biopsy specimens or scrapings from oral lesions are most satisfactory for histological examination and culture. Demonstration of the presence of the fungus in tissue is diagnostic of disease (culture is not), but cultures must be set up to determine the specific etiologic agent. Mucoraceous fungi are readily seen by using the H&E stain and GMS, but their detection rate when stained with the conventional specialized fungal stains Gridleys and PAS is uneven and barely adequate. One useful stain is Cresyl Fast Violet, which stains zygomycete walls brick red and other fungi blue or purple.

Hyphal elements of mucoraceous fungi consist of broad, twisted, ribbonlike hyphae, which are variable in width (up to 50 μm) and often devoid of cytoplasm and have collapsed walls. True septa are not usually present, although wrinkles and folds in the cell wall may appear as septa unless the microscope is focused up and down on the apparent boundary. The hyphae are irregularly and haphazardly branched, and a lateral mycelium often grows at nearly 90 degrees. Although hyphae are broad, their widths may vary greatly and the hyphal walls also vary in thickness. The side branches may be much narrower than the main hyphal axis. The mucoraceous fungi can be mistaken for sclerosed capillaries or nematodes, and they may also be confused with empty spherules of *Coccidioides immitis* in regions where this organism is endemic. The small amount of fungus present in tissue sections and the oblique angles at which the sections are cut make the identification of fungus by histological morphology alone difficult. In some tissue areas, hyphae and hyphal fragments may appear to be septate and constricted at prominent septations. Often hyphae may have terminal bulbous swellings and lateral oval to spherical structures.

The pathology does not vary with the site of infection. Gangrene involvement is common. Surfaces show massive hemorrhage with recent emboli, and in older lesions the appearance is puttylike. The tissue reaction is not consistent. In some cases no cellular response is seen, but usually there are various degrees of edema and necrosis with neutrophil accumulations, plasma cells, and some giant cells. The acute reaction shows the diffuse infiltration of polymorphonuclear leukocytes. Eosinophils are not often seen. Tissues often exhibit suppurative reactions but may show granulomatous changes. The characteristic feature is the involvement of the blood vessels, with mucoraceous fungi invading the walls of arteries, causing thrombosis and necrosis of the adjacent tissue. The lumens and walls of blood vessels often contain hyphae, chlamydoconidia, fibrin, and inflammatory cells.

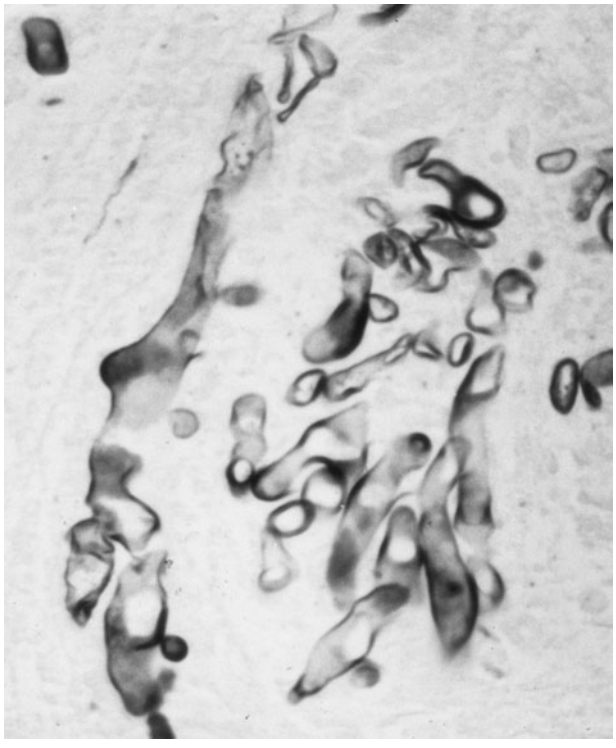


FIGURE 3 Hyphae of *Rhizopus oryzae* in an H&E-stained section of the soft palate from a patient with rhinocerebral mucormycosis. Note the irregular shapes and diameters of the hyphae. Magnification, ×400.

Another pertinent feature is the presence of spherical to ovoid, 15- to 30-μm-diameter, thick-walled, hyaline chlamydospores. Chlamydospores of mucoraceous fungi are PAS and H&E positive but stain either poorly or not at all with GMS. Because of fixation and processing of tissues, the chlamydospore cytoplasm can retract from the plasma membrane, creating an artificial clear space between the condensed cytoplasm and the thick, rigid cell walls. Some chlamydospores stain entirely, and others can appear as empty rings. Chlamydospores are seen in the vicinity of mucoraceous hyphae. Detached chlamydoconidia can resemble the yeast form cells of *Blastomyces dermatitidis* or the immature spherules and arthroconidia of *Coccidioides immitis*. In pulmonary mucormycosis, chlamydospores are most often observed in alveolar spaces, the lumens of bronchioles, and the visceral pleura. In cutaneous mucormycosis, chlamydospores may be seen within necrotic tissue on or near a wound surface.

Invasive mucormycosis is rapidly progressive and is associated with angioinvasion and infarction. In addition to being able to identify the morphological features of zygomycetes in histopathological preparations, it is important to recognize the preferential sites of growth and the host response (21). Medical record and histological, review in a single institution identified 20 patients with mucormycosis. Diagnostic criteria included the presence of typical ribbonlike hyphae and positive culture, a clinical history of invasive mucormycosis, or both. The clinical review in reference 21 is helpful. Fungus ball (15%), rhinocerebral (55%), and pulmonary (30%) disease were the types of diseases represented. The inflammatory responses were predominantly neutrophilic (50%), predominantly granulomatous (5%), pyogranulomatous (25%), or absent (20%). Invasive disease was characterized by prominent infarcts (94%), angioinvasion (100%), and prominent perineural invasion (90%) in biopsy specimens that contained nerves for evaluation. Notably, hyphal septa were always seen (100%), and most branches (95%) varied from 45 to 90 degrees. Furthermore, perineural invasion is a common finding in invasive mucormycosis, as are angioinvasion and infarcts. Therefore, prior to excluding the presence of mucormycosis agents in biopsy specimens suspected to contain zygomycetes, the perineural space should be carefully examined.

Quite often there may be a delay in obtaining fixed and histopathologically stained biopsy sections. One alternative to expedite the work-up is to use frozen sections. The visualization of hyphal elements in frozen sections has been associated with improved outcomes in two recent case series (27, 31).

In cases of conidiobolomycosis, microscopic examination of smears or tissue from the nasal mucosa will reveal broad, nonseptate, thin-walled mycelial filaments. In basidiobolomycosis, direct microscopy will reveal wide, irregular hyphae or fragments thereof with few septa.

In cases of basidiobolomycosis, biopsy specimens will show fibroblastic proliferation and an inflammatory reaction with lymphocytes, plasma cells, histiocytes, eosinophils, and giant cells. Broad, thin-walled hyphae with occasional septa, branched at right angles, are seen. The Splendore-Hoeppli phenemonon may be seen. The histopathological features of basidiobolomycosis are very similar to those of conidiobolomycosis.

Antigen Detection

There are no routinely available antigen detection formats for the diagnosis of mucormycosis.

Nucleic Acid Detection Techniques

Multiplex PCR has been used to identify *Rhizopus* species in paraffin sections and in sera from three mucormycosis patients (55). *Rhizopus oryzae* and *Rhizopus azygosporus* DNA was amplified from clinical material, which correlated with the identification of the isolated strains.

In a case of pulmonary mucormycosis associated with acute lymphocytic leukemia caused by *Cunninghamella bertholletiae,* retrospective analysis of stored serum, sputum, and necrotic lung tissue samples enabled subspecies identification by PCR, direct DNA sequencing of the PCR products, and a homology search with nucleotide basic local alignment search tools (44). A further case of *Cunninghamella bertholletiae* infection diagnosed by panfungal PCR is reported by Rickerts and colleagues (75).

ISOLATION PROCEDURES

Isolation of the causative agents of mucormycosis has been achieved in only a small proportion of cases, but specimens should be sent for culture if the clinician suspects mucormycosis. Direct detection of fungus in tissues is significantly more sensitive than culture. The underlying reason for this is unclear. Typically, even if a large amount of tissue is removed at surgery, a relatively small piece is submitted for fungal culture. Infections may be quite localized and may not be detected in all portions of the specimen submitted for analysis. Infarcted areas and areas into which there has been extensive bleeding are the best choices of sites for culture. To maximize the recovery of zygomycetes from tissue, the material should be cut up into small fragments and inoculated onto Sabouraud dextrose agar.

Because the Mucorales are common contaminants, isolation of these organisms from sputum, aspirated material from the sinuses, or bronchial washings must be interpreted with

caution. However, if the patient is diabetic or immunosuppressed, the isolation should not be ignored. Isolation of the causal agents of disease has been achieved in a small proportion of cases. Sputum culture is highly unreliable. In two case series, sputum and bronchoalveolar lavage fluid cultures were negative in 18 of 19 cases of biopsy-proven pulmonary mucormycosis (71, 88). The agents of mucormycosis can be grown on most laboratory media, but glucose-peptone agar (glucose, 4%; mycological peptone, 1%; agar, 2%) or 4% malt extract agar (malt extract, 4%; agar, 2%) is recommended because typical morphology can be demonstrated. Media containing cycloheximide should not be used because cycloheximide inhibits the growth of these fungi. Small portions of biopsy material should be point inoculated onto glucose-peptone agar. Cultures of swabs from the nose or palate are usually negative and are not recommended. Cultures should be incubated at room temperature and 37°C. Tissue material should not be homogenized, as this procedure will kill viable hyphal elements of mucoraceous fungi. Refrigeration may also reduce the viability of some of these agents.

In suspected cases of entomophthoromycosis, tissue obtained by biopsy or curettage should be cultured on Sabouraud dextrose agar. Positive cultures are difficult to obtain, and the pathological material must be inoculated onto the largest possible number of mycological media plates to ensure culture growth if the organism is present in the tissues; the plates should be incubated at between 25 and 35°C. Identifiable colonies should be obtained in less than 1 week.

IDENTIFICATION

General Description of Fungi Most Likely Isolated and Basis of Identification

Identification of most zygomycetes is based primarily on the morphology of the sporangia, i.e., the arrangement and number of sporangiospores and the shape, color, and presence or absence of columellae and apophyses, as well as the arrangement of the sporangiophores and the presence or absence of rhizoids. Growth temperature studies can also be especially helpful in identifying and differentiating members of the genera *Rhizopus, Rhizomucor,* and *Absidia.* The Mucorales are characterized by the production of nonseptate hyphae. However, occasional septa are formed in older cultures. Some genera produce rhizoids, rootlike anchors (Fig. 4) which are connected by hyphae called stolons. The Mucorales reproduce asexually by producing spores in sporangia which are borne on sporangiophores. The sporangiophores of some genera swell where they meets the sporangium, forming the structure called an apophysis (Fig. 5 and 6). Typically, a sporangium is a globose swelling at the apex of a sporangiophore. The sporangium contains a central, variously shaped collumella which is separated from the spore-containing sac by a membrane. The cytoplasm of the sporangium is cleaved into hundreds of sporangiospores during maturation (Fig. 5 and 6). In addition, some genera of Mucorales produce small, several-spored sporangia called sporangioles. Finally, a few genera, for example, *Cunninghamella,* produce sporangia which contain a single spore; these are called conidia. Sexual reproduction in the Mucorales is characterized by the formation of zygospores. However, detection of the presence of zygospores is not a part of a routine laboratory identification scheme.

Several excellent monographs and book chapters on the taxonomy and detailed identification of zygomycete fungi have been published (17, 72, 86). The interested worker is referred to these sources for information to complement those features highlighted here and for data on routine isolates not corresponding to the descriptions below. World Wide Web sites and CD-ROM resources include http://www.doctorfungus.org and Organism Central (Lippincott Williams and Wilkins, Philadelphia, Pa.) and the CD-ROM version of *Atlas of Clinical Fungi,* 2nd ed. (http://www.cbs.knaw.nl) (17).

Description of Causal Agents

The descriptions given below of the causal agents of zygomycoses are based almost entirely on their asexual characteristics.

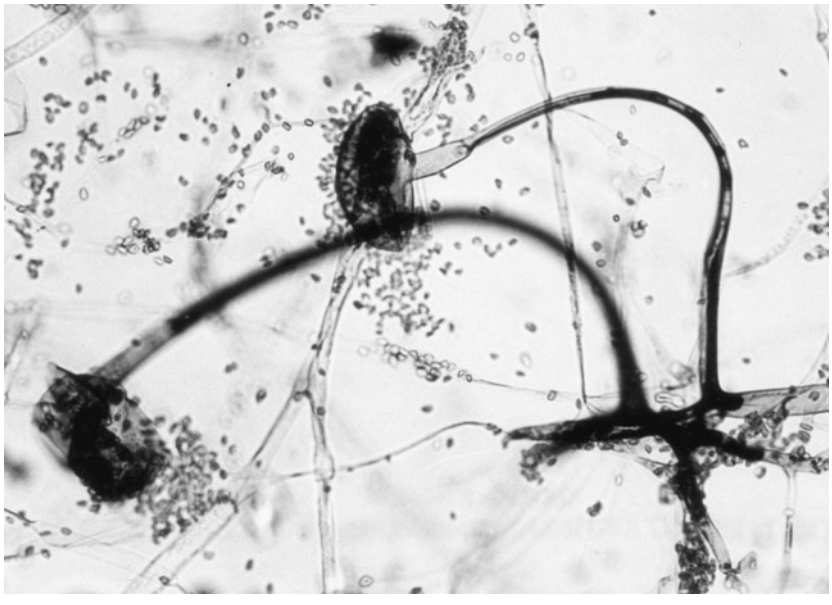
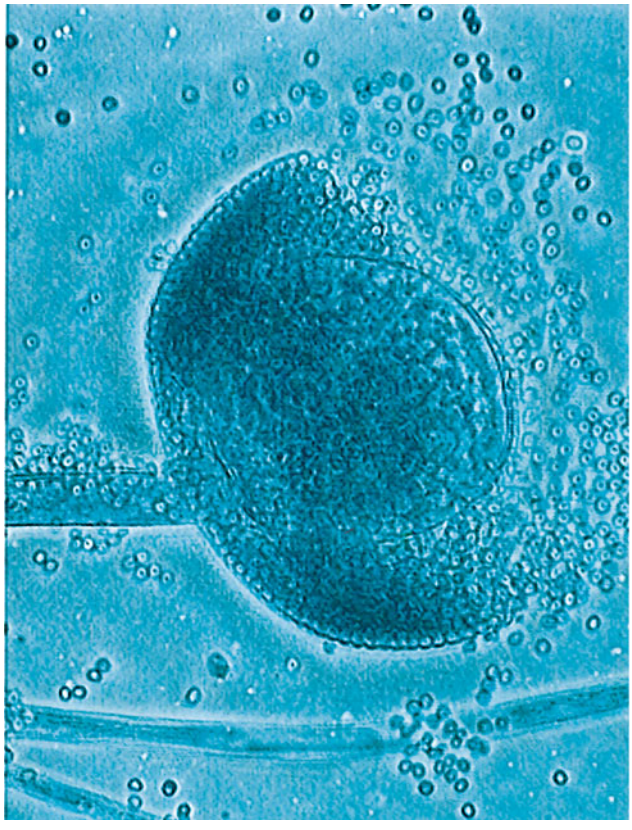


FIGURE 4 *Rhizopus oryzae* (NCPF 2634). Note the simple sporangiophores borne singly opposite rhizoids. Some sporangia have collapsed, and the sporangial wall has dissolved. Magnification, ×400.



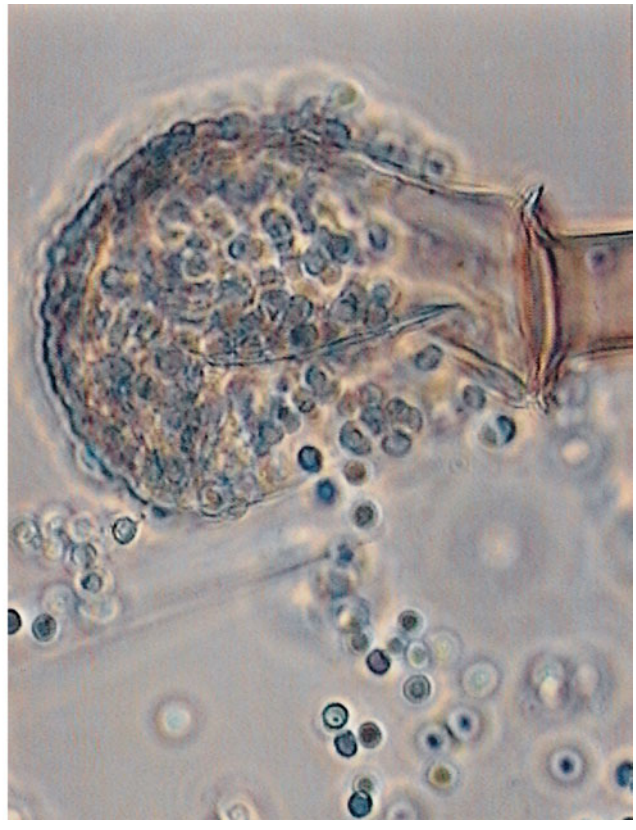


FIGURE 5 (left) Microscopic appearance of sporangium of *Rhizopus* species.

FIGURE 6 (right) Microscopic appearance of sporangium of *Rhizopus oryzae*.

The differentiation of these fungi to the species level is difficult, but a scheme of identification based on a combination of their asexual morphology and biochemical characteristics has made it more feasible for the clinical laboratory. The descriptions given below are based on the colony appearances and microscopic morphologies of individual isolates (some deposited in the National Culture Collection of Pathogenic Fungi, Mycology Reference Laboratory, Public Health Laboratory, Bristol, United Kingdom) cultured on 4% malt agar as a primary isolation medium and subcultured on glucose-peptone agar without the addition of cycloheximide. The maximum incubation temperature is indicated, assuming that primary isolation plates for clinical specimens from suspected cases of fungal infection are routinely incubated at a range of temperatures, e.g., 28, 37, and 45°C.

Rhizopus oryzae

Found in soil, decaying fruit and vegetables, animal feces, and old bread, *Rhizopus oryzae* is the most prevalent agent of mucormycosis.

Colony Morphology

Rhizopus oryzae grows as a rapidly growing, white, cottony colony at 28°C, filling a 9-cm-diameter petri dish within 2 to 3 days and reaching up to about 8 mm in height. The fungus will grow at temperatures of up to 45°C if routine primary isolation plates are incubated at this temperature. Colonies tend to collapse, becoming brownish gray to blackish gray, depending on the amount of sporulation. Growth is inhibited by cycloheximide.

Microscopic Morphology

The single most useful characteristic in identifying members of the genus *Rhizopus* is the presence of simple sporangiophores borne singly or in groups opposite rhizoids (Fig. 4). With *Rhizopus oryzae,* broad, nonseptate hyphae are seen. Sporangiophores are seen and are up to 3,500 μm in length and 18 μm wide, smooth walled, nonseptate, and simple with a single axis or branched, arising from stolons opposite brownish rhizoids, usually in groups of three or more.

Grayish black sporangia are globose, often with a flattened base, powdery in appearance, and up to 200 μm in diameter. Columellae are ellipsoidal on a truncate base, mouse gray in color, and up to 130 μm in length. Sporangiospores vary in shape and size, having a rhomboidal, lemon shape, with ridges on the surface.

Rhizopus microsporus var. *rhizopodiformis*

The second most common species associated with mucormycosis, causing nearly 15% of all culturally proven cases of the disease in humans, is *Rhizopus microsporus* var. *rhizopodiformis*, which is isolated mainly from cutaneous lesions (reviewed in references 19 and 53).

Colony Morphology

Colonies range from gray to dark grayish brown.

Microscopic Morphology

Rhizoids are hyaline to subhyaline. Sporangiophores are shorter (maximum length, 1,000 μm) than those of *Rhizopus oryzae* and are produced singly or in groups of up to four (Fig. 7). Sporangia average 100 μm in diameter. Columellae

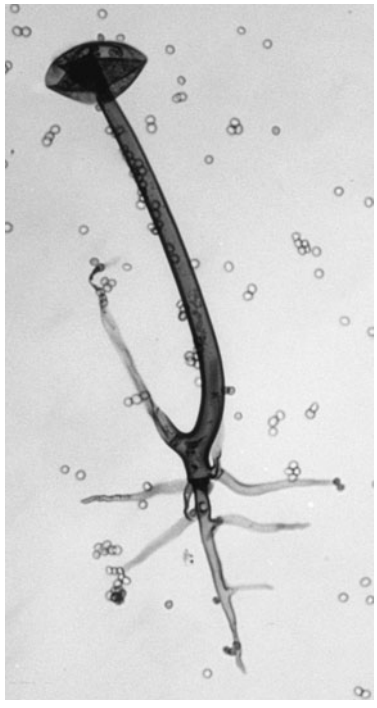


FIGURE 7 *Rhizopus microsporus* var. *rhizopodiformis* (NCPF 2955). Note the short sporangiophore compared with that of *Rhizopus oryzae* and the branched rhizoids. Magnification, ×400.

are pyriform or somewhat elongated, with distinct angular apophyses. Sporangiospores (4 to 6 μm) are uniform in size and spherical or slightly elongated, with fairly striated surfaces. The fungus is thermotolerant, growing well at up to 50°C.

Rhizopus microsporus var. *microsporus*

Rhizopus microsporus var. *microsporus* is an emerging pathogen and has been reliably identified in two patients with cutaneous infections for whom amputation of the affected area was required (96).

Colony Morphology

Rhizopus microsporus var. *microsporus* may be distinguished from other pathogenic variants of the microsporus group by growth at 46°C but not at 50°C.

Microscopic Morphology

The sporangiophores of *Rhizopus microsporus* var. *microsporus* are angular to broadly ellipsoidal, striated, and up to 6.5 μm in length.

Rhizomucor pusillus

Rhizomucor pusillus is found in decaying fruits and vegetables and in the soil. Very few reports of human disease caused by this organism have been published. *Rhizomucor pusillus* has been isolated in cases of pulmonary, rhinofacial, and disseminated infection in immunocompromised patients and also in occasional cases of cutaneous infections and endocarditis. This fungus can also cause mycotic abortion in cows, horses, and pigs.

Colony Morphology

Colonies are rapidly growing and appear white at first, turning dark smoke gray or brown as sporulation occurs. The colony texture is floccose to woolly. Growth is inhibited by cycloheximide. The maximum growth temperature of 54 to 58°C is a useful distinguishing feature.

Microscopic Morphology

Rhizomucor pusillus forms broad, sparsely septate, hyaline hyphae. Sporangiophores are mainly monopodially branched and anchored to the substratum by short rhizoids (Fig. 8). Sporangia are globose and dark brown and measure up to 100 μm in diameter. *Rhizomucor* differs from *Rhizopus* by forming sporangia without apophyses. Columellae are subglobose to pyriform. The sporangium wall dissolves to leave a collar around the sporangiophore. Globose to subglobose, hyaline sporangiospores are released (3 to 5 μm in diameter).

Absidia corymbifera

Absidia corymbifera is an infrequent agent of mucormycosis in humans (19, 65). It is found worldwide in soil, compost, and decaying vegetation. Documented cases of *Absidia corymbifera* infection include a case of meningitis in an immunocompetent patient that followed a penetrating head injury (65), a case of cutaneous infection in an immunocompetent patient, and cases of infection in AIDS patients and, more recently, in patients with hematological malignancies (62). Infection is most probably acquired by inhalation of sporangiospores. *Absidia corymbifera* is the only human pathogenic species of the genus *Absidia* and is also an agent of bovine mycotic abortions.

Colony Morphology

Absidia forms rapidly growing colonies that quickly fill the petri dish. It grows well at 25 to 45°C, and some isolates can grow at 50°C. The very high colonies are light gray to grayish brown. The reverse side is colorless to very slightly yellow to buff with age. The colony may originate from stolons between

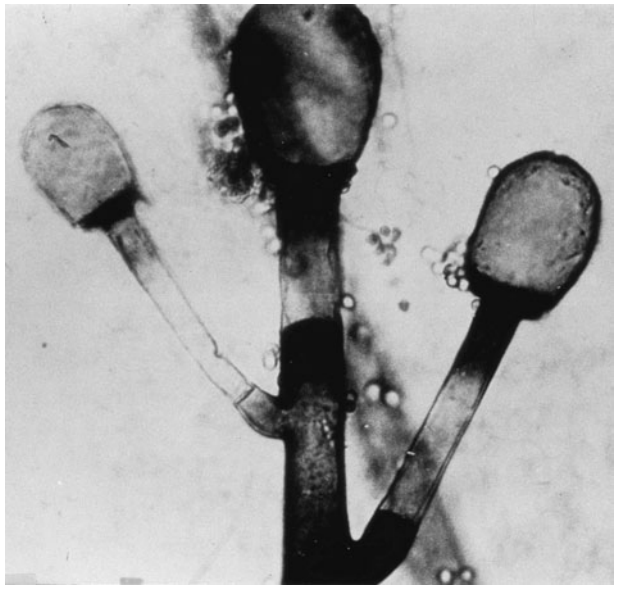


FIGURE 8 *Rhizomucor pusillus*. Note the monopodially branched sporangiophores, pyriform columellae, and small sporangiospores. A collarette can be seen around the sporangiophore. Magnification, ×262. (Reprinted with permission of Kaminski's Teaching Slides on Medical Mycology, Adelaide Children's Hospital, Adelaide, Australia.)

rhizoids or from finely branched aerial hyphae. *Absidia corymbifera* does not grow in the presence of cycloheximide.

Microscopic Morphology

Absidia corymbifera forms broad (often 10-μm or more), sparsely septate hyphae with stolons and internodal rhizoids. Sporangiophores are hyaline to light gray and branched simply or in a corymbiform pattern. Sporangia are pyriform, 100 to 150 μm in diameter, and hyaline when young, but they become grayish brown to greenish beige. A swollen apophysis is seen at the point where the sporangiophore merges with the sporangium (Fig. 9). Columellae are hemispherical to short-ovoidal. Sporangiospores have a greenish yellow tinge and are variable in shape and size, with dimensions ranging from 3.2 to 3.9 by 3.1 to 3.5 μm to those of a long, ellipsoidal shape, 4.1 to 4.9 by 2.3 to 2.9 μm.

Cunninghamella bertholletiae

In the genus *Cunninghamella,* only the species *Cunninghamella bertholletiae* has been verified as a human pathogen. *Cunninghamella bertholletiae* is widely distributed in decaying vegetation and animal matter and in the soil and has been recovered from cheese and Brazil nuts. Infection is acquired by inhalation of conidia. Mating reactions have been very important in classifying the species of *Cunninghamella.*

Cunninghamella spp., in the class Zygomycetes and the order Mucorales, are unusual opportunistic pathogens that have been identified with increased frequency in immunocompromised patients. Infections with this group of organisms have been seen most frequently in patients with hematologic malignancies. Thirty or so cases of invasive *Cunninghamella* infection have been reported in the literature (reviewed by Darrisaw and colleagues in reference 14; for recent case reports, see references 7, 60, 69). A series of cases highlight the ineffectiveness of prophylaxis and the difficulties of treatment (74).

Colony Morphology

Cunninghamella bertholletiae is rapidly growing, filling a petri dish within 2 to 3 days. Colonies are white at first, becoming dark gray and powdery with development. The reverse side is colorless to buff. Primary isolates will grow at temperatures of up to 42°C. This organism is sensitive to cycloheximide.

Microscopic Morphology

The sporangiophores of *Cunninghamella bertholletiae* are up to 20 μm wide and are straight with verticillate or single branches (Fig. 10). Terminal vesicles are subglobose to pyriform, with the terminal ones up to 40 μm and the lateral ones 10 to 30 μm in diameter. Sporangiola are globose (7 to 11 μm in diameter) or ellipsoidal (9 to 13 by 6 to10 μm), verrucose or echinulate, and hyaline when single but brownish in mass (Fig. 10). *Cunninghamella bertholletiae* grows optimally between 25 and 30°C, and the maximum temperature tolerated is 50°C.



FIGURE 9 *Absidia corymbifera*. Note the relatively fine sporangiophore, pyriform sporangium, hemispherical columella, and long, conical apophysis. Sporangiospores vary in shape from globose to long ellipsoidal. Magnification, ×400. (Reprinted with permission of Kaminski's Teaching Slides on Medical Mycology, Adelaide Children's Hospital, Adelaide, Australia.)

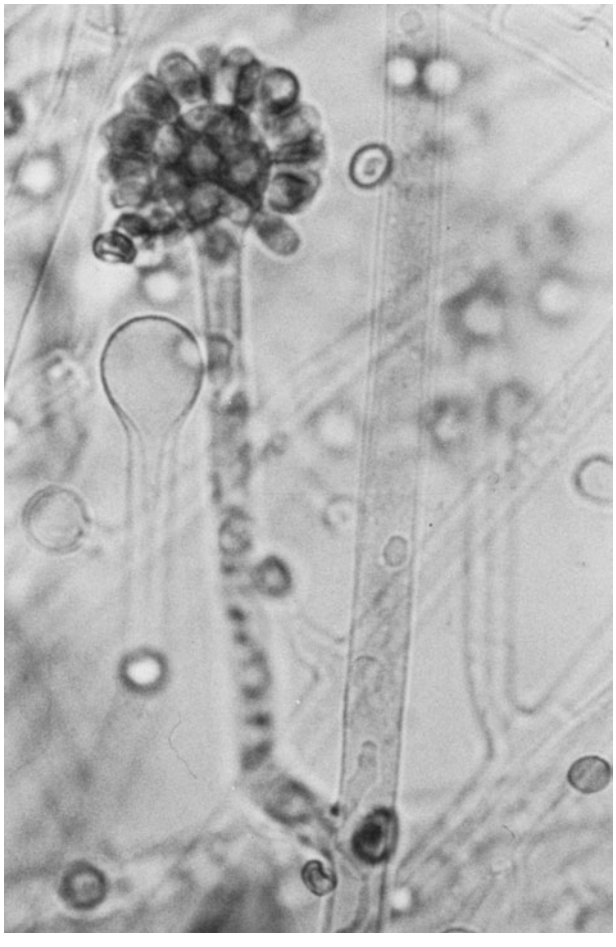


FIGURE 10 *Cunninghamella bertholletiae*. Note the single sporangiophore branching off a main hyphal trunk and subglobose to pyriform vesicle bearing ellipsoidal sporangiola. Magnification, ×400. (Reprinted with permission of Kaminski's Teaching Slides on Medical Mycology, Adelaide Children's Hospital, Adelaide, Australia.)

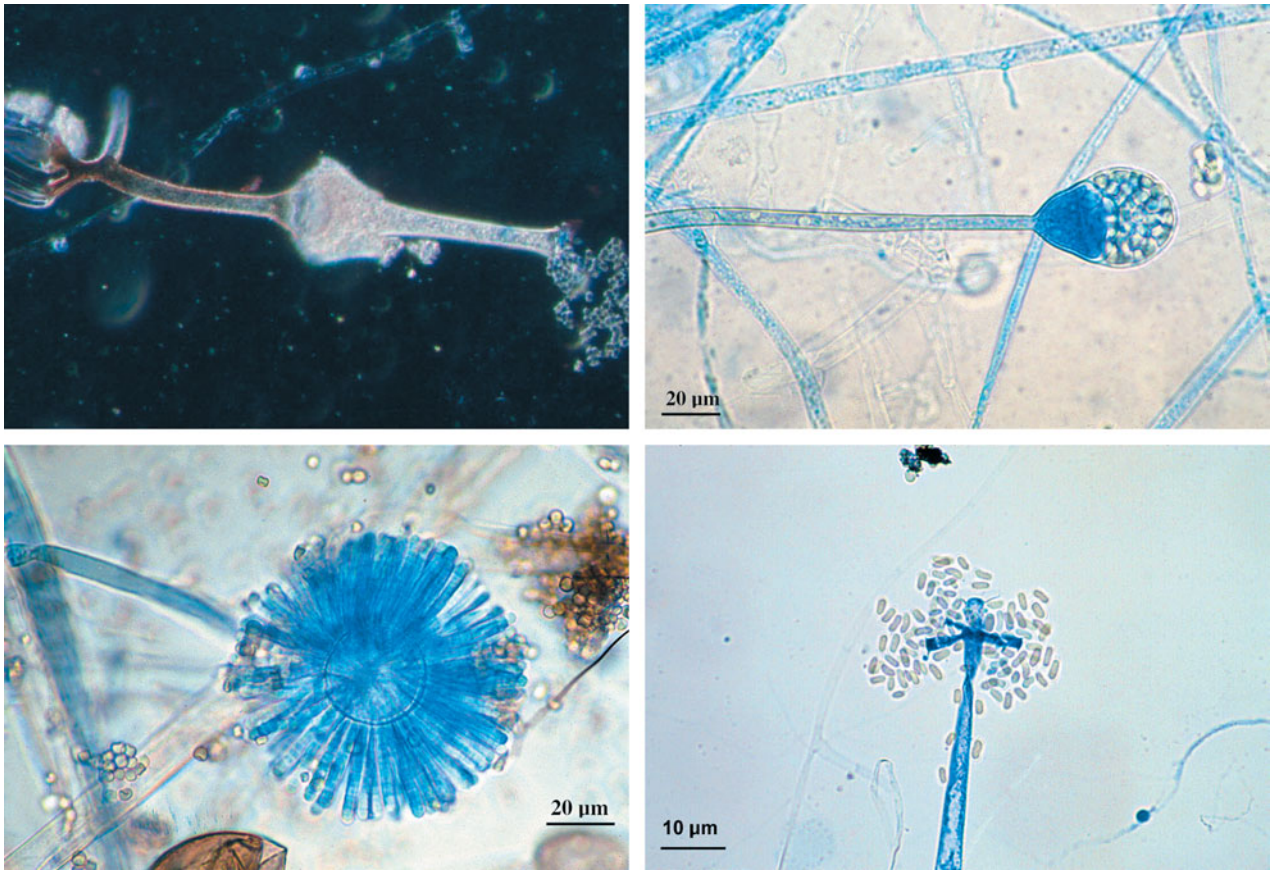


FIGURE 11 (top left) *Saksenaea vasiformis* isolated from a tsunami patient. Note the flask-shaped sporangium with a distinct, globose columella and small, smooth, elongated sporangiospores.

FIGURE 12 (top right) Young sporangium of *Apophysomyces elegans* showing distinct funnel-shaped apophyses and a conspicuous pigmented subapical thickening below the apophysis. (Reprinted with permission of Kaminski's Teaching Slides on Medical Mycology, Adelaide Children's Hospital, Adelaide, Australia.)

FIGURE 13 (bottom left) Microscopic morphology of *Syncephalastrum racemosum* showing a terminal vesicle, fingerlike merosporangia, and sporangiospores. (Reprinted with permission of Kaminski's Teaching Slides on Medical Mycology, Adelaide Children's Hospital, Adelaide, Australia.)

FIGURE 14 (bottom right) Microscopic morphology of *Mortierella wolfii* showing a typical erect, delicate sporangiophore, wide at its base, with a compact cluster of acrotonous (terminal) branches. On dehiscence of the sporangia, a conspicuous collarette is usually present. Columellae are generally lacking, and sporangiospores are single-celled, short cylinders with a double membrane. (Reprinted with permission of Kaminski's Teaching Slides on Medical Mycology, Adelaide Children's Hospital, Adelaide, Australia.)

Saksenaea vasiformis

Saksenaea vasiformis is widespread in tropical soils and is a known agent of subcutaneous zygomycosis (8). At least 21 cases of invasive infection have been reported, occurring in both immunocompromised and immunocompetent individuals. Many of these cases have been reviewed by Holland (32). Disseminated infection is rapidly fatal. *Saksenaea vasiformis* is a rare cause of rhinocerebral mucormycosis.

Colony Morphology

The colonies of *Saksenaea vasiformis* are rapidly growing and appear downy white with no reverse pigment.

Microscopic Morphology

Saksenaea vasiformis forms broad, nonseptate hyphae and flask-shaped sporangia (Fig. 11) with a distinct spherical venter (that portion of a phialide that lies between the base of the phialide and the conidiogenous locus) and long necks arising singly or in pairs from dichotomously branched, darkly pigmented rhizoids. The collumellae are prominent and dome shaped. Small, oblong sporangiospores, 1 to 2 by 3 to 4 μm in size, are discharged through the necks of the sporangia following the dissolution of an apical mucilaginous plug.

Laboratory identification may be difficult or delayed because of the mold's failure to sporulate on primary isolation media or upon subsequent subculture onto potato

dextrose agar. Sporulation may be stimulated by the use of nutrient-deficient media, such as cornmeal-glucose-sucrose-yeast extract agar.

Saksenaea vasiformis usually grows easily on routine mycological media, but it often fails to sporulate on those media. It usually produces sporangia when it is grown on nutrition-deficient media, such as inoculated agar blocks containing hyphal growth on sterile distilled water with sterilized yeast extract solution added to it, Czapek's agar, and 1 to 1.5% agar in saline. In most reported cases, sporulation was successfully induced by using the sterile distilled water method, although this method may fail occasionally, and on rare occasions, sporulation may occur on routine media, such as Sabouraud dextrose agar and cornmeal agar. In our experience gained from the isolation of *Saksenaea* from Finnish tsunami patients, subculture on Sabouraud glucose agar induced sporulation.

Refer to the review by Holland for additional details on the laboratory diagnosis of *Saksenaea vasiformis* infection (32).

Mucor Species

Mucor species are infrequently the causative agents of mucormycosis. The genus contains 49 recognized species, many of which have a widespread occurrence and are of considerable importance. However, few species have been recovered in well-documented cases of mucormycosis, and infections due to members of this genus are rare.

Identification of *Mucor* species beyond the genus level is difficult because the pathogenic species lack distinctive characteristics. However, molecular typing has been described previously (70). Emerging pathogens within the *Mucor* group are *Mucor circinelloides* and *Mucor ramosissimus*. Very few cases of human disease have been associated with *Mucor circinelloides* and have, to date, been limited to cutaneous infections. Similarly, very few cases of human disease caused by *Mucor ramosissimus* have been reported in the literature; such cases are possibly underreported owing to inaccurate or incomplete identification. *Mucor ramosissimus* has been associated with peritoneal dialysis-associated peritonitis, septic arthritis, and mucocutaneous and rhinocerebral disease.

Colony Morphology

Mucor species are rapidly growing organisms. Colonies are white and cottony at first but become gray to brown as sporulation occurs. *Mucor* species are inhibited by the concentration of cycloheximide usually incorporated into routine isolation media. Most species grow poorly, if at all, at 37°C.

Microscopic Morphology

The genus *Mucor* can be differentiated from *Absidia, Rhizopus,* and *Rhizomucor* by the absence of stolons and rhizoids. Species of *Mucor* produce simple or sympodially branching sporangiophores from nonseptate hyphae. There is no swelling where the sporangiophore and the sporangium merge. The gray-brown sporangia themselves are globose and contain globose to ellipsoidal sporangiospores.

Apophysomyces elegans

Originally described after detection in specimens from India, *Apophysomyces elegans* was found in soil samples from a mango orchard, and subsequently, 27 definite cases of mucormycosis caused by this fungus have been described, resulting from traumatic implantation, contamination of burns, or disseminated infection (8, 9, 20, 32). *Apophysomyces* species closely resemble *Absidia* species in their morphology.

Colony Morphology

Apophysomyces elegans grows rapidly. Colonies are white initially, becoming creamy white to buff with age and downy, with no reverse pigment. The fungus grows well at temperatures of up to 42°C. The growth of *Apophysomyces elegans* is not inhibited by cycloheximide.

Microscopic Morphology

Apophysomyces elegans does not sporulate on routine mycological media; it forms only broad, nonseptate hyphae. Water culture containing 10% yeast extract is required to induce sporulation. Characteristic identifying features include sporangiophores arising singly from aerial hyphal segments. Thin-walled, hyaline, light grayish brown rhizoids are produced opposite sporangiophores. A foot cell (resembling that seen in *Aspergillus* species) is present at the union of the sporangiophore with the surrounding hyphae. Sporangia are pyriform in shape (20 to 50 μm in diameter) with prominent funnel- or wine glass-shaped apophyses and hemispherical columellae produced singly at the tips of sporangiophores (Fig. 12). Sporangiospores are smooth, mostly oblong (5.4 to 8.0 by 4.0 to 5.7 μm), occasionally globose, and light brown.

Basidiobolus ranarum

Colony Morphology

Colonies are moderately fast growing at 30°C and are flat, yellowish gray, and glabrous, becoming radially folded and covered by a fine, powdery, white surface mycelium. Satellite colonies are often formed by germinating conidia ejected from the primary colony.

Microscopic Morphology

Large, vegetative hyphae (8 to 20 μm in diameter) forming round, smooth, thick-walled zygospores (20 to 50 μm in diameter) that have two closely appressed beaklike appendages are characteristic. Two types of asexual spores are formed: primary and secondary. Primary spores are globose, one celled, and solitary and are forcibly discharged from a sporophore. The sporophore has a distinct swollen area just below the spore that actively participates in the discharge of the spore. Secondary spores are clavate and one celled and are passively released from a sporophore. These sporophores are not swollen at their bases. The apex of the passively released spore has a knoblike adhesive tip. These spores may function as sporangia, producing several sporangiospores. See reference 6 for additional details.

Conidiobolus coronatus

Colony Morphology

Colonies of *Conidiobolus coronatus* grow rapidly and are flat, cream colored, and glabrous and become radially folded and covered by a fine, powdery, white surface mycelium and conidiophores. The lid of the petri dish soon becomes covered with conidia, which are forcibly discharged by the conidiophores. The color of the colony may become tan to brown with age.

Microscopic Morphology

Conidiophores are simple, forming solitary, terminal conidia which are spherical, 10 to 25 μm in diameter, and single celled and have a prominent papilla. Conidia may also produce hairlike appendages called villae. Conidia germinate to produce either (i) single or multiple hyphal tubes that may also become conidiophores which bear secondary conidia or

(ii) multiple short conidiophores, each bearing a small secondary conidium.

Rare Causes of Mucormycosis

Rare causes of mucormycosis include *Cokeromyces recurvatus, Syncephalastrum racemosum* (Fig. 13), *Mortierella* species (Fig. 14), and *Actinomucor elegans* (6, 16).

TYPING SYSTEMS

The phenotype-based identification methods routinely used for the identification of zygomycetes require expertise. Isolates are identified by recognition of colonial morphology and microscopic reproductive structures. A number of molecular methods for identification of pathogenic fungi have been validated for use in clinical settings (87). Many of these methods have focused on sequence diversity of the internal transcribed spacer regions (ITS1 and ITS2). Voigt et al. (94) used 18S and 28S sequences and the actin and EF-1alfa genes (95) to describe the phylogeny of medically important zygomycetes. More recently, it has been suggested that DNA sequence analysis of multiple loci should be useful for rapidly identifying medically important molds. Rakeman et al. (70) generated a phenotypically validated database of 118 diagnostic alleles from 210 fungal strains which included four isolates of *Rhizopus oryzae* and various *Mucor* species. Sequence analysis permitted identification of all species tested. The MicroSeq D2 large-subunit ribosomal DNA sequencing kit (Applied Biosystems, Foster City, Calif.) has become commercially available for the identification of filamentous fungi. In an extensive study, this system correctly identified only 67.5% of over 200 clinical isolates identified by using standard morphological criteria (28). Of the 17 zygomycetes identified in this series, only 6 were correctly identified by using this molecular assay. It is noteworthy that this system misidentified all of the *Absidia, Rhizopus, Cunninghamella,* and *Apophysomyces* isolates and some of the *Mucor* isolates.

Molecular methods have been applied to determine the genetic variability of *Rhizomucor* species. In one study, 23 *Rhizomucor* isolates were characterized by random amplified polymorphic DNA-PCR with 10-bp oligonucleotide primers (92). These data were used for numerical analyses to obtain information on the intraspecies genetic polymorphism of *Rhizomucor* species. The extents of genetic variability in *Rhizomucor pusillus* and *Rhizomucor miehei* isolates were found to differ; the latter revealed less intraspecies polymorphism. The different levels of genotypic diversity suggest a correlation with the different forms of mating behavior of these species. *Rhizomucor tauricus* displayed amplification patterns similar to those of the investigated *Rhizomucor pusillus* strains.

A recent study by Nagao and colleagues reported a multiplex PCR designed to identify five *Rhizopus* species, namely, *Rhizopus oryzae, Rhizopus microsporus* (var. *microsporus* and var. *rhizopodiformis*), *Rhizopus azygosporus, Rhizopus stolonifera,* and *Rhizopus schipperae* (55). Strains of *Apophysomyces elegans* isolated from patients in northern India were assigned to groups based on microsatellite and PCR fingerprinting (9).

ANTIFUNGAL SUSCEPTIBILITIES

There are four factors that are critical for treating mucormycosis: rapid diagnosis, reversal of underlying predisposing factors, appropriate surgical debridement of infected tissue, and appropriate antifungal therapy. Guidelines for the management of zygomycoses can be found in Richardson and Warnock (73), Spellberg et al. (84), and numerous case reports (3, 23, 29, 62).

A number of new antifungal compounds are under development, including voriconazole, posaconazole, ravuconazole, and the echinocandins. Voriconazole has fungistatic activity against many yeasts and filamentous fungi but minimal activity against zygomycetes. Caspofungin is an echinocandin and has been licensed for the salvage treatment of confirmed cases of invasive aspergillosis and the treatment of invasive candidiasis and for empiric treatment of febrile neutropenic patients. In vitro antifungal susceptibility data suggest that caspofungin has little activity against zygomycetes. The investigational drug posaconazole appears to be active against various species of zygomycetes. A distinct advantage of posaconazole is that for the first time there is an oral treatment for zygomycoses (36, 42).

Treatment of conidiobolomycosis is difficult, but patients often respond to oral itraconazole, ketoconazole, or saturated potassium iodide. Treatment should be continued for at least 1 month after the lesions have cleared. The therapy of choice for basidiobolomycosis remains saturated potassium iodide solution.

Most of the information available in the literature about the susceptibility profiles of the zygomycetes is based on the methods detailed in the NCCLS (now the Clinical and Laboratory Standards Institute [CLSI]) document M38-A, which was published as the accepted standard in 2002 (57) and reviewed by Greenberg et al. (25).

The CLSI standard recommends the use of a broth microdilution assay for antifungal susceptibilities, with *Rhizopus arrhizus (Rhizopus oryzae)* being the only zygomycete addressed specifically. Interpretative breakpoints have not been published at this time, and the CLSI protocol states that the clinical relevance of testing of this group of fungal pathogens remains uncertain. The number of reports on antifungal susceptibility tests of zygomycetes is limited, with only a few publications describing testing of 35 or more isolates (11, 12, 13, 85).

The sensitivity of *Cunninghamella bertholletiae* to amphotericin B determined by using the CLSI standard has been described by Carey et al. (7). The MIC for the isolate was 4 μg/ml. The other reported MICs for *Cunninghamella bertholletiae* range from 1 to 100 μg/ml. Although the available data represent only a few patients, it is interesting to note that the patients who survived had isolates for which MICs were 1 to 2 μg/ml.

Sun and colleagues (85) compared the activity of posaconazole with those of amphotericin B, itraconazole, voriconazole, and fluconazole against 37 clinical isolates of zygomycetes, namely, *Mucor* species, *Rhizopus* species, isolates of *Absidia corymbifera*, *Cunninghamella* species, isolates of *Apophysomyces elegans*, and isolates of *Cokeromyces recurvatus* and *Saksenaea vasiformis*. The MICs of posaconazole at which 50 and 90% of the isolates were inhibited (MIC_{50} and MIC_{90}) were 0.25 and 4 μg/ml, respectively. Posaconazole was significantly more active than voriconazole and fluconazole and slightly more active than itraconazole.

By using the CLSI broth dilution method, amphotericin B and posaconazole have been found to be the most effective drugs against the zygomycetes in vitro (11, 12, 13). The MIC_{50} of amphotericin B for all isolates was 0.125 μg/ml. The MIC_{50} of posaconazole was 0.25 μg/ml. Voriconazole demonstrated a MIC of 2 μg/ml or greater, with an overall MIC_{50} of 32 μg/ml. Zygomycetes are variably susceptible to itraconazole and to terbinafine (11).

The CLSI method has more recently been applied to an extended range of zygomycetes (80). In summary, the in vitro susceptibilities of 15 strains of six genera of zygomycetes, including *Rhizopus oryzae* (*Rhizopus arrhizus*), *Rhizopus stolonifer*, *Mucor circinelloides* (three strains), *Absidia corymbifera* (three strains), *Rhizomucor pusillus* (three strains), *Cunninghamella bertholletiae* (two strains), and *Syncephalastrum racemosum* (two strains), to nine antifungal agents were determined by the CLSI M38-A broth microdilution method. Geometric means of the MICs were as follows: amphotericin B, 0.07 μg/ml (range, 0.03 to 0.5 μg/ml); nystatin, 0.83 μg/ml (range, 0.25 to 4 μg/ml); itraconazole, 0.59 μg/ml (range, 0.03 to >8 μg/ml); voriconazole, 6.50 μg/ml (range, 2 to >8 μg/ml); ciclopiroxolamine, 1.59 μg/ml (range, 0.5 to 4 μg/ml); and amorolfine, 9.19 μg/ml (range, 1 to >16 μg/ml). All strains were resistant to 5-flucytosine, fluconazole (MIC, >64 μg/ml), and caspofungin (MIC, >16 μg/ml).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

The microscopic demonstration of the presence of Mucorales in clinical material taken from necrotic lesions or in sputum or bronchoalveolar lavage fluid is more significant than the isolation of these fungi in culture. These organisms can be distinguished from other molds, such as *Aspergillus* species, by their characteristic broad, nonseptate hyphae with right-angled branching.

Isolation in culture is required for specific identification of the Mucorales. Despite the predilection of these organisms for angioinvasion and hematogenous dissemination, blood cultures in all forms of mucormycosis are always negative. Nasal, palatal, and sputum cultures are seldom helpful. Because the Mucorales are common contaminants, isolation of these organisms from material obtained from a necrotic lesion or from sputum or bronchoalveolar lavage fluid must be interpreted with caution. However, if the patient is diabetic or immunosuppressed, the isolation should not be ignored and treatment should be initiated immediately.

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Histoplasma, Blastomyces, Coccidioides, and Other Dimorphic Fungi Causing Systemic Mycoses*

MARY E. BRANDT AND DAVID W. WARNOCK

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TAXONOMY

The dimorphic fungi causing systemic disease belong to the class Euascomycetes, order Onygenales. This contains three families of medical importance, the Onygenaceae (dimorphic pathogens), the Arthrodermataceae (dermatophytes), and the Gymnoascaceae (agents of onychomycosis and dermatomycosis). The Onygenales share several general characteristics: their sexual stages (teleomorphs) form rudimentary asci surrounded by a network of hyphae, which may have complex appendages; and their asexual (anamorph) species generally possess one of two forms, either unicellular aleurioconidia or arthroconidia in chains of alternately viable and nonviable cells.

The family Onygenaceae was originally defined on the basis of morphological characteristics of the teleomorphs and of the conidia of these organisms. The teleomorph genus *Ajellomyces* contains three species, *A. dermatitidis, A. capsulatus,* and *A. crescens,* the anamorphs of which are placed in the genera *Blastomyces, Histoplasma,* and *Emmonsia,* respectively (60). The teleomorphs of *Coccidioides* and *Paracoccidioides* have not been discovered, although genetic data suggest that *Coccidioides* is recombining in nature (12).

Molecular phylogenetic studies have indicated that species of the Onygenales are divided into several clades (descendants of a common ancestor). The family name Ajellomycetaceae has recently been proposed for one of these clades (72), which includes *Emmonsia crescens* and *E. parva, H. capsulatum, P. brasiliensis, B. dermatitidis,* and *Lacazia loboi,* the agent of lacaziosis (34, 60). A second clade, which retains the family name Onygenaceae, includes the pathogen *Coccidioides immitis* with, among others, species of *Aphanoascus, Chrysosporium, Nannizziopsis,* and *Uncinocarpus* (72). The Onygenaceae and Ajellomycetaceae are well separated from the other families, Gymnoascaceae and Arthrodermataceae. In all these phylogenetic trees, pathogenic organisms are interspersed with nonpathogenic relatives. This suggests that the capacity to infect humans has arisen numerous times during the evolution of the Onygenales (8).

This chapter covers the families Onygenaceae and Ajellomycetaceae, which include the classical dimorphic fungi *B. dermatitidis, H. capsulatum, P. braziliensis,* and *C. immitis* as well as *Emmonsia* species and nonpathogenic species of *Chrysosporium, Uncinocarpus,* and other related genera. It should be noted that, because the three species with *Ajellomyces* teleomorphs appear in the same clade, an argument has been made that all the anamorphs should be placed in the same genus. At this time, this proposal has not been implemented, in part due to the widespread usage of the anamorph names.

Histoplasma capsulatum

Historically, *H. capsulatum* was divided into three varieties: *H. capsulatum* var. *capsulatum,* a human pathogen found in North and South America; var. *duboisii,* a human pathogen found in Africa; and var. *farciminosum,* a pathogen of horses and mules found in parts of northern Africa and the Middle East. Recent phylogenetic studies have defined at least eight clades within *H. capsulatum:* North American class 1, North American class 2, Latin American group A, Latin American group B, Australian, Netherlands (Indonesian), Eurasian, and African clades (40). Seven of these eight clades comprise genetically distinct geographical populations that can be regarded as phylogenetic species. The single exception, the Eurasian clade, originated from within the Latin American group A clade. *H. capsulatum* var. *farciminosum* was placed within the Eurasian clade. In addition to the seven phylogenetic species, another seven lineages represented by single isolates from Latin America were identified (40). These may represent additional phylogenetic species.

At this time, African histoplasmosis, as defined by var. *duboisii,* is considered a distinct entity, but this view has been called into question by the finding of one var. *capsulatum* isolate from South Africa that was placed in the African (var. *duboisii*-containing) clade (40). This extends the results of earlier studies which had shown that var. *duboisii* had mitochondrial DNA restriction patterns identical to those of var. *capsulatum* strains (73).

Blastomyces dermatitidis

The genus *Blastomyces* is represented by the single species *B. dermatitidis*. Phylogenetic data show that, within the single branch occupied by the anamorphs of the three dimorphic *Ajellomyces* species, *B. dermatitidis* is most closely related to the genus *Emmonsia*. Isolates of *E. parva* are closer to *B. dermatitidis* than they are to *E. crescens*. The recognition

* This chapter contains information presented in chapter 118 by Thomas J. Walsh, Davise H. Larone, Wiley A. Schell, and Thomas G. Mitchell in the eighth edition of this Manual.

of this close relationship between *Blastomyces* and *Emmonsia* has suggested that they be consolidated into one genus. However, this proposal has not been implemented.

Coccidioides Species

Phylogenetic studies have led to the recognition of two species within the genus. The species name *C. immitis* is now restricted to isolates from California, while the new name *C. posadasii* has been proposed for all other isolates belonging to this genus (23). Zimmerman et al. (85) first divided *C. immitis* into two groups, named group I (non-CA) and group II (CA), after comparing restriction fragment length polymorphisms (RFLPs) within total genomic DNA. The genealogies of five nuclear genes were later used to confirm that *C. immitis* consists of two taxa, non-CA (Arizona, Texas, Mexico, and Argentina) and CA (California) (43). These two taxa did not interbreed, although genetic recombination had occurred between individuals within each of the two groups. Later, a more extensive population sample, including isolates from Venezuela, Mexico, and Brazil, was studied using a set of nine microsatellite markers (23). This study showed that two major clades could be distinguished. These clades, now named *C. posadasii* and *C. immitis,* respectively, correspond to the previous group I (non-CA) and group II (CA).

Paracoccidioides brasiliensis

This species has no known teleomorph, but sequence data suggest that, if one is found, it will be in the genus *Ajellomyces*. On phylogenetic trees showing the family Onygenaceae, Peterson and Sigler (60) and Bialek et al. (2), using internal transcribed spacer (ITS) and 18S sequences, respectively, were able to place this genus between *E. crescens* on one side and *E. parva* and *B. dermatitidis* on the other. Comparison of 18S and chitin synthetase sequences has indicated that *P. brasiliensis* is related to the uncultivable pathogen *Lacazia loboi,* the agent of lacaziosis (34). However, these sequences demonstrated sufficient differences that *L. loboi* was kept as an independent genus (34).

Emmonsia Species

The genus *Emmonsia* currently includes three species: *E. crescens, Emmonsia parva,* and *E. pasteuriana* (29, 60). The last organism has thus far been recovered from only a single case of disseminated cutaneous infection in an AIDS patient (29). *E. crescens* is known to form a sexual stage in the genus *Ajellomyces,* but *E. parva* has no known teleomorph.

DESCRIPTION OF AGENTS

Histoplasma capsulatum

H. capsulatum is a thermally dimorphic fungus, displaying a filamentous mold form in the environment and in culture at temperatures below 35°C and a yeast phase in tissue and at temperatures above 35°C. The mold phase may contain two types of conidia (Fig. 1). Macroconidia are thick-walled, with a diameter of 8 to 15 μm, and display characteristic tubercles or projections on their surfaces. Microconidia, smooth-walled with a diameter of 2 to 4 μm, are the infectious particles. The yeast phase develops as small oval budding cells with a diameter of 2 to 4 μm, often within macrophages (Fig. 2 and 3). The yeast cell found in African histoplasmosis is thick-walled and larger at 8 to 15 μm.

H. capsulatum is found in soils throughout the world. It grows best in soils with a high nitrogen content, particularly those enriched with bird or bat guano. Birds do not become colonized or infected with *H. capsulatum* (due to their high body temperatures), and their droppings are primarily a nutrient source. Soil samples from sites where birds have roosted have been found to remain contaminated for at least 10 years after the roost has been cleared.

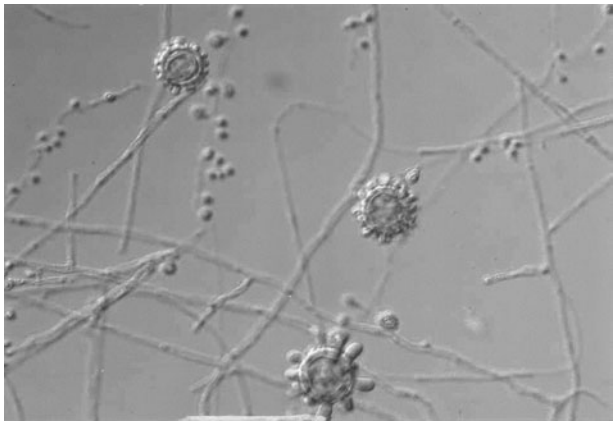


FIGURE 1 Mycelial phase of *H. capsulatum* showing tuberculate macroconidia and microconidia. Lactophenol cotton blue stain. Magnification, ×244.

Histoplasmosis is the most common endemic mycosis in North America, but it is also found throughout Central and South America. In the United States, the disease is most prevalent in states surrounding the Mississippi and Ohio Rivers, but foci of endemicity exist throughout the eastern half of the continent. Other regions of endemicity include parts of Africa, Australia, and eastern Asia, in particular India and Malaysia.

Blastomyces dermatitidis

B. dermatitidis is thermally dimorphic, converting from the mold phase to the yeast phase under appropriate conditions of temperature and nutrition. At room temperature, a floccose white mold can be recovered (Fig. 4). The microconidia are oval or pyriform (pear shaped), with a diameter of 2 to 10 μm; no macroconidia are produced. Large round thick-walled yeast cells, 5 to 15 μm in diameter, with broad-based budding daughter cells, are found in tissue and on appropriate media at 37°C (Fig. 5). Yeast cells may occur inside or outside macrophages.

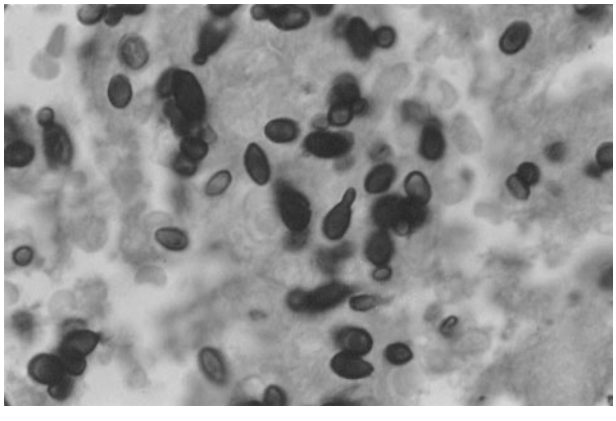


FIGURE 2 GMS stain showing blastoconidia of *H. capsulatum*. Magnification, ×285.

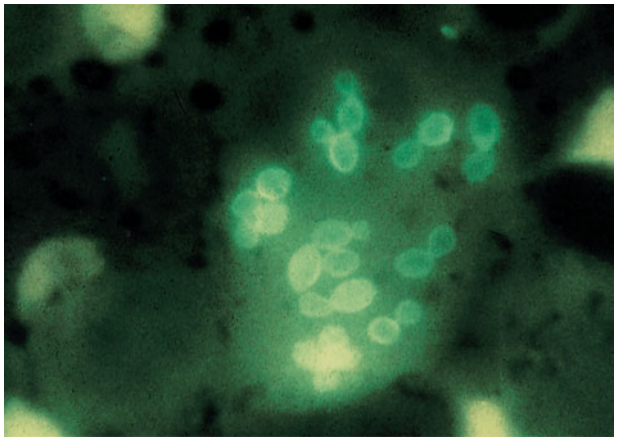


FIGURE 3 Calcofluor white wet mount of sputum showing blastoconidia of *H. capsulatum*. Magnification, ×475. (American Society of Clinical Pathology.)

The natural habitat of *B. dermatitidis* is the soil. It appears to survive best in moist acidic soils that contain a high nitrogen and organic content. Higher soil temperatures and recent rainfall facilitate growth of the fungus.

The largest number of cases of blastomycosis has been reported from North America, but the disease is also endemic in Africa and parts of Central and South America. In the United States, the organism is most commonly found in states surrounding the Mississippi and Ohio Rivers; in Canada, the disease occurs in the provinces that border the Great Lakes.

Coccidioides Species

In the environment and in culture at room temperature, the fungus exists as a mold producing septate hyphae and arthroconidia that usually develop in alternate hyphal cells (Fig. 6). As the arthroconidia mature, the alternating disjunctor cells undergo lytic degradation, releasing the barrel-shaped arthroconidia, approximately 2 to 5 μm, which are the infectious particles. Inside the host and on special media, the arthroconidia transform into a structure called a spherule. A spherule is a large (up to 120 μm in diameter),

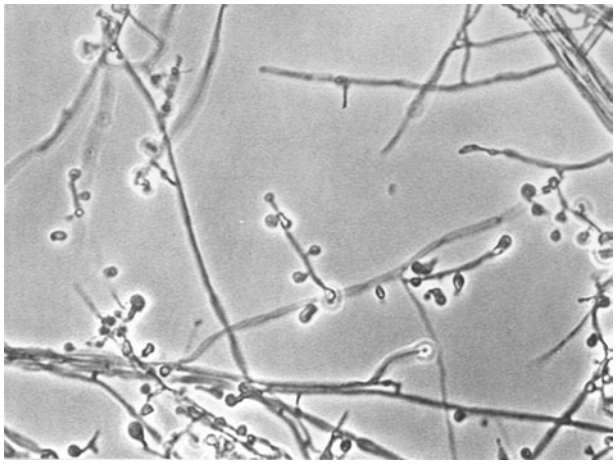


FIGURE 4 Mycelial phase of *B. dermatitidis*. Magnification, ×240.

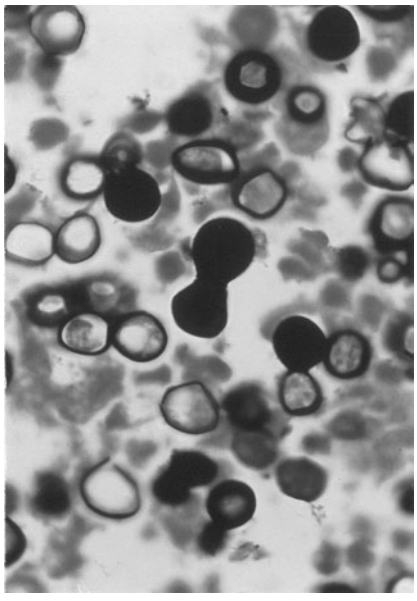


FIGURE 5 Blastoconidia of *B. dermatitidis*. GMS stain. Magnification, ×285.

thick-walled spherical structure containing many internal endospores, each approximately 2 to 4 μm, which can be released if the spherule ruptures (Fig. 7). Each endospore can develop into a spherule as well, continuing the process within the host.

Coccidioides is a soil-inhabiting fungus with a restricted geographical distribution. It is confined to regions of the Western Hemisphere that correspond to the lower Sonoran desert life zone. In the United States, the region of endemicity includes central and southern California, southern Arizona, southern New Mexico, part of Utah, and western Texas. The region of endemicity extends southwards into the desert regions of northern Mexico and parts of Central and South America.

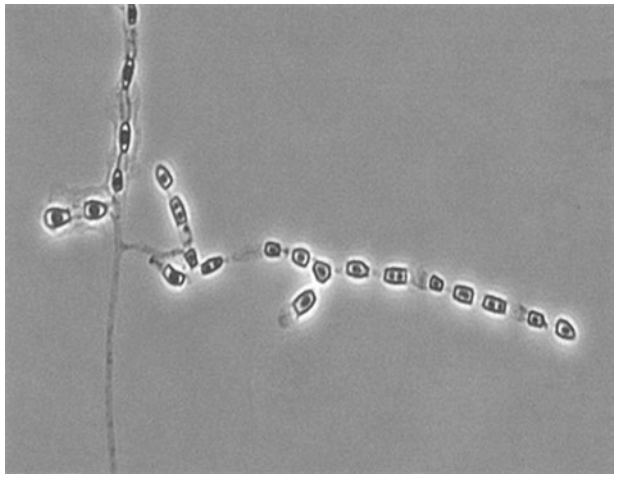


FIGURE 6 Mycelial phase of *Coccidioides immitis* showing alternating arthroconidia. Lactofuchsin stain. Magnification, ×248.

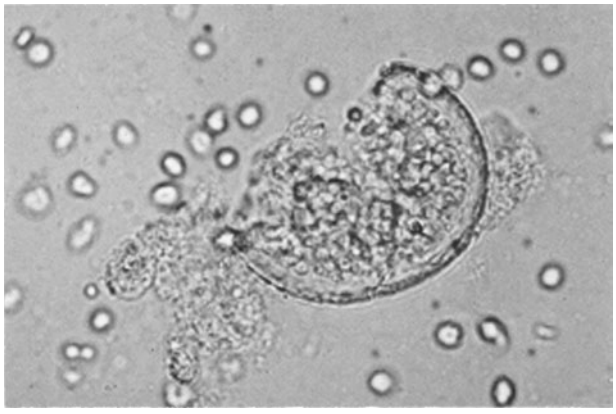


FIGURE 7 KOH preparation of pus from a lesion showing *Coccidioides* spherule and endospores. Magnification, ×285. (Reprinted from S. Verghese, D. Arjundas, K. C. Krishnakumar, P. Padmaja, D. Elizabeth, A. A. Padhye, and D. W. Warnock, *Med. Mycol.* **40:**307–309, 2002, with permission.)

Paracoccidioides brasiliensis

This organism is a thermally dimorphic fungus. At room temperature, *P. brasiliensis* grows as a mold. Growth requires a lengthy incubation of up to 30 days. Isolates incubated on rich media produce thin septate hyphae with sparse terminal and intercalary chlamydospores, but no conidia. Under conditions of nutritional deprivation, some isolates produce conidia, which vary in structure from arthroconidia to microconidia of less than 5 μm. Conidia transform into yeast cells under appropriate nutritional conditions and at a temperature of approximately 37°C. Yeast cells are mostly oval and characteristically display a mother cell surrounded by multiple buds, referred to as a "pilot's wheel" appearance (Fig. 8).

Although *P. brasiliensis* has been isolated from soil, understanding of its precise environmental habitat remains limited. The region of endemicity extends from Mexico to Argentina, but the disease is more prevalent in South America than Central America. The greatest number of reported cases has come from Brazil, Venezuela, and Colombia. The disease is most frequently acquired in regions classified as subtropical upland rain forests, such as the Amazon river basin in Brazil.

Emmonsia Species

In the environment and in culture at room temperature, *Emmonsia* species exist as a mold that produces small, single-celled conidia (about 4 μm) on the sides of the hyphae or on short side branches. Inside the host, the conidia of *E. crescens* and *E. parva* transform into structures termed adiaspores, which resemble the spherules of *Coccidioides* species (Fig. 9). Although adiaspores enlarge to become enormous, thick-walled structures, no endospores are produced and the spores eventually die. Adiaspores can also be generated in vitro using rich media. *E. crescens* and *E. parva* are largely indistinguishable: the former produces large (20- to 140-μm) multinucleate adiaspores at 37°C, whereas the latter produces smaller (8- to 20-μm) adiaspores at 40°C. *E. pasteuriana* does not produce adiaspores in tissue; rather, at 37°C on rich media it produces structures that resemble budding yeast cells.

The natural habitat of *Emmonsia* species is the soil. Infection has occurred in numerous species of burrowing mammals. Human cases are rare but have been reported from North, Central, and South America, as well as from several European and Asian countries.

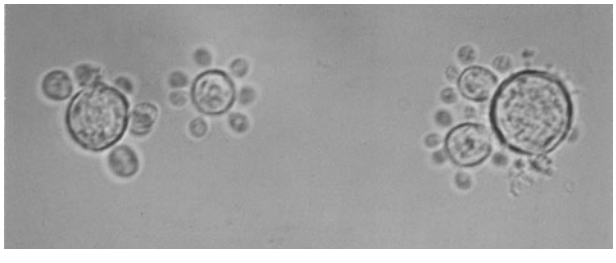


FIGURE 8 Wet mount showing blastoconidia of *P. brasiliensis*. Magnification, ~×290.

EPIDEMIOLOGY AND TRANSMISSION

Histoplasmosis

Inhalation of microconidia of *H. capsulatum* is the usual mode of infection in humans. The incubation period is 1 to 3 weeks. In cases of reinfection, the incubation period appears to be shorter (4 to 7 days after exposure). Histoplasmosis is not contagious, but there have been occasional reports of transmission from an organ donor to a recipient (45).

The major risk factor for infection is environmental exposure. The risk depends on several factors including the nature of the environmental site, the activities performed, and the duration and degree of dust or soil exposure. Longer and more intense exposures usually result in more severe pulmonary disease. Most reported outbreaks have been associated with exposures to sites contaminated with *H. capsulatum* or have followed activities that disturbed accumulations of bird or bat guano.

Persons with underlying illnesses are at increased risk for some forms of histoplasmosis. Disseminated infection is more common among individuals with underlying cell-mediated immunological defects, including those with human immunodeficiency virus (HIV) infection, and transplant recipients. Immunocompromised persons with histoplasmosis have a higher mortality rate than those who are not immunosuppressed.

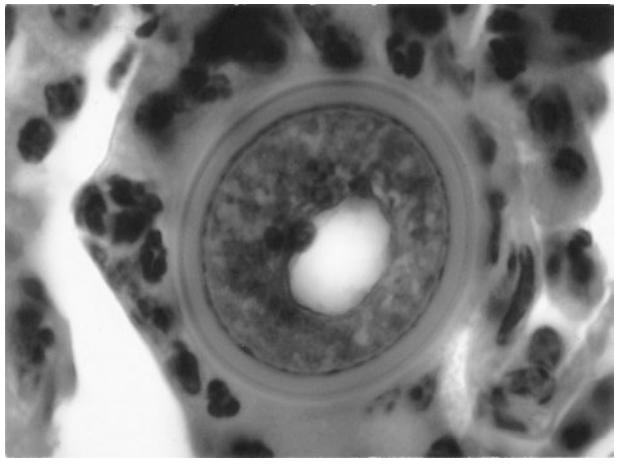


FIGURE 9 *E. parva* adiaspore in mouse tissue. PAS stain. Magnification, ~×280.

Blastomycosis

Inhalation of conidia is the usual mode of infection leading to blastomycosis. The incubation period has been estimated to be 4 to 6 weeks. Blastomycosis is not contagious.

Outbreaks have been associated with occupational and recreational activities, often along streams or rivers, and have resulted from exposures to moist soil enriched with decaying vegetation. Apart from outbreaks, blastomycosis is more commonly seen in adults than children. More men than women are affected. The disease often occurs in individuals with an outdoor occupation or recreational interest.

B. dermatitidis is uncommon as an opportunistic pathogen, but it causes more aggressive disease in persons with underlying cell-mediated immunological defects, such as those with HIV infection and transplant recipients. Immunocompromised persons with blastomycosis have a higher mortality rate than those who are not immunosuppressed.

Coccidioidomycosis

Inhalation of arthroconidia is the usual mode of infection leading to coccidioidomycosis in humans. The incubation period is 1 to 3 weeks. In contrast to what is observed with histoplasmosis, once individuals have recovered from *Coccidioides* infection, they are usually immune to reinfection. The infection is not contagious, but occasional person-to-person spread has occurred via contaminated fomites (20) or by transmission from an organ donor to a recipient (83).

The major risk factor for infection is environmental exposure. The risk depends on a number of factors including the nature of the environmental site, the activities performed, and the duration and degree of dust or soil exposure. Infection has been associated with ground-disturbing activities, such as building construction, landscaping, farming, archaeological excavation, and numerous recreational pursuits. Natural events that result in the generation of dust clouds, such as earthquakes and windstorms, have been associated with an increased risk of infection and have resulted in large outbreaks.

Disseminated infection is more common among those of black, Asian, or Filipino race and among pregnant women in the third trimester. Individuals with underlying cell-mediated immunological defects, such as those with AIDS and those receiving immunosuppressive medications, are also at increased risk of disease dissemination.

Paracoccidioidomycosis

Inhalation of conidia is the usual mode of infection leading to paracoccidioidomycosis in humans. The incubation period is unknown, but it is clear that the fungus can remain dormant for very long periods in the lymph nodes following asymptomatic primary infection.

Paracoccidioidomycosis is rare in children, accounting for around 5% of all reported cases. More than 90% of cases of symptomatic disease occur in men, most of whom have outdoor occupations: estrogen-mediated inhibition of the mold-to-yeast transformation could help to account for this (61). In recent years there has been an increase in the number of cases involving immunocompromised patients, including persons with HIV infection.

Adiaspiromycosis

Inhalation of conidia is the usual mode of infection leading to adiaspiromycosis in animals and humans. The incubation period is unknown. No risk factors have been identified.

CLINICAL SIGNIFICANCE

Histoplasmosis

There is a wide spectrum of clinical manifestations of histoplasmosis, ranging from a transient pulmonary infection which subsides without treatment to chronic pulmonary infection or to more widespread disseminated disease. Many healthy individuals develop no symptoms when exposed to *H. capsulatum* in a setting of endemicity. Higher levels of exposure result in an acute symptomatic and often severe flu-like illness. The symptoms, which include fever, chills, headache, nonproductive cough, myalgia, pleuritic chest pain, loss of appetite, and fatigue, usually disappear within a few weeks. The most severe and rarest form of this disease is disseminated histoplasmosis. The clinical manifestations range from an acute illness that is fatal within a few weeks if left untreated (often seen in infants, persons with AIDS, and solid-organ transplant recipients) to an indolent, chronic illness that can affect a wide range of sites.

Hepatic infection is common in nonimmunosuppressed individuals with disseminated histoplasmosis, and adrenal gland destruction is a frequent problem. Mucosal ulcers are found in over 60% of these patients. The mouth and throat are often affected, but lesions also occur on the lip, nose, and other sites. Central nervous system (CNS) disease occurs in 5 to 20% of patients, presenting as chronic meningitis or focal brain lesions. In persons with AIDS, disseminated histoplasmosis is usually associated with low CD4 T-lymphocyte counts and presents with nonspecific symptoms, such as fever and weight loss. Mucosal lesions are uncommon, but multiple cutaneous lesions may be present. CNS involvement occurs in 10 to 20% of cases.

African Histoplasmosis

The clinical manifestations of *H. capsulatum* var. *duboisii* infection (African histoplasmosis) differ from those of var. *capsulatum* infection. The illness is indolent at the onset, and the predominant sites affected are the skin and bones. Those with more widespread infection involving the liver, spleen, and other organs have a febrile wasting illness that is fatal within weeks or months if left untreated. Multiple cutaneous lesions often develop on the face and trunk. These often enlarge and ulcerate. Osteomyelitis occurs in about 30% of patients. The infection may spread into contiguous joints, causing arthritis, or into adjacent soft tissue, causing a purulent subcutaneous abscess.

Blastomycosis

There is a wide spectrum of clinical manifestations of blastomycosis, ranging from a transient pulmonary infection which subsides without treatment to chronic pulmonary infection or to more widespread disseminated disease. Acute pulmonary blastomycosis usually presents as a nonspecific influenza-like illness, similar to that seen with histoplasmosis or coccidioidomycosis. Most otherwise healthy persons recover after 2 to 12 weeks of symptoms, but some return months later with infection of other sites. Other patients with acute blastomycosis fail to recover and develop chronic pulmonary disease or disseminated infection.

The skin and bones are the most common sites of extrapulmonary disease. The skin is involved in more than 70% of cases, the lesions presenting either as raised verrucous lesions with irregular borders or as ulcers. The latter can also appear on the mucosa of the nose, mouth, and throat. Osteomyelitis occurs in about 30% of patients with disseminated infection,

the spine, ribs and long bones being the most common sites of involvement. Arthritis occurs in about 10% of patients. Meningitis is rare, except in immunocompromised individuals. Other organs, such as the male genitourinary tract, adrenal glands, thyroid, liver, spleen, and gastrointestinal tract, are sometimes involved.

Coccidioidomycosis

There is a wide clinical spectrum of manifestations of coccidioidomycosis, ranging from a transient pulmonary infection which resolves without treatment to chronic pulmonary infection or to more widespread disseminated disease. About 40% of newly infected persons develop an acute symptomatic and often severe flu-like illness. Higher rates of symptomatic infection (50 to 100%) have often been reported during outbreaks. The symptoms include fever, headache, chills, nonproductive cough, myalgia, and pleuritic chest pain. Up to 50% of patients develop a mild, diffuse erythematous or maculopapular rash, covering the trunk and limbs, within the first few days of the onset of symptoms. More dramatic and persistent is the rash of erythema nodosum or erythema multiforme, which occurs in about 5% of infected persons but is more common in women. Most otherwise healthy persons will recover without treatment, their symptoms disappearing in a few weeks.

Fewer than 1% of infected individuals develop disseminated coccidioidomycosis. This is a progressive disease that usually develops within 3 to 12 months of the initial infection, although it can occur much later, following reactivation of a quiescent infection in an immunosuppressed individual. The clinical manifestations range from a fulminant illness that is fatal within a few weeks if left untreated to an indolent chronic disease that persists for months or years. One or more sites may be involved, but the skin, soft tissue, bones, joints, and meninges are most commonly affected. Cutaneous and subcutaneous lesions are common. Osteomyelitis occurs in about 40% of patients, with the spine, ribs, cranial bones, and ends of the long bones being the most common sites. The disease may spread into contiguous joints, causing arthritis, or into adjacent soft tissue, causing subcutaneous abscess formation. Meningitis is the most serious complication of coccidioidomycosis, occurring in 30 to 50% of patients with disseminated disease. Without treatment, it is almost always fatal.

Paracoccidioidomycosis

The lungs are the usual initial site of *P. brasiliensis* infection, but the organism then spreads through the lymphatics to the regional lymph nodes. In most cases the primary infection is asymptomatic. However, children and adolescents sometimes present with an acute disseminated form of infection in which superficial and/or visceral lymph node enlargement is the major manifestation. This presentation is also seen in immunocompromised patients. It has a poor prognosis.

Most adults with paracoccidioidomycosis present with chronic progressive infection. In 80% of cases the disease involves the lungs. In some, it spreads to involve the mucosa, skin, and other organs. The disease is slowly progressive and may take months or even years to become established. Ulcerative mucocutaneous lesions of the face, mouth, and nose are the most obvious presenting sign. Other sites of infection include the small or large intestine, liver and spleen, adrenal glands, bones and joints, CNS, and male genitourinary tract.

Adiaspiromycosis

In most cases, adiaspiromycosis is a self-limited, localized pulmonary infection with few or no symptoms. Because the adiaspores enlarge but do not reproduce, symptoms and clinical signs appear to depend upon the number of conidia inhaled. Some patients have presented with nonproductive cough, dyspnea, and fever. A few cases of fatal, multifocal pulmonary disease have been reported.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Clinical Specimens

Methods of collection, transport, and storage of specimens are detailed in chapter 116. Tissue samples should be obtained when appropriate and should be divided and submitted for microbiological and histopathological examination. If possible, special tissue stains should be requested, such as the Grocott-Gomori methenamine silver (GMS) and periodic acid-Schiff (PAS) stains. Since colonization with the dimorphic fungal pathogens does not occur, their microscopic detection and/or isolation in culture is consistent with a firm diagnosis.

Histoplasma capsulatum

H. capsulatum can be isolated from sputum or bronchoalveolar lavage fluid specimens in 60 to 85% of cases of chronic pulmonary histoplasmosis if multiple specimens are tested (77). In disseminated disease, useful specimens for culture include blood, urine, lymph node, and bone marrow samples. Bone marrow cultures are positive in over 75% of cases. Blood cultures collected with the Isolator lysis-centrifugation system (Wampole Laboratories, Princeton, N.J.) or Bactec Mycolytic bottles (BD Diagnostic Systems, Franklin Lakes, N.J.) show the greatest sensitivity. Biopsy specimens of oral, cutaneous, and gastrointestinal lesions, adrenal glands, or liver and spleen have also provided a diagnosis. *Histoplasma* meningitis is difficult to diagnose, cerebrospinal fluid (CSF) cultures being positive in no more than two-thirds of cases. The best results are obtained when large volumes of CSF (10 to 20 ml) are cultured on multiple occasions.

Blastomyces dermatitidis

Sputum samples, bronchoalveolar lavage fluid, or lung biopsy specimens may be submitted for isolation of *B. dermatitidis*. Skin biopsy specimens are useful in the diagnosis of cutaneous disease. Collection of urine after prostatic massage may be helpful in the diagnosis of genitourinary blastomycosis.

Coccidioides Species

In addition to lower respiratory tract samples, material for microscopy and culture can be collected from suppurative cutaneous and soft tissue lesions for isolation of *Coccidioides* species. Organisms can be recovered only infrequently from CSF, and usually only after culture of large volumes (10 to 20 ml).

Paracoccidioides brasiliensis

In addition to lower respiratory samples, material can be collected from oral or pharyngeal lesions, cutaneous lesions, lymph nodes, adrenal glands, and the gastrointestinal tract for detection of *P. brasiliensis.*

Emmonsia Species

Sputum and bronchoalveolar lavage fluids are seldom culture positive in patients with disease caused by *Emmonsia* species. The diagnosis is usually based on histopathologic demonstration of adiaspores in lung tissue.

DIRECT EXAMINATION

Microscopy

Direct microscopic examination of clinical materials may provide a rapid presumptive diagnosis of a systemic fungal infection. However, it is important to appreciate that tissue form cells of *H. capsulatum* and *B. dermatitidis* can appear similar to each other, as well as to yeast cells of various *Candida* species, *Cryptococcus neoformans* and *Penicillium marneffei,* and endospores of *C. immitis*. It is often helpful to stain fresh, wet preparations of sputum, bronchoalveolar lavage fluid, CSF, urine, pus, or other material with calcofluor white, a fluorescent compound that binds to the fungal cell wall.

Histoplasma capsulatum

Giemsa and Wright's stains can be used to detect yeast cells of *H. capsulatum* in blood or bone marrow smears. These cells can also be seen in tissue sections stained with GMS or PAS, but usually not with hematoxylin and eosin (H&E). Detection of the small (2- to 4-μm) oval budding yeasts allows a presumptive diagnosis of histoplasmosis. Organisms can be found within macrophages or free in the tissues. It is unusual to find yeasts on cytological examination of sputum or other respiratory tract fluids. The thick-walled, narrow-based budding yeast cells of *H. capsulatum* var. *duboisii,* approximately 10 to 15 μm in diameter, are about fourfold larger than those of *H. capsulatum* var. *capsulatum* in tissue sections.

Blastomyces dermatitidis

Direct calcofluor white or KOH mounts or Gram stains of sputum, tissues, and exudates will often permit the detection of the large round yeast cells of *B. dermatitidis*. The broad-based buds often attain the same size as the parent cells before becoming detached. Histopathologic examination using PAS or GMS stains also can be of value.

Coccidioides Species

Tissue sections should be stained with PAS, GMS, or H&E to permit the detection of the characteristic large, thick-walled spherules of *Coccidioides* species. Microscopic examination of wet preparations of sputum, bronchoalveolar lavage fluid, pus, etc., treated with KOH is also helpful but less sensitive. *Prototheca wickerhamii* may resemble small spherules, and *Rhinosporidium seeberi* may simulate larger ones.

Paracoccidioides brasiliensis

The characteristic yeast cells with multiple buds of *P. brasiliensis* can often be found on direct microscopic examination of sputum, bronchoalveolar lavage fluid, pus from draining lymph nodes, or tissue biopsy specimens. Staining of wet preparations with lactophenol cotton blue, methylene blue, Gram's stain, or calcofluor white can be helpful. Tissue sections can be stained with PAS, GMS, or H&E.

Emmonsia Species

Tissue sections stained with PAS or GMS are most helpful in demonstrating the characteristic adiaspores of *Emmonsia* species in lung tissue. It is important to appreciate that in the chronic stage, the organism may collapse and thus form various shapes that may resemble other fungi, helminths, or pollen grains. Adiaspores must also be distinguished from the spherules of *Coccidioides*. Adiaspores do not contain endospores.

Antigen Detection

Histoplasma capsulatum

The *Histoplasma* polysaccharide antigen (HPA) test is a microtitration plate-based double-antibody sandwich enzyme immunoassay (EIA) to detect antigenuria and antigenemia in cases of disseminated histoplasmosis (18). This test is also useful in the early diagnosis of acute pulmonary histoplasmosis (82). No kits or reagents are commercially available for purchase, but this test is performed on a fee-for-service basis by MiraVista Diagnostics (Indianapolis, Ind.; http://www.miravistalabs.com).

Ten milliliters of urine, 5 ml of serum, or 1 ml of CSF is the preferred volume for testing, although a minimum volume of 0.5 ml of any specimen is required for a single test. To obtain maximum test sensitivity, it is recommended that both serum and urine specimens be tested in parallel. Treatment success or failure may be assessed by collecting specimens at least 14 days after starting treatment and testing the newly acquired samples in parallel with the last specimen that was positive before initiation of treatment.

Blastomyces dermatitidis

The *Blastomyces* antigen test is a microtitration plate-based double-antibody sandwich EIA to detect antigenuria and antigenemia in disseminated blastomycosis (19). No kits or reagents are commercially available for purchase, but this test is performed on a fee-for-service basis by MiraVista Diagnostics (http://www.miravistalabs.com). The sample requirements are the same as those for the HPA test.

Coccidioides Species

Galgiani et al. (24) reported the detection of circulating antigen in sera from 35 patients with coccidioidomycosis, 33 of whom had no detectable antibodies to *C. immitis* at that time. Antigen was detected most frequently in sera obtained within the first month of the onset of symptoms. At present, coccidioidomycosis antigen testing is not available as a routine diagnostic test.

Paracoccidioides brasiliensis

Mendez-Giannini et al. (53) demonstrated that a 43-kDa glycoprotein antigen of *P. brasiliensis* could be detected in the sera of patients with both the acute and chronic forms of paracoccidioidomycosis. Later, Gomez et al. (25) described an inhibition enzyme-linked immunosorbent assay (ELISA), which utilized a monoclonal antibody directed against an 87-kDa heat shock protein antigen of *P. brasiliensis*. Several reports have described the detection of *P. brasiliensis* antigen in urine, CSF, and bronchoalveolar fluid samples (47, 65). Others have noted that antigen levels in serum diminished or even disappeared during successful treatment (26, 48). At present, paracoccidioidomycosis antigen testing is not available as a routine diagnostic test.

Emmonsia Species

No serologic tests exist at this time for the diagnosis of adiaspiromycosis in humans.

Nucleic Acid Detection in Clinical Materials

No commercially available systems exist for the detection of fungal nucleic acid in clinical materials. However, several methods are currently under investigation. Conserved regions of the rRNA genes have been used as targets in a number of PCR-based detection assays. It is important to appreciate that amplification of conserved genes can result

in products derived both from pathogenic fungi and from genetically related nonpathogenic fungal species. These nonspecific nucleic acids could arise from colonization of the original sample with saprophytic organisms, from contamination during sample collection, or from contamination of PCR reagents with fungal DNA. It is very important that the identity of amplicons detected using conserved genes be verified by direct sequencing (7, 54). In more recent studies, genes specific for the fungus of interest are now being chosen as PCR targets, thus eliminating this specificity problem.

Histoplasmosis

Several publications have described the use of PCR to detect *H. capsulatum* DNA in fixed paraffin-embedded tissue samples (64, 67, 71), in blood or tissue samples (9), and in clinical materials from three patients with histoplasmosis (49). Imhof et al. (37) used a real-time PCR to detect *H. capsulatum* DNA in a culture-positive bone marrow sample; the amplicon was then sequenced to verify the specificity of the product. Bialek et al. designed a nested PCR that targeted the gene coding for a unique 100-kDa protein of *H. capsulatum* (4). This assay was also used to detect *H. capsulatum* DNA in several clinical samples from a patient with disseminated histoplasmosis (63).

Blastomycosis

A nested-PCR assay targeting the WI-1 gene has been described for the detection of *B. dermatitidis* DNA in paraffin-embedded tissue (5). The PCR amplified DNA from 8 of 13 tissue samples in which yeast cells were detected by microscopy, and all products were homologous to the WI-1 gene on direct sequencing.

Coccidioidomycosis

A PCR assay using *Coccidioides*-specific primers derived from the ITS region of rRNA was used to amplify DNA from sera of mice infected with *C. posadasii* and from sera of seropositive patients (39). In another study, a PCR directed at the antigen 2/proline-rich antigen gene was used to detect *C. posadasii* DNA in three human paraffin-embedded tissue samples that were microscopically positive for spherules (6).

Paracoccidioidomycosis

A nested-PCR assay targeting the immunogenic *gp43* gene was evaluated for the detection of *P. brasiliensis* DNA in lung homogenates from infected and uninfected mice (3).

ISOLATION PROCEDURES

Biosafety

Laboratory-associated infections with *Coccidioides* species, *H. capsulatum,* and *B. dermatitidis* have occurred in healthy immunocompetent individuals after exposure to airborne conidia (74). These can be released from contaminated soil samples as well as from sporulating mold phase cultures of the organisms. Even the mere lifting of a culture plate lid is often sufficient to cause the release of large numbers of conidia into the air. Should a sporulating culture be dropped, millions of conidia will be dispersed. It is also important to note that local infections including granuloma formation have been reported following accidental percutaneous inoculation during injection of laboratory animals, or while performing autopsies of humans with histoplasmosis, coccidioidomycosis, or blastomycosis. No cases of laboratory-acquired *P. brasiliensis* infection have been reported.

All procedures involving the manipulation of sporulating cultures of *Coccidioides* species and *H. capsulatum* and for processing soil or other environmental materials known or likely to contain these organisms should be performed inside a class II biological safety cabinet under conditions of biosafety level 3 containment. Biosafety level 2 practices and facilities are recommended for handling and processing clinical specimens and animal tissues (62). Hyaline (colorless) molds of unknown identity should always be examined and manipulated inside a class II biosafety cabinet. Such molds should never be handled on an open laboratory bench.

Biosecurity

In the United States, both species of *Coccidioides* are classified as select agents. The select agent regulations (42 C.F.R. Part 73, 7 C.F.R. Part 331, and 9 C.F.R. Part 121) govern the possession, use, and transfer of these agents. Any entity in the United States that possesses, uses, or transfers isolates that are known to be *Coccidioides* species must have a certificate of registration issued by the Secretary of the Department of Health and Human Services (DHHS). Isolates that are known to be *Coccidioides* may be transferred only between registered laboratories. In addition, individuals may not handle or possess isolates that are known to be *Coccidioides* species unless they have been approved by DHHS based on a security risk assessment from the U.S. Attorney General. Clinical or diagnostic laboratories that possess, use, or transfer an isolate suspected to be *Coccidioides* that is contained in a specimen presented for diagnosis, verification, or confirmation are exempt from these requirements. These exempt laboratories must destroy or transfer the isolate within seven calendar days of identification of the agent. The exempt laboratory, as well as the registered laboratory, must submit a completed APHIS/CDC Form 4 within seven calendar days of identification, specifying the final status of the isolate. Application forms, Form 4, and a copy of the regulations can be found at http://www.cdc.gov/od/sap.

Culture for Mold Phase

In general, the organisms discussed in this chapter can be readily cultivated in the mold phase on general fungal media such as Sabouraud dextrose agar or potato dextrose agar incubated at 25°C. Incubation at 37°C is also helpful to recover the yeast phase of dimorphic organisms. Media containing antibiotics such as chloramphenicol or gentamicin should be used when culturing clinical materials, such as sputum, that may be contaminated with bacteria. Media containing cycloheximide are useful to inhibit saprophytic fungi and to provide a useful differential tool in identification. Many unrelated saprophytic soil fungi fail to grow on media containing cycloheximide, fail to grow altogether at 37°C, or fail to convert to the yeast phase at 37°C. Screw-capped slants are preferable to plates for culturing dimorphic fungi. If plates are used, they should be sealed so that mold spores cannot escape into the ambient air. Seals that are permeable to air such as Shrink Seals (Scientific Device Laboratory, Des Plaines, Ill.) are useful for this purpose. In general, colonies develop within 3 to 7 days, but some strains of *H. capsulatum* may require incubation for as much as 4 to 6 weeks.

Culture for Yeast Phase

H. capsulatum, B. dermatitidis, and *P. brasiliensis* can be recovered in the yeast phase by using appropriate media incubated at 37°C. *Coccidioides* species do not have a yeast phase. *E. parva* and *E. crescens* produce adiaspores at elevated temperatures.

Histoplasma capsulatum

The yeast phase of *H. capsulatum* can be recovered using rich media such as brain heart infusion agar (BHI) or BHI with blood (BHIB). Plates or slants should be incubated at 37°C under aerobic conditions for at least 4 weeks.

Blastomyces dermatitidis

The yeast phase of *B. dermatitidis* can be recovered using rich media such as BHI, BHIB, Pine's medium, or Kelley's agar, incubated at 37°C under aerobic conditions. Yeasts are usually visible within 1 week, but media should be held for at least 4 weeks before being discarded.

Paracoccidioides brasiliensis

The yeast phase can be recovered using media such as BHI, Pine's medium, or Kelley's agar, incubated at 37°C. The organism grows slowly, and plates or slants should be held for at least 4 weeks.

Emmonsia Species

E. parva and *E. crescens* produce adiaspores in vitro when cultivated on phytone yeast extract agar, BHI, or BHIB at 37 to 40°C depending on the species. *E. pasteuriana* produces yeast-like cells after culture on BHI at 37°C for approximately 10 days.

IDENTIFICATION

In general, these organisms are identified by their characteristic morphologic features, by conversion to the yeast phase, and/or by DNA probe testing. Slide cultures should not be performed for suspected dimorphic isolates due to the possibility of laboratory infection from accidental inhalation of infectious conidia.

Histoplasma capsulatum

The mold phase of *H. capsulatum* can be recovered after incubation at 25°C. The colony is initially white or buff-brown. Both types may be isolated from the same patient, and eventually the brown type may convert to the white type. The brown type generally produces more of the characteristic tuberculate macroconidia than the white type. On subculture, only about 30% of macroconidia show tubercles. Microconidia are abundant in fresh isolates of *H. capsulatum*. After multiple subcultures, the production of both macroconidia and microconidia may be diminished. The presence of both macroconidia and microconidia is not required for identification of *H. capsulatum,* as authentic *H. capsulatum* isolates that fail to produce either macroconidia or microconidia have been recognized. Macroconidia but no microconidia can also be seen in the saprophytic fungus *Sepedonium,* as well as in the related fungus *Renispora flavissima*. Authentic *H. capsulatum* isolates can be recognized by their thermal dimorphism, as well as by their growth on inhibitory mold agar.

Once the characteristic morphology has been recognized, mold phase *H. capsulatum* isolates can be confirmed by conversion to the yeast phase. The isolate is transferred to BHI or BHIB agar and incubated at 37°C for at least 7 to 10 days. Hyphal cells may form buds directly or develop enlarged, transitional cells that subsequently begin to bud. The microconidia may also convert to budding yeast cells. Complete conversion rarely is achieved, and multiple transfers to fresh BHI or BHIB medium may be required. The colony will develop a white, smooth, yeast-like appearance, and microscopic examination will reveal oval, budding yeasts approximately 1 to 3 by 3 to 5 μm. The cells have a narrower base of attachment between the bud and parent cell than do those of *B. dermatitidis*.

The Gen-Probe AccuProbe test (GenProbe, San Diego, Calif.) can also be used to confirm isolates as *H. capsulatum.* This test requires actively growing cultures: mold phase cells not more than 4 weeks of age or yeast cells no older than 1 week. Isolates can be taken from solid media or broth cultures. A portion of the isolate or the broth culture is added to lysis and hybridization buffers in a tube containing glass beads. After sonication and denaturation steps, the mixture is incubated with the acridinium ester-labeled *H. capsulatum* hybridization probe, which binds to *H. capsulatum* rRNA if the latter is present in the sample. Following a short incubation to degrade unbound probe, formation of specific DNA-RNA hybrids is quantitated in relative light units by using a luminometer. Extracts that display relative light unit values greater than 50,000 are considered positive.

The GenProbe test has largely replaced exoantigen testing for identification of *H. capsulatum*. Several studies have shown that the GenProbe method is sensitive and specific for identification of *H. capsulatum* (31, 35, 56, 68), although false-positive results can be obtained when isolates that are genetically related to the Ajellomycetaceae are tested (10).

The colonial morphology of *H. capsulatum* var. *duboisii* is indistinguishable from that of non-African isolates of *H. capsulatum*. Isolates of *H. capsulatum* var. *duboisii* also display positive results in the GenProbe test for *Histoplasma capsulatum* (56). Histopathologic examination of tissue forms is essential to distinguish the two varieties.

Blastomyces dermatitidis

At 25 to 30°C, isolates of *B. dermatitidis* produce a variety of forms ranging from a fluffy white colony that is visible within 2 to 3 days to a glabrous, tan, nonconidiating colony that grows more slowly. Microscopic examination shows microconidia that are oval or pyriform, usually smooth-walled, and formed on short lateral or terminal branches along the hyphae. *B. dermatitidis* also grows readily on inhibitory media containing cycloheximide. Conidia of the hyaline hyphomycete *Scedosporium apiospermum,* of some *Chrysosporium* species, and of the dermatophyte *Trichophyton rubrum* are morphologically similar and can be mistaken for *B. dermatitidis*. These species either fail to grow at 37°C (some *Chrysosporium* species) or grow as mold when incubated at 37°C *(S. apiospermum* and *T. rubrum)*.

The identification can be confirmed by conversion to the yeast phase. Generally, isolates of *B. dermatitidis* convert readily to the yeast phase on BHI, BHIB, or Pine's or Kelley's medium incubated at 37°C. Yeast cells are hyaline, smooth- and thick-walled, generally 8 to 15 μm in diameter, with the bud connected to the parent cell by a broad base of up to 4 to 5 μm in diameter. Conversion can be accomplished in 2 to 3 days, although occasional isolates may take several weeks.

Identification can also be confirmed using the GenProbe AccuProbe reagent for *B. dermatitidis*. False-positive *Blastomyces dermatitidis* Gen-Probe results were obtained with *P. brasiliensis* (57), so care must be taken to distinguish these organisms. An isolate of *Gymnascella hyalinospora* also cross-reacted with the *B. dermatitidis* probe (38).

Coccidioides Species

At 25 to 30°C, isolates of *Coccidioides* species display considerable variation in colonial morphology. Colonies can range from moist, glabrous, and grayish to abundant, floccose, and white. Colonies may become tan with age. Microscopic

examination shows hyphae that are thin and septate, with fertile (spore-producing) hyphae usually arising at right angles. Arthroconidia are hyaline, one celled, short, cylindrical to barrel shaped, moderately thick walled, smooth walled, and 2 to 8 by 3 to 5 μm. Arthroconidia alternate with thin-walled empty disjunctor cells. At maturity, the disjunctor cells undergo lytic degradation, releasing the arthroconidia. After this fragmentation, the arthroconidia may display frill-like remains of the disjunctor cells on each end. Isolates of *Coccidioides* grow well on inhibitory mold agar containing cycloheximide, which helps to distinguish this organism from similar soil saprophytes such as *Malbranchea* species. It is also important to distinguish true alternate arthroconidia from aging mycelia that, due to cytoplasmic shrinkage, display an appearance that can be misinterpreted as arthroconidia formation. True arthroconidia eventually fragment and disperse; aging mycelia do not. The two species of *Coccidioides* cannot be readily distinguished morphologically, although some strains of *C. posadasii* display slower growth on media containing high salt concentrations.

When incubated at 37°C, no yeast form is produced. The organism can produce spherules in vitro by using appropriate media and increased CO_2 tension incubated at 37 to 40°C, but this is rarely performed with routine clinical isolates.

The GenProbe AccuProbe for *Coccidioides* may be used for confirmation of unknown isolates as *Coccidioides* species. This test is generally sensitive and specific, although pretreatment of isolates with formaldehyde leads to false-negative results (30). The GenProbe test does not distinguish between the two species of *Coccidioides*.

Paracoccidioides brasiliensis

When incubated at 25 to 30°C, *P. brasiliensis* isolates display slow growth and produce a variety of forms ranging from a glabrous, leathery, brownish, flat colony with a few tufts of aerial mycelium to a wrinkled, folded, floccose to velvety, and white to beige form. The colonies are very similar in appearance to those of *B. dermatitidis*. Most strains grow for long periods of time without the production of conidia. Microscopic examination shows intercalary chlamydospores and coiled hyphae. Sporulation can be enhanced on yeast extract agar, and some isolates may produce pyriform conidia and arthroconidia.

When cultures are transferred to 37°C on rich media such as BHI or Pine's or Kelley's agar, colonies are generally folded (cerebriform) in appearance. Mycelial elements may be seen mixed with yeast cells. Conversion to the yeast phase also is quite slow. Yeasts are 2 to 30 μm in diameter and are oval or irregular in shape, displaying the characteristic "pilot's wheel" appearance of multiple thin-necked round buds developing around the parent cell. The walls are thinner than those of *B. dermatitidis* yeast cells, and the buds are not broad based.

Identification is usually confirmed by demonstrating thermal dimorphism. There is no commercial DNA probe test for *P. brasiliensis*. The GenProbe test for *B. dermatitidis* cross-reacts with *P. brasiliensis* (57), so care must be taken when employing this test with isolates that may actually be *P. brasiliensis*. Slow-growing, nonsporing isolates are more characteristic of *P. brasiliensis* than of *B. dermatitidis*.

Emmonsia Species

At 25°C, *Emmonsia* species grow as glabrous (waxy), colorless colonies, which produce yellowish white aerial mycelia in time. Some display pale orange to grayish orange aerial mycelia. The colonies often have areas that alternate between tufted mycelium and a glabrous consistency. Reverse pigmentation is pale gray to grayish brown. On microscopic examination, the hyphae are septate and branching. Sporulation is enhanced on potato dextrose or Pablum cereal agar. Numerous conidia are produced either directly from the sides of the hyphae or on short stalks that branch at right angles from the hyphae. Each stalk bears a single terminal conidium. Sometimes the swollen end may bear one to three secondary spine-like pegs, which in turn form a secondary conidium in a flower-like arrangement (66). The conidia are round, oval, or pyriform and measure 2 to 4 μm by 3 to 5 μm. The conidial wall is smooth but may roughen with age. *E. parva* and *E. crescens* are indistinguishable in morphology and appearance at 25°C.

E. crescens displays no hyphal growth at 37°C and forms larger adiaspores (20 to 140 μm in diameter) on BHI or phytone yeast extract agar at this temperature. *E. parva* produces hyphae at 37°C and produces smaller adiapores (8 to 20 μm in diameter) at 40°C. *E. pasteuriana* displays features at 25°C that are similar to those of *E. crescens* and *E. parva*. At 37°C on BHIB, this species produces yeast-like cells that are oval or lemon shaped, budding on a narrow base, and 2 to 4 μm. The colonies are creamy and smooth. This species does not produce adiaspores in vitro or in vivo (29).

TYPING SYSTEMS

In general, typing systems have been used to show geographic differences among isolates and cryptic species. In some genera, diversity among isolates collected from a single geographic area can be shown.

Histoplasma capsulatum

Isolates can be divided into at least eight clades as described earlier (40). This typing system can be used to place an unknown isolate into one of these major worldwide geographic groupings. In earlier studies, types based on variation in mitochondrial DNA or on the yeast phase-specific nuclear gene *yps-3* were shown to be consistent for U.S. isolates from persons without AIDS. Central American isolates and U.S. isolates from AIDS patients have a different *yps-3* genotype and several different mitochondrial DNA subtypes (reviewed in reference 69). Carter et al. (14) analyzed 30 isolates from Indianapolis, Ind., using a set of 11 biallelic markers, and found that every isolate had a distinct multilocus genotype. Taylor et al. (70) investigated the genetic polymorphisms of *H. capsulatum* isolates recovered from infected bats captured in Mexico by using random amplified polymorphic DNA (RAPD) PCR. The pattern of DNA polymorphisms appeared to reflect the geographic areas from which the bats originated. A major relatedness between bat and human isolates was shown, in contrast to blackbird isolates.

Blastomyces dermatitidis

RFLPs in mitochondrial DNA were used to type 19 isolates of *B. dermatitidis* from Little Rock, Ark. (84), dividing these isolates into three classes. When these isolates were typed in an RAPD assay with arbitrary primers, the classes could be further subdivided. Significant genetic diversity was observed. In another study, 55 isolates from the United States, India, and Africa were divided into three major groups based on RFLP analysis. These groups were further divided using RAPD fingerprinting into 5, 15, and 12 types, respectively, that correlated with the geographic origin of the isolate (51). Interestingly, these studies showed that soil isolates collected from an outbreak of blastomycosis in Eagle

River, Wis., were not responsible for the majority of cases of disease in that outbreak.

Coccidioides Species

Two major clades, corresponding to the two species *C. immitis* and *C. posadasii,* were defined when an extensive population sample, including isolates from Venezuela, Mexico, and Brazil, was studied using a set of nine microsatellite markers (23). Typing of isolates to *C. immitis* (California) or *C. posadasii* (non-California) species can be accomplished by examining any of 17 sites fixed for alternate alleles (43).

Paracoccidioides brasiliensis

The first genetic study of this organism compared ITS 1 and ITS 2 DNA sequences from armadillo and human clinical isolates (33) and suggested the existence of two genetic groups within this species. In a later study, microsatellite sequences were compared as markers to discriminate among a set of *P. brasiliensis* human isolates causing either chronic or acute disease (55). These authors did not observe any clustering of isolates associated with either acute or chronic disease.

Emmonsia Species

Based on ITS sequences, isolates of *E. crescens* fall into two phylogenetic groups, North American and Eurasian, depending on the continents from which the isolates were obtained (60). Isolates of *E. parva* separate into two groups as well, one group isolated from the North American prairies and the second group from the desert southwest of the United States or from Italy.

SEROLOGIC TESTS

Histoplasmosis

Serologic tests have an important role in the rapid diagnosis of several forms of *H. capsulatum* infection but are most useful for persons with chronic pulmonary or disseminated histoplasmosis (46). Of the different methods that have been developed, the immunodiffusion (ID), complement fixation (CF), and latex agglutination (LA) tests are the most popular. The principal antigen used in these tests is histoplasmin, a soluble filtrate of mycelial-phase broth cultures. Histoplasmin contains two glycoprotein components of particular interest: the H antigen, against which antibodies are formed during the acute phase of histoplasmosis, and the M antigen, against which antibodies are produced during all phases of the disease. In contrast to the other components of histoplasmin, such as the C antigen, which is a heat-stable galactomannan polysaccharide that is cross-reactive with *B. dermatitidis* and *C. immitis*, both the H and M antigens were once thought to be specific proteins for the detection of anti-*H. capsulatum* antibodies. The M antigen, however, was found to be a catalase that was not specific unless used in a deglycosylated form (32). The H antigen has been identified as a β-glucosidase (15).

The ID test is a qualitative method that detects precipitins to the H and M glycoprotein antigens of *H. capsulatum* present in histoplasmin. Both serum and CSF can be used. Patients with negative serum reactions during the acute phase of infection should have additional samples taken 3 to 4 weeks later. ID test kits and reagents are available from Gibson Laboratories (Lexington, Ky.), Immuno-Mycologics, Inc. (Norman, Okla.), and Meridian Diagnostics (Cincinnati, Ohio). Commercial kits include mycelial-phase culture filtrates containing *H. capsulatum* H and M antigens, positive control sera containing antibodies against both H and M antigens, and ID plates. Positive control sera must be included each time the test is performed and must react with both the H and M antigens. The ID test is a useful screening procedure or adjunct to the CF test. It is more specific but less sensitive than the CF test.

The CF test is a quantitative procedure in which two antigens are employed: histoplasmin and a suspension of intact merthiolate-killed *H. capsulatum* yeast phase cells. The latter is more sensitive (~80% versus ~20%) but less specific (~90% versus ~99%) than histoplasmin. Serum, peritoneal fluid, or CSF can be used in the CF test. Patients with negative serum reactions during the acute phase of infection should have additional samples taken 3 to 4 weeks later. No commercial kits are available, but antigens, antisera, and other reagents can be purchased from several commercial sources (Immuno-Mycologics, Inc., and Meridian Diagnostics). Negative control sera and positive control sera from human histoplasmosis cases demonstrating a CF titer of 1:32 or greater with the homologous antigen should be tested each time the CF test is performed.

An LA test, using histoplasmin as antigen, is commercially available (LA-Histo Antibody system; Immuno-Mycologics, Inc.). This semiquantitative test detects immunoglobulin M (IgM) antibodies and is used primarily for the presumptive diagnosis of acute histoplasmosis. It is less helpful for the detection of chronic infection.

Blastomycosis

The most useful serologic test is ID, but CF and ELISA procedures have also been developed and evaluated. Substantial improvement in the performances of these tests has been achieved by the use of a purified surface antigen of *B. dermatitidis,* termed the A antigen (36) or WI-1 antigen (42). Both are released from the yeast phase of *B. dermatitidis* by autolysis and can be recovered from culture filtrates. Immunological comparison of the two antigens has shown that they are very similar, but WI-1 is a 120-kDa protein that is not glycosylated whereas the A antigen is a 135-kDa glycosylated protein.

The ID test is a qualitative method that detects precipitins to the A antigen of *B. dermatitidis*. Patients with negative serum reactions during the acute phase of infection should have additional samples taken 3 to 4 weeks later. ID test kits and reagents are available from Gibson Laboratories, Immuno-Mycologics, Inc., and Meridian Diagnostics. Commercial kits include purified *B. dermatitidis* A antigen, positive control serum containing antibodies against A antigen, and ID plates. The positive control serum must be included each time the test is performed and must react with the homologous reference antigen to form the A precipitin line.

Coccidioidomycosis

Despite the fact that sensitive procedures, such as ELISA, have been developed, the ID and CF tests remain the most reliable methods for the serologic diagnosis of coccidioidomycosis (46). The principal antigen used in these tests is coccidioidin, a soluble filtrate of mycelial-phase broth cultures.

The simultaneous use of heated and unheated coccidioidin antigens permits the ID test to be employed to detect either IgM or IgG antibodies on a single plate. The immunodiffusion tube precipitin (IDTP) test utilizes heated coccidioidin as antigen, detects IgM, and gives results comparable to those obtained with the classical tube precipitin test. It is most useful for diagnosing recent infection.

The immunodiffusion complement fixation (IDCF) test utilizes unheated coccidioidin, detects IgG antibodies, and gives results comparable to those of the CF method (see below). It is less sensitive but more specific than the CF test (59). Commercial kits are available (Gibson Laboratories, Immuno-Mycologics, Inc., and Meridian Diagnostics) and include unheated and heat-treated coccidioidin antigens, positive control sera containing IgM or IgG antibodies, and ID plates. The sensitivity of the IDTP and IDCF tests can be improved by 10-fold concentration of serum prior to testing. Positive control sera must be included each time the test is performed and must react with the homologous reference antigen to form a precipitin line. The ID test is useful for initial screening of specimens and can be followed by other tests if positive.

The CF test is a sensitive quantitative method in which unheated coccidioidin is used to detect IgG antibodies. The major disadvantage of CF is that it is a laborious and time-consuming procedure that requires experienced personnel for optimum performance. No commercial kits are available, but reagents for in-house use can be obtained from several commercial sources (Immuno-Mycologics, Inc., and Meridian Diagnostics). Negative control serum and a positive control serum from a human case of coccidioidomycosis (with a titer of ≥1:32) should be included each time the test is performed. Anticomplementary activity in serum samples can occur and may be resolved by subsequent ID testing. In addition to serum, the CF test can be performed on CSF, pleural, and joint fluid samples.

A qualitative LA test, using heat-treated coccidioidin as antigen, is available from several commercial sources (LA-Cocci Antibody system; Immuno-Mycologics, Inc., and Coccidioides Latex Agglutination system; Meridian Bioscience, Inc.). This test is simple and rapid to perform. It detects IgM antibodies and is more sensitive than the IDTP test in detecting early infection. However, it has a false-positive rate of 5 to 10% and the results should be confirmed using the IDTP and/or CF methods. It is not recommended for screening CSF specimens because false-positive reactions can occur (58).

The Premier *Coccidioides* EIA (Meridian Bioscience, Inc.) is a qualitative test for detection of IgM and IgG antibodies in serum or CSF specimens. The antigen used in this test is a mixture of purified TP and CF antigens. Published evaluations suggest that this test has a sensitivity of >95% and a specificity of ~95% (41, 50). However, false-positive reactions have been obtained with some sera from patients with blastomycosis. EIA results should be confirmed by the IDTP and IDCF tests.

Paracoccidioidomycosis

The most popular serologic methods are ID and CF, but other tests, such as ELISA, have also been employed. The principal antigens used in these tests are derived from culture filtrates of mycelial or yeast phase broth cultures of *P. brasiliensis*. The major diagnostic antigen found in these preparations is a 43-kDa glycoprotein. Cell wall antigens have proved less useful than culture filtrate antigens, largely because they are dominated by cross-reactive galactomannan (11).

The ID test is a qualitative method that can be performed on a fee-for-service basis by Cerodex Laboratories, Inc., Washington, Okla. Commercial mycelial-form culture filtrate antigen can be obtained for in-house use from Immuno-Mycologics, Inc. No commercial kits are available for this test. The CF test is performed with *P. brasiliensis* yeast-form culture filtrate antigen. No commercial kits or reagents are available.

Adiaspiromycosis

No serologic tests are available for the diagnosis of adiaspiromycosis.

ANTIMICROBIAL SUSCEPTIBILITIES

Established treatment options for *H. capsulatum, B. dermatitidis, Coccidioides* species, and *P. brasiliensis* include amphotericin B, ketoconazole, itraconazole, and fluconazole, although no comparative trials of these agents have been performed. Table 1 lists the in vitro susceptibilities of these molds to established and investigational antifungal agents as reported in studies that were performed in accordance with Clinical and Laboratory Standards Institute (formerly National Committee for Clinical Laboratory Standards) document M38-A guidelines. Of note, *H. capsulatum* has been reported to become resistant to fluconazole during treatment, and this has accounted for failure in some patients (81).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Histoplasmosis

The definitive diagnosis of histoplasmosis can be accomplished by direct microscopic detection of *H. capsulatum* in clinical specimens or its isolation in culture. However, isolation and identification may take 2 to 4 weeks.

Antigen detection complements other diagnostic methods for histoplasmosis and is particularly useful for immunocompromised patients with more extensive disease, often providing a rapid diagnosis before positive cultures can be identified. HPA has been detected in serum, urine, CSF, and bronchoalveolar lavage fluid specimens obtained from individuals with disseminated histoplasmosis. Antigen levels are higher in urine than in serum. For patients with AIDS and disseminated histoplasmosis, sensitivity is 95% in urine and 86% in serum (82). Specificity is about 98%.

Antigen levels in the urine and serum decline with effective treatment, becoming undetectable in most patients (80). Failure of antigen concentrations to fall during treatment suggests therapeutic failure. For patients who have responded to treatment and in whom antigen levels have previously fallen, an increase in antigen in the urine or serum is suggestive of relapse (79). Antigen has been detected in the CSF of patients with meningitis caused by *Histoplasma*.

The LA test for *Histoplasma* antibodies is most useful for the diagnosis of acute infection, positive results being obtained within 2 to 3 weeks after exposure. An LA titer of 1:16 is presumptive evidence of infection, and a titer of ≥1:32 is considered strong presumptive evidence of active or recent infection (46). Because false-positive reactions can occur, the results should be confirmed by the ID test. Low-titer results from single specimens should be interpreted with caution. In such cases, the test should be performed on another specimen collected 4 to 6 weeks later.

In the ID test, precipitins to the M antigen of *H. capsulatum* are the first to appear (4 to 8 weeks after exposure) and can be detected in up to 75% of persons with acute histoplasmosis. However, they can also be found in nearly all individuals with chronic pulmonary infection, as well as in those who have undergone a recent skin test with histoplasmin. Precipitins to the H antigen are specific for active disease

TABLE 1 In vitro susceptibilities of dimorphic fungi to antifungal agents

Fungus	Antifungal agent	MIC range (μg/ml)	MIC_{90} range (μg/ml)	Reference(s)
B. dermatitidis	Amphotericin B	≤0.03–1	0.5	21, 44, 52
	Fluconazole	1–64	NR[a]	52
	Itraconazole	≤0.03–1	0.125	21, 44, 52
	Posaconazole	≤0.03–0.06	NR	22
	Voriconazole	≤0.03–16	0.25	21, 44, 52
	Anidulafungin	2–8	NR	22
	Caspofungin	0.5–8	NR	22
Coccidioides spp.	Amphotericin B	0.125–2	0.5–1	27, 44, 52
	Fluconazole	2–64	64	27, 52
	Itraconazole	0.125–2	1	44, 52
	Posaconazole	0.25–1	1	28
	Voriconazole	≤0.03–0.5	0.25	44, 52
	Caspofungin	8–64	32	27
H. capsulatum	Amphotericin B	≤0.03–2	0.25	21, 44, 52
	Fluconazole	≤0.125–64	NR	52
	Itraconazole	≤0.03–8	0.06	21, 44, 52
	Posaconazole	≤0.03–0.06	NR	22
	Voriconazole	≤0.03–2	0.25	21, 44, 52
	Anidulafungin	2–4	NR	22
	Caspofungin	0.5–4	NR	22
P. brasiliensis	Amphotericin B	0.125–4	NR	52
	Fluconazole	≤0.125–64	NR	52
	Itraconazole	≤0.03–1	NR	52
	Voriconazole	≤0.03–2	NR	52

[a] NR, not reported.

but occur in fewer than 20% of cases (76). They usually disappear within the first 6 months of infection and are seldom, if ever, found in the absence of M precipitins. The presence of precipitins to both H and M antigens is highly suggestive of active histoplasmosis, regardless of other serologic test results.

The CF test is useful in the diagnosis of acute, chronic, disseminated, and meningeal forms of histoplasmosis. In acute infections, antibodies to the yeast antigen are the first to appear (about 4 weeks after exposure) and the last to disappear after resolution of the infection. Antibodies to histoplasmin appear later and reach lower titers than those observed for the yeast antigen. In contrast, histoplasmin titers are usually higher in persons with chronic histoplasmosis. CF test results can be difficult to interpret because cross-reactions can occur with sera from persons with blastomycosis, coccidioidomycosis, and other fungal infections. In such instances, titers usually range between 1:8 and 1:32 and occur mainly against the yeast-form antigen. However, many serum samples from culture-confirmed cases of disseminated histoplasmosis give titers in the same range. CF titers of 1:8 or greater with either antigen are considered presumptive evidence of histoplasmosis. Titers above 1:32 or rising titers in serial samples offer stronger evidence of infection.

Titers of CF antibodies to *H. capsulatum* decrease following resolution of the infection but increase in individuals with chronic progressive disease. However, clinical and microbiological findings should also be considered in assessing the patient's prognosis or making treatment decisions. In some patients, positive CF titers decline slowly and persist long after the disease has been cured. The significance of persistently elevated or fluctuating CF titers is unclear, as is the effect of antifungal treatment on antibody clearance (78).

Serologic tests are particularly useful for patients with *Histoplasma* meningitis. The detection of precipitins to H and M antigens in CSF specimens is sufficient to make a diagnosis in the appropriate clinical setting and often is the only positive diagnostic test (75).

Blastomycosis

Although microscopic examination and culture remain the most sensitive means of establishing the diagnosis of blastomycosis, serologic tests can also provide useful information. A positive reaction in an ID test using the A antigen of *B. dermatitidis* is specific and diagnostic for blastomycosis (46). However, a negative ID test does not rule out the diagnosis because the sensitivity of this method has been reported to range from ~30% for cases of localized infection to ~90% for cases of disseminated blastomycosis. In established cases of the disease, a decline in the number or the disappearance of precipitin lines is evidence of a favorable prognosis.

With urine specimens, the *Blastomyces* antigen test has been reported to have a sensitivity of 89% for disseminated infection and 100% for pulmonary disease (19). However, cross-reactive antigens occurred in urine samples from all patients with paracoccidioidomycosis and 96% of patients with histoplasmosis (19).

Coccidioidomycosis

Although the definitive laboratory diagnosis of coccidioidomycosis depends on microscopic examination and

culture, serologic tests are of proven usefulness in diagnosis and management. A positive IDTP test result is indicative of acute coccidioidomycosis. IDTP-reactive IgM antibodies can be detected in up to 75% of cases within 1 week of symptom onset, and ~90% are positive within 3 weeks. Although infrequent, a positive IDTP test result with CSF is indicative of acute meningitis. In cases where the IDTP test is negative but the CF test is positive, patients should be investigated for microbiologic or histopathologic evidence of histoplasmosis or blastomycosis. In addition, further sera should be obtained at 3-week intervals and examined by CF and ID tests for coccidioidomycosis, histoplasmosis, and blastomycosis. False-positive IDTP reactions have been reported to occur in 15% of sera obtained from cystic fibrosis patients in the absence of a positive culture for *C. immitis* (17).

A positive IDCF test result is presumptive evidence of recent or chronic infection. IDCF-reactive IgG antibodies can usually be detected within 2 to 6 weeks after onset of symptoms. Although the IDCF test is generally not performed as a quantitative test, it can be used in this manner after serial dilution of patient serum to obtain an endpoint titer. Titers obtained using the quantitative IDCF test are not identical to titers obtained from the CF test, but the observed trends are comparable.

The LA test is more sensitive than the IDTP test in detecting acute infection, but it is less specific. For this reason, a positive test result with undiluted serum should be confirmed by the ID and/or CF tests.

The CF test does not become positive until about 4 to 12 weeks after infection, but CF antibodies persist for long periods in individuals with chronic pulmonary or disseminated coccidioidomycosis. Testing of serial specimens to detect rising or falling titers can reveal the progression or regression of illness and the response to antifungal treatment. A CF titer to coccidioidin at any dilution should be considered presumptive evidence of coccidioidomycosis. In most instances, the titer is proportional to the extent of the infection, and failure of the CF titer to fall during treatment of disseminated coccidioidomycosis is an ominous sign (59). Titers of 1:2 or 1:4 are usually indicative of early, residual, or meningeal disease; however, similar titers are sometimes found in individuals without coccidioidomycosis. The parallel use of the IDCF and the CF tests can help to confirm or refute the diagnosis. CF titers of >1:16 should lead to a careful assessment of the patient for possible spread of the disease beyond the respiratory tract. More than 60% of patients with disseminated coccidioidomycosis have CF titers of >1:32. However, false-negative results can occur for immunocompromised individuals, such as persons with AIDS (1). Patients with clinical presentations consistent with coccidioidomycosis and whose sera give negative or low CF titers should be retested at 3- to 4-week intervals.

The detection of CF antibodies in the CSF is usually diagnostic of coccidioidal meningitis and remains the single most useful test for diagnosis of that infection. However, ~5% of CSF specimens from patients with coccidioidal meningitis are negative in the CF test.

Paracoccidioidomycosis

The definitive diagnosis of paracoccidioidomycosis depends on microscopic examination and culture. However, isolation and identification of *P. brasiliensis* from clinical specimens may take up to 4 weeks.

Serologic tests are useful for the rapid presumptive diagnosis of paracoccidioidomycosis, particularly in cases of disseminated infection. The ID test with yeast-form culture filtrate antigen is highly specific and is positive in 65 to 100% of cases of acute or chronic pulmonary infection or of disseminated paracoccidioidomycosis (13). The CF test with yeast-form culture filtrate antigen is less specific than the ID test, and cross-reactions can occur with cases of histoplasmosis (16). However, CF titers of ≥1:8 are considered presumptive evidence of paracoccidioidomycosis. Low CF titers are usually associated with localized infection, while higher titers are found for patients with multifocal disease (14, 19). Falling CF titers are often predictive of successful treatment, and high or fluctuating CF titers are suggestive of a poor prognosis (13, 16). Some reports, however, have indicated that ID and CF results do not correlate well with the clinical status of the patient (26, 48).

Adiaspiromycosis

The definitive diagnosis of adiaspiromycosis can be accomplished by direct microscopic detection of *Emmonsia* species in clinical specimens or their isolation in culture.

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Trichophyton, Microsporum, Epidermophyton, and Agents of Superficial Mycoses

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TAXONOMY

The etiologic agents of dermatophytosis are classified, along with some nonpathogenic relatives, in three anamorphic genera: *Trichophyton, Microsporum,* and *Epidermophyton*. Those dermatophytes capable of reproducing sexually, i.e., producing ascomata with asci and ascospores, are classified in the teleomorphic genus *Arthroderma* (113) in the family Arthrodermataceae of the order Onygenales (21), phylum Ascomycota. The recorded connections between teleomorphic (sexual) and anamorphic (asexual) states of the dermatophytes as well as the dermatophytoids (i.e., the soil-borne *Trichophyton* and *Microsporum* species that are best not called "dermatophytes" [Greek for "skin plants"] because they are not pathogenic [3, 105]) are given in Table 1.

Recent phylogenetic studies have tended to support the ongoing classification of dermatophytes in the three traditional anamorph genera mentioned above. Some normally nonpathogenic, geophilic dermatophytoids currently placed in *Trichophyton,* e.g., *T. ajelloi* and *T. terrestre,* appear to belong to a distinct clade in most phylogenies based on the ribosomal internal transcribed spacer (ITS) region sequences, but the degree of bootstrap support for this clade does not support its distinction as a separate genus (15, 33, 34). Further study of this matter with additional genes is necessary.

As part of the ongoing molecular revolution in biology, fungal taxonomy is ever more strongly influenced by our greatly increased understanding of population genetics (16). Dermatophytes show two population genetics patterns differing among species having "population hosts" (43) in different zoological families, orders, or classes (101, 102). (A population host, which is the normal epidemiologic reservoir of the species, is distinguished from "occasional host" species that may acquire infection but that do not support ongoing populations; for example, *Microsporum canis* has mostly feline population hosts, and while humans are frequently infected by feline carriers, the species is seldom directly transmitted from human to human, making our species only an occasional host.) Some dermatophyte species, including pathogens with population hosts in the rodent, rabbit, pig, dog, and cat families, are potentially sexual, with sexual reproduction occurring only off the host, i.e., on hair or other keratinous debris in contact with the ground. Other species, particularly those with human, ungulate, equine, or avian population hosts, have no access to a soil-based location suitable for sexual reproduction and host reinfection; not surprisingly, these species are found on investigation to be asexual and clonal. They consist, in all known cases, of genetically highly uniform isolates (36, 72, 83) sharing, where known, a single mating type factor (101, 107). Most appear to have evolved from a single strain of a sexual ancestral species that was able to make the rare, successful switch to ongoing contagious infection of a new animal host. A few epidemiological and phenotypic characters in the clonal species appear to have undergone accelerated evolution due to strong selection for increased compatibility with the new host. This process has tended to produce differences allowing relatively easy laboratory identification of these species. At the same time, the basic cellular housekeeping genes investigated in phylogenetic taxonomic studies have evolved at a normal rate and thus strongly tend to resemble forms seen in ancestral species complexes or in sibling species. This has led to considerable recent confusion about species concepts. Long-recognized dermatophyte species such as *Trichophyton equinum* and *Microsporum gallinae* that have distinct epidemiologies and that are easily identified in the routine diagnostic laboratory have nonetheless been suggested not to be distinct species as traditionally conceived (33, 35), since their ITS and some other sequences are strongly similar to those of related species (e.g., *Trichophyton tonsurans* and *Arthroderma grubyi,* respectively, for the examples mentioned). The ITS sequences of the dermatophytes proposed for synonymy, however, generally show a small number of consistent sequence polymorphisms when compared with sequences of the species with which synonymy has been proposed. This suggests that further genetic study will tend to support the species in question as recently diverged but nonetheless separate lineages. The present chapter takes a relatively cautious approach to the ongoing debates in this area (118), synonymizing traditionally recognized species primarily when there is unequivocal, multigene molecular evidence that they are not supported at the species level. Notwithstanding recent literature, in no case is a clonal species of a given mating type (such as the horse dermatophyte *T. equinum,* + mating type) recognized as synonymous with a phenotypically and epidemiologically distinct species evolutionarily derived from the opposite ancestral mating type (such as the human tinea capitis agent *T. tonsurans,* – mating type), since such a difference in mating types confirms independent evolutionary origin from a common ancestor. The list of recognized species is in Table 1.

TABLE 1 Important characteristics of clinically isolated dermatophytes and dermatophytoids[a]

Dermatophyte species (teleomorph name[s] if formation of sexual state is known)	Growth	Macromorphology	Micromorphology	BCPMSG medium pH results (for 7–10 days)	Urea test (7 days, broth)	Hair perforation	Other comments
Epidermophyton floccosum[b]	Moderately rapid	Flat, slightly granular at first, soon developing white puffs of degeneration; sandy to olive-brown (Fig. 18e); reverse pale to yellowish	Macroconidia abundant, club shaped with broadly rounded apex, usually with fewer than 6 cells (Fig. 12); no microconidia formed; many chlamydospores in primary isolates	Alkaline	Pos	Neg	Invades skin and nails, rarely hair; no microconidia
Microsporum spp.							
M. audouinii[c]	Moderately rapid	Flat to velvety, thin, pale salmon to pale brownish reverse	Rare, deformed macroconidia, often with beak, constricted midregion, and at least trace granulation (Fig. 13); drop-shaped microconidia and aerial arthroconidia may be present; pectinate branching, apiculate terminal chlamydospores often seen	No pH change or alkaline	Neg	Neg	Poor growth and no or brownish pigment on polished rice medium; usually connected with patient or index patient in or recently from Africa; only children typically infected
M. canis[b] *(Arthroderma otae)*	Rapid	Flat to velvety, thin, pale to yellow (Fig. 18g), with yellow (rarely pale) reverse	Macroconidia thick walled, roughened and beaked (Fig. 14); microconidia drop shaped	No pH change; macroconidia often abundant	Pos	Pos	Good growth and yellow pigment on polished rice medium; human infection usually from cat or dog; *M. canis* "distortum" phenotype has macroconidia distorted, bizarrely shaped; "*M. equinum*" phenotype from horses has few, short macroconidia
M. cookei[c] *(A. cajetani)*	Moderately rapid	Granular to velvety; reverse wine red	Macroconidia rough, thick walled with cellular compartments rather than true cross walls (Fig. 15); microconidia drop shaped	No pH change	Pos	Pos	Probably nonpathogenic; existing case reports poorly substantiated

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TABLE 1 Important characteristics of clinically isolated dermatophytes and dermatophytoids[a] *(Continued)*

Dermatophyte species (teleomorph name[s] if formation of sexual state is known)	Growth	Macromorphology	Micromorphology	BCPMSG medium pH results (for 7–10 days)	Urea test (7 days, broth)	Hair perforation	Other comments
M. ferrugineum[d]	Slow	Flat or folded, waxy to slightly velvety; surface and reverse yellow, rusty or pale	No conidia, coarse, straight "bamboo" hyphae with prominent septa may be present	No pH change	Neg	Neg	Yellow colony on Lowenstein-Jensen medium (compare *T. soudanense*); geographically restricted to parts of Africa, Asia, and Eastern Europe
M. gallinae[d]	Moderately rapid	Flat to velvety; surface white tinged with pink; reverse red; red pigment diffuses into agar	Macroconidia smooth to slightly rough, often bent and with thickest cells near the apex, sometimes slightly rough; microconidia drop shaped	No data	Neg	Neg	Rare; human infection usually from chicken. Species may be an asexual phenotype within *A. grubyi* according to Gräser et al. (33)
M. gypseum complex[b] (*A. gypseum*, *A. incurvatum*, *A. fulvum*)	Rapid	Granular, sandy in color, or occasionally light cinnamon or rosy buff; reverse usually pale to brownish	Macroconidia abundant, thin walled, fusoid (tapered at both ends), roughened, with up to 6 septa (Fig. 16); microconidia drop shaped, mostly formed along sparsely branched hyphae (a feature only noted if *M. racemosum* is queried)	No pH change	Pos	Pos	Human infection usually from soil contact
M. nanum[c] (*A. obtusum*)	Moderately rapid	Powdery, sandy in color; reverse often reddish brown	Macroconidia rough, usually only 1–3 cells long, egg shaped to ellipsoidal	No data	Pos	Pos	Human infection usually from pig; now rare
M. persicolor[c] (*A. persicolor*)	Rapid	Powdery, sandy in color; reverse pale to yellowish, sometimes with rosy tones	Macroconidia fusoid (tapered on both ends), often absent or smooth walled on Sabouraud agar but usually common and rough walled on Sabouraud with added salt (3 or 5% NaCl) (Fig. 17); microconidia formed on pedicels (must be checked within 5 days)	No pH change	Pos	Pos	Usually poor growth at 37°C in vitro; rose to wine red reverse on sugar-free media, e.g., glucose-free Sabouraud agar; human infection usually from soil (fomites from voles)

M. praecox[c]	Rapid	Powdery, sandy in color, reverse yellow	As *M. gypseum*	No pH change	Pos	Neg	Uncommon
M. racemosum[d] (*A. racemosum*)	Rapid	Powdery, sandy in color, reverse red	Macroconidia as *M. gypseum;* microconidia mostly formed in densely branched formations structured like grape clusters (racemes)	No pH change	Pos	Pos	Rare
M. vanbreuseghemii[d] (*A. grubyi*)	Rapid	Powdery, pinkish or buff; pale to yellow reverse	Macroconidia rough, thick walled, cylindrical, often more than 8 cells long, with cellular compartments rather than true cross walls	No data	Pos	Pos	Rare; anamorph may be conspecific with phenotypically and epidemiologically different *M. gallinae* according to Gräser et al. (33)
Trichophyton spp.							
T. ajelloi[c] (*A. uncinatum*)	Moderately rapid	Powdery, rich tan to medium orange-brown in color; reverse pale, brownish or with purple-black pigment	Macroconidia smooth, thick walled, cylindrical, often more than 7 cells long, with cellular compartments rather than true cross walls (Fig. 5)	No data	Pos	Pos	Nonpathogenic in humans
T. concentricum[d]	Slow	Folded, honey brown to reddish brown, glabrous or slightly velvety colony	No conidia	No data	Pos or neg	Neg	Only from indigenous Asian, Austronesian/Melanesian, or indigenous Central and South American people with distinct tinea imbricata infection
T. equinum[c]	Moderately rapid	Flat to velvety colony with cream-colored surface and yellow to red-brown reverse	Macroconidia uncommon, cylindrical to club shaped, smooth; microconidia abundant, on small pedicels (examine before 5 days)	Alkaline	Pos	Usually neg, sometimes pos	Human infection usually from horse; has a nicotinic acid requirement except in autotrophic variant from Australia and New Zealand
T. erinacei hedgehog form[c] (*A. benhamiae*)	Rapid	Granular to powdery, yellow-cream to buff surface, yellow reverse	Macroconidia uncommon, club shaped, smooth; microconidia nearly spherical, abundant, mostly produced in dense tufts; spiral appendages present	Alkaline	Neg (European/ New Zealand form); pos (African form)	Pos	Human infection usually from hedgehog or its fomites; therefore, mostly restricted to regions with wild hedgehogs or to pet hedgehog owners
T. megninii[c]	Moderately rapid	Cottony, with white down sometimes suffused with rosy pigment; reverse red to red-brown	Macroconidia seldom seen, pencil shaped; microconidia drop shaped	Alkaline	Pos or weak pos	Neg	Region of endemicity in Portugal and nearby areas; requires histidine; considered a subtype of *T. rubrum* by Gräser et al. (33)

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TABLE 1 Important characteristics of clinically isolated dermatophytes and dermatophytoids[a] *(Continued)*

Dermatophyte species (teleomorph name[s] if formation of sexual state is known)	Growth	Macromorphology	Micromorphology	BCPMSG medium pH results (for 7–10 days)	Urea test (7 days, broth)	Hair perforation	Other comments
T. mentagrophytes complex (zoophilic)[b] inclusive of *T. mentagrophytes* sensu stricto (= the former *T. mentagrophytes* var. *quinckeanum*) plus animal-adapted forms of *T. interdigitale* and the *Trichophyton* anamorph of *A. benhamiae* (*A. vanbreuseghemii*, *A. benhamiae*)	Rapid	Granular to powdery, yellow-cream to buff surface (Fig. 18b), pale to red-brown reverse	Macroconidia uncommon, club shaped, smooth; microconidia nearly spherical, abundant, mostly produced in dense tufts; spiral appendages present	Alkaline (rarely weak)	Pos	Pos	Human infection usually from rodent or rabbit. Macroconidia induced on SGA + 3 or 5% sodium chloride (Fig. 6)
T. mentagrophytes complex (anthropophilic)[b] inclusive of human-adapted forms of *T. interdigitale* and possibly of the *Trichophyton* anamorph of *A. benhamiae* (*A. vanbreuseghemii*, *A. benhamiae*)	Rapid	Powdery to cottony, yellow-cream to buff or white surface, pale to red-brown reverse	Macroconidia uncommon, club shaped, smooth; microconidia nearly spherical or drop shaped, abundant, produced mainly in dense tufts when round and on sparsely branched hyphae when drop shaped; spiral appendages present but rare in very cottony isolates	Alkaline	Pos	Pos	Macroconidia often induced on SGA + 3 or 5% sodium chloride
T. mentagrophytes ("nodular" variant[c] formerly called *T. krajdenii*, now known to be a distinct morph of *T. interdigitale*)	Moderately slow	Cottony, cream to white surface often with yellow marginal zone (Fig. 18a), intense yellow reverse	Macroconidia rare, microconidia usually drop shaped, sometimes also round; coiled, yellow "nodular bodies" and yellow pigment granules present in submerged mycelium; spiral appendages seldom seen	Alkaline	Pos	Pos	Although usually very different in morphology, this variant so far is not genetically distinguishable from other anthropophilic isolates of *T. interdigitale*
T. rubrum[b] (cosmopolitan variant)	Moderately slow	Cottony to velvety, white to reddish surface (Fig. 18c), typically wine red reverse (Fig. 18d) but yellow variants occasional; red	Macroconidia seldom seen, pencil shaped (Fig. 8); microconidia drop shaped, abundant, scanty or not formed;	No pH change (alkalinity after 14 days)	Neg (rarely weak)	Neg	Melanoid variants secreting brown pigment rarely seen

		color poorly formed in presence of common bacterial contamination	lateral hyphal projections often present				
T. rubrum (Afro-Asiatic variant[c] formerly called *T. raubitschekii, T. fluviomuniense,* or, when microconidia absent, *T. kanei*)	Moderately slow	Powdery to low velvety, cream to deep red; reverse wine red	Macroconidia abundant (Fig. 7), club shaped, sometimes with "rat-tail" extension; microconidia drop shaped to round; many chlamydospores in primary isolates	No pH change (alkalinity after 14 days)	Pos	Neg	Although usually very different in morphology, this variant so far is genetically distinguished from typical *T. rubrum* only at microsatellite markers. It is often from upper body infection (tinea corporis, tinea cruris)
T. schoenleinii[d]	Slow	Convoluted, slightly velvety whitish colony	No conidia seen; "favic chandeliers" or "nailhead hyphae" present	Alkaline	Variable	Neg	Very rare; associated with clinically recognizable "favus" lesions; now extirpated except in rural central Asia, rural Africa
T. simii[c] *(A. simii)*	Rapid	Granular to powdery, yellow-cream to buff surface, pale to red-brown reverse	Macroconidia abundant, often with some cells swollen as chlamydospores; microconidia drop shaped	Alkaline	Pos	Pos	Endemic to India and Africa; similar to zoophilic *T. mentagrophytes* but macroconidial number and shape are atypical; reference distinction is by mating or molecular study
T. soudanense[c]	Moderately slow	Flat, bright yellow to (less commonly) wine red colony with radial striations and starlike margin; uncommonly cottony; reverse yellow to wine red	Macroconidia not seen, microconidia drop shaped, scarce or absent; reflexive hyphal branches in radial striations	Alkaline, with small zone of clearing	Usually neg, occasionally pos	Neg	Endemic to sub-Saharan Africa but widely disseminated in cosmopolitan parts of Europe and Americas.Dark colony on Lowenstein-Jensen medium (compare *M. ferrugineum*); may or may not grow on growth factor test media, considered a subtype of *T. rubrum* by Ohst et al. (77)
T. terrestre complex[c] *(A. lenticulare, A. quadrifidum, A. insingulare)*	Moderately rapid	Powdery white to cream or pinkish surface; pale or rarely yellow to red reverse	Macroconidia numerous, mostly small (5 or fewer cells) intergrading with large club-shaped micro-conidia in a continuous series (Fig. 9), so that 3-, 2-, and 1-celled conidia are present	Alkaline	Pos	Pos	No growth at 37°C in vitro; nonpathogenic

(Continued on next page)

TABLE 1 Important characteristics of clinically isolated dermatophytes and dermatophytoids[a] *(Continued)*

Dermatophyte species (teleomorph name[s] if formation of sexual state is known)	Growth	Macromorphology	Micromorphology	BCPMSG medium pH results (for 7–10 days)	Urea test (7 days, broth)	Hair perforation	Other comments
T. tonsurans[b]	Moderately slow	Powdery to velvety, white to yellowish or red-brown surface; reverse chestnut, red-brown (Fig. 18f), and/or sulfur yellow, rarely pale	Macroconidia uncommon, small pencil or club shaped; microconidia abundant (Fig. 10), often on broad "match-stick" pedicels; "balloon forms" and "filiform branches" may be seen	Alkaline, sometimes weak	Pos	Usually neg, sometimes pos	Stimulated by thiamine; a form producing only macroconidia has been described
T. vanbreuseghemii[d] *(A. gertleri)*	Moderately slow	Buff colony, leathery, finely grainy; reverse whitish or pale yellow	Abundant macroconidia with cells of uneven length, tending to fragment into single cells; microconidia small, boxy	No data	Pos	Pos	Geophilic; very rare in clinical laboratory
T. verrucosum[b]	Slow	Convoluted, slightly velvety, whitish or less commonly tan to ochraceous colony	Macroconidia seldom seen, with "rat-tail extension"; microconidia round to drop shaped; chains of symmetrical chlamydospores seen on milk solids media at 37°C (Fig. 11)	Alkaline (may be weak) with broad zone of clearing	Neg	Neg	Human infection usually from cattle; growth stimulated at 37°C
T. violaceum[c]	Slow	Glabrous (bald-looking), smooth or convoluted colony; purple-red, sometimes with white sectors; some East African isolates purely whitish	Macroconidia seldom seen; microconidia drop shaped, formed mostly on thiamine medium or on sporulation media; chains of asymmetrical chlamydospores seen on milk solids media at 37°C	No pH change or weak alkaline with small to broad zone of clearing (always broad after 14 days)	Pos or weak	Neg	Endemic to North Africa and Middle East but widely disseminated in cosmopolitan parts of Europe, Americas, and South Africa

[a] Abbreviations: Pos, positive; Neg, negative.
[b] Common.
[c] Uncommon but likely to be seen by large laboratories in the Americas and Europe.
[d] Unlikely to be seen except in regions of endemicity, proficiency test, or soil isolation experiment.

EPIDEMIOLOGY AND TRANSMISSION

Dermatophytes are keratinophilic fungi that are capable of invading the keratinous tissues of living animals. They are grouped into three categories based on host preference and natural habitat (Table 2) (2). Anthropophilic species almost exclusively infect humans; animals are rarely infected. Geophilic species are soil-associated organisms, and soil per se or soil-borne keratinous debris (e.g., shed hairs or molted feathers) is a source of infection for humans as well as other animals. Zoophilic species are essentially pathogens of nonhuman mammals or, rarely, birds; however, animal-to-human transmission is not uncommon. Understanding this ecological classification for case isolates may be helpful in determining the source of infection; e.g., human infections caused by *Microsporum canis* are often the result of contact between susceptible children and newly acquired or stray kittens (64). Clinical species identification of dermatophytes assists in controlling infections that may have a family pet or other domesticated animal as an ongoing source of inoculum.

Some dermatophytes, e.g., *Trichophyton rubrum,* are cosmopolitan, whereas others, e.g., *Trichophyton concentricum,* are geographically limited (87). *T. concentricum* is found only in the Pacific Islands and regions in Southeast Asia and Central and South America.

Anthropophilic fungi are usually transmitted either directly through close human contact or indirectly through sharing of clothes, combs, brushes, towels, bedsheets, etc. Tinea capitis is highly contagious and may spread rapidly within a family, institution, or school. Transmission of tinea cruris is associated with shared clothing, towels, and sanitary facilities. The transmission of tinea pedis and tinea unguium often involves communal showers, baths, or other aquatic facilities but may depend on both environmental and host factors (91, 115). Acquisition of chronic *T. rubrum* tinea pedis has been suggested to require a dominant autosomal susceptibility gene (121).

Infections with geophilic dermatophytes involve transmission of soil-borne inoculum to humans or other mammals. Outbreaks originating from infected soil with secondary human-to-human transmission have been reported (5). Infections by zoophilic species result from animal-to-human contact (cats, dogs, cattle, laboratory animals, etc.) or from indirect transmission involving fomites. The fungi may then be transmitted among humans to a limited extent, especially in institutions (96).

TABLE 2 Grouping of dermatophytes on the basis of host preference and natural habitat[a]

Anthropophilic	Zoophilic	Geophilic
Epidermophyton floccosum	*Microsporum* spp.	*Microsporum* spp.
	M. canis	*M. gypseum* complex
Microsporum spp.	*M. gallinae*	*M. praecox*
M. audouinii	*M. nanum*	*M. racemosum*
M. ferrugineum	*M. persicolor*	*M. vanbreuseghemii*
	Trichophyton spp.	
Trichophyton spp.	*T. equinum*	*Trichophyton vanbreuseghemii*
T. concentricum	*T. erinacei*	
T. megninii	*T. mentagrophytes* complex (granular isolates)	
T. mentagrophytes complex (velvety and cottony isolates)	*T. simii*	
	T. verrucosum	
T. rubrum		
T. schoenleinii		
T. soudanense		
T. tonsurans		
T. violaceum		

[a] Normally nonpathogenic, soil-associated dermatophytoids such as *T. terrestre* and *M. cookei* are not included in this table.

CLINICAL SIGNIFICANCE

The dermatophytoses (tinea or ringworm) generally manifest as infections of the keratinized tissues (hair, nails, skin, etc.) of humans, other mammals, and birds. Cutaneous infections resembling dermatophytoses may be caused by yeasts or by unrelated filamentous fungi that are normally saprobes or plant pathogens; these infections are referred to as opportunistic dermatomycoses (115).

Dermatophytes are among the very few fungal species that cause contagious, directly host-to-host-transmissible diseases of humans and animals. The transmission of these fungi is usually carried out by arthroconidia that have formed in or on infected host tissue. These conidia may be spread by direct skin-to-skin contact or via fomites containing free arthroconidia, shed skin scales, or hairs. Typical fomites include such divergent materials as hats, shoes, shower room floors, bedding, clothing of nursing staff in chronic care institutes, and farm fenceposts used by animals for scratching. Tissue invasion is normally cutaneous; dermatophytes are usually unable to penetrate deeper tissues as a result of nonspecific inhibitory factors in serum (60), inhibition of fungal keratinases (24), and other immunological barriers (76, 116), including a Th1 reaction mediated in part by $CD4^+$ lymphocytes (61). Dermatophytes tend to grow in an annular fashion on most affected skin regions, producing a "ringworm" infection form with a more or less raised and erythematous active area at the periphery and a relatively scurfy inactive zone at the center of established lesions. The organism can often be isolated only from the active, peripheral ring. Infection may range from mild to severe, partly as a consequence of the reaction of the host to the metabolic products of the fungus. Also important in determining the severity of infection are the virulence of the infecting strain, the anatomic location of the infection, the status of the host's immune system, and local environmental factors. Occasionally, especially in immunocompromised patients, subcutaneous tissue may be invaded, e.g., in Majocchi's granuloma, kerion, mycetoma-like processes (11, 117), or, more rarely, a generalized systemic infection (8). A *T. rubrum* infection suggestive of cutaneous blastomycosis has been reported in an immunocompromised patient (97).

In synopsis, the principal current risk factors for common forms of dermatophytosis are age (youth for tinea capitis or advanced age for onychomycosis), family history of chronic dermatophytosis, participation in athletics featuring extensive body contact (e.g., wrestling or judo) or foot maceration (e.g., marathon running), barefoot use of communal aquatic facilities (showers and swimming areas), exchange of headgear and footwear or inadequately cleaned bedding, contact with feral domestic animals (especially street kittens) or animals recently supplied by en masse breeding operations

(especially cats, guinea pigs, rabbits, laboratory rats, and cattle), inhabitation of rodent-infested dwellings, and, especially for children in developing countries, contact with livestock suffering from untreated dermatophytosis and contact with barbering instruments that have not been effectively disinfected (1, 7, 19, 42, 48, 62, 66, 89, 116, 121).

Anatomic Specificity

Infections caused by dermatophytes are named according to the anatomic location involved, e.g., tinea barbae (beard and moustache), tinea capitis (scalp, eyebrows, and eyelashes), tinea corporis (face, trunk, and major limbs), tinea cruris (groin, perineal, and perianal areas), tinea pedis (soles and toe webs), tinea manuum (palms), and tinea unguium (nails). Different dermatophyte species may produce clinically identical lesions; conversely, a single species may infect many anatomic sites.

Tinea barbae, usually caused by zoophilic fungi, e.g., *Trichophyton verrucosum* and granular, zoophilic forms of the *T. mentagrophytes* complex, is typically highly inflamed and may present as acute pustular folliculitis that can progress to suppurative boggy lesions (kerion). A less severe form that appears as dry, erythematous, scaly lesions also occurs. Tinea capitis may vary from highly erythematous, patchy, scaly areas with dull gray hair stumps to highly inflamed lesions with folliculitis, kerion formation, alopecia, and scarring. *Trichophyton tonsurans* and *M. canis* are the most common agents (87). Favus (tinea favosa), usually caused by *Trichophyton schoenleinii* but also potentially caused by *Trichophyton violaceum* or *Microsporum gypseum,* is a now very rare chronic infection of the scalp and glabrous skin characterized by the formation of cup-shaped crusts (scutula) resembling honeycombs. Tinea corporis, which can be caused by any dermatophyte but is often associated with zoophiles, classically manifests as circular, erythematous lesions with scaly, raised, active, and often vesicular borders. Chronic lesions on the trunk and extremities usually are caused by *T. rubrum,* in particular by Afro-Asiatic forms of this fungus (formerly often called *Trichophyton raubitschekii*). Tinea cruris ("jock itch"), usually caused by *T. rubrum* or *Epidermophyton floccosum,* typically appears as scaly, erythematous to tawny brown, bilateral and asymmetric lesions extending down to the inner thigh and exhibiting a sharply marginated border frequently studded with small vesicles. Tinea pedis varies in appearance: the most common manifestation is maceration, peeling, itching, and painful fissuring between the fourth and fifth toes, but an acute inflammatory condition with vesicles and pustules can also occur, as can a hyperkeratotic chronic infection of the sole ("moccasin foot"). Members of the *T. mentagrophytes* complex frequently cause the more inflammatory type of infections, whereas *T. rubrum* usually causes the more chronic type. Infection of the sole by human-adapted forms of *Trichophyton interdigitale,* one of the members of the *T. mentagrophytes* complex, can be recognized by the formation of bullous vesicles in the thin skin of the plantar arch and along the sides of the feet and heel adjacent to the thick plantar stratum corneum (120). Tinea unguium, or nail infection by dermatophytes, is a subcategory of the more general phenomenon of onychomycosis, fungal nail infection. It is most often caused by *T. rubrum* and usually appears as thickened, deformed, friable, discolored nails with accumulated subungual debris. This type of presentation results from invasion of the underside of the distal nail and is therefore termed "distal-subungual onychomycosis." A less common infection type usually caused by *T. interdigitale* typically manifests, especially in its earlier stages, as "superficial white onychomycosis," i.e., white patches in the superficial portions of the nail. "Proximal-subungual tinea unguium" may also occur. This infection, in which the nail is subungually infected beginning near its point of origin in the area of the lunula, is usually caused by *T. rubrum* and often signals immunosuppression, e.g., AIDS (22).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Preliminary Patient Examination

In areas where tinea capitis caused by *Microsporum canis* is common, patients may be examined with a Wood's lamp (filtered UV light with a wavelength of 365 nm) in a darkened room for the presence of bright green fluorescent hairs. These are ideal for collection as laboratory specimens, although in some cases diagnosis may be done by Wood's light alone. The fluorescent hairs, considered "Wood's light positive," typically show a small-arthroconidial, ectothrix type of hair invasion in direct microscopy. Apart from *M. canis, M. audouinii* and *M. ferrugineum* also cause this type of fluorescent ectothrix infection. Hairs infected with *T. schoenleinii* may show a dull green color (89). The Wood's lamp can also be used to differentiate between dermatophytosis and nonfungal skin conditions that may be similar clinically, e.g., erythrasma. In erythrasma, the skin fluoresces orange to coral red, whereas in dermatophytosis, the skin is not fluorescent.

Sampling Preparations and Practice

Sufficient clinical material should be collected for both direct microscopic examination and culture. Whenever feasible, an aseptic technique should be used to minimize contamination. The following equipment should be available for the collection and transport of specimens: forceps for epilating hairs, sterile no. 15 scalpel blades or sharp curette, sterile nail clippers, scissors, sterile gauze squares, 70% alcohol for disinfection, sterile water for cleansing painful areas, and clean pill packets or clean paper envelopes to contain and transport the clinical specimens such as hairs, skin scrapings, or nail clippings. Black photographic paper or strong black paper may be used for collecting and better visualizing scrapings. After collection, the paper is folded, tightly taped in the corners, and placed in an envelope for transport. Closed tubes are not recommended for specimens, since they retain moisture, which may result in an overgrowth of bacterial as well as fungal contaminants. Disposable sterilized brushes have been recommended for collection of specimens from the scalp or from the fur of animals (68). If histopathological processing is done, the nail plate may be placed in a 4% formaldehyde solution (98). Culture media (detailed below) may be inoculated directly on collection.

Hairs from the scalp should be epilated with sterile forceps. If the specimen is Wood's light positive, epilate only fluorescent hairs. Nonfluorescent hairs, especially those infected with endothrix fungi such as *T. tonsurans,* may need to be dug out with the tip of a sterile scalpel blade because the hairs often break off at the scalp level and are thus difficult to grasp with a forceps. Rubbing with a sterile moistened swab has been successful with pediatric patients (47). In the rare event that favus is seen, the scutulum at the mouth of the hair follicle is suitable for culture and microscopic examination. In lower-body dermatophytoses, lesions with defined borders should be preliminarily disinfected with alcohol or cleansed with sterile water, and then active

border areas should be scraped with a scalpel to collect epidermal scales. If borders are not visible to indicate the area of maximal fungal activity, as in tinea manuum, the preliminarily cleansed infected area can be broadly scraped in order to obtain specimen from a variety of areas that may imperceptibly differ in current fungal activity. In vesicular tinea pedis, the tops of the vesicles can be removed with sterile scissors for direct examination and culture. Culture of the vesicle fluid is not recommended.

Nails should be disinfected with alcohol gauze squares. The most desirable material for culture in typical subungual onychomycosis is the waxy subungual debris, which contains the fungal elements. The highest proportion of viable elements for culturing is often found close to the juncture of the nail bed. In order to remove contaminating saprobic fungi and bacteria, the crumbly debris directly underneath the nail near the tips is removed with the scalpel before material is collected for culture. Some investigators clip the nail short first and perform this scraping away of contaminated material on the clipping. If the dorsal nail plate is diseased (superficial white onychomycosis), scrape and discard the outer surface before the underlying material is removed for culture. In rare cases with a presentation consistent with *Trichophyton soudanense* endonyx nail infection (110), in which the internal strata of the nail plate are milky white but the upper and lower nail surfaces appear relatively unaffected, clippings may be taken or the milky area may be exposed and scraped.

Any specimen needing to be transported, or for any other reason not processed immediately, should be retained in the paper packets described above. Closed tubes are not recommended for specimens since they may retain moisture and may result in an overgrowth of contaminants. Dermatophytes in dry specimens of skin, hair, and nails may be stored for years in viable condition, provided the material is not subjected to temperature extremes.

LABORATORY TESTING OF SPECIMENS

Direct Microscopic Examination

Direct microscopic examination of skin, hair, and nails is the most rapid method of determining fungal etiology and is traditionally accomplished by examining the clinical material in 10% potassium hydroxide (KOH) (freely interchangeable with the cheaper sodium hydroxide [NaOH]) (59, 90). Another common procedure is to use 25% potassium or sodium hydroxide mixed with 5% glycerin to impede desiccation (59). Addition of fluorescent brighteners such as calcofluor white may significantly increase accuracy, especially if the staff are not highly experienced in visual detection of fungal elements (86). In nails, histopathology based on staining nail biopsy material with periodic acid-Schiff (PAS) stain has been shown to have potential for generating results more accurate than those afforded by hydroxide-based direct microscopy (63). The suggestion, however, that the biopsy-PAS method can also replace culturing (63) is unsound, because this technique does not permit reliable distinction of dermatophytes from other nail-invading species (103).

Nail clippings should be aseptically cut into smaller fragments and, where possible, pounded with a heavy object inside their collection packet in order to release friable, flaky material containing the greatest amount of dermatophyte inoculum. Skin or nail scrapings, nail fragments, or hair roots are placed in 1 or 2 drops of one of the above-mentioned KOH or NaOH solutions on a clean glass slide. A coverslip is placed on top, and the preparation is heated gently (short of boiling) by being passed rapidly over a Bunsen burner three or four times, and then it is allowed to sit at room temperature for a few minutes for clearing. The exact time needed depends on the concentration of hydroxide used, the thickness of specimen fragments, and the exact amount of heat imparted by contact with flame. Clearing is evident to the naked eye as a pronounced decrease in the opacity of the scraping. Alternatively, a slide warmer set at 51 to 54°C may be used to heat the slides for 1 h (59). Laboratories using a 10% KOH or NaOH solution for skin may find that nail scrapings may require a stronger alkali solution (up to 25% KOH or NaOH). Demonstration of fungal elements may be facilitated by use of glucan-binding fluorescent brighteners such as calcofluor white (90) or Congo red (59). These require use of a fluorescent microscope set up to visualize the specific fluorescence obtained. Calcofluor white is added directly to the KOH drop on the slide as an approximately equal drop of 0.1% solution (69). All preparations should be examined under low power and confirmed under high power.

Skin and nails infected by dermatophytes may reveal one or more of the following: hyaline hyphal fragments; septate, often branched hyphae; and chains of arthroconidia (Fig. 1).

The appearance of infected hairs depends on the invading dermatophyte species. Hyphae invade the hairs, and arthroconidia are formed by fragmentation of these hyphae. The appearance and locations of the arthroconidia may suggest the infecting genera or species (Table 3), as may the sizes (89). Three main types of colonizations (ectothrix, endothrix, and favic) are observed by direct microscopic examination. The terms "ectothrix" and "endothrix" refer to the location of the arthroconidia in relation to the hair shaft (ecto- and endo- meaning outside and inside, respectively), while favic refers to the distinctive infection caused by *T. schoenleinii,* a fungus that is now extremely rare except in some parts of central Asia and the African Sahel**.**

In ectothrix colonization, arthroconidia appear as a mosaic sheath around the hair or as chains on the surface of the hair shaft (Fig. 2). In *M. canis, M. audouinii,* and *M. ferrugineum* infections, colonized hairs fluoresce green under Wood's lamp; other ectothrix infections (Table 3) are nonfluorescent. Endothrix hair invasion is observed as chains of arthroconidia filling the insides of shortened hair stubs (Fig. 3). Hairs are

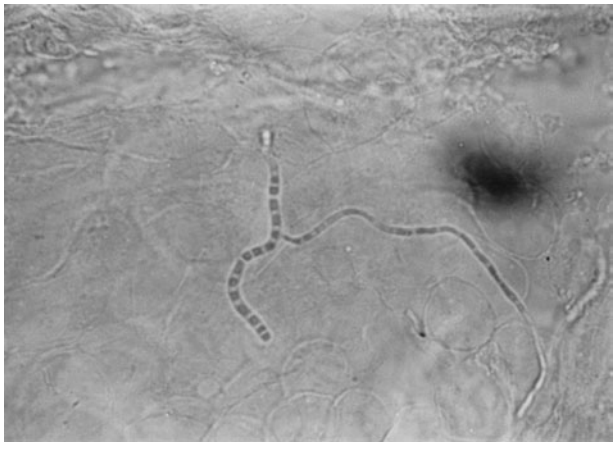


FIGURE 1 Dermatophyte hyphae in skin scraping. NaOH mount. Magnification, × 400.

TABLE 3 Hair invasion by dermatophytes on the human host

Site and organism
Ectothrix
M. audouinii
M. canis
M. ferrugineum
M. gypseum complex
M. praecox
T. megninii
T. mentagrophytes complex
T. verrucosum
Endothrix
T. soudanense
T. tonsurans
T. violaceum
Favic
T. schoenleinii

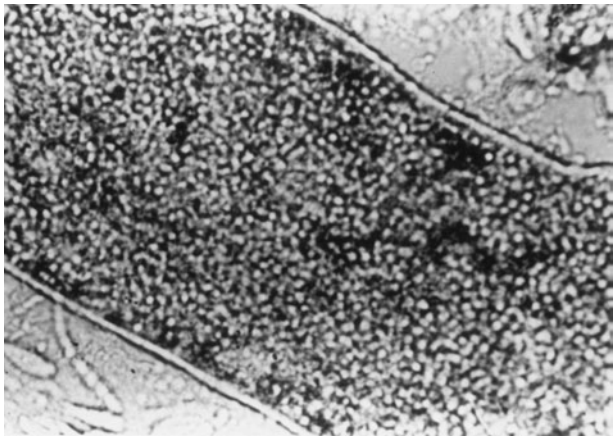


FIGURE 3 *T. tonsurans,* endothrix type of hair invasion. Magnification, ×1,000.

Wood's lamp negative. In favic hairs, hyphae, air bubbles, or tunnels and fat droplets are observed within the hair (Fig. 4). These hairs are dull green under the Wood's lamp. In general, infected hairs from all infection types show hyphae within the hair shaft at some time during the course of infection, usually during the early stages.

Isolation Procedures

Scrapings, hairs, and other materials collected as outlined above for direct examination are plated on selected isolation media and incubated at 32°C for optimal growth; temperatures between 24 and 32°C are also acceptable if the total incubation time is suitably adjusted to compensate for the slower outgrowth expected at lower temperatures. Generally from 5 to 15 skin or nail fragments are planted per plate or tube used for isolation, and these fragments are separated so that antibiotic-resistant mold or bacterial contaminants from one piece cannot overgrow the others. Hairs are also well separated. Cultures on primary isolation medium are routinely incubated at 25 to 30°C and examined weekly for up to 4 weeks.

The most common medium used for the isolation of dermatophytes is Sabouraud glucose agar (SGA) (original formulation with 4% glucose or Emmons' modification with 2% glucose), amended with chloramphenicol and cycloheximide to inhibit bacterial and saprobic fungal contamination. This type of medium is available commercially as, for example, Mycobiotic agar (BD Diagnostic Systems, Sparks, Md.; Remel, Lenexa, Kans.), Mycosel (BD), or Dermasel Selective Supplement (Oxoid, Basingstoke, Hampshire, United Kingdom; note that Remel is currently the U.S. distributor for Oxoid). An alternative medium promoting more rapid conidiation and colony pigmentation development is potato flakes agar amended with cycloheximide and chloramphenicol (Hardy Diagnostics, Santa Maria, Calif.; also available as Rapid Sporulation Medium, Bacti-Lab, Mountain View, Calif.). For the isolation of cycloheximide-susceptible fungi that cause clinical infections resembling dermatophytosis, any of the SGA with chloramphenicol (Remel, BD), inhibitory mold agar (BD, Remel), or Littman oxgall agar (BD) may be recommended (90). Inhibitory mold agar and Littman oxgall agar have

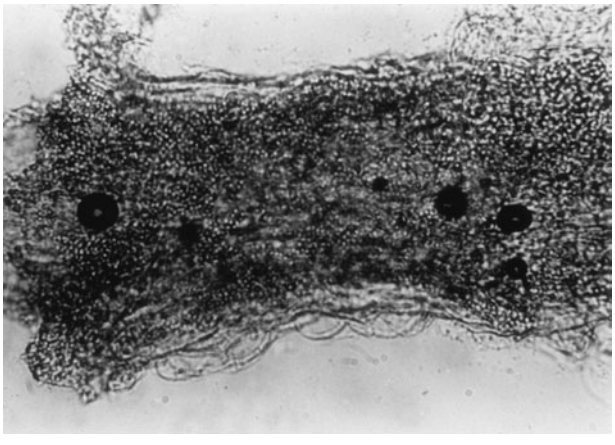


FIGURE 2 *M. audouinii,* ectothrix type of hair invasion. Magnification, ×400.

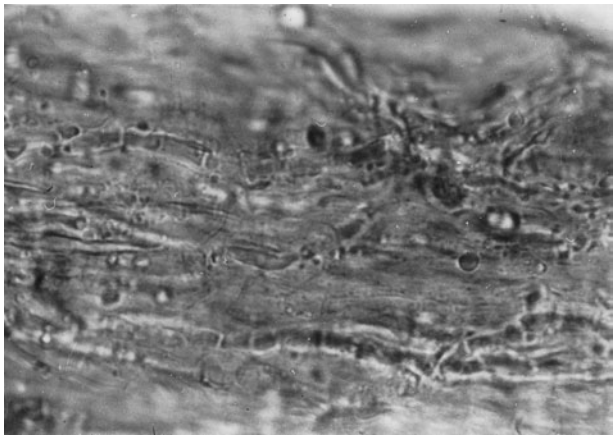


FIGURE 4 Hair infected by *T. schoenleinii* from a patient with favus. Magnification, ×1,000.

the advantage of restricting contaminant colony diameters. For all media, the addition of gentamicin is recommended for specimens heavily contaminated by bacteria (108). SGA with cycloheximide, chloramphenicol, and gentamicin (CCG) is routinely used as an isolation medium in some laboratories (59).

Additional and alternative media may be used in special circumstances. For example, vitamin-free Casamino acids (BD)-erythritol-albumin agar medium plus CCG may be used for filament-positive skin and nail specimens, especially from body sites where *Candida* overgrowth may be a problem (e.g., groin or fingernails). This medium (currently not commercially available to our knowledge) prevents the common suppression of dermatophyte outgrowth by heavy inoculum of *Candida albicans, C. parapsilosis,* and related biotin-requiring yeasts (28). Dermatophyte species with vitamin requirements (very uncommon in these types of cases) may grow poorly on it, and it is always used in combination with a cycloheximide-containing SGA.

Another primary isolation medium that may be used is dermatophyte test medium (DTM; available commercially from BD and Remel). This selective medium screens for the presence of dermatophytes in heavily contaminated material (nails, etc.). The growth of dermatophytes causes a rise in pH, thus changing the phenol red indicator from yellow to red (109). The use of DTM should be combined with morphological study, since dermatophytoids such as *Trichophyton terrestre,* as well as various *Chrysosporium* species and other nondermatophytic fungi, can grow and turn the medium red (59, 70, 93). Rapid sporulation medium (Bacti-Lab), mentioned above (amended potato flakes agar), contains a pH indicator that works on a similar principle but turns from yellow to blue-green, leaving the red reverse pigment of typical *T. rubrum* visible (6). DTM may uncommonly give false-negative results with some *Microsporum* isolates (73).

Nucleic Acid-Based Direct Detection Techniques

Numerous techniques have been published for directly detecting dermatophyte DNA in tissue, but as yet none is established as a routine diagnostic procedure. This is due mainly to the relatively high cost-effectiveness of traditional procedures. However, in terms of accuracy, traditional procedures are by no means optimal; in onychomycosis, for example, they disclose at best circa 85% of true-positive cases from the initial patient specimen, and this percentage is much lower in many laboratories (103). This has given strong incentive for molecular development. A combination of amplifications with ribosomal PCR primer pairs TR1/TR2 and N5/N6, followed by HaeIII restriction enzyme digestions, was shown to amplify dermatophytes and nondermatophytes from infected nails and distinguish dermatophytes as a group from other fungi (10). Dermatophyte species could not be consistently identified based on the patterns seen. Similar results were also seen in a study that followed up the same procedures with LightCycler melting curves for the N5/N6 amplicon, which offered greater resolution than several other amplicons tested (46). In a deep dermatophytosis case in which all conventional tests had given negative results, *Trichophyton rubrum* was identified by means of a nested-PCR study directed to amplifying a portion of the ITS region from paraffin-embedded sections (74). Some additional studies dealing with direct amplification of dermatophyte materials are discussed by Gutzmer et al. (46). It is beyond the scope of the present chapter to extensively review studies that are preliminary in terms of implemented diagnostic procedure. Some molecular culture identification methods for dermatophytes are mentioned below under "Identification."

IDENTIFICATION

At present, the great majority of dermatophytes are identified phenotypically. Identification is often based on (i) colony characteristics in pure culture on SGA and (ii) microscopic morphology. These criteria alone, however, may be insufficient, since colonial appearance may vary or be similar for different species. Characteristic pigmentation may fail to appear, and isolates, especially *Trichophyton* spp., may not sporulate. Special media may be required to stimulate pigment production; it may be necessary to use sporulation and physiologic tests in conjunction with morphology to identify the species correctly. Various molecular identification mechanisms have also been described (discussed below) but are seldom practically applicable except in high-level reference laboratories. The majority of isolates are easily identified when visual examination is combined with any needed testing for characteristic growth factor requirements; the consequence of this is that phenotypic studies remain the least expensive option for routine identification.

Colony Characteristics

In observing gross colony morphology, note the color of the surface and the reverse of the colony, the texture of the surface (powdery, granular, woolly, cottony, velvety, or glabrous), the topography (elevation, folding, margins, etc.), and the rate of growth.

Microscopic Morphology

Microscopic morphology, especially the appearance and arrangement of the conidia (macroconidia or microconidia) and other structures, may be determined by teased mounts or slide culture preparations mounted in lactophenol cotton blue (LCB), in lactophenol aniline blue (phenol, an ingredient of LCB and lactophenol aniline blue, is listed as a hazardous chemical; therefore, solutions containing phenol should be prepared, stored, and used in an approved chemical safety cabinet), in lacto-fuchsin (59), or in more permanent mounting fluids (112). Sometimes a special medium such as cornmeal or cornmeal-glucose agar, potato-glucose agar, SGA plus 3 to 5% NaCl (56, 58), pablum cereal agar (59), rapid sporulation medium (6), or lactrimel agar (14, 53) may be required to stimulate sporulation.

Physiological Tests

In Vitro Hair Perforation Test

The in vitro hair perforation test distinguishes between atypical isolates of the *T. mentagrophytes* complex and *T. rubrum* (4). It may also be used to assist in making other distinctions such as *M. canis* versus *Microsporum audouinii* and *Microsporum praecox* versus *M. gypseum* (81). Hairs exposed to *T. mentagrophytes, M. canis,* and *M. gypseum* show wedge-shaped perforations perpendicular to the hair shaft (a positive test result), whereas *T. rubrum, M. audouinii,* and *M. praecox* do not form these perforating structures.

Place short strands of human hair (ideally hair from a child under 18 months old) in petri dishes, and autoclave the dishes at 121°C for 10 min; add 25 ml of sterile distilled water and 2 or 3 drops of 10% sterilized yeast extract. Inoculate these plates with several fragments of the test fungus that has been grown on SGA; incubate the plates at

25°C, and examine them at regular intervals over a period of 21 days. Hairs may be examined microscopically for perforations by removing a few segments and placing them in a drop of LCB mounting fluid. Gently heating the mounts aids in the detection of the fungus. A positive control test should always be run with a known perforating species; *M. canis* is recommended. Some hair samples may prove unsuitable for unknown reasons, e.g., possible prior contact with shampoos containing antifungal inhibitors.

Special Nutritional Requirements

Nutritional tests aid in the routine identification of *Trichophyton* species that seldom produce conidia or that resemble each other morphologically (30). Certain species have distinctive nutritional requirements, whereas others do not. The method employs a Casamino acids basal medium that is vitamin free (*Trichophyton* agar 1 [T1]) and to which various vitamins are added, i.e., inositol (T2), thiamine + inositol (T3), thiamine (T4), and nicotinic acid (T5). In addition, the series includes an ammonium nitrate basal medium (T6) to which histidine is added (T7). These media are available commercially in dehydrated form from BD Biosciences and in prepared form from Remel. A small fragment (about the size of the head of a pin) from the culture to be tested is placed on the surface of the basal medium (controls) and the media containing the vitamin and amino acid additives. Care must be taken to avoid transferring agar from the fungal inoculum to the nutritional media. Cultures are incubated at room temperature (or 37°C if *T. verrucosum* is suspected) and read after 7 and 14 days. The amount of growth is graded from 0 to 4+. Commonly observed reactions are summarized in Table 4.

Urea Hydrolysis

The ability to hydrolyze urea provides additional data to aid in distinguishing the typical, cosmopolitan form of *T. rubrum* (urease negative) from members of the *T. mentagrophytes* complex (typically urease positive) and from the urease-positive Afro-Asiatic or "granular" form of *T. rubrum,* formerly often called *T. raubitschekii* (92, 95). Christensen urea agar and broth may both be used; the broth appears to be the more sensitive of these alternatives (57). After the urea medium is inoculated, it is incubated at 25 to 30°C up to 7 days. The tubes should be examined every 2 to 3 days for the color change from orange or pale pink to purple-red that indicates the presence of urease, a positive test result. Negative and positive controls should always be done with new batches of these media. See Table 1.

Growth on BCPMSG

BCP-milk solids-glucose medium (BCPMSG) is available commercially as Dermatophyte Milk Agar (Hardy). The type of growth (profuse versus restricted) and a change in the pH indicator (BCP) indicating alkalinity are especially useful for distinguishing *T. rubrum* from the *T. mentagrophytes* complex and *T. mentagrophytes* from *Microsporum persicolor* (58, 59, 106). *T. rubrum* shows restricted growth and produces no alkaline reaction on BCPMSG, whereas members of the *T. mentagrophytes* complex typically show profuse growth and an alkaline reaction. Although *M. persicolor* shows profuse growth, it does not result in an alkaline reaction. Other tests for distinguishing *T. mentagrophytes* and allies from *M. persicolor* are described elsewhere (78).

Cultures to be tested are inoculated onto slants of BCPMSG and examined for pH change and growth characteristics at the end of a 7-day incubation at 25°C. A color change from pale blue to violet-purple indicates an alkaline reaction.

Growth on Polished Rice Grains

Unlike most dermatophytes, *M. audouinii* grows poorly on rice grains and produces a brownish discoloration of the rice (18, 59). This is a useful test for differentiating this species from *M. canis* and from other dermatophytes that typically grow and sporulate on rice grains. It may be especially useful in areas where *M. audouinii* is still endemic, e.g., sub-Saharan

TABLE 4 Dermatophyte nutritional response as elucidated by *Trichophyton* agars[a]

Species	Response by vitamin tests					Response by amino acid tests	
	1 Vitamin free	2 Inositol	3 Thiamine + inositol	4 Thiamine	5 Nicotinic acid	6 Amino acid free	7 Histidine
M. gallinae						4	4
T. concentricum, 50%	4	4	4	4	4		
T. concentricum, 50%	2	2	4	4	2		
T. equinum var. *equinum*	0	0	0	0	4		
T. equinum var. *autotrophicum*	4	4	4	4	4		
T. megninii						0	4
T. soudanense	v	v	v	v	v	v	v
T. tonsurans	1	1	4	4	1		
T. verrucosum, 84%	1	2	4	2	1		
T. verrucosum, 16%	1	1	4	4	1		
T. violaceum (typical)	1	1	4	4	1		
T. violaceum (rare "*T. yaoundei*" form)	4	4	4	4	4		

[a] Only the growth responses for organisms with growth factor requirements and the selected organisms that must be most closely compared with them are included in this table. The numbers in the table body indicate the relative degree of growth according to traditional 1+ to 4+ visually approximated scale: 0, no growth; 1, slight growth, strongly nutrient-deprived colony morphology (very sparse, subsurface colonial growth only or colony diameter strongly reduced compared to Sabouraud agar control); 2, partially stimulated growth, but still significantly suppressed compared to that of the control; 4, growth comparable to that of the control (the table includes no 3+ reactions); v, variable; blank spaces in the table indicate growth responses that are not customarily examined but that are insignificantly different from control growth responses on Sabouraud agar.

Africa, or where immigrants or travellers from such areas are reintroducing it.

The medium is prepared in 12-ml flasks by mixing 1 part raw unfortified rice grains and 3 parts water (18) or 8.0 g of rice grains and 125 ml of distilled water. Autoclave at 15 lb/in^2 for 15 min. Inoculate the surface of the rice, and incubate the sample for 2 weeks at 25 to 30°C.

Temperature Tolerance and Temperature Enhancement

Tests for temperature tolerance and enhancement are useful for distinguishing the *T. mentagrophytes* complex from *Trichophyton terrestre* (79), *T. mentagrophytes* from *M. persicolor* (58), *T. verrucosum* from *T. schoenleinii* (89), and *Trichophyton soudanense* from *M. ferrugineum* (114). At 37°C, members of the *T. mentagrophytes* complex show good growth, whereas *T. terrestre* does not grow and *M. persicolor* generally grows poorly or not at all (a single atypical isolate with good growth has been observed); growth of *T. verrucosum* and *T. soudanense* is enhanced, but that of *T. schoenleinii* and *M. ferrugineum* is not.

Inoculate two slants of SGA with equivalent fragments of the culture. Incubate one slant at room temperature (25 to 30°C) and one at 37°C. Compare the growths at the two temperatures when mature colonies appear at room temperature. Appropriate controls are recommended and should be compared first.

Molecular Identification Techniques

Numerous methods for rapid nucleic acid-based distinction of dermatophyte species have been described in recent years (51). Methods used in recent studies have largely been based on arbitrarily primed PCR (34, 67) or restriction fragment length polymorphisms generated from PCR products (13). An oligonucleotide probe for *T. rubrum* has been designed (27). Recently, several additional techniques have been shown to have promise, in particular a rather complex amplification of the DNA topoisomerase II gene followed by nested PCR and restriction fragment digestion (54, 55). Repetitive-sequence-based PCR performed via the patented DiversiLab system (Spectral Genomics, Houston, Tex.) has also shown promise but has so far been tested only on a reduced set of species (82). It is currently unclear whether any techniques based on electrophoretic banding patterns of amplicons or restriction fragments will be able to compete with routine diagnostic sequencing of the ITS region (84) or with forthcoming oligonucleotide-based array and bead technologies (104).

DESCRIPTION OF ETIOLOGIC AGENTS

Characteristic features of dermatophyte species are presented in Table 1. The table also includes data on some similar but rarely or never pathogenic *Microsporum* and *Trichophyton* species that must be distinguished from pathogenic species.

Two types of conidia may be produced on the aerial mycelium of the dermatophytes: large multicellular, smooth or rough, thin- or thick-walled macroconidia and smaller unicellular, smooth-walled microconidia. The three genera are classically grouped according to the presence or absence of these two types of conidia and the appearance of the surface of the macroconidia, i.e., rough versus smooth. However, these conceptual distinctions must be mapped onto a reality in which, when atypical isolates are taken into account, a morphologic continuum is found based on overlapping characteristics (80). For this reason, it is often more convenient to identify isolates directly at the species level than it is to try to identify them at the genus level first. Identification of species is based on the microscopic appearance and arrangement of the conidia (Fig. 5 through 17), colonial morphology on SGA (Fig. 18), and physiological tests (Table 5).



FIGURE 5 Smooth-walled macroconidia of *Trichophyton ajelloi.* Magnification, ×400.

Trichophyton Species

Macroconidia have smooth, thin to thick walls, are variable in shape (clavate, fusiform to cylindrical), vary in number of septa (1 to 12) and in size (8 to 86 by 4 to 14 μm), and are borne singly or in clusters. Microconidia, which are usually present and more numerous than macroconidia, may be globose, pyriform, or clavate and are borne singly along the sides of hyphae or in grapelike clusters. Though species in this genus generally produce microconidia more readily than macroconidia, two lineages producing macroconidia but lacking microconidia have been described: (i) "*Trichophyton kanei*" (100), now known to be a variant of the Afro-Asiatic genotype of *T. rubrum*, and (ii) a variant all-macroconidial form of *T. tonsurans* (80). Fresh isolates of the dermatophytoid *T. ajelloi* may also produce many macroconidia and few

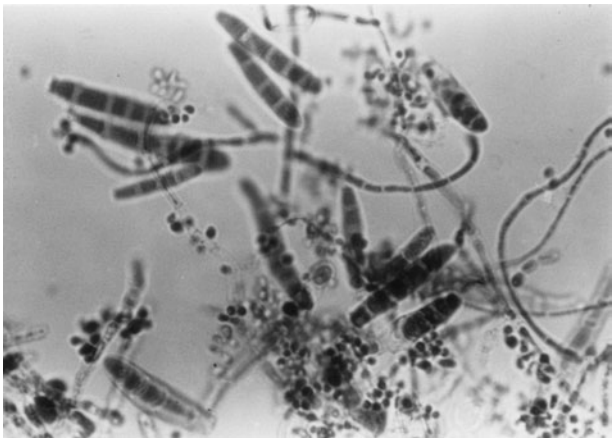


FIGURE 6 Macroconidia and microconidia of *Trichophyton mentagrophytes* complex on SGA with 5% NaCl. Magnification, ×400.

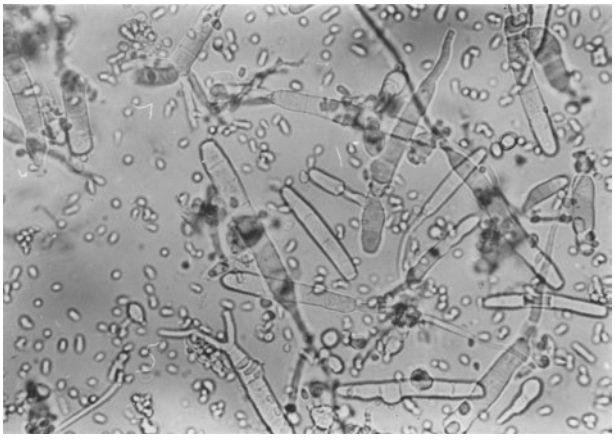


FIGURE 7 Smooth-walled macroconidia of Afro-Asiatic type *Trichophyton rubrum* (= *T. raubitschekii*) from primary isolate on SGA. Magnification, ×1,000.

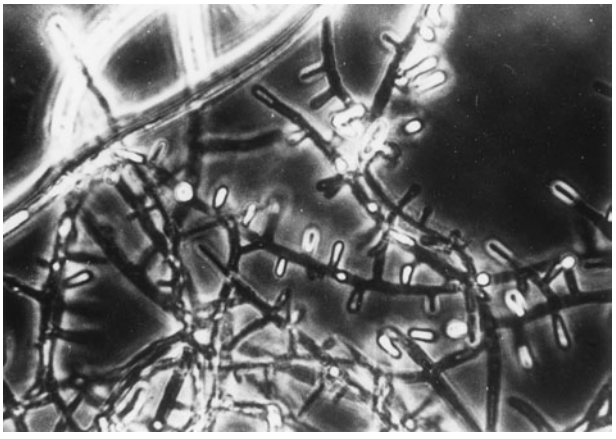


FIGURE 9 Clavate macroconidium, microconidia, and intermediate conidia of *Trichophyton terrestre*. Phase contrast; magnification, ×400.

or no microconidia. Some species such as *T. schoenleinii* rarely produce conidia of any kind in culture, and nonsporulating isolates of normally conidial species, especially *T. rubrum,* may also be encountered.

Microsporum Species

Microsporum species produce macroconidia and microconidia that may be rare or numerous, depending on the species and the substrate. The distinguishing characteristic is the macroconidium, which is typically rough walled (varying from minutely to strongly roughened). Macroconidia also vary in shape (obovate, fusiform to cylindrofusiform), number of septa (1 to 15), size (6 to 160 by 6 to 25 μm), and width of the cell wall. Microconidia are pyriform or clavate and usually are arranged singly along the sides of the hyphae. *Microsporum* species invade skin, hair, and, rarely, nails. Biological (teleomorphic) species within the *M. gypseum* complex can be provisionally recognized on the basis of colonial and microscopic features on Takashio's medium (25), but these species are best distinguished by mating or molecular testing.

Epidermophyton floccosum

In *E. floccosum,* microconidia are lacking; only smooth-walled, broadly clavate macroconidia are produced. They have one to six septa, are 20 to 40 μm long by 7 to 12 μm wide, and are borne singly or in clusters of two or three. *E. floccosum* is currently the only recognized *Epidermophyton* species; the former *E. stockdaleae* is now considered a synonym of *Trichophyton ajelloi* (33).

STRAIN TYPING SYSTEMS

Dermatophyte strains within anthropophilic species tend to be very closely related, making epidemiological analysis difficult, but strains of *T. rubrum* have been distinguished by polymorphisms in the numbers of subrepeat elements in the ribosomal nontranscribed spacer region (45, 50, 85, 119). Similar techniques can also be applied to distinguish *Trichophyton interdigitale* and *T. tonsurans* isolates (29, 45, 71). In addition, the Afro-Asiatic lineage of *T. rubrum* can be distinguished from the now-cosmopolitan epidemic *T. rubrum* form by means of a microsatellite marker designated T1 (77).

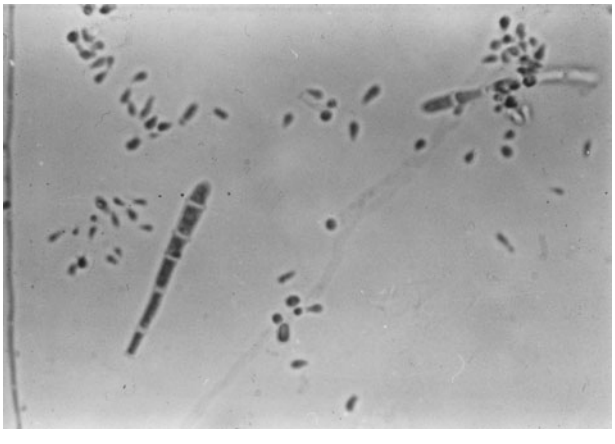


FIGURE 8 Long, narrow macroconidium and clavate to pyriform microconidia of *T. rubrum*. Magnification, ×400.

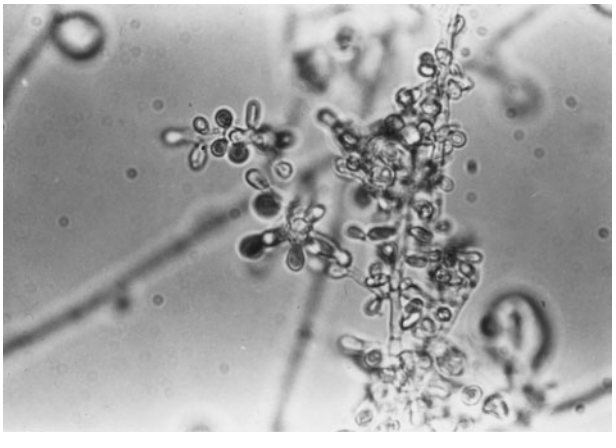


FIGURE 10 Microconidia with typical refractile cytoplasm of *Trichophyton tonsurans*. Magnification, ×400.



FIGURE 11 Characteristic chlamydospores produced by *Trichophyton verrucosum* or BCP-milk solids-yeast extract agar. Magnification, ×400.



FIGURE 13 Macroconidia of *Microsporum audouinii* on SGA with 3% NaCl. Magnification, ×400.

ANTIMICROBIAL SUSCEPTIBILITIES

Dermatophytes can in principle be tested for susceptibility to antifungal drugs by using the general NCCLS (now CLSI) M38-A standard for molds (75). A trial has shown that this methodology can be applied with good inter- and intralaboratory reproducibility to the commonly used drugs ciclopirox, fluconazole, griseofulvin, itraconazole, posaconazole, terbinafine, and voriconazole (31). Treatment failures in dermatophytoses, however, are almost always due to factors other than drug resistance (44), and potentially burdensome requests for dermatophyte susceptibility testing should be closely screened for scientific and clinical appropriateness.

EVALUATION, INTERPRETING, AND REPORTING OF LABORATORY RESULTS

For nonimmunocompromised patients, positive direct microscopy compatible with dermatophytosis is conventionally interpreted as presumptively indicating this condition in hair and in skin specimens other than those from nails, soles, and palms. The positive microscopic report itself conveys this information; it should be issued within two working days of specimen receipt. With soles and palms, persons who have lived in tropical areas or who are of south Asian heritage may have dermatophytosis-like *Scytalidium* infections, which are similar to dermatophytoses both clinically and in direct microscopy (59); no presumptive diagnosis can be inferred until the culture result is available. The direct microscopic result, however, is still reported immediately. For patients without risk factors for *Scytalidium,* qualified physicians may make presumptive diagnoses of dermatophytosis for sole and palm skin as for other skin sites. In onychomycosis, over 35 different mold species may be involved in producing conditions clinically and microscopically resembling tinea unguium (59), particularly in geriatric patients. Microscopic results are still reported promptly, but culture results are of high interest. At the same time, outgrowth of known onychomycosis-causing nondermatophytes may or may not be significant, excepting the nail-infecting *Scytalidium* species, which are always considered significant when grown. The complexities of accurately reporting nondermatophytes from nail specimens are beyond the scope of this

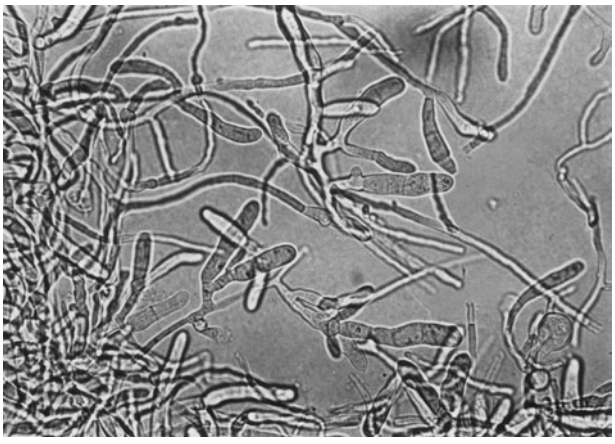


FIGURE 12 Macroconidia of *Epidermophyton floccosum* on SGA. Note the absence of microconidia. Magnification, ×400.



FIGURE 14 Macroconidia of *Microsporum canis* with rough thick walls. Magnification, ×400.



FIGURE 15 Macroconidia of *Microsporum cookei,* showing thick walls and pseudosepta. Magnification, ×400.

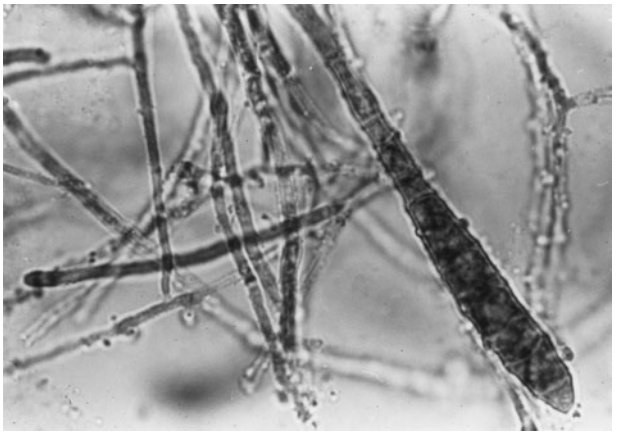


FIGURE 17 Rough-walled macroconidium of *Microsporum persicolor* on SGA with 3% NaCl. Magnification, ×1,000.

chapter but are discussed in light of the first-ever rigorous validation studies on this topic by Summerbell et al. (103).

Isolation of a dermatophyte culture from lesional skin, hair, or nails is interpreted as diagnostic whether or not fungal elements are seen in the initial direct microscopy examination. Note that dermatophytoids such as *Microsporum cookei* and *Trichophyton terrestre* are presumed to be contaminants until proven otherwise; they are, however, reported along with the comment "normally nonpathogenic" when grown. These fungi have been the subjects of numerous false and questionable case reports in the literature, and there may be some confusion about their status. If such a fungus were to infect human epidermis, the "gold standard" for scientifically evidencing the case would be three successive, consistent repeat isolations of the fungus from the lesion in specimens collected on different days, plus demonstration of compatible fungal elements in direct microscopy of the affected tissue. No inferential diagnoses from lower-quality evidence should be accepted as conclusive, even if the patient is said to have been successfully treated. A common dermatophyte such as *T. rubrum* that failed to grow out in initial culture(s) is very likely to be the actual etiologic agent in such cases (103). In onychomycosis in particular, true causal dermatophytes have only at best a circa 75% chance of being grown from the initial specimen taken from any given patient; another circa 15% can be recovered from a second specimen, while circa 10% can be detected only by doing three or more serial specimens, something very seldom done in practice (103). Cycloheximide-tolerant dermatophytoids that grow (in any quantity whatsoever) from single, otherwise unproductive specimens should not be assumed to be causal simply because they share a genus name with dermatophyte species.



FIGURE 16 Macroconidia of *Microsporum gypseum*. Magnification, ×400.

SUPERFICIAL MYCOSES

In the superficial mycoses, the causative fungi colonize the cornified layers of the epidermis or the suprafollicular portion of the hair. There is little tissue damage, and cellular response from the host generally is lacking. The diseases are largely cosmetic in impact, involving changes in the pigmentation of the skin (tinea versicolor or tinea nigra) or formation of nodules along the distal hair shaft (black piedra and white piedra).

In contrast to agents of the dermatophytoses, the etiologic agents are diverse and unrelated.

Tinea Versicolor (Pityriasis Versicolor)

Tinea versicolor is an infection of the stratum corneum caused by a group of closely similar lipophilic yeast species of the *Malassezia furfur* complex. Members of this complex infecting human skin were often treated in former times as a single species but were then shown by molecular, physiological, and serotyping studies to be separate (39). The complex includes *M. furfur* (synonyms, *Pityrosporum furfur* and *P. ovale* pro parte), *M. sympodialis, M. globosa* (probable synonym, *Pityrosporum orbiculare*), *M. restricta, M. slooffiae, M. obtusa, M. dermatis, M. japonica,* and *M. yamatoensis* (39, 99). Some additional species have been reported from animals. In routine clinical reporting, referring to these organisms as members of the *M. furfur* complex is normally sufficient. *Malassezia pachydermatis,* which causes animal ear infections and occasional human iatrogenic fungemias, is not considered a member of the *M. furfur* complex and, if reported, is reported under its individual species name. It is

easily distinguished from *M. furfur* complex members by its ability to grow on ordinary laboratory media such as Sabouraud agar (see comments on *Malassezia* culture below). It is insignificant when grown from human skin except if investigations of catheter-related problems are involved.

Tinea versicolor lesions appear as scaly, discrete or concrescent, hypopigmented or hyperpigmented (fawn, yellow-brown, brown, or red) patches chiefly on the neck, torso, and limbs. The infection is largely cosmetic, becoming apparent when the skin fails to tan normally. The disease has a worldwide distribution: in tropical climates 30 to 35% of the population may be affected, while incidence in areas of temperate climate is much lower, with only 1.0 to 4.0% of the population affected. *M. furfur* and related yeasts are found on the normal skin and elicit disease only under conditions, local or systemic, that favor the overgrowth of the organism.

The *M. furfur* complex has been associated with folliculitis (9), obstructive dacryocystitis (88), systemic infections in patients receiving intralipid therapy (23), and seborrheic dermatitis, especially in patients with AIDS (37). Excellent reviews are available on human infections caused by *Malassezia* spp. and on the characteristics of the genus (12, 41, 94). Additional information is also found in chapter 119 of this Manual.

Direct Examination

The fungi are observed readily when scrapings are mounted in 10% KOH plus ink (17), 25% NaOH plus 5% glycerin, calcofluor white, or Kane's formulation (glycerol, 10 ml; Tween 80, 10 ml; phenol, 2.5 g; methylene blue, 1.0 g; distilled water, 480 ml). In cases where skin scrapings from unspecified body sites are examined, Kane's formulation has the advantage of vividly staining both fungi and the differential-diagnostic organisms causing erythrasma and pitted keratolysis. (These two bacterial infections, often confused with superficial mycosis, are mainly from intertriginous sites and foot soles, respectively.) Tinea versicolor is signaled in microscopy by the presence of "spaghetti and meatballs," i.e., short, septate, occasionally branching filaments 2.5 to 4 μm in diameter and of variable lengths intermingled with clusters of small, unicellular, oval or round budding yeast cells (Fig. 19). The yeasts show the presence of a collarette between mother and daughter cells (budding is phialidic and unipolar) and average 4 μm (up to 8 μm) in size.

Isolation and Culture

Culture is not essential for identification unless the findings of direct microscopic examination are atypical, or unless full species identification is desired for research purposes. Also, *M. furfur* complex members are part of the normal flora of the skin and positive culture does not indicate infection. The species require exogenous lipid and do not grow on routine mycology media. If culture is desired, scrapings may be inoculated on Leeming-Notman medium, which uses whole milk as a major lipid source (65), on Dixon agar (20), or on modified Dixon agar, consisting of malt extract (3.6%), Mycological Peptone (Oxoid) (0.6%), desiccated ox bile (Bile Salts, Oxoid) (2%), Tween 40 (1%), glycerol (0.2%), oleic acid (0.2%), and agar (1.2%). Growth of the yeasts is slow; colonies are cream colored, glossy or rough, and raised (Fig. 20), later becoming dull, dry, and tan to brownish. Only budding yeast cells generally appear in culture (Fig. 21).

Tinea Nigra

Tinea nigra is characterized by the appearance, primarily on the palms of the hands and less commonly on the dorsa of the feet, of flat, sharply marginated, brownish black, nonscaly macules that may resemble melanoma (94).

The disease, almost always caused by *Hortaea werneckii* (= *Phaeoannellomyces werneckii*, = *Exophiala werneckii*, = *Cladosporium werneckii*), is most common in tropical areas (88) but has been contracted occasionally in coastal areas in and near the southeastern United States (94, 111). Cases diagnosed outside the area of endemicity have mostly resulted from travel to the American tropics or the Caribbean islands (88). *H. werneckii* is related to the ascomycetous order Dothideales (49).

Direct Microscopic Examination

Microscopic examination of skin scrapings in KOH or NaOH reveals numerous light brown, frequently branching septate filaments 1.5 to 5 μm in diameter (Fig. 18h); short, sinuous filaments; and budding cells, some septate.

Isolation

On SGA with or without antibiotics *H. werneckii* grows slowly and usually appears within 2 to 3 weeks as moist, shiny olive to greenish black yeast-like colonies. The yeast-like cells are usually two-celled when reproductive and, instead of budding, produce new yeast cells from thick (up to 2 μm in diameter) distinctly annellated (multiply ringed as if wearing several bracelets) pegs. After 7 or more days, colonies may develop a fringe of thick, dark, conspicuously septate hyphae that also bear annellated fertile structures. These produce conidia that are indistinguishable from young yeast cells. The micromorphology of *H. werneckii* is described in more detail in chapter 125.

Black Piedra

Black piedra is a fungal infection of the scalp hair, less commonly of the beard or moustache, and rarely of axillary or pubic hairs. The disease is characterized by the presence of discrete, hard, gritty, dark brown to black nodules adhering firmly to the hair shaft (Fig. 22). It is found mostly in tropical regions in Africa, Asia, and Central and South America. Humans as well as other primates are infected (32, 88, 94).

The etiologic agent in humans is *Piedraia hortae*, an ascomycete (order Dothideales) forming nodules that serve as ascostromata containing locules that harbor the asci and ascospores.

Direct Microscopic Examination

Hair fragments containing one or more black nodules are placed in 25% KOH or NaOH with 5% glycerol. The preparation is heated gently and carefully squashed so as not to break the coverslip, as the nodules are very hard. A squashed preparation of a mature nodule should reveal compact masses of dark, septate hyphae and round to oval asci containing two to eight hyaline, aseptate banana-shaped (fusiform) ascospores that bear one or more appendages. The preparation should first be observed under low-power objective to reveal the dark mass of compacted hyphae around the surface of the hair and then examined under the high-power objective to observe the asci and ascospores.

Isolation, Culture, and Identification

When ascospores are seen in direct specimen microscopy, culture is unnecessary. Otherwise, SGA with chloramphenicol and SGA with chloramphenicol and cycloheximide may be used for isolation. Some reports have indicated that cycloheximide may be inhibitory; however, others have used

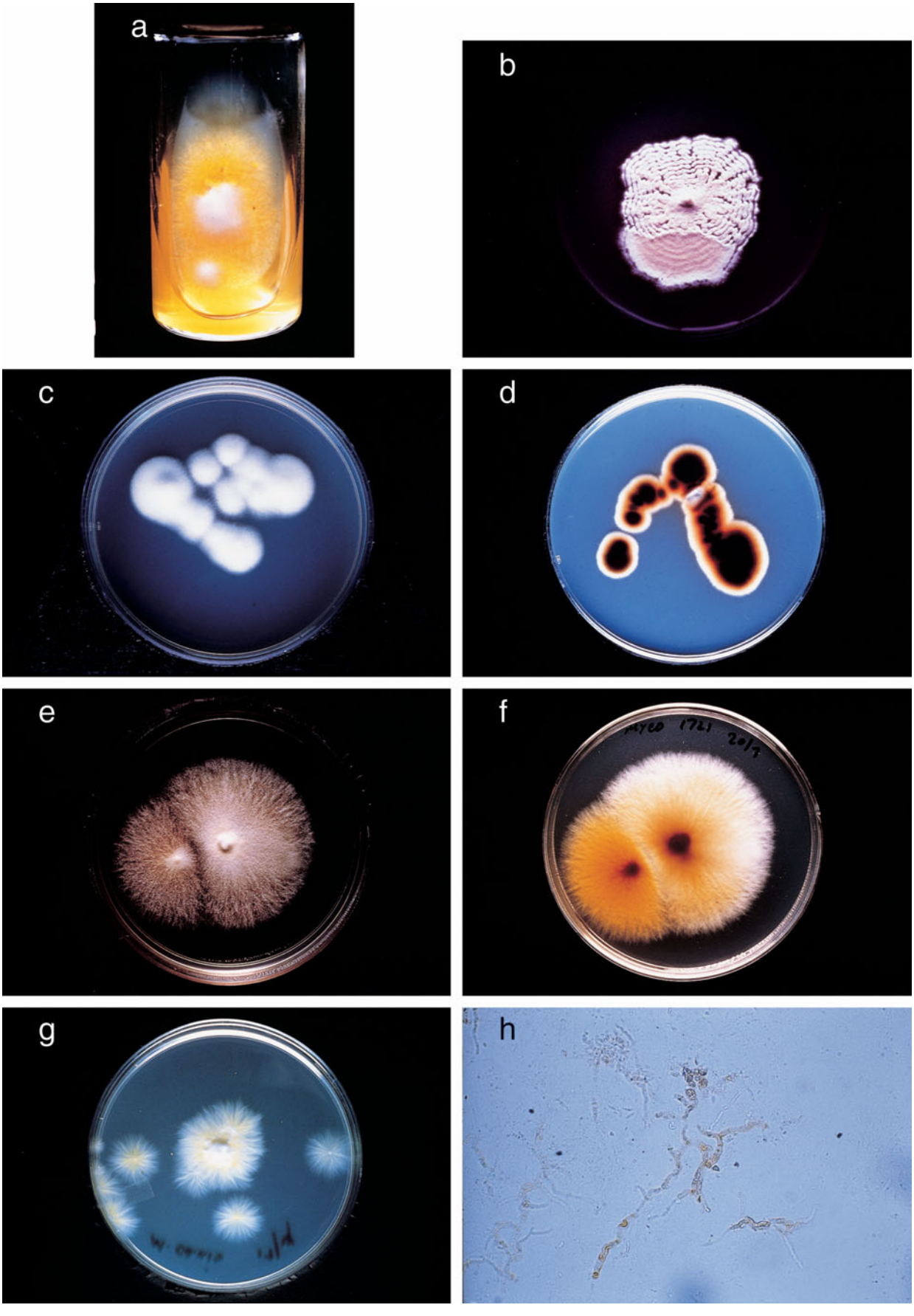

a
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h

TABLE 5 Sequence of procedures for phenotypic identification of dermatophytes in pure culture[a]

1. Examine colony at day 7 and, if necessary, day 14 for colors of surface and reverse, topography, texture, and rate of growth. Proceed to step 2.
2. Prepare tease mounts and search for identifying microscopic morphology, especially presence, appearance, and arrangement of macroconidia and microconidia (consult Fig. 5 to 18 and Table 1). If the results are inconclusive, proceed to step 3.
3. Prepare slide cultures or transparent tape mounts and examine for characteristic morphology as indicated above if tease mounts do not provide sufficient information. Consider special media if sporulation is absent (potato glucose agar, lactrimel, BCPMSG, SGA with 3–5% NaCl). At the same time, proceed to step 4.
4. Perform as many of the following physiologic and other special tests as necessary for identification.
   a. Urease (ensure culture is bacteria-free!).
   b. Nutritional requirements if a *Trichophyton* is suspected.
   c. Growth on rice grains if an unusual *Microsporum* sp. is suspected.
   d. Elevated temperature response (37°C on SGA or, if *T. verrucosum* is suspected, BCPMSG).
   e. Special differentiation media: e.g., BCPMSG to distinguish *T. mentagrophytes* complex from *T. rubrum* and *M. persicolor;* Lowenstein-Jensen or BCPMSG to distinguish *T. soudanense* from *M. ferrugineum;* SGA + 3–5% NaCl to distinguish *T. mentagrophytes* complex from *M. persicolor* and atypical *T. rubrum;* DTM to distinguish nonsporulating dermatophytes from most other nonsporulating hyaline fungi.
   f. In vitro hair perforation test.
   g. Molecular or mating studies (to be performed in reference laboratories).

[a] It may be necessary to incubate cultures on brain heart infusion agar or BCPMSG to ensure absence of antibiotic-resistant bacterial contamination before proceeding to step 4. Procedures are adapted from Weitzman and colleagues (115) and Kane et al. (59).

this antibiotic successfully. SGA amended with chloramphenicol alone may be used for successful isolation.

Colonies are very slow growing, appear dark brown to black, are glabrous at first, and later are covered with short dark brown-to-black aerial mycelium. They tend to be heaped in the center, with a flat periphery. Some colonies produce a reddish brown diffusible pigment on the agar. Microscopic examination reveals only highly septate dark hyphae and swollen intercalary cells. Conidia and ascospores are usually not found on routine mycological media.

White Piedra

White piedra is a fungal infection of the hair shaft characterized by the presence of soft white, yellowish, beige, or greenish nodules found chiefly on facial, axillary, or genital hairs (Fig. 23) and less commonly on scalp, eyebrows, and eyelashes. Nodules may be discrete or more often coalescent, forming an irregular transparent sheath.

The infection occurs sporadically in North America and Europe and more commonly in South America, Africa, and

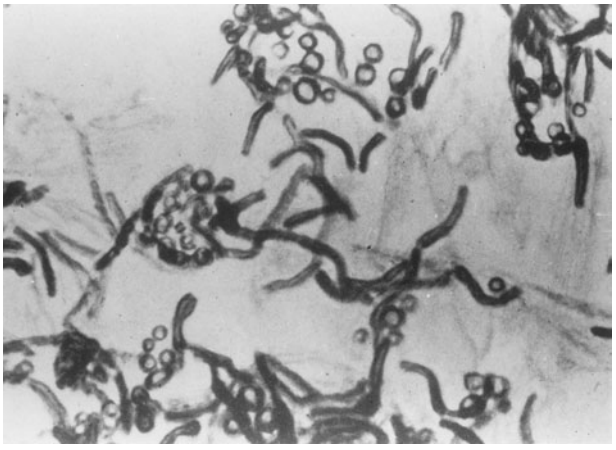


FIGURE 19 *Malassezia furfur* in skin scrapings from a lesion of tinea versicolor (Kane's stain). Magnification, ×1,000.

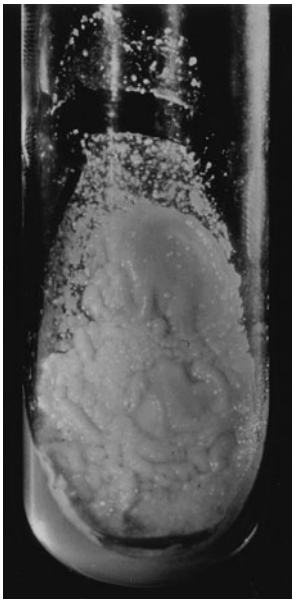


FIGURE 20 Culture of *Malassezia furfur* on Littman oxgall agar overlaid with oil.

FIGURE 18 (a) *Trichophyton mentagrophytes* complex: "nodular" variant of *Trichophyton interdigitale* (formerly *Trichophyton krajdenii*), SGA, 12 days, showing typical bright yellow pigmentation. (b) *Trichophyton mentagrophytes* complex: granular, zoophilic type *Trichophyton interdigitale* (mating tester strain of *Arthroderma vanbreuseghemii*), 14 days. (c) *Trichophyton rubrum*, SGA, 10 days, surface showing cottony white mycelium. (d) *Trichophyton rubrum,* SGA, 10 days, reverse showing typical red pigment. (e) *Trichophyton tonsurans,* SGA, 14 days, surface showing low velvety texture, mixed white and brownish mycelium. (f) *Trichophyton tonsurans,* SGA, 14 days, reverse showing mixture of mahogany red-brown and sulfur yellow coloration. (g) *Microsporum canis,* SGA, 10 days, relatively flat colony showing pale striate margin and yellowish pigment near colony center. (h) Brown filaments of *Hortaea werneckii* in NaOH mount of scraping from tinea nigra. Magnifications, ×400.

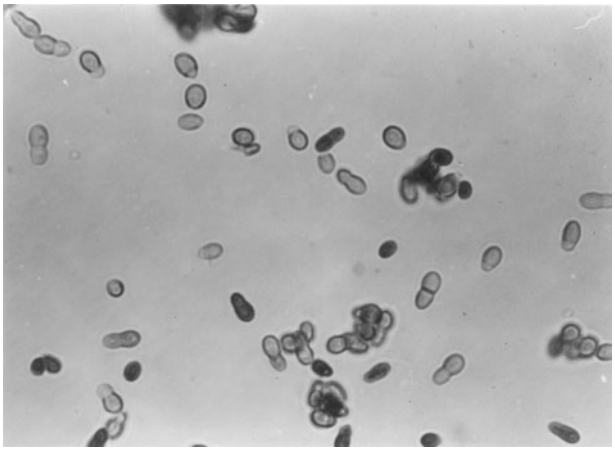


FIGURE 21 Microscopic appearance of *Malassezia furfur* yeast cells on Littman oxgall overlaid with olive oil. Magnification, ×400.

the Orient (88). Although white piedra is an uncommon infection, genital white piedra is occasionally but regularly seen in certain populations (52, 94).

Microscopic examination of hairs containing the adherent nodules mounted in 10% KOH or 25% NaOH–5% glycerin and squashed under a coverslip reveals intertwined hyaline septate hyphae, hyphae breaking up into oval or rectangular arthroconidia 2 to 4 μm in diameter (Fig. 24), occasional blastoconidia, and bacteria that may surround the nodule as a zooglea.

The isolates were formerly described as *Trichosporon beigelii* or *T. cutaneum* but are now correctly identified in most cases as *Trichosporon ovoides* (which causes scalp hair white piedra), *T. inkin* (which causes most cases of pubic white piedra), and *T. asahii* (26, 40). As with the *M. furfur* complex, these species form a complex of difficult-to-identify species whose distinction, except in demonstrated cases of piedra, is not known to have strong clinical implications in dermatologic mycology. They are also very common skin contaminants. Ordinarily, they may be reported from skin, hair, and nails of nonneutropenic patients simply as members of the genus *Trichosporon,* based on the production of budding yeast cells and of arthroconidia and a positive urease test, unless a research level identification of a proven etiologic agent is attempted. The causal agents of white piedra may be readily isolated on SGA with chloramphenicol or other isolation media containing antibacterial antibiotics. The isolation medium should not contain cycloheximide, since this drug is inhibitory to some of the species. Growth is rapid, yielding white to cream-colored colonies that exhibit a variety of colonial morphologies depending on the species. A description of the genus and characteristics of the species involved in white piedra are given in papers by Guého et al. (38, 40). More information about *Trichosporon* is found in chapter 119 of this Manual.

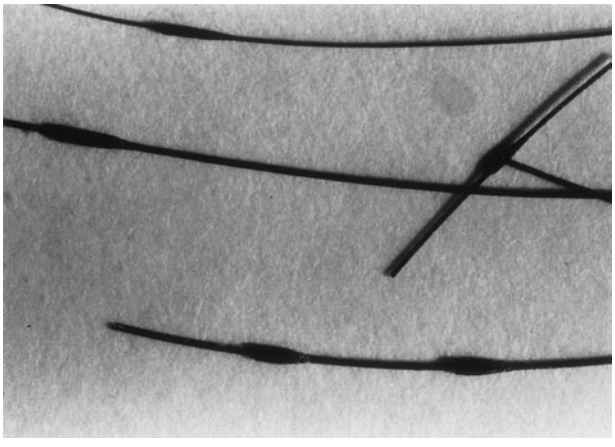


FIGURE 22 Black piedra nodules on scalp hair. NaOH mount. Magnification, ×100.

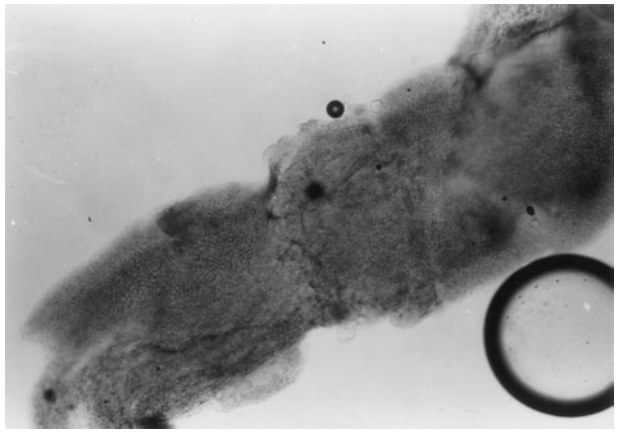


FIGURE 23 White piedra nodule on hair from the groin. Magnification, ×1,000.

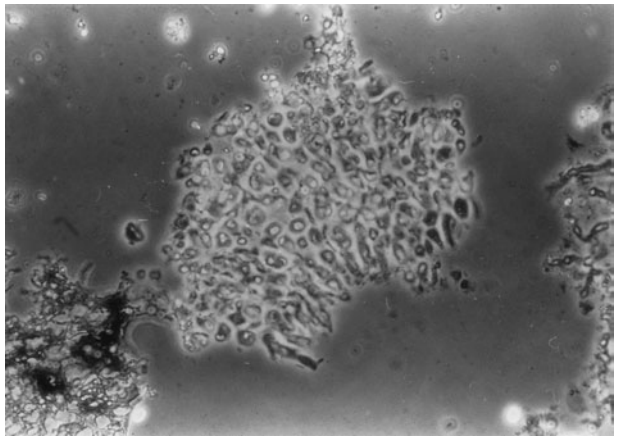


FIGURE 24 Arthroconidia from a crushed nodule of white piedra. Magnification, ×1,000.

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Bipolaris, Exophiala, Scedosporium, Sporothrix, and Other Dematiaceous Fungi*

G. SYBREN DE HOOG AND ROXANA G. VITALE

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The melanization of vegetative cells, conidia, or both, which results in colony coloration that ranges from olive or gray to black, is caused by the deposition of dihydroxynaphthalene melanin in cell walls. This property occurs commonly among species classified in very different parts of the fungal kingdom. The amount of melanin expressed in host tissue may be very small. The use of the Fontana-Masson stain (127, 134) for demonstrating the presence of melanin is therefore recommended as a routine to distinguish fungi with melanized hyphae from, e.g., *Aspergillus*. This does not apply for *Scedosporium* and *Sporothrix*, which are not melanized but are able to produce melanized conidia in vitro. *Sporothrix* has a characteristic yeast form in tissue; the recognition of *Scedosporium* (causing "hyalohyphomycosis") rests upon other methods than histopathology.

The genera discussed in this chapter belong to the ascomycetous orders Chaetothyriales, Calosphaeriales, Dothideales, Microascales, Ophiostomatales, Pleosporales, and Sordariales (Table 1). It is often incorrectly believed that all these fungi are ubiquitous and cosmopolitan on living and dead plant material and infect humans and animals occasionally. But depending on their phylogenetic position, very divergent degrees of predisposition for infection of vertebrate hosts are noted (48). While members of Pleosporales and Dothideales, such as *Alternaria, Aureobasidium, Bipolaris,* and *Curvularia*, are indeed ubiquitous saprobes, members of Chaetothyriales (*Exophiala* and relatives) can be characterized as microextremophiles, living on special microhabitats such as toxic compounds (186). In this review the genera are therefore treated according to their ordinal relationships.

The term "dematiaceous" applies to fungi with black hyphae in general (69, 70), but it has been recommended in medical mycology to reserve this term for the rapidly growing members of Pleosporales only (48), as these are very different from, for example, Chaetothyriales in all respects. Also the term "phaeoid" indicates brown hyphae (177); "phaeohyphomycoses" is an umbrella term for any fungus present in tissue with brownish yeastlike cells, pseudohyphae, or hyphae or a combination of these forms. The term "black yeasts and relatives" is preferably used to indicate members of Chaetothyriales and Dothideales only (48). The "black yeasts" are not a formal taxonomic group, but the term is applied to a wide range of unrelated ascomycetous and basidiomycetous fungi that are able to produce budding cells at some stage in their life cycle.

Pleoanamorphism is particularly striking in members of the black yeasts and in the genus *Scedosporium.* These fungi are frequently seen in the clinical laboratory to produce more than one asexual form of propagation, so-called "anamorphs," which may bear separate names. For example, the preponderant morphology of *Scedosporium apiospermum* consists of solitary annellated conidiogenous cells, but an additional, synnematous anamorph, known as *Graphium fructicola,* may be exhibited. When a single fungus produces more than one anamorph, the term "synanamorph" is used to designate any of the concurrently existing forms. In addition, some isolates of *S. apiospermum* have the ability to produce a sexual form ("teleomorph"), characterized by the formation of fruiting bodies with meiotic ascospores. In each organism only a single teleomorph can be produced. The fungus involving all its forms of propagation is preferably referred to by its teleomorph name, which is in the present example *Pseudallescheria boydii*.

Among the specific disease entities in the melanized fungi is chromoblastomycosis, a cutaneous disease with often extended acanthosis and in which the fungus is present as muriform cells. Sporotrichosis characteristically develops with nodular abscesses along the lymphatic system, while mycetoma is a subcutaneous infection in which the fungus is present in the form of grains that are expelled from the host through sinuses. When darkened hyphae are present in tissue, the umbrella term "phaeohyphomycosis" is frequently used, but this is confusing because the melanized fungi may not be black or may not be hyphal in tissue.

TAXONOMY

Calosphaeriales

Recently some melanized phialidic fungi were segregated from *Phialophora* on molecular grounds, and they now constitute small islands of clinical significance in the order Calosphaeriales that otherwise contains plant-associated molds. A close relationship to teleomorph genera such as *Togninia* and *Pleurostoma* was revealed (235). Infections are mostly of traumatic nature and are considered to originate directly from the woody plant material of the fungal habitat.

*This chapter contains information presented in chapter 120 by Wiley A. Schell, Ira F. Salkin, and Michael R. McGinnis in the eighth edition of this Manual.

TABLE 1 Overview of clinically most relevant species and their attribution to the ordinal level

Order, genus, and species	Synanamorph	Teleomorph	Obsolete name
Calosphaeriales			
Phaeoacremonium			
inflatipes			
parasiticum			*Phialophora parasitica*
rubrigenum			
Phialemonium curvatum			
Pleurostomophora			
repens			*Phialophora repens*
richardsiae			*Phialophora richardsiae*
Chaetothyriales			
Exophiala			
attenuata			
bergeri			
dermatitidis	Unnamed *Phialophora*		*Wangiella dermatitidis*
jeanselmei			
oligosperma			
phaeomuriformis	*Sarcinomyces phaeomuriformis*		
spinifera	Unnamed *Phialophora*		
Cladophialophora			
arxii			
bantiana			*Xylohypha bantiana*, *Cladosporium trichoides*
boppii			
carrionii	Unnamed *Phialophora*		
devriesii			
emmonsii			
Cyphellophora			
laciniata			*Pseudomicrodochium suttonii*, *Pseudomicrodichium fusarioides*
pluriseptata			
Fonsecaea			
monophora			
pedrosoi	Unnamed *Phialophora*		*Fonsecaea compacta*
Ochroconis gallopava			*Dactylaria gallopava*, *D. constricta* var. *gallopava*
Phialophora			
americana		*Capronia semiimmersa*	
europaea			
verrucosa			
Ramichloridium			
mackenziei			*Ramichloridium obovoideum*
Rhinocladiella			
aquaspersa			
similis	Unnamed *Exophiala*		
Dothideales			
Aureobasidium pullulans		*Discosphaerina fulvida*	
Cladosporium			
cladosporioides			
herbarum		*Davidiella tassiana*	
Hormonema dematioides		*Sydowia polyspora*	
Hortaea werneckii			*Exophiala werneckii*, *Phaeoannellomyces werneckii*
Lasiodiplodia theobromae		*Botryosphaeria theobromae*	*Botryodiplodia theobromae*
Nattrassia mangiferae	*Scytalidium dimidiatum*		*Hendersonula toruloidea*, *Scytalidium hyalinum*
Microascales			
Scedosporium			
apiospermum	*Graphium fructicola*	*Pseudallescheria boydii*	*Polycytella hominis*
prolificans			*Scedosporium inflatum*
Ophiostomatales			
Sporothrix schenckii			

(Continued on next page)

TABLE 1 Overview of clinically most relevant species and their attribution to the ordinal level *(Continued)*

Order, genus, and species	Synanamorph	Teleomorph	Obsolete name
Pleosporales			
Alternaria			
alternata			*Alternaria tenuissima*
infectoria		*Lewia infectoria*	
Bipolaris			
australiensis		*Cochliobolus australiensis*	*Drechslera australiensis*
hawaiiensis		*Cochliobolus hawaiiensis*	*Drechslera hawaiiensis, Dissitimurus exedrus*
spicifera		*Cochliobolus spicifera*	*Drechslera spicifera*
Curvularia lunata		*Cochliobolus lunatus*	
Exserohilum rostratum		*Setosphaeria rostrata*	*Exserohilum longirostratum, Exserohilum mcginnisii*
Sordariales			
		Achaetomium strumarium	*Chaetomium strumarium*
		Chaetomium globosum	

Phaeoacremonium

Phaeoacremonium (44) is morphologically characterized by slender, tubular, and often tapering or inflated, brown phialides. Hyaline, slimy conidia are produced through inconspicuous collarettes. Six species have been described, three of which are known from opportunistic infection in humans. *Phaeoacremonium parasiticum* (Fig. 1) is a well-documented agent of phaeohyphomycosis (75) and mycetoma (110, 201), while deep and disseminated infections occur in debilitated patients (104). *P. inflatipes* was implicated in a mycetoma (5) and in a phaeohyphomycosis (173). *P. rubrigenum* caused subcutaneous infection in an immunosuppressed patient (152).

Phialemonium

The genus *Phialemonium* is only poorly melanized, and its phylogenetic position is unknown. Colonies are white, with some strains having a diffusible yellow-green pigment or a faint vinaceous hue (84, 128). Reported cases of infection by *P. curvatum* involve subcutaneous cysts, disseminated infection, endocarditis, peritonitis, and fungemia (84, 97, 128, 207). Proia et al. (187) reported several cases of infection after hemodialysis, some of which were fatal.

Pleurostomophora

This is a genus of mainly wood-inhabiting fungi (235). Hyphae are dark and bear pale, tapering phialides that may be single or aggregated in dense brushes; hyaline, slimy conidia are produced through small or large collarettes. *Pleurostomophora repens*, until recently known as *Phialophora repens*, was occasionally reported from subcutaneous infections in humans (105, 160). *Pleurostomophora richardsiae* (Fig. 1) (syn. *Phialophora richardsiae*) is a soft-rot fungus on wood (242); human infections caused by this species involve mostly subcutaneous cysts in which the fungus is encapsulated by a collagen layer (98), occasionally with bone involvement (232). Most patients have some underlying disorder such as diabetes (184) or immunodepression (119).

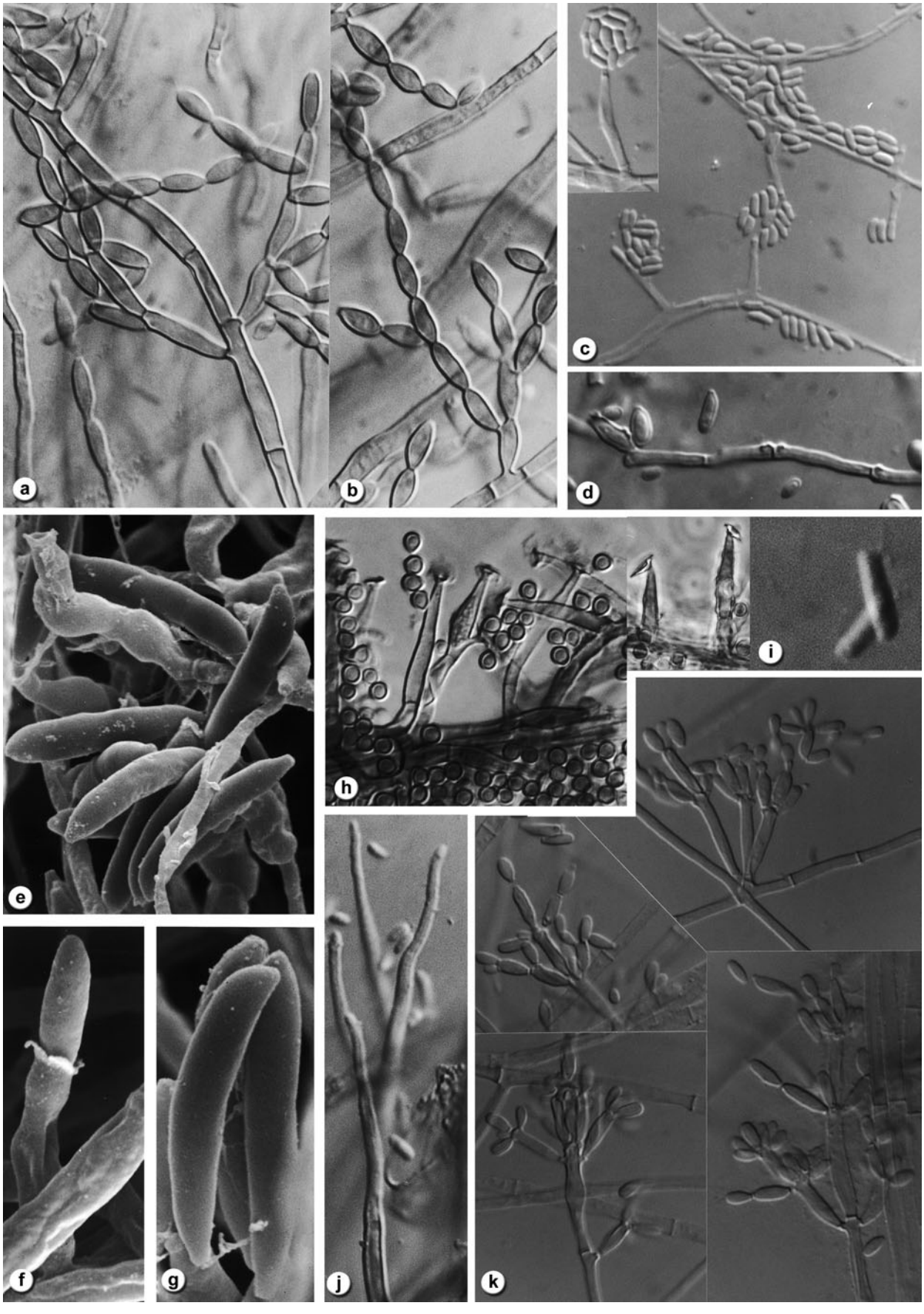
Chaetothyriales

This rather small order is clinically highly relevant, because about one-half of the species known to date are able to cause infections in humans. Infections vary from traumatic inoculations into otherwise healthy individuals and largely asymptomatic colonization of cavities to a wide range of opportunistic disorders and systemic, mainly cerebral or disseminated disease. The deep clinical syndromes are mostly fatal if untreated and frequently occur in patients with no known immune disorder. The Chaetothyriales contain some of the most dangerous fungi known to date, which should be handled with great care. The natural ecology of most species is practically unknown. A remarkable tendency is observed in growth with xenobiotics, such as creosote-treated utility poles (242), xylene (42), or toluene (186).

Among the traumatic infectious syndromes is chromoblastomycosis, with a small number of potential etiologic agents, all of which are members of Chaetothyriales. The disorder occurs in otherwise healthy patients and is characterized by chronic, cutaneous to subcutaneous lesions, frequently with marked hyperplasia. Typically, spherical and mostly cruciately septate muriform (sclerotic) bodies are present within infected tissue. The bodies in tissue are a hallmark of chromoblastomycosis. Three fungal species account for virtually all cases of chromoblastomycosis: *Cladophialophora carrionii, Fonsecaea pedrosoi,* and *Phialophora verrucosa*.

Another disease entity that is largely confined to Chaetothyriales is primary cerebral infection in immunocompromised or immunocompetent individuals, i.e., cerebritis in which the first symptoms of disease are of neurological nature. Hyphal elements that show melanization either directly or after Fontana-Masson staining are observed in abscesses in the brain parenchyma. The portal of entry may be the lung, but frequently symptoms are confined to the brain. Six fungal species account for most nontraumatic brain infections: *Cladophialophora bantiana* (Fig. 1), *C. modesta, Exophiala dermatitidis, Fonsecaea monophora, Ochroconis gallopava,* and *Ramichloridium mackenziei*.

FIGURE 1 (a and b) *Cladophialophora bantiana;* (c) *Phialemonium curvatum;* (d) *Exophiala dermatitidis;* (e to g) *Cyphellophora pluriseptata;* (h and i) *Pleurostomophora richardsiae;* (j) *Phaeoacremonium parasiticum;* (k) various pictures of *Fonsecaea pedrosoi*. Reproduced from de Hoog et al. (48).


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Occasionally systemic dissemination is observed in patients with or without proven immune disorder; these infections are fatal after a chronic course if untreated. Secondary cutaneous lesions often lead to marked eruptions with high morbidity. The disorder has been observed repeatedly with *Cladophialophora devriesii, Exophiala dermatitidis,* and *E. spinifera*.

Cladophialophora

Catenate, dry conidia and the absence of differentiated conidiophores characterize *Cladophialophora*. This genus contains eight species, seven of which are nearly exclusively known from humans and other warm-blooded animals. *C. bantiana* is a neurotropic mold that has caused numerous cases of cerebral phaeohyphomycosis (63, 111, 123, 192), in about one-third of the cases in otherwise healthy individuals. If untreated, the infections are fatal within 1 to 6 months. The species is recognizable by very long, coherent, poorly branched conidial chains and by an ability to grow at 40°C. It has a characteristic 558-bp intron at position 1768 of the small subunit ribosomal operon (87). *C. devriesii, C. modesta,* and *C. arxii* are known from scattered cases of chronic, fatal deep or disseminated mycoses (92, 156, 163, 230). *C. emmonsii,* with branched conidial chains and a maximum growth temperature of 38°C, has thus far been seen only in cutaneous infections (176). *C. carrionii,* with small conidia in profusely branched chains, is a common agent of chromoblastomycosis in arid climates (72, 140). The disease is probably acquired via traumatic inoculation of cactus spines (255). *Cladophialophora boppii* is a rare agent of mild cutaneous infections (22).

Cyphellophora

This is a rare group of infective agents characterized by slender, curved, mostly 1-3-septate conidia. Cultures are evenly melanized and show limited expansion growth; budding cells are absent. Conidia are produced from poorly developed collarettes alongside the hyphae. *C. laciniata* (Fig. 1), also known under the generic name *Pseudomicrodochium,* and *C. pluriseptata* are occasionally isolated from human skin and nails (60, 61), rarely from other body sites (3, 226), but their etiology has not unambiguously been proven.

Exophiala

Exophiala (including *Wangiella*) is the main genus of clinically relevant black yeasts. Strains show high degrees of morphological diversity. Most isolates initially grow in a yeast form that is succeeded by a hyphal anamorph. As a result, colonies are moist and slimy at first, becoming velvety to woolly with age. The process of conidium production is annellidic, from narrow, inconspicuous scars or extensions. Occasionally a very slow growing, meristematic morphology ("*Sarcinomyces*") is preponderant. Fresh isolates or strains cultivated on nutritionally deficient media frequently produce catenate conidia (53) and may produce scattered or compacted phialides with huge collarettes (54). The species are uncommon in the environment and occupy hitherto-unrevealed microhabitats. Some thermotolerant species seem to be associated with environmental xenobiotics (42) or occur in animal feces (G. S. de Hoog and M. Sudhadham, unpublished data); others are found on slightly osmotic surfaces such as fruit (150). Psychrophilic species that cause infections in cold-blooded vertebrates are frequent in water, either ocean water (136, 137), municipal drinking water (95), or hospital water (185). The most frequently identified species, *E. jeanselmei,* has recently been subdivided into a number of taxa on molecular grounds (56, 236). *E. oligosperma* is a common etiologic agent of subcutaneous infections in immunosuppressed, elderly, diabetic, or otherwise debilitated individuals (56, 212, 224). The two species with capsule-like extracellular polysaccharides around yeast cells, *E. spinifera* and *E. dermatitidis* (Fig. 1) (251), have the highest virulence to humans. The rare infections by *E. spinifera* can be localized, but in about one-half of the cases fatal, disseminated mycoses are observed in adolescents (reviewed by de Hoog et al. [52]). The species can be recognized by the presence of large, stiff conidiophores. Recently a similar species with reduced conidiogenous cells, *E. attenuata,* was described (236). *Exophiala (Wangiella) dermatitidis* is the most commonly encountered species in clinical settings, causing infections of cutaneous and subcutaneous tissue (108) in mostly immunocompromised patients; recurrent continuous ambulatory peritoneal dialysis-related infections are also seen (239). It regularly occurs asymptomatically in the lungs of patients with cystic fibrosis (102, 114), as well as in the stool of patients with diarrhea (50). A striking number of fatal, neurotropic infections have been described in young, healthy individuals, all in Asia (35, 107, 153). In the indoor environment the fungus has a focus in Turkish steam baths (150). *E. dermatitidis* is recognizable morphologically by "phialides without collarettes," which are wide, scar-like, very short annellated zones. In addition, growth at 40°C and the inability to assimilate nitrate are characteristic (172). The related species *E. phaeomuriformis* differs by having a growth maximum at 38°C (151).

Fonsecaea

The two species known in the genus *Fonsecaea,* characterized by conidia produced in chains of maximally four, are both human pathogens. In culture *Fonsecaea* species are mostly monomorphic but may produce additional phialides with collarettes releasing balls of one-celled conidia. No budding cells are produced on routine media. *Fonsecaea pedrosoi* (Fig. 1; including the mutant *F. compacta*) is one of the etiologic agents of human chromoblastomycosis (13, 161, 219). The second species, *F. monophora* (47), has a more diverse clinical spectrum which includes brain infections (170, 225). The etiologic agents in cases of phaeohyphomycosis published under the name *F. pedrosoi* (17, 166) should therefore be reconsidered.

Ochroconis

The phylogenetic position of the genus *Ochroconis* is unclear. All species have rust brown to olivaceous colonies and produce 1-3-septate conidia from small, open denticles inserted in low numbers on sympodial cells. *O. gallopava* (Fig. 2) is an etiologic agent of neurotropic infections in immunocompromised humans, and it is the cause of epizootic encephalitis in flocks of turkeys and chickens (112, 145, 215). It has hyaline, clavate conidia and grows well at 40°C. Other, nonthermotolerant species have been observed to cause disseminated infections in cold-blooded animals such as fish (205).

Phialophora

When phialides are the only form of propagation in chaetothyrialean fungi, the generic name *Phialophora* is applied. Dematiaceous phialidic fungi belonging to other orders are now classified in genera such as *Cadophora, Phaeoacremonium,* and *Pleurostomophora* (83, 235). No budding cells are produced in *Phialophora. P. verrucosa,* with darkened, funnel-shaped collarettes (Fig. 2), is a leading cause of chromoblastomycosis in tropical climatic zones (81, 233). Occasionally, opportunistic infections were reported, including

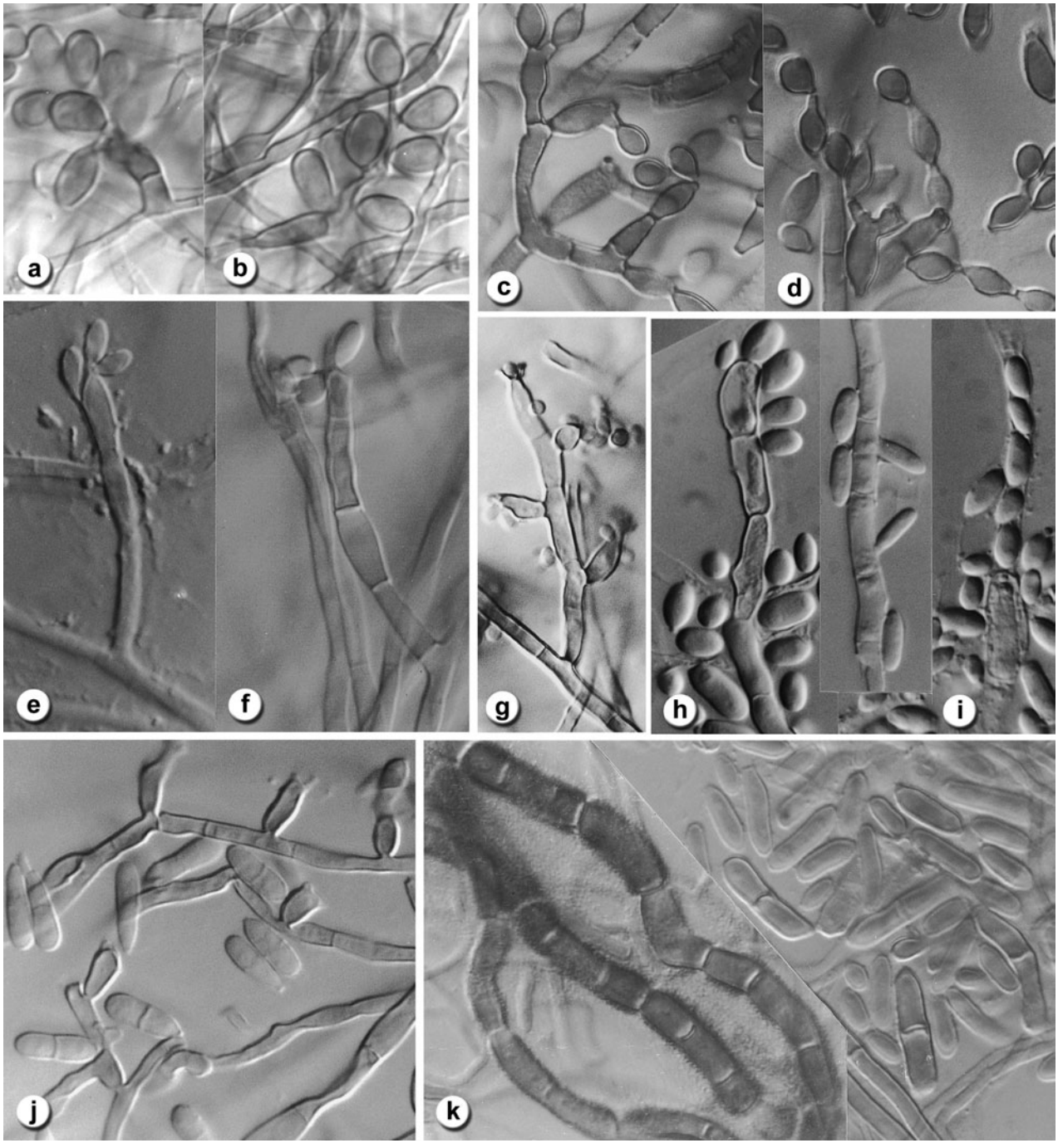


FIGURE 2 (a and b) *Ramichloridium mackenziei;* (c and d) *Cladosporium cladosporioides;* (e and f) *Rhinocladiella aquaspersa;* (g) *Phialophora verrucosa;* (h and i) *Aureobasidium pullulans;* (j) *Ochroconis gallopavum;* (k) *Hormonema dematioides*. Reproduced from de Hoog et al. (48).

endocarditis, keratitis, and osteomyelitis (65, 231). It is possible that some of these infections are attributable to *P. americana,* which differs mainly by having vase-shaped collarettes (57). Given their close molecular similarity, there is no consensus as to whether *P. verrucosa* and *P. americana* constitute two species or one (57, 214, 246). *P. europaea,* with very short collarettes (51), is fairly commonly involved in mild skin and nail infections, but due to its slow growth it is frequently overlooked.

Ramichloridium

Ramichloridium is morphologically characterized by stout, mostly erect conidiophores bearing noncatenate conidia on denticles. *Ramichloridium mackenziei* (Fig. 2) is a remarkable fungus because it is exclusively known from fatal brain infections in the Middle East or from immigrants from that region (29, 122, 227). Several patients had no known immune disorder; the environmental niche of the species is unknown.

The species is morphologically similar to *R. obovoideum,* an organism described as occurring on leaf litter and long thought to be conspecific, but on the basis of rRNA gene data it is distantly related, not even belonging to the same order (G. S. de Hoog, unpublished data).

Rhinocladiella

The genus *Rhinocladiella* has less conspicuously differentiated conidiophores, the sympodial conidia being produced from a loosely branched system. In addition to the sympodial anamorph, annellidic and phialidic cells can be observed. *Rhinocladiella aquaspersa* (Fig. 2) is a rare agent of chromoblastomycosis in South America (11, 182, 206, 216). *R. atrovirens* has been reported to cause cerebral phaeohyphomycosis in an AIDS patient (59) and mycetoma of the foot (167), but because *R. atrovirens* in a modern, molecular circumscription is limited to isolates growing on conifer wood in Northern countries, these cases probably concerned the sibling species *R. similis* (56).

Dothideales

The order Dothideales comprises numerous saprobes which thrive well under conditions of decreased water activity, such as on sugary leaf surfaces, rock, glass, and moist medical devices. Many are extremotolerant and are found in Antarctic or Mediterranean rock (209, 222) or in hypersaline ponds (254) and have the ability of morphological transition to a tolerant, meristematic ecotype consisting of clumps of amorphous, thick-walled, highly melanized cells. *Cladosporium* species are very common airborne laboratory contaminants.

Aureobasidium and *Hormonema*

The genus *Aureobasidium* contains a single, ubiquitous, and highly polymorphic species, *A. pullulans* (Fig. 2). It has a yeast phase that colonizes moist surfaces of, for example, insufficiently sterilized instruments or prosthetic devices. It can maintain by converting to a meristematic morphology, i.e., it forms isodiametrically expanding cells that become subdivided in all directions and may eventually fall apart into small cell clumps. Colonies in vitro are often pink due to the preponderance of wide, hyaline, oligokaryotic hyphae bearing synchronously produced conidia and containing scattered endoconidia. *Aureobasidium* is often confused with *Hormonema* (Fig. 2), which, however, produces conidia in a basipetal succession (58). In addition, the internal transcribed spacer (ITS) rRNA gene sequences of the two species are clearly different (252). *A. pullulans* has been implicated as an opportunistic agent of mycoses in humans and other animals, having been isolated from peritonitis and disseminated infection (31, 38, 79, 80, 106, 117, 191, 202). Cutaneous phaeohyphomycosis and peritonitis have been reported as caused by *H. dematioides* (40, 213).

Cladosporium

Species of *Cladosporium,* especially *C. cladosporioides* (Fig. 2) and *C. sphaerospermum*, are extremely common contaminants in the clinical laboratory. They are also associated with allergic disease in the indoor environment. Some (sub)cutaneous (100, 142, 180, 199, 234) and even deep (124, 134) infections have been reported, but there remains some doubt about the pathogenic role of the *Cladosporium* isolates.

Hortaea

Members of the genus *Hortaea* have rather wide hyphae which become profusely septate during growth of the fungus, and they have annellidic conidiogenesis from broad scars. Two species are recognized to date, which have a predilection for acidic (109) and salty (254) environments, respectively, and do not show any pathogenicity for humans. The halophilic species, *H. werneckii* (Fig. 3), lives in evaporation ponds at the subtropical seashore and can adhere to human hands when these are exceptionally salty (95), causing a syndrome in the dead keratin layers known as tinea nigra (210). Therapy is by the application of keratin-dissolving agents.

Lasiodiplodia

Lasiodiplodia is a coelomycete genus characterized by spherical fruiting bodies filled with asexual conidia. The conidia initially are ellipsoidal and hyaline but gradually become brown and develop a median septum at maturation. *L. theobromae* (Fig. 3) is occasionally encountered as a cause of keratitis following injury to the cornea (190). Rare cases of subcutaneous infection have also been reported (148).

Nattrassia

N. mangiferae is a pycnidial fungus. Inside multilocular fruiting bodies hyaline, ellipsoidal conidia develop, which in part become brownish and 1-2-septate. In culture usually only a rapidly growing, jet black, hairy anamorph with dark arthroconidia (*Scytalidium dimidiatum;* Fig. 3) is seen; the pycnidia are produced only after 2 months of growth on a moistened plant leaf. Melaninless mutants (195), which also show reduced conidiation, were until recently referred to as *Scytalidium hyalinum*. The fungus is a common plant pathogen in the tropics, is regularly involved in syndromes very similar to dermatophytosis on skin and nails (68, 141, 164, 253), is rarely involved in subcutaneous tissue (194, 218), and has caused endophthalmitis (6). Deep infections are very rare in immunocompromised patients (19).

Microascales

The order Microascales comprises *Scopulariopsis,* with its *Microascus* teleomorphs, but also *Scedosporium,* linked to *Pseudallescheria* and *Petriella* teleomorphs. *Scopulariopsis* has dry, airborne conidia and is commonly involved in superficial infections, although some *Microascus* species were found to be involved in deep infections (131, 147). In contrast, *Scedosporium* has sticky conidia and the species cause subcutaneous infections, while deep and disseminated infections are noted in immunosuppressed or otherwise debilitated patients.

Scedosporium/Pseudallescheria

Scedosporium comprises three clinically relevant species (89). The genus is characterized by hyaline, Fontana-Masson-negative hyphae from which slimy, pale brown conidia are produced in basipetal succession from inconspicuous annellides (66). *S. prolificans*, with flask-shaped conidiogenous cells, is monomorphic; the complete life cycle of *S. apiospermum* (Fig. 3), with cylindrical conidiogenous cells, comprises a *Graphium fructicola* synanamorph and a *Pseudallescheria boydii* teleomorph (Fig. 3). The latter fungus has become one of the most frequently misidentified organisms in medical mycology, largely due to the differential abundance of each of the phenotypes of *P. boydii*. This has led to a large number of new introductions in totally unrelated genera (48). The most recent synonym is *Polycytella hominis* (28), a near-sterile phenotype that was found to cover degenerate *P. boydii* strains lacking annellidic conidia (23).

Scedosporium apiospermum was discovered in 1889 as an agent of human otitis (217). The reported clinical spectrum of the fungus has changed over time, from prevalently chronic mycetoma in otherwise healthy patients between 1911 and 1980 to systemic opportunistic infection after 1980 (96). Systemic infections after solid organ transplant are relatively frequent (85, 143); occasionally infections are noted with the

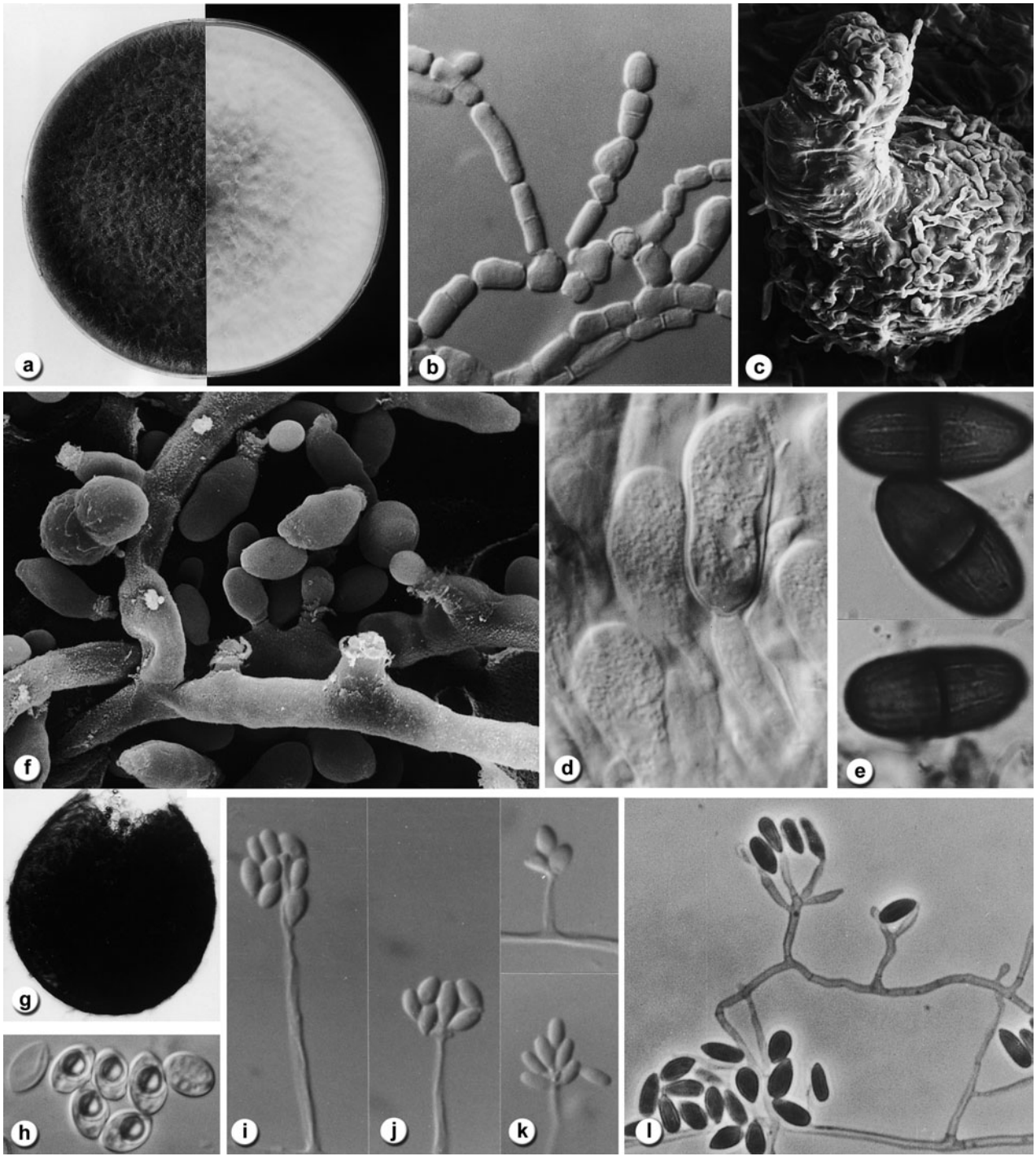


FIGURE 3 (a and b) *Scytalidium* synanamorph of *Nattrassia mangiferae;* (c to e) *Lasiodiplodia theobromae;* (f) *Hortaea werneckii;* (g and h) *Pseudallescheria boydii;* (i to l) *Scedosporium* anamorph of *Pseudallescheria boydii*. Reproduced from de Hoog et al. (48).

application of invasive devices (118, 211). Osteomyelitis (15) and arthritis (91) are frequent. Fisher et al. (76) were the first to describe the near-drowning syndrome by *S. apiospermum*, which characteristically leads to delayed, potentially fatal brain infection after the patient has recovered from the primary effects of aspiration of polluted water. The species' neurotropism was stressed by Berenguer et al. (20). In severely compromised patients, cerebral dissemination may take place from local foci (113). Although Creitz and Harris (43) had already noted the occurrence of *P. boydii* as a colonizer of the lungs of patients with pulmonary disorders and numerous cases have been reported since then (196), its consistent occurrence in patients with cystic fibrosis has only recently received proper attention (37).

Despite its rather frequent occurrence in clinical settings, the history of the second species, *Scedosporium prolificans*, is

remarkably short (96). The first clinical cases were described by Malloch and Salkin (144) after disseminated infections in immunocompromised hosts. Since then, numerous strains have been recovered, mostly from clinical cases with major immunosuppression, and since no older reports of cases in immunocompetent patients are known, the species is a truly emerging opportunist (20). The species has been reported from bone and soft tissue infections (203, 244, 245) as well as from (secondary) cutaneous infection, fungemia, and endocarditis (24, 32, 90). The fungus may disseminate to visceral organs in immunocompromised individuals (7, 20, 169, 183). A nosocomial outbreak has been described (99).

Ophiostomatales

Ophiostomatales is a large order of plant-pathogenic fungi. Most are blue-stain fungi in wood and live in association with bark beetles. Only a single species has a clinical potential. The main teleomorph genus is *Ophiostoma*.

Sporothrix

Sporotrichosis, caused by *Sporothrix schenckii,* is a cutaneous-to-subcutaneous chronic infection that can undergo lymphatic spread. Musculoskeletal involvement and disseminated infection are relatively rare, as are the nasal and pulmonary infections that may arise from inhalation of conidia (34, 39, 93). Most infections originate from traumatic implantation of the fungus. Some disseminated cases were observed in AIDS patients (21, 64). In addition, infections have been described to be transmitted by animals (8, 204). In sporotrichosis, the narrow-based yeast cells in tissue are characteristic, but their numbers are usually very small and may easily be missed upon microscopy. Gomori methenamine silver or periodic acid-Schiff staining is therefore recommended; Fontana-Masson staining is negative.

Hyphae of *S. schenckii* are colorless and bear a cluster of thin denticles with hyaline, tear-shaped conidia at their tips. Additional spherical conidia may or may not be present; this is the only melanized part of the fungus. Strains lacking these brown conidia have been shown to be avirulent in mice (62). The mold can be transformed in vitro to a stable yeast at 37°C on enriched media such as chocolate agar or brain heart infusion agar. Occasional isolates are difficult to convert and may require multiple subcultures and extended incubation. Transition is observed in environmental as well as clinical isolates.

Three isolates of *S. schenckii* var. *luriei* have been reported (2, 4, 175). The appearance of this variety differs from that of *S. schenckii* var. *schenckii* by large, often septate budding cells. Its separation is supported by rRNA ITS sequence data (G. S. de Hoog and R. C. Summerbell, unpublished data).

Pleosporales

Most members of the order Pleosporales have rapidly expanding, hairy colonies, which show optimal conidium production on media poor in nutrients, such as potato carrot agar. The large conidia are then mostly visible with the aid of a stereomicroscope. Very rarely, however, preponderantly meristematic cultures are produced, which show limited expansion growth and produce amorphous clumps of black cells as the only form of conidiation, reported in the literature as *Phaeosclera* and *Botryomyces*.

Conidia are generally produced on septate, dark brown, erect conidiophores. They consist of several compartments. Different types of septation, characterizing the genera, are observed. True septa are found in those conidia where outer wall and septum are continuous; such conidia are euseptate (in *Alternaria* and *Curvularia*). False septa are observed in those conidia where only the inner wall layers are involved in septation and the outer wall forms a sac-like structure around the individual cells; such conidia are distoseptate (in *Bipolaris* and *Exserohilum*).

Members of the Pleosporales are mainly plant pathogens with a restricted host range. The few saprobic members in each of the genera mentioned are occasionally observed in human disease. Two main clinical entities are recognized, namely, cutaneous infections in immunosuppressed patients *(Alternaria)* and allergic sinusitis with occasional cerebral involvement in otherwise healthy individuals *(Bipolaris, Curvularia,* and *Exserohilum)*.

Alternaria

Species of *Alternaria* are fairly frequent agents of mainly cutaneous infections in patients on long-term steroid usage, tacrolimus, or other immunosuppressive agents (88, 181, 238, 243), cases of sinusitis being rare. Nearly all cases were caused by *Alternaria infectoria* (139, 200) and *A. alternata* (Fig. 4) (155), the two main saprobic species of the genus (49). Infections attributed to the host-specific plant pathogen *A. dianthicola* (162) have not been confirmed by molecular methods. Infections attributed to *A. chlamydospora* (16, 221) probably concerned meristematic segregants of *A. alternata*. *A. longipes* (86) is a host-specific pathovar of *A. alternata* on tobacco, with identical diagnostic features. Several cutaneous infections have been attributed to *Alternaria tenuissima* (33, 197, 198), which is only doubtfully separate from *A. alternata* (49).

Since clinical strains are very likely to belong to only two species of *Alternaria*, isolates are easily identified down to the species level. Available criteria comprise characteristics of growth, sporulation, and morphology and very large differences in rRNA gene ITS, which can be displayed by sequencing or by restriction fragment length polymorphism (49). *Alternaria infectoria* in tissue may present with hyaline yeast cells rather than melanized hyphae. Cultures of *A. infectoria*, in contrast to those of *A. alternata*, frequently exhibit creamish patches and show reduced sporulation. Conidia of *A. infectoria* bear long apical beaks which serve as secondary conidiophores.

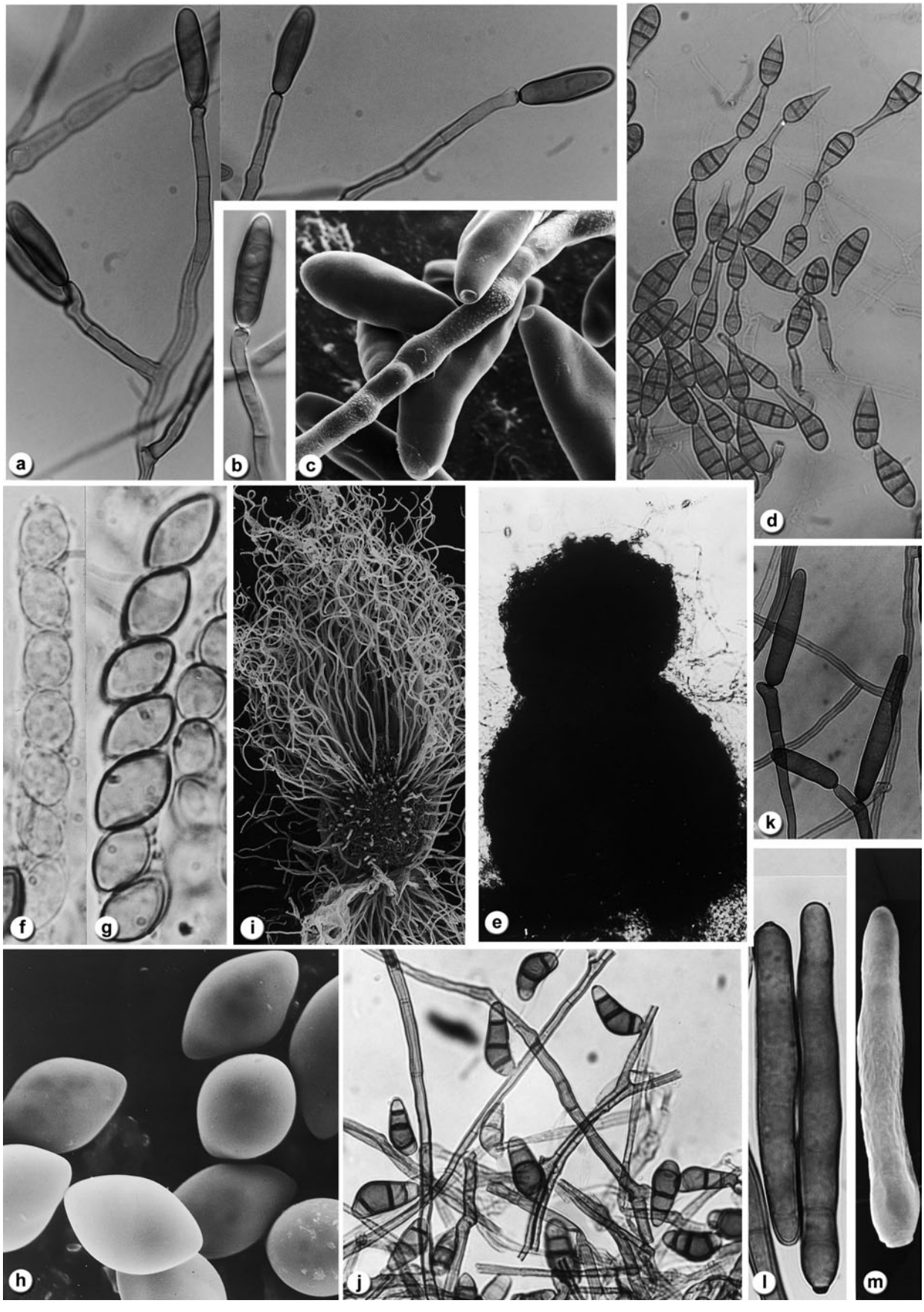
Bipolaris

Preponderant species of the *Bipolaris* genus are *Bipolaris australiensis, B. hawaiiensis* (Fig. 4), and *B. spicifera,* which are infrequently involved in allergic sinusitis (77, 138, 157, 179). Cerebral complication takes place, which is rapidly fatal if untreated (82, 250). Simmons et al. (220) reported such a case caused by the single member of the genus *Dissitimurus*, which probably concerned a degenerate strain of *Bipolaris hawaiiensis* (55).

Curvularia

Most species of this genus are easily recognizable on the basis of conidial characteristics (69, 70). Saprobic members of *Curvularia,* particularly *C. geniculata* and *C. lunata* (Fig. 4),

FIGURE 4 (a to c) *Bipolaris hawaiiensis;* (d) *Alternaria alternata;* (e to h) *Achaetomium strumarium;* (i) *Chaetomium globosum;* (j) *Curvularia lunata;* (k to m) *Exserohilum rostratum*. Reproduced from de Hoog et al. (48).


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have been identified as potential agents of fungal sinusitis, in humans as well as in cattle (18, 193). Cerebral involvement may occur (135), which may be fatal if untreated. A wide spectrum of opportunistic infections is noted, including endocarditis, pulmonary infection, cerebral infection, and subcutaneous phaeohyphomycosis (26, 67, 74, 120, 132, 193, 247).

Exserohilum

Exserohilum (5, 157) is characterized by very long, distoseptate conidia with a distinct, protruding hilum at the basal scar. Three species have been recognized as opportunistic agents (*E. longirostratum, E. mcginnisii,* and *E. rostratum*; Fig. 4), but given their close molecular similarities the possibility is not excluded that these are morphological variants of a single species (48). Prevalent etiology is sinusitis with eventual brain involvement (14). Exceptionally, phaeohyphomycosis of the skin, subcutaneous tissue, and cornea has been documented (10, 115, 149, 157, 171).

Sordariales

Sordariales is a large and diverse order of ascomycetes. *Madurella mycetomatis* was recently proven to be a further member of Sordariales (46); this species is treated in chapter 126.

Chaetomium

Chaetomium species are very common saprobic decomposers of all sorts of organic materials. The few human infections, particularly those caused by *C. globosum,* may involve blood, lung, and brain in both healthy and immunocompromised patients (Fig. 4) (9, 101, 223, 229, 248). Cutaneous infections are extremely rare (223). The related species *Achaetomium strumarium* (Fig. 4) has been reported from cerebral infections in several patients (1). *Chaetomium* species have sexual fruiting bodies containing brown, one-celled ascospores produced in deliquescent asci. The fruiting bodies are covered with often elaborately branched and ornamented hairs. Anamorphs are not produced.

CLINICAL SIGNIFICANCE

Disorders caused by the fungi described in this chapter are mainly localized and occur in otherwise healthy hosts. The infections arise mostly after traumatic inoculation of contaminated material from the environment, or, less frequently, e.g., in the case of sinusitis, otitis, or growth in the mucous lungs of patients with cystic fibrosis, asymptomatic or mildly symptomatic colonization of cavities is observed. They are typical opportunists, infections increasing in severity in individuals with impaired innate immunity and metabolic diseases such as diabetes. The infections also arise in patients with severe immunological disorders such as neutropenic patients, solid organ transplantation recipients, or patients undergoing long-term corticosteroid therapy. Some species of Chaetothyriales are able to cause deep or disseminated infections in hosts with no known immune disorder. Such infections may take, if untreated, a chronic, fatal course after a destructive disease process. The frequency of all infections described above is relatively to extremely low, but given the potential severe course of the disease, as well as the sometimes very high degrees of resistance to antifungal drugs, attentiveness to these fungi is mandatory. *Sporothrix* (order Ophiostomatales) is the only agent of infection that increases in severity with defects of acquired cellular immunity, such as AIDS.

IDENTIFICATION

Direct Microscopic Examination

There are numerous procedures for treating the specimen to enhance fungal detection, including treatment with potassium hydroxide or calcofluor white and Giemsa, Wright, or Gram stains. Histopathology procedures using periodic acid-Schiff, hematoxylin and eosin, or methenamine silver enhance observation. Fontana-Masson staining is useful to detect melanization in cells that appear hyaline with light microscopy. Below some characteristic features are summarized.

Skin Infections

Pigmented hyphal fragments, occasionally melanized yeast-like cells, are visible in infected skin tissue.

Subcutaneous Infections

Mycetoma, a chronic subcutaneous infection, is characterized by the formation of large aggregates of fungal elements in the form of grains. The grains are black when *Exophiala* is the etiologic agent but white in the case of *Pseudallescheria*. Occasionally the infection is encapsulated by a collagen layer. Otherwise, the prevalent form is hyphal, less commonly yeast-like. Hyphae and yeasts are generally melanized, but frequently melanization is observed only after specific staining.

Sporotrichosis

In sporotrichosis, hyaline yeast-like cells are present in low frequency, bearing slender daughter cells at very narrow bases.

Chromoblastomycosis

The hallmark of chromoblastomycosis disease is the presence of muriform cells, which are swollen, spherical, dark brown cells which often develop a septum and may finally become cruciately septate. The skin usually is verrucose and through acanthosis may develop cauliflower-like tumors.

Sinusitis

In sinusitis, the sinuses are occluded by amorphous fungus balls.

Cerebral Infection

In cases of cerebral infection, the hyphae are often poorly colored and scantily branched. In advanced cases abscesses are formed.

Disseminated Infection

Pigmented or poorly colored fungal elements such as hyphae or yeast-like cells can be observed in cases of disseminated infection.

Isolation Procedures

The use of nutritionally minimal media such as 2% water agar, moistened sterile wooden sticks, or moistened filter paper stimulates the formation of conidia. In the case of infection due to more than one agent, the strain with limited expansion growth may pass unnoticed. Therefore, a loopful of cells suspended in 0.1% Tween and streaked onto a fresh culture plate is useful to select an individual colony for identification. Slide culture preparations (157) using potato dextrose agar or cornmeal dextrose agar, to be handled only within a biological safety cabinet, are ideal for determining conidiogenesis. For morphological features of the fungi treated in this chapter, the reader is referred to de Hoog et al. (48).

Nucleic Acid Detection Techniques

Molecular identification of most species is currently performed by sequencing of ribosomal genes and comparison with dedicated databases. Care should be taken when GenBank is used for the purpose of identification in less-known fungal groups, because over 10% of the sequences may be incorrect (49). Sequences should be evaluated not only from a technical point of view but also nomenclaturally, i.e., by comparison with ex-type strains.

When using ribosomal genes, the phylogenetic position of taxa can be established by sequencing the nuclear small subunit rRNA gene. This gene is mostly invariant between closely related species. In most fungal groups, species diagnostics is possible with ITS sequences. But application of this technique as a "gold standard" for melanized fungi is still in dispute. In some genera, such as *Alternaria*, the numerous species described on the basis of morphology prove to be invariant with ITS. In this case the question as to whether ITS shows insufficient polymorphism or whether simply too many species have been introduced remains pending. In contrast, ITS-based species distinction with black yeasts provides satisfactory results.

Serologic Tests

Serologic tests can detect the presence of antibodies or antigens. A latex agglutination test is commercially available (Immuno-Mycologics Inc., Norman, Okla.) for detecting antibodies against *S. schenckii*, particularly in disseminated cases, but despite a study (27) reporting sensitivity and specificity of 90 to 94% and 95 to 100%, respectively, the test has not been used widely. However, the detection of antibodies was demonstrated to be useful in the diagnosis of central nervous system sporotrichosis in several patients when culture-based diagnosis had failed (208). The remaining serologic tests consist of fluorescent-antibody conjugates for differential staining of histologic sections (125) and the exoantigen test for identification of cultures (126, 178). None of these reagents are commercially available.

ANTIMICROBIAL SUSCEPTIBILITIES AND RECOMMENDED TREATMENTS

In this section an overview of in vitro susceptibility testing and current therapies for the fungi described above is given (Table 2). The optimal therapy for infections caused by black fungi is controversial. Generally, surgical excision, antifungal monotherapy, or application of drug combinations has been used, but prolonged treatment is needed because relapses often occur.

The main etiological agents of chromoblastomycosis are *Cladophialophora carrionii, Fonsecaea pedrosoi, F. monophora,* and *Phialophora verrucosa.* Clinical trials for the treatment of the disease have not been performed. Patients with small lesions mostly respond better than those with progressive disease. Good results were obtained with topical heat. Cryosurgery has been recommended, but subsequent administration of antifungal agents is necessary. The use of terbinafine and itraconazole alone or combined with flucytosine seems to be the best option for treatment. The use of the new triazoles, voriconazole and posaconazole, might be promising since low in vitro MIC values were observed, but their effectiveness for treatment of chromoblastomycosis remains to be determined (71, 146, 188).

(Sub)cutaneous phaeohyphomycoses are caused by a diversity of black fungi belonging to unrelated genera such as *Alternaria, Aureobasidium, Bipolaris, Curvularia, Exophiala, Phaeoacremonium,* and *Phialemonium* (Fig. 1) (36, 97, 104, 110, 128, 174, 249). In vitro test results have shown that itraconazole, voriconazole, and terbinafine were active for these groups of fungi (121, 236, 237, 241, 249). Itraconazole is used as well as amphotericin B alone or in combination with flucytosine (241, 249).

For *Exophiala* species, the use of itraconazole alone or in combination with flucytosine has been reported to be effective and the new triazole, posaconazole, was shown to be active (36, 168), whereas the use of ketoconazole or miconazole appeared to be inadequate (129). The in vitro data for *Exophiala* species have demonstrated low MIC values for itraconazole, voriconazole, and terbinafine (236, 237).

The primary neurotropic dematiaceous fungi with a pronounced predilection for the central nervous system include *Cladophialophora bantiana, Exophiala dermatitidis, Ochroconis gallopava,* and *Ramichloridium mackenziei.* In vitro data have shown that amphotericin B, itraconazole, voriconazole, and terbinafine are active against *C. bantiana* and *O. gallopava* (121, 158). For treatment the following drugs have been used successfully: amphotericin B, liposomal amphotericin B, and the combination of itraconazole or voriconazole with

TABLE 2 Summary of diseases and generally applied treatments

Disease	Etiologic agents	Recommended treatments[a]	Reference(s)
Chromoblastomycosis	*Cladophialophora carrionii, Fonsecaea pedrosoi, F. monophora, Phialophora verrucosa*	TBF, ITZ, 5FC + TBF or ITZ, cryosurgery	73, 188
Subcutaneous mycosis	*Alternaria* spp., *Aureobasidium pullulans*, *Bipolaris* spp., *Curvularia* spp., *Exophiala* spp., *Phaeoacremonium parasiticum*, *Rhinocladiella similis, Phialemonium* spp.	ITZ, AMB + 5FC, ITZ + 5FC, PCZ, surgical excision	36, 168, 241
Cerebral mycosis	*Cladophialophora bantiana, Ochroconis gallopava, Ramichloridium mackenziei*	ITZ, AMB + 5FC, L-AMB, surgical excision, new triazoles (investigational)	192, 241
Sinusitis	*Bipolaris* spp., *Exserohilum* spp.	AMB, ITZ, surgical curettage, corticosteroids (allergic sinusitis)	25
Scedosporiosis	*Scedosporium apiospermum, S. prolificans*	VCZ + TBF, L-AMB, ABZ (investigational)	94
Sporotrichosis	*Sporothrix schenckii*	ITZ, TBF, AMB, VCZ, PCZ (investigational), potassium iodide solution, thermotherapy	188

[a] Abbreviations: AMB, amphotericin B; 5FC, 5-fluorocytosine; ITZ, itraconazole; L-AMB, liposomal amphotericin B; PCZ, posaconazole; TBF, terbinafine; VCZ, voriconazole.

amphotericin B. Ketoconazole, miconazole, or fluconazole is not recommended. In addition, surgical excision of the lesion has proven useful (78, 192).

Pleosporalean agents of sinusitis mostly are *Bipolaris, Curvularia,* or *Exserohilum*. For these infections, surgical curettage and initial treatment with amphotericin B is advised. Itraconazole should be given after surgery in order to prevent recurrence of the infection and has also led to improvement in patients who failed to respond to amphotericin B. Allergic sinusitis may require the administration of corticosteroids after surgery (25, 241).

In vitro data on *Scedosporium apiospermum, S. prolificans,* and *Sporothrix schenckii* have shown that amphotericin B was more active than itraconazole or voriconazole (121). Lymphocutaneous sporotrichosis can be treated effectively with orally administered itraconazole and potassium iodide solution. Thermotherapy is also useful. For sporotrichosis, itraconazole, amphotericin B, or the new triazoles are applicable, although the last require more investigation (189). *Scedosporium* infections are difficult to treat because these pathogens are often resistant to amphotericin B and several azoles, and thus, the therapeutic strategies are limited. The mortality rate among transplant recipients with scedosporiosis was reported to be 58%, and voriconazole was associated with a trend towards better survival than that obtained with amphotericin B (116). Voriconazole or itraconazole combined with terbinafine was reported to be synergistic in vitro, despite high MIC values for the drugs alone (94, 159). A successful outcome was observed in an orthopedic infection due to *S. prolificans* in a nonimmunocompromised host following treatment with the combination of voriconazole plus terbinafine, without radical surgery (94). The combination of new triazoles and amphotericin B at high doses seems to be better than monotherapy. Nevertheless, reversal of predisposing factors such as neutropenia remains essential (240). Cytokines and immunomodulation may be applicable, as was reported previously (241). One of the latest drugs, albaconazole, was evaluated in an immunocompetent rabbit model of systemic infection due to *Scedosporium prolificans* and showed promising efficacy exceeding that of amphotericin B at a dose of 50 mg/kg of body weight (30).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

More than 100 species of melanized fungi have caused infection in humans and animals, and many of these are relatively rare as etiologic agents. As a result, few clinicians are familiar with these fungi and they are frequently overlooked. Infections caused by dematiaceous fungi are being diagnosed increasingly among healthy as well as compromised patients. Concurrently, the expanding diversity of etiology within this group of fungi is becoming apparent.

Determining whether a particular dematiaceous fungus is involved in a disease process can be difficult because most of these fungi occasionally are recovered as contaminants from clinical specimens. Repeated recovery of a suspected etiologic agent is significant, while sequence identity of clinical material and the isolate is highly supportive. Isolation of a dematiaceous fungus from a normally sterile body site should not be dismissed as contamination, particularly if colonies are numerous or more than one culture vessel shows growth. The isolation from a nonsterile pulmonary specimen, such as skin, sputum, or bronchial lavage fluid, of well-documented opportunists from genera such as *Cladophialophora, Fonsecaea, Ochroconis,* or *Scedosporium,* which are not seen as contaminants, also is highly indicative.

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Fungi Causing Eumycotic Mycetoma

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TAXONOMY

All fungi known to cause eumycetomata are supposed to belong to the division Ascomycota, although for many species no ascigerous state is known (23). Ordinal relationships are then hypothesized on the basis of ribosomal small subunit DNA sequences (21) (Table 1; see also listing per order below). The etiologic agents described in this chapter are species that are isolated most commonly from human or lower-animal mycetomata.

DESCRIPTION OF AGENTS

Curvularia geniculata (Pleosporales)

The common saprobe *Curvularia geniculata* (Fig. 2) has occasionally been reported as an etiologic agent of mycetoma in dogs in the United States (13). Its grains are black to dark brown, firm, and 0.5 to 1.0 mm or more in size. In tissue sections, grains are spherical, ovoid, or irregularly shaped and are often surrounded by a zone of epithelioid cells. The periphery of the grain is a dense, interwoven mass of dematiaceous mycelium and thick-walled, chlamydospore-like cells embedded in a cement-like substance. The interior of the grains is vacuolar and consists of a loose network of septate, hyphal filaments.

In culture, *C. geniculata* develops a rapidly growing, floccose to downy, olive-gray to black colony. Microscopically, the melanized, septate hyphae bear solitary, geniculate conidiogenous cells. The conidia are without protruding hila. They are smooth walled, predominantly five-celled, curved, with the swollen median cell pale to dark brown, in contrast to the lighter end cells.

Curvularia lunata (Pleosporales)

The ubiquitous saprobe *Curvularia lunata* (Fig. 3) has exceptionally been described as an etiologic agent of mycetoma in humans in Senegal and Sudan (51, 82). Its grains resemble those of *C. geniculata* in their morphologic characteristics. In culture, *C. lunata* produces geniculate conidiophores that are sympodial in their development. Conidia are without protruding hila. They are smooth and predominantly four-celled, with the penultimate cell being pale to dark brown.

Exophiala jeanselmei (Chaetothyriales)

E. jeanselmei (Fig. 4) has been reported as an agent of eumycetoma in India (86, 87), Malaysia, Thailand (71), Argentina (66), and the United States (67), but only a few cases (46, 65) have been confirmed by molecular data (25). In host tissue, *E. jeanselmei* produces dark sclerotia that are brown to black, irregular in shape, and fragile. Detached portions or fragments of the sclerotia often are found within giant cells. When extruded through fistulae, the grains often look like worms (vermiform) because of their elongated shapes and irregular surfaces. In tissue sections, they appear as hollow structures or as sinuous bands that are vermiform. The external surface is composed of brown, thick-walled hyphae and thick-walled chlamydospore-like cells. The grains are cement free. Within the hollow grains, smaller, degenerated hyphal fragments with leukocytes and giant cells may be seen.

Initially, the colonies of *E. jeanselmei* may be yeast-like and black, gradually spreading, becoming raised or dome shaped, with limited expansion growth. After 2 weeks on Sabouraud dextrose agar (SDA), the colonies are covered with short aerial hyphae and appear olive-gray with an olive-black reverse. Microscopically, annellidic, rocket-shaped conidiogenous cells are inserted at right angles on undifferentiated hyphae. The septate mycelium is sometimes toruloid, branched, and pale brown. The conidia, which aggregate in masses at the tips of annellides, tend to slide down the conidiophore or along the hyphae. The smooth conidia are exogenous, nonseptate, subspherical, and ellipsoidal to cylindrical, measuring 1.5 to 2.8 μm.

Recently Guillot et al. (35) reported a canine mycetoma caused by a related fungus, *Cladophialophora bantiana* (Chaetothyriales), a species that in humans causes nearly exclusively brain infections (41).

Fusarium falciforme (Hypocreales)

Fusarium falciforme (85) (Fig. 1), previously known as *Acremonium falciforme,* is occasionally found as a cause of mycetoma in the United States (38, 60). It was also reported as the cause of an opportunistic infection in a renal transplant recipient (30). The grains of *F. falciforme* are white to pale yellow, soft, and 0.2 to 0.5 mm in diameter. They are composed of slender, polymorphic, septate hyphae 1.5 to 2.0 μm in diameter with irregular bulbous swellings and peripheral cementing material.

Colonies of *F. falciforme* on SDA are slow growing, reaching 60 to 65 mm in diameter in 2 weeks. They are downy and gray-brown, becoming gray-violet. The reverse of the colony

TABLE 1 Overview of main species causing eumycotic mycetoma

Species	Geography	Color of grains	Phylogeny
Curvularia geniculata	Worldwide	Black	Pleosporales
Curvularia lunata	Worldwide	Black	Pleosporales
Exophiala jeanselmei	Worldwide	Black	Chaetothyriales
Fusarium falciforme	Worldwide	Whitish	Hypocreales
Leptosphaeria senegalensis	West Africa, India	Black	Pleosporales
Madurella grisea	South America, India	Black	Pleosporales
Madurella mycetomatis	East Africa, Middle East	Black	Sordariales
Neotestudina rosatii	Central Africa	Whitish	Sordariales
Phaeoacremonium spp.		Black	Diaporthales
Pseudallescheria boydii	North and South America	Whitish	Microascales
Pyrenochaeta mackinnonii	Central and South America	Black	Pleosporales
Pyrenochaeta romeroi	Arid subtropics	Black	Pleosporales

develops a violet-purple pigment. The hyphae are hyaline, septate, smooth, branched, and 1.5 to 2.5 μm in diameter. They bear erect, undifferentiated, unbranched, repeatedly septate conidiophores. The conidia are borne at the tip of phialidic conidiogenous cells, where they accumulate in mucoid clusters. The conidia are allantoid, slightly curved, and nonseptate to monoseptate and measure 7 to 8.5 by 2.7 to 3.2 μm. The intercalary or, rarely, terminal chlamydospores are smooth, thick walled, and 5 to 8 μm in diameter.

Leptosphaeria senegalensis (Pleosporales)

L. senegalensis and the related species *L. tompkinsii* cause mycetomata in the northern tropical portion of West Africa, especially in Senegal and Mauritania, and in India. The grains of the two species are indistinguishable from each other. They are black, 0.5 to 2 mm in size, and firm to hard. In tissue sections, the grains are round to polylobulated, with large vesicles. At the periphery, the mycelium is embedded in a black, cement-like substance. The central portion of each grain consists of a loose network of hyphae.

In culture, *L. senegalensis* and *L. tompkinsii* grow rapidly and produce gray-brown colonies. On cornmeal agar, both species produce ascostromata that are nonostiolate, scattered, immersed or superficial, globose to subglobose, black, and covered with brown, flexuous hyphae. The asci, produced after prolonged incubation on plant stems, are eight-spored, clavate to cylindrical, and double walled. The major difference between the two species is found in the ascospores, which differ in size, shape, septation, and the nature of the gelatinous sheath that surrounds them (27, 28).

Madurella grisea (Pleosporales)

M. grisea occurs as an etiologic agent of black grain mycetoma in South America, India, Africa, and North and Central America (50, 51). In the United States, five cases caused by *M. grisea* have been described (14, 61). As the fungus is strictly sterile, molecular confirmation is overdue for all these cases.

The grains are black, 0.3 to 0.6 mm in diameter, and soft to firm. In tissue sections, the grains are oval, lobulated or reniform, and sometimes vermiform. They are composed of a dense network of hyphae weakly pigmented in the center and brown to blackish brown in the peripheral region as the result of the presence of a brown, cement-like interstitial material.

In culture, *M. grisea* forms slow-growing, velvety colonies that are cerebriform, radially furrowed or smooth, and dark gray to olive-brown to black. The reverses of the colonies are black. Microscopically, the hyphae are septate, light to dark brown, 1 to 3 μm in diameter, and nonsporulating. Chlamydospores are rare. Large moniliform hyphae 3 to 5 μm in diameter are often present. Some isolates of *M. grisea* have been described as producing abortive or fertile pycnidia (56, 81). Such isolates are morphologically indistinguishable from *Pyrenochaeta mackinnonii* (11, 75), but rRNA internal transcribed spacer (ITS) sequence data (21) show large differences between these species.

Madurella mycetomatis (Sordariales)

Madurella mycetomatis is common in the arid climatic zone of West Africa (Sudan, Mali, and Djibouti) and is occasionally encountered in the Middle East and India. The grains produced by *M. mycetomatis* are reddish brown to black. They may reach 5 mm or more in diameter and are firm to hard. In tissue sections, the sclerotia are compact, variable in size and shape, and frequently multilobulated. They are composed of hyphae 1.2 to 5 μm in diameter that terminate in enlarged hyphal cells at the periphery of the grains, which measure 12 to 15 μm in diameter. The cell wall pigment is minimal, but hyphal cells contain brown particles. The hyphae are embedded in a conspicuous brown matrix that is characteristic of *M. mycetomatis*. Some grains are vesicular and more regular in size and shape. The vesicles are predominantly visible in the peripheral zone in a dense, brown, cement-like matrix.

In culture, *M. mycetomatis* shows wide variation. Colonies are slow growing and white at first, becoming olivaceous, yellow, or brown; flat or dome shaped; and velvety to glabrous, often with a rust brown diffusible pigment. On nutritionally deficient media, sclerotial bodies 750 μm in diameter develop. These are black and consist of undifferentiated polygonal cells. On SDA, the mycelium is sterile. On nutritionally poor media, such as soil extract or hay infusion agar, about 50% of the isolates produce round to pyriform conidia 3 to 4 μm in diameter at the tips of phialides. The phialides are tapering, ranging from 3 to 15 μm in length, often with an inconspicuous collarette (Fig. 5).

M. mycetomatis grows better at 37 than at 30°C, with a maximum of 40°C, whereas *M. grisea* grows better at 30 than at 37°C. *M. mycetomatis* is slowly proteolytic and utilizes glucose, maltose, and galactose but not sucrose. It utilizes potassium nitrate, ammonium sulfate, asparagine, and urea, and it hydrolyzes starch. *M. grisea,* on the other hand, is weakly

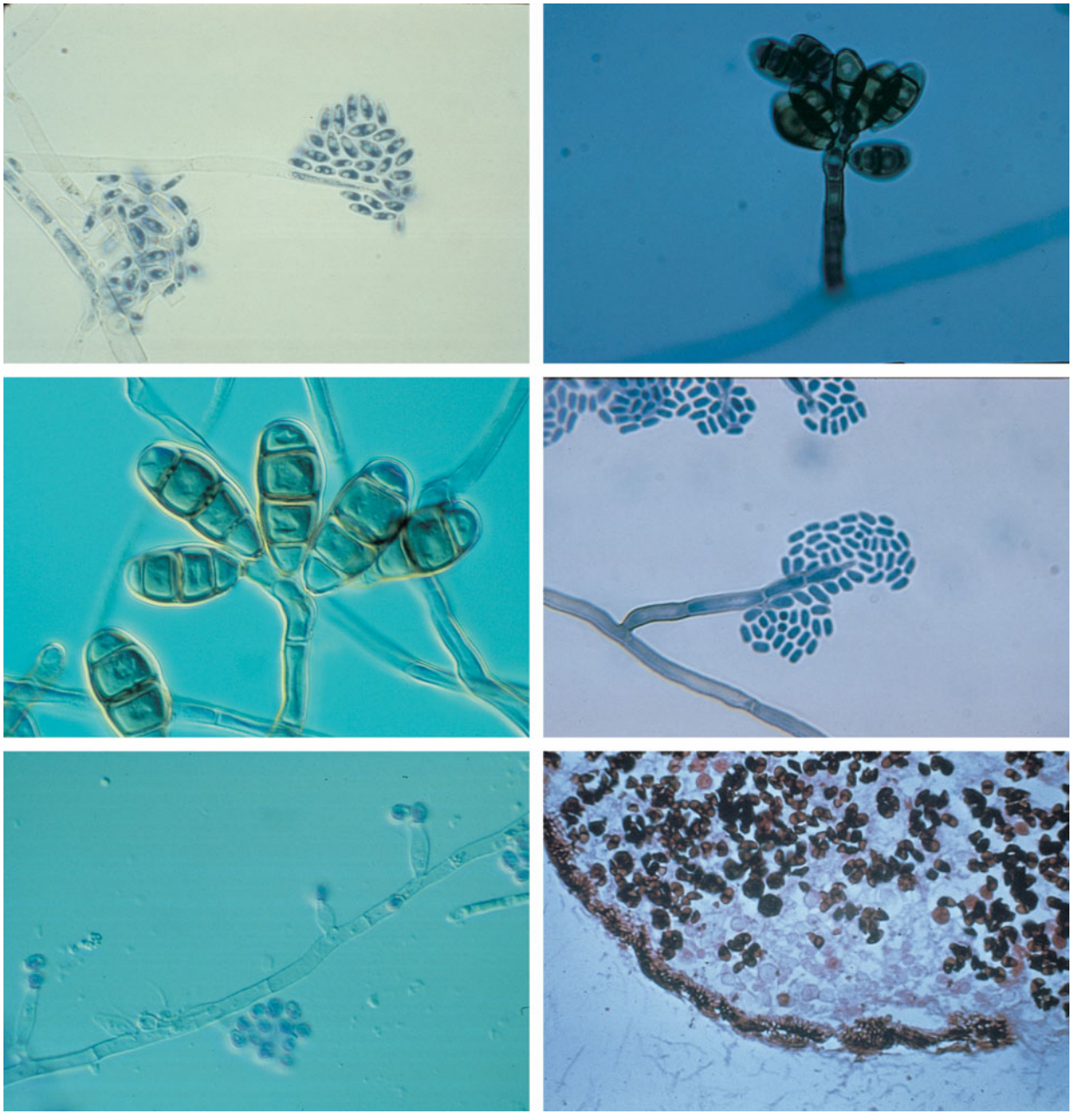


FIGURE 1 (top left) *F. falciforme.* Slide culture on SDA showing erect, septate conidiophore, phialidic conidiogenous cell, and slightly curved conidia. Magnification, ×160.

FIGURE 2 (top right) *C. lunata.* Slide culture on potato dextrose agar showing geniculate conidiogenous cell and septate, curved conidia. Magnification, ×160.

FIGURE 3 (middle left) *C. lunata.* Geniculate conidiogenous cell bearing smooth, predominantly four-celled conidia. Magnification, ×160.

FIGURE 4 (middle right) *E. jeanselmei.* Lateral, septate conidiophore bearing closely annellated conidiogenous cell producing smooth, nonseptate, ellipsoidal to cylindric conidia. Magnification, ×160.

FIGURE 5 (bottom left) *M. mycetomatis*. Slide culture on soil extract agar showing lateral phialides and globose conidia. Magnification, ×250.

FIGURE 6 (bottom right) *N. rosatii.* Cross section of an ascocarp showing the ascomatal wall of interwoven hyphae and containing dark asci and ascospores. Magnification, ×250.

proteolytic and assimilates glucose, maltose, and sucrose but not lactose. Phylogenetic studies suggest that *M. mycetomatis* and *M. grisea* are distantly related. *M. mycetomatis* is probably a member of the order Sordariales, while *M. grisea* clusters with known members of the Pleosporales (21).

Neotestudina rosatii (Sordariales)

The sclerotia of *N. rosatii* are white to brownish white, 0.5 to 1.00 mm in diameter, and soft. In tissue sections, the sclerotia appear to be polyhedral to subregular and consist of hyphae, which are embedded, in the peripheral cementing material. The sclerotia manifest an eosinophilic border. The central portion of each sclerotium consists of more or less disintegrated mycelium and chlamydospores. Mycetomata caused by *N. rosatii* have been described in Australia, Cameroon, Guinea, Senegal, and Somalia (54, 80).

In culture, colonies of *N. rosatii* are slow growing, attaining diameters of 25 to 28 mm in 2 weeks, and have an aerial mycelium that is grayish black to brownish black. On potato-carrot or cornmeal agar incubated at 30°C, most of the ascomata are submerged. The ascostromal walls are smooth and are surrounded by interwoven brown to hyaline hyphae. The eight-spored asci, 12 to 35 by 10 to 25 μm, are scattered in the central part of the ascostroma (Fig. 6) and are globose to subglobose, thick walled, and bitunicate, becoming evanescent as the ascospores mature. The ascospores vary in size (9 to 12.5 by 4.5 to 8.0 μm) and shape, ranging from ellipsoidal to bicampanulate, asymmetrical, or slightly curved; are constricted at the median transverse septum; and have brown smooth walls.

Phaeoacremonium (Diaporthales)

Members of the genus *Phaeoacremonium,* morphologically characterized by slender, gradually tapering, melanized conidiophores with narrow phialide openings producing ellipsoidal to reniform conidia, are occasionally reported as agents of mycetoma. de Albornoz (19) described a case of infection by *P. inflatipes,* Rowland and Farrar (76) and Mostert et al. (63) described cases of infection by *P. krajdenii,* and Hood et al. (40) described a case of infection by *P. parasiticum.*

Pseudallescheria boydii (Anamorph: *Scedosporium apiospermum*) (Microascales)

Grains in tissue are white to yellowish white and soft to firm; they vary from globose to subglobose or lobulated and are 0.2 to 2.0 mm in diameter. They are composed of hyaline hyphae 1.5 to 5.0 μm in diameter that radiate from the center into terminal thick-walled cells 15 to 20 μm in diameter at the peripheries. The central portion of each grain consists of loosely interwoven hyaline mycelium.

Colonies grow rapidly and are floccose and white at first, becoming gray as conidia are produced. With age, the colonies become dark grayish brown. Submerged ascocarps (cleistothecia) (Fig. 9) may be produced when isolates are grown on cornmeal agar and are visible macroscopically as small black dots. They are globose, nonostiolate, 140 to 200 μm in diameter, and often covered with brown, thick-walled, septate hyphae 2 to 3 μm wide. They have a wall 4 to 6 μm thick that is composed of two or three layers of interwoven, flattened, dark brown cells, each 2 to 6 μm wide. The cleistothecia open at maturity by an irregular rupture of the wall. The eight-spored asci are ellipsoidal to nearly spherical and 12 to 18 by 9 to 13 μm. The ascospores are ellipsoidal to oblate, symmetrical or slightly flattened, and 6 to 7 by 3.5 to 4.0 μm; are straw colored; and have two germ pores.

The anamorph, *Scedosporium apiospermum* (Fig. 7), produces conidia that are oval to clavate, truncate, and subhyaline, becoming pale gray to pale brown in mass. Conidia are produced singly. They remain attached at the tips of annellides. Annellations can be detected at the tips of conidiogenous cells as swollen rings. Some isolates also produce a *Graphium* synanamorph (Fig. 8), which is characterized by rope-like bundles of hyphae with annellidic conidiogenesis. The hyphae are fused into long stalks known as synnemata. The conidia produced are hyaline, cylindric to clavate, and truncate at the base. A fungus consisting of elongate, multicellular conidia and described as *Polycytella hominis* (15) was recently proven to be a degenerate anamorph of *P. boydii* (12).

P. boydii is the most common agent of mycetoma in humans as well as lower animals in temperate climates (16), occurring mostly in the limbs (91). Subcutaneous infections frequently lack the formation of grains when the patients' immunity is impaired (70). In addition, arthritis is frequently observed, the species having a predilection for cartilaginous tissues (34, 39, 48, 68). More often it is encountered as an opportunist in respiratory specimens from patients with lung disease (84) and is particularly observed in lungs of patients with cystic fibrosis (20), where it is often concealed by the abundant presence of *Aspergillus fumigatus*. Although its virulence is low, cases of dissemination from lungs to vital organs have been observed (96). Of particular significance is the species' neurotropism, potentially leading to fatal brain abscesses (42, 43). This syndrome is frequently noted after severe debilitation, e.g., after near-drowning, but interestingly also after dissemination from a traumatic, mycetoma-like infection (41).

Pyrenochaeta romeroi (Pleosporales)

Mycetomata caused by *P. romeroi* (Fig. 10) have been reported from Somalia, India, and South America (9, 44, 54). *P. romeroi* produces soft to firm black sclerotia that are oval, lobulated, and sometimes vermiform. They resemble those of *M. grisea* (81).

In culture, colonies of *P. romeroi* are fast growing and floccose to velvety, with a gray surface and whitish margin. The reverse of the colony is black, with no diffusible pigment. On nutritionally deficient media such as oatmeal agar under near-UV light, pycnidia develop after 3 to 4 weeks. The conidiomata are subglobose, 80 to 160 μm in diameter, and dark brick to fawn, later becoming dark brown with short necks. They are ostiolate, and setae around ostioles are septate and roughened and measure 80 to 100 by 3 μm. The conidiophores inside the conidiomata are sparse and have lateral branches. Conidiogenous cells are hyaline and are borne on branches or arise directly from cells lining the conidiomatal cavity, which produce hyaline to yellowish, shortly cylindrical conidia measuring 1 to 2 by 1.0 μm.

The close resemblance of the granules produced by the coelomycete *P. romeroi* and the gray cultures of that species suggests that *Madurella grisea* may be a nonsporulating counterpart of *P. romeroi*. However, Murray and Buckley (64) found serologic differences that allow differentiation between the two species. Romero and Mackenzie (75) studied culture filtrate and cellular antigens prepared from 14 agents of black sclerotial mycetoma by double diffusion and immunoelectrophoresis. They found up to 90% cross-reactivity among *M. grisea* isolates and between *M. grisea* and *Pyrenochaeta mackinnonii* but not between *M. grisea* and *P. romeroi.* Molecular data of rRNA ITS (21) show that all these species are very different from one another.

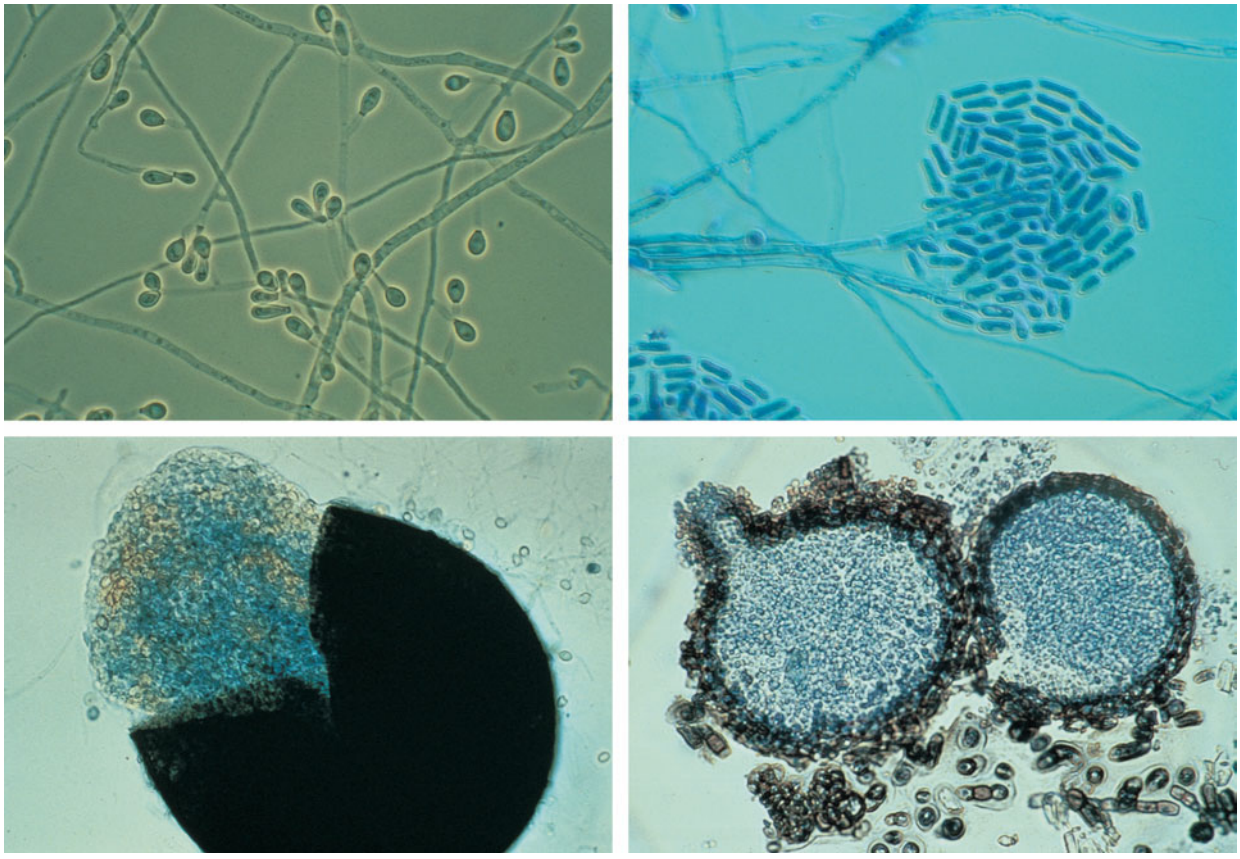


FIGURE 7 (top left) *S. apiospermum* anamorph of *P. boydii.* Slide culture on potato dextrose agar showing lateral, single, egg-shaped to clavate, truncate conidia. Magnification, ×160.

FIGURE 8 (top right) *Graphium* synanamorph of *P. boydii.* Slide culture on potato dextrose agar showing rope-like bundles of hyphae producing cylindric, smooth conidia. Magnification, ×160.

FIGURE 9 (bottom left) *P. boydii.* Globose, nonostiolate, ruptured cleistothecium containing ellipsoid to oblate ascospores. Magnification, ×160.

FIGURE 10 (bottom right) *P. romeroi.* Ostiolate conidiomata (pycnidia) containing numerous cylindric conidia. Magnification, ×250.

EPIDEMIOLOGY AND TRANSMISSION

The causal agents of eumycetoma are supposed to be saprobes that live on hard plant materials, such as various types of thorns and spines, associated with soil (5, 8, 29, 49). Segretain and coworkers (80, 82), using specific isolation media and techniques, showed that *L. senegalensis* and *L. tompkinsii* could be recovered from about 50% of the dry thorns of *Acacia* trees that they examined, particularly those that had been stained by mud during the rainy season. *N. rosatii* was reportedly isolated from sandy ground (54), and *M. mycetomatis* was isolated from soil and anthills (78, 79, 89). It should be noted, however, that the identity of these fungi has not been verified with molecular methods; the possibility that environmental strains do not always belong to the same species as the clinical strains cannot be excluded. *M. mycetomatis* is a common agent of mycetoma in the arid climate zones of East Africa but is difficult to isolate directly from the environment (1). Some predilection for human invasion must exist.

Borelli (10) isolated another sterile fungus, reported as *Madurella grisea* from soil in Venezuela; the ecological niche and clinical potential of this rare species have not yet been determined. *P. boydii* is associated with manure and polluted environments (24, 32) and has been known as an agent of human mycetoma in temperate climates since the 1920s (83).

Mycetoma may infect all people living in areas of endemicity but develops more commonly among persons who are in contact with contaminated materials, such as field workers, farmers, and fishermen. Areas of endemicity are particularly located in tropical climate zones. *M. mycetomatis* is limited to semiarid to arid climates, while *Leptosphaeria* species are found in the rain forest. Locally acquired mycetomata in temperate climates invariably concern *P. boydii*. Cases observed in the United States and Europe caused by species other than *P. boydii* are imported by immigrants from tropical countries. For example, de Hoog et al. (22) reported cases in The Netherlands originating from Indonesia and Suriname, while cases of *M. mycetomatis* in France originated from Mali (2).

Climate has a definite influence on the prevalence and distribution of mycetomata. Rivers that flood each year during the wet season in many countries of Africa and Asia influence

the distribution of the causal agents. Rainfall also aids the spread of the etiologic agents on organic matter (26).

CLINICAL SIGNIFICANCE

A mycetoma (plural, mycetomata) (58) is a localized, chronic, noncontiguous, granulomatous infection involving cutaneous and subcutaneous tissues and eventually, in some cases, bones. Mycetomata are generally confined to either the feet or the hands but occasionally affect sites such as the back, shoulders, buttocks, and, rarely, the scalp. The lesions contain granulomata and abscesses that suppurate and drain through fistulae to the skin surface. The pus contains grains (57) that vary from approximately 0.2 to over 5 mm in diameter. The size, color, shape, and internal architecture of the sclerotia vary depending on the species of the etiologic agent. Mycetomata caused by actinomycetes are called actinomycetomata, and those incited by filamentous fungi are referred to as eumycetomata. Eumycetomata, in contrast to actinomycetomata, have a hyphal substructure. The disease is more commonly seen in humans than in lower animals. Only a few cases of mycetoma involving such animals as dogs, horses, and goats have been described in the literature (6, 13, 45, 52).

Fungus balls or fungomata in preexisting lung cavities are sometimes inappropriately called mycetomata (31, 36). In the absence of well-organized grains, they should be referred to, depending on the etiologic agent, as aspergillomata, or simply as fungus balls (55). Similarly, mycelial aggregates formed by dermatophytes in cutaneous or subcutaneous tissues differ from grains of mycetomata by lacking granule ontogeny, a distinct Splendore-Hoeppli reaction (an immune reaction seen histologically as an eosinophilic deposit) surrounding the mycelial aggregates, and the entry of the fungus from the hair follicles into deeper tissue following the rupture of the follicular epithelium. Such infections caused by dermatophytes have been referred to as pseudomycetomata (7).

A mycetoma develops after a traumatic injury by microbe-contaminated thorns, splinters, fish scales or fins, snake bites, insect bites, farm implements, and knives. Most cases occur in otherwise healthy patients. The development of mycetoma after an accidental implantation of the etiologic agent following surgery (69) and in a renal transplant recipient (30) has been reported. The initial lesion is often characterized by a feeling of discomfort and pain at the point of inoculation. Weeks or months later, the subcutaneous tissue at the site of inoculation becomes indurated, abscesses develop, and fistulae may drain to the surface. Mycetomata are characterized by swelling, granulomata, abscesses, and sinuses from which serosanguinous fluid containing fungal grains is discharged. The mycetoma develops slowly beneath thick fibrosclerous tissue. The subsequent phase of proliferation involves the invasion of muscles and intramuscular layers by portions of the sclerotium that break free from their parent structure. The granulomatous lesions can extend as deep as bone, where severe bone destruction, formation of small cavities, and complete remodeling may occur. Early osteolytic damage includes loss of the cortical margin and external erosion of the bone. As the infection progresses, blood, lymphatic vessels, and nerves may be damaged. Frequently, secondary bacterial infections and osteomyelitis producing total bone destruction occur. Pain is often associated with the development of multiple fistulae. Regardless of the etiologic agent involved, the organisms develop in the form of soft or hard, compact mycelial masses (grains) within the infected tissue. The hallmark of a mycetoma is a grain composed of compact mycelium. These structures frequently contain host tissue. Weathered et al. (92) described the cement surrounding grains of *M. mycetomatis* as being an amorphous, electron-dense material with areas containing different-size membrane-bound vesicular inclusions. Some of the host material, especially at the periphery of the grain, provides a protective barrier for the fungus against antifungal agents and humoral immunological responses such as antibodies.

DIRECT EXAMINATION

The grains of eumycetomata are composed of septate mycelial filaments at least 2 to 4 μm in diameter. The mycelium may be distorted and unusual in form and size, and the cell walls of the fungi, especially toward the periphery of the grains, are thickened. Vesicles are frequently present, especially at the periphery of the grain. The mycelium of the grains may be embedded in a cement-like substance, depending on the species involved. Often the sclerotia elicit an immunologic response, known as the Splendore-Hoeppli reaction (antigen-antibody) (17, 58). This reaction is seen histologically in the form of a deposit of amorphous material around the grain. A similar tissue reaction has been described in experimental eumycetoma in mice (4).

Grains are white or yellow-brown with agents mostly producing hyaline mycelium, and black in the case of melanized etiologic agents. Melanin is a high-molecular-weight compound that is anchored to extracellular proteins. A precursor for production of melanin in ascomycetous fungi is 1,8-dihydroxynaphthalene (57, 58). Reports of hyaline fungi such as *Acremonium kiliense* and *Fusarium solani* var. *coeruleum* forming black grains (88) are probably erroneous. Etiologic agents with melanized hyphae or conidia such as *Phaeoacremonium inflatipes* and *Pseudallescheria boydii* produce whitish grains in tissue (18, 19, 49, 59). Melanin production in *Phaeoacremonium* is facultative, while in *P. boydii* dark pigmentation is limited to the conidia. Although the gross and microscopic characteristics of the grains provide insight into the identity of the etiologic agent or a particular group to which it belongs (74), definitive identification of the etiologic agent should be based on isolation of the same fungus from several grains. Physicians should avoid planning a patient's therapy based on grain morphology in clinical materials alone; rather, results of laboratory diagnosis should be awaited.

Nucleic Acid Detection Techniques

Molecular diagnostics has been developed for a number of selected agents. The black yeast *Exophiala jeanselmei* is recognizable by sequence data of the rRNA ITS region (25). Agents of the genus *Phaeoacremonium* can be identified using the same marker (62, 63). Molecular phylogeny and diagnostics of members of the genus *Madurella* based on the ribosomal operon showed that the genus encompasses a hidden diversity beyond the two currently recognized species (21). rRNA restriction fragment length polymorphism was used for species distinction by Ahmed et al. (2, 3), and a specific ITS-PCR was used by Ahmed et al. (3). Van de Sande et al. (90) in an amplified fragment length polymorphism study revealed clones differing in virulence and geographic distribution.

Also the genus *Pseudallescheria* comprises a larger number of species than supposed originally (33, 72). On the basis of multigene sequence data, Gilgado et al. (33) described several new taxa. The ITS region can be used as a marker. PCR-based assays for rapid diagnosis of *Scedosporium apiospermum*

infections from infected tissue are useful (37, 53). Wedde et al. (93) developed specific primers based on ITS rRNA sequences. Given the large infraspecific variability of the species, a less variable region, such as the 26S rRNA operon (77), might also provide successful detection of *S. apiospermum*-like genotypes. Willinger et al. (95) used this marker for detection of *S. apiospermum* and similar organisms from fungus balls in the maxillary sinus. Subspecific typing of *S. apiospermum* at the population level is unproblematic because of the large degree of heterogeneity. Rainer et al. (73) found with M-13 fingerprinting that nearly all strains analyzed belonged to another genotype; several genotypes could be recovered from a single sampling site. The high degree of polymorphism enabled Zouhair et al. (96) to monitor the dissemination of a strain colonizing the lungs of a patient with cystic fibrosis to cutaneous locations by using multilocus enzyme electrophoresis and randomly amplified polymorphic DNA. In a longitudinal study, Defontaine et al. (20) analyzed nine patients with cystic fibrosis, none of which were found to share the same genotype.

Isolation Procedures

To maximize the chances of obtaining pure cultures of the etiologic agents, grains from the eumycotic mycetomata should be washed several times with saline containing antibacterial antibiotics such as penicillin and streptomycin. The grains are then cultured on SDA containing chloramphenicol (50 mg/liter) and SDA containing chloramphenicol and cycloheximide (500 mg/liter) in petri dishes. Plates should be incubated at 25 and 37°C. Because many of the fungi that cause mycetoma grow slowly, culture plates should be incubated for 4 weeks before being discarded as negative. Identification of the isolated fungus is based on gross morphology of fruit bodies and conidia, if present. Certain species *(M. grisea, M. mycetomatis, N. rosatii, Pyrenochaeta mackinnonii,* and *P. romeroi)* do not sporulate readily but can be recognized by molecular techniques, particularly sequencing of the rRNA ITS region (3, 93).

ANTIMICROBIAL THERAPY

The treatment of eumycotic mycetoma is challenging. Antifungal therapy often has to be combined with debridement (surgical removal of infected tissue) when possible (94). Prolonged antifungal therapy usually is necessary to enhance the prognosis of these patients. Amphotericin B is commonly used to treat patients with eumycotic mycetoma; however, this drug is associated with several adverse reactions. Amphotericin B can be administered either intravenously or by inhalation (36). Itraconazole (36, 47) and ketoconazole (94) have been used successfully to treat eumycotic mycetoma patients and in general are associated with less severe adverse reactions than those observed with amphotericin B (36, 47). Fluconazole has been used to treat some patients but has shown poor results (94).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Because agents of eumycotic mycetoma are soil or plant saprobes, their etiologic role in mycetoma must be carefully established. A definitive diagnosis is based on the demonstration of grains in tissue, which are expelled through draining sinuses. Grains may become entangled on gauze bandages placed over fistulae.

Pus, exudate, or biopsy material should be examined for the presence of grains that are detectable with the naked eye. Their color, internal architecture, size, and shape give a fair indication of the identity of the possible etiologic agents. Actinomycotic mycetomata are differentiated from eumycotic mycetomata by the examination of crushed, Gram-stained grains. Actinomycotic grains, as well as coccoid and bacillary forms, are composed of gram-positive, interwoven, thin filaments, 0.5 to 1.0 μm in diameter. Grains of the eumycotic agents, on the other hand, are composed of broader, interwoven, septate hyphae, 2 to 5 μm in diameter, with many unusual-shaped, swollen cells up to 15 μm in diameter, especially at the periphery of the grains. In many species, the grains are embedded in cement-like material.

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Mycotoxins

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Mycotoxins are nonvolatile secondary metabolites produced by many different species of ubiquitous fungi having adverse effects on humans and animals. For many years, interest in mycotoxins centered on contamination of agricultural products with mycotoxin-producing fungi and the health effects of consumption of or direct contact with these products. Recently, two events brought interest in mycotoxins to the forefront: (i) the report of black mold as the possible cause of acute idiopathic pulmonary hemosiderosis in newborn infants in Cleveland, Ohio, which suggested that the presence of fungi in buildings (termed sick building syndrome [SBS]) could have deleterious health effects, and (ii) the flooding of New Orleans, La., and the Gulf Coast as a result of Hurricane Katrina. This flooding led to extensive fungal proliferation in homes and businesses and may require the demolition of affected buildings. In this chapter we review various mycotoxins and their relevance to food safety, SBS, veterinary problems, and bioterrorism. We do not describe the health effects of mycotoxins, as this topic has been reviewed extensively elsewhere (15, 30, 33, 38, 44, 61, 62, 70). The reader is also referred to an excellent review on mycotoxins by Bennett and Klich (6).

TAXONOMY

Fungi are eukaryotic organisms with cell walls containing chitin and/or cellulose that belong to the kingdom Fungi. A large number of fungi produce mycotoxins, but the majority of these belong to the anamorphic form-class Hyphomycetes. They may be either hyaline (colorless), such as *Aspergillus, Fusarium,* and *Penicillium* spp., or dematiaceous (darkly pigmented), like *Stachybotrys chartarum*.

MAJOR CATEGORIES OF MYCOTOXINS

Not all toxic compounds produced by fungi are classified as mycotoxins; for example, compounds mainly toxic to bacteria are termed antibiotics, while those toxic to plants are called phytotoxins (6). There are some 300 to 400 compounds, toxic to vertebrates in low concentrations, which are currently recognized as mycotoxins. Table 1 provides a listing of some of the most commonly encountered mycotoxin-producing fungi, many of which produce more than one mycotoxin class.

Aflatoxin

The aflatoxin class of mycotoxins is one of the first to be discovered and studied. Some of the most common aflatoxins (Fig. 1) (6) are aflatoxins B_1, B_2, G_1, and G_2 (based on their blue or green fluorescence under UV light) (86), with aflatoxin B_1 being the most potent natural carcinogen known (74).

Aflatoxins are produced by several species of *Aspergillus,* in particular, *Aspergillus flavus* and *Aspergillus parasiticus*. *A. flavus* is a common contaminant of agricultural products, among which are cereals, rice, figs, nuts, and tobacco (17, 18, 71). Contamination of crops can occur in the fields before harvest, especially in times of drought (18, 36), or during storage, depending upon the moisture content of the substrate and the relative humidity of the storage conditions (17, 82).

Aflatoxin contamination can be the cause of a variety of economic and health problems. For instance, the presence of aflatoxin in grain significantly lowers the grain's value as feed or an export commodity because of the toxin's link to increased mortality in farm animals (72). Further, ingestion of aflatoxin by dairy cows can lead to the presence of aflatoxin M_1 (a hydoxylated form of B_1) in the milk (79).

There are substantial differences in the susceptibilities of different vertebrate species to aflatoxin exposure. One of the first indications of the effects of aflatoxins was observed in 1960, when more than 100,000 turkey poults died from aflatoxin-contaminated feed, an outbreak named "turkey X" disease (1, 8). Other outbreaks have occurred in ducklings and chickens (3), swine (26, 45), and calves (46), due mostly to contaminated Brazilian peanut meal used as feed. The most recent and notable outbreak of acute aflatoxicosis occurred in Kenya, Africa, in 2004 in humans (4, 10, 43). This outbreak illustrates the need for a regulatory body to monitor the amount of mycotoxins present in foods meant for human consumption, a luxury not normally available to developing countries.

Citrinin

Citrinin (Fig. 2) (6) is a simple, low-molecular-weight compound that crystallizes as lemon-colored needles. It had been tested for use as an antibiotic (41, 42, 87) and as a treatment for ulcers (41) before its mycotoxic effects were discovered.

The most common organisms producing citrinin are *Penicillium citrinum, Penicillium expansum, Penicillium viridicatum, Penicillium camembertii* (used to produce cheese), *Aspergillus niveus, Aspergillus oryzae* (used to produce sake and

TABLE 1 Simplified taxonomy of mycotoxin-producing fungi

Mycotoxin class	Genus(era)	Representative species
Aflatoxin	*Aspergillus*, *Penicillium*	*A. fumigatus, A. flavus, A. parasiticus, P. puberulum*
Citrinin	*Penicillium*	*P. citrinin, P. expansum*
Ergot alkaloid	*Aspergillus*, *Balansia*, *Claviceps*, *Epichloë*, *Neotyphodium*	*A. fumigatus, C. purpurea*
Fumonisin	*Fusarium*	*F. verticillioides* (syn. *F. moniliforme*), *F. proliferatum*
Ochratoxin	*Aspergillus*, *Penicillium*	*A. ochraceus, P. verrucosum*
Patulin	*Aspergillus*, *Byssochlamys*, *Penicillium*	*A. clavatus, B. fulva, B. nivea, P. griseofulvum, P. expansum*
Trichothecene	*Stachybotrys*	*S. chartarum*
Zearalenone	*Fusarium*	*F. crookwellense, F. culmorum, F. graminearum, F. semitectum*

soy sauce), *Aspergillus terreus,* and *Monascus* spp. (used to produce red food dyes) (11).

Citrinin, first isolated from *P. citrinum,* has been associated with Japanese yellow rice disease (68). It has also been found in various other grains, peanuts, and fruit, and there is limited evidence of it surviving unchanged in cereal food products (6). Citrinin has demonstrated nephrotoxic effects on all animal species tested (9) and was shown to inhibit dehydrogenase activity in rats' kidneys, liver, and brain (28). However, there have been no reported outbreaks of human citrinin poisoning, and its relevance to human health is unknown.

Ergot Alkaloids

Ergot alkaloids, produced by the ergot fungus *Claviceps purpurea,* are the causative agent of ergotism (also called St. Anthony's fire), which can manifest in either a gangrenous or convulsive condition following ingestion of contaminated grains (especially rye) (5). Two main classes of ergot alkaloids exist: lysergic acid derivatives and clavines. Both are indole alkaloids derived from a tetracyclic ergoline ring system. Lysergic acid, a component common to all ergot alkaloids, often forms amide, amino acid, or peptide derivatives (e.g., ergine, ergonovine, lysergic acid diethylamide [LSD], and ergovaline). The clavines contain the ergoline structure but do not have amino acid or peptide components (e.g., pergolide and lisuride) (5). A representative chemical structure, ergotamine, is shown in Fig. 3 (6).

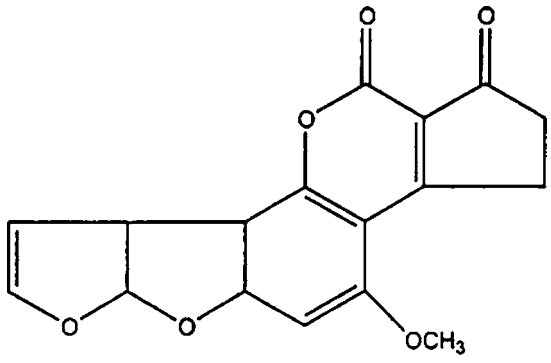


FIGURE 1 Aflatoxin B_1. (Reprinted from reference 6 with permission.)

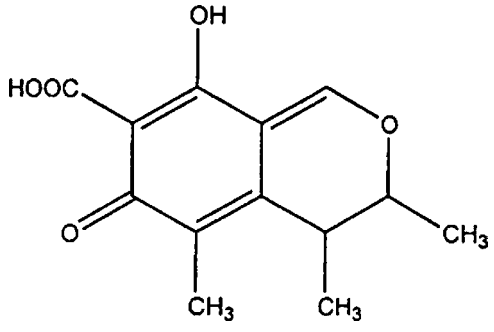


FIGURE 2 Citrinin. (Reprinted from reference 6 with permission.)

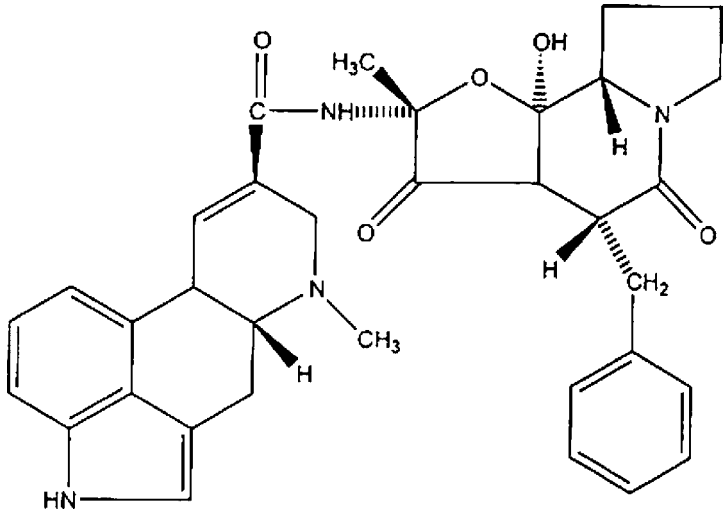


FIGURE 3 Ergotamine. (Reprinted from reference 6 with permission.)

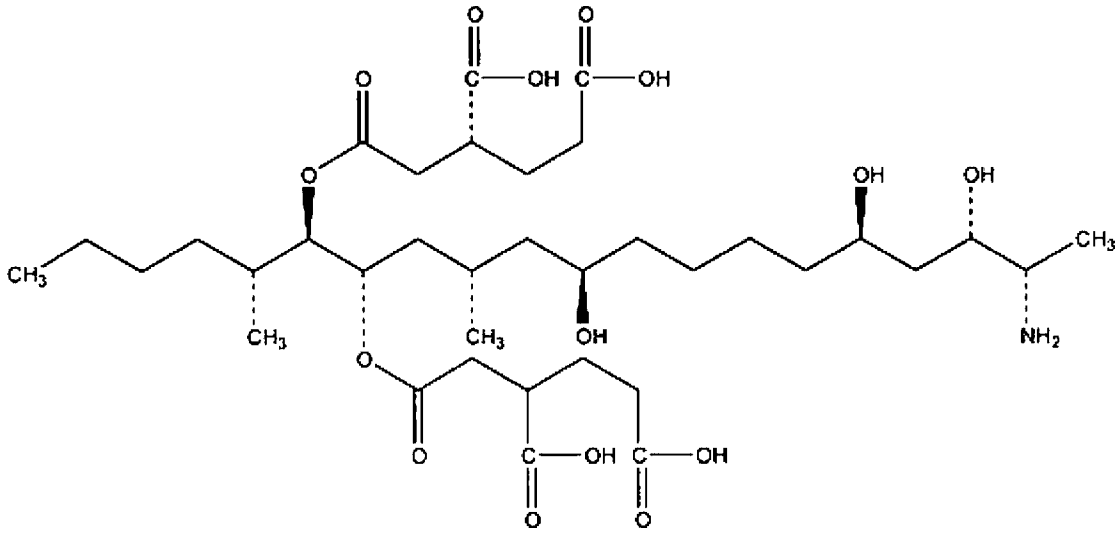


FIGURE 4 Fumonisin B_1. (Reprinted from reference 6 with permission.)

Semisynthetic ergot alkaloids have been developed through decades of research, which began with the series of lysergic acid derivatives that included the infamous hallucinogen LSD (21). Most recently, ergot alkaloids have been investigated for their anticancer potential (22), as well as their effect on serotonin and serotonin receptors (63, 85). However, definition of the mechanisms of action for the myriad of ergot alkaloids is beyond the scope of this chapter.

Aspergillus, Balansia, Claviceps, Epichloë, and *Neotyphodium* spp. have been found to produce ergot alkaloids. *Aspergillus fumigatus* has been shown to produce fumigaclavines A, B, and C and festuclavine (first described for *C. purpurea* and later for *Neotyphodium* spp.) (13, 60).

Modern methods of cleaning grains have all but eliminated the threat to the human food chain. However, ergotism remains an important veterinary concern, as symptoms of gangrene, convulsions, and abortion in cattle, sheep, pigs, and chickens mimic those in humans (47).

Fumonisin

Fumonisins (Fig. 4) (6) are among the most recently discovered mycotoxins. In 1988, Gelderblom et al. (25) determined that fumonisins produced by *Fusarium verticillioides* (then called *F. moniliforme*) were the causative agent of leukoencephalomalacia in horses (50) and hepatocarcinoma in rats (35, 51). As of 2002, there were 28 known fumonisin analogs, separated into four main groups: A, B, C, and P (64).

Fusarium proliferatum, Fusarium nygamai, Alternaria alternata, and *Gibberella fujikuroi*, the teleomorph (sexual form) of *F. verticillioides,* also produce these mycotoxins (16). The reader is referred to Rheeder et al. (64) for a review of the taxonomy of fumonisin-producing fungi.

Besides the syndromes mentioned above, fumonisins have also been shown to cause pulmonary edema and hydrothorax in swine (27). *F. verticillioides,* the major producer of fumonisins, can have an important economic effect on the supply of corn, as it can cause a variety of blights and rots depending on environmental conditions (57).

Ochratoxin

Ochratoxins A and B (OTA and OTB) (Fig. 5) (6) are produced by *Penicillium verrucosum* and many species of *Aspergillus,* especially *Aspergillus ochraceus*. These toxins can be carcinogenic, immunosuppressive, and nephrotoxic (39, 73).

OTA, first discovered to be toxic to animals in 1965 (78), is more common than OTB and can be metabolized after ingestion by cytochrome P450 liver enzymes (89).

Ochratoxins are found most often in contaminated barley but may also be present in oats, rye, wheat, and coffee beans (6, 80). They are of particular concern because of their ability to be carried through the food chain, especially in milk and in the tissues of pork (20, 52). Scandinavian countries (Denmark in particular) have had a high incidence of porcine nephropathy and high levels of OTA contamination (37).

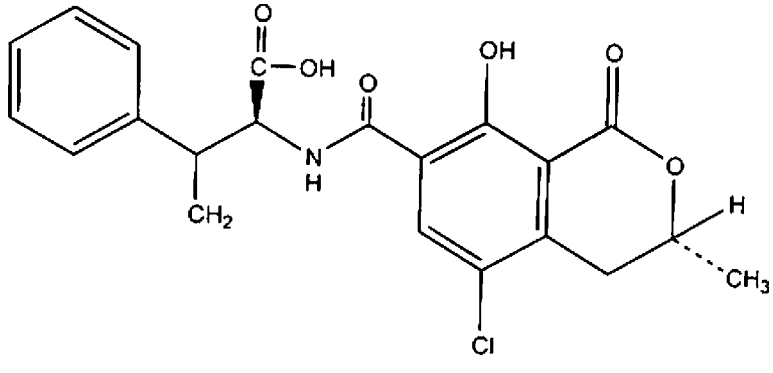


FIGURE 5 OTA. (Reprinted from reference 6 with permission.)

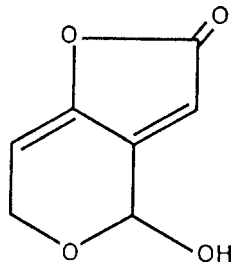


FIGURE 6 Patulin. (Reprinted from reference 6 with permission.)

Patulin

Patulin (Fig. 6) (6) was first isolated in the 1940s from *Penicillium griseofulvum,* and efforts were made to mass-produce this mycotoxin for its antibiotic effects (58). However, patulin was reclassified as a mycotoxin in the 1960s, following the discovery of its toxicity to plants and animals (6).

One of the most common occurrences of patulin is in unfermented juices made from fruit contaminated with *P. expansum* (77). Patulin is toxic at high concentrations in vitro, but natural poisoning in humans has yet to be proven (6).

Trichothecenes

Trichothecene mycotoxins are secondary metabolites of a variety of *Fusarium* spp., but also of *Myrothecium, Phomopsis, Stachybotrys, Trichoderma,* and *Trichothecium,* among others. Some of the most common and well-studied *Fusarium* trichothecenes (Fig. 7) (6) are T-2 toxin, HT-2 toxin, diacetoxyscirpenol, nivalenol, and deoxynivalenol (also called DON or "vomitoxin"). Deoxynivalenol is one of the most common mycotoxins found in grain, including barley, oats, rye, and wheat. When ingested in large quantities by agricultural animals, it can cause nausea, vomiting, and diarrhea; ingestion of smaller quantities results in weight loss and feed refusal (67). DON can also suppress bovine and porcine neutrophil function in vitro (76).

S. chartarum produces several macrocyclic trichothecenes, including verrucarins B and J; roridin E; satratoxins F, G, and H; and isosatratoxins F, G, and H (32, 34). *Stachybotrys*-contaminated straw was first described as causing a highly fatal equine disease (24) and has since become better known as a factor in SBS, discussed below.

Zearalenone

Zearalenone (Fig. 8) (6) is a nonsteroidal estrogen or phytoestrogen produced by various species of *Fusarium*, including *Fusarium roseum* and *Fusarium tricinctum* (19). Zearalenones are common contaminants of cereal crops worldwide and have been implicated in the hyperestrogenism of farm animals as a result of digestion of moldy corn and grains (29, 54, 55). Some of the symptoms of hyperestrogenism are enlargement of the uterus and nipples, vaginal prolapse, and infertility (56).

MAJOR HEALTH RISKS ASSOCIATED WITH MYCOTOXINS

Food Safety

Consumption of moldy food products is the leading cause of mycotoxicoses in agricultural animals and humans alike.

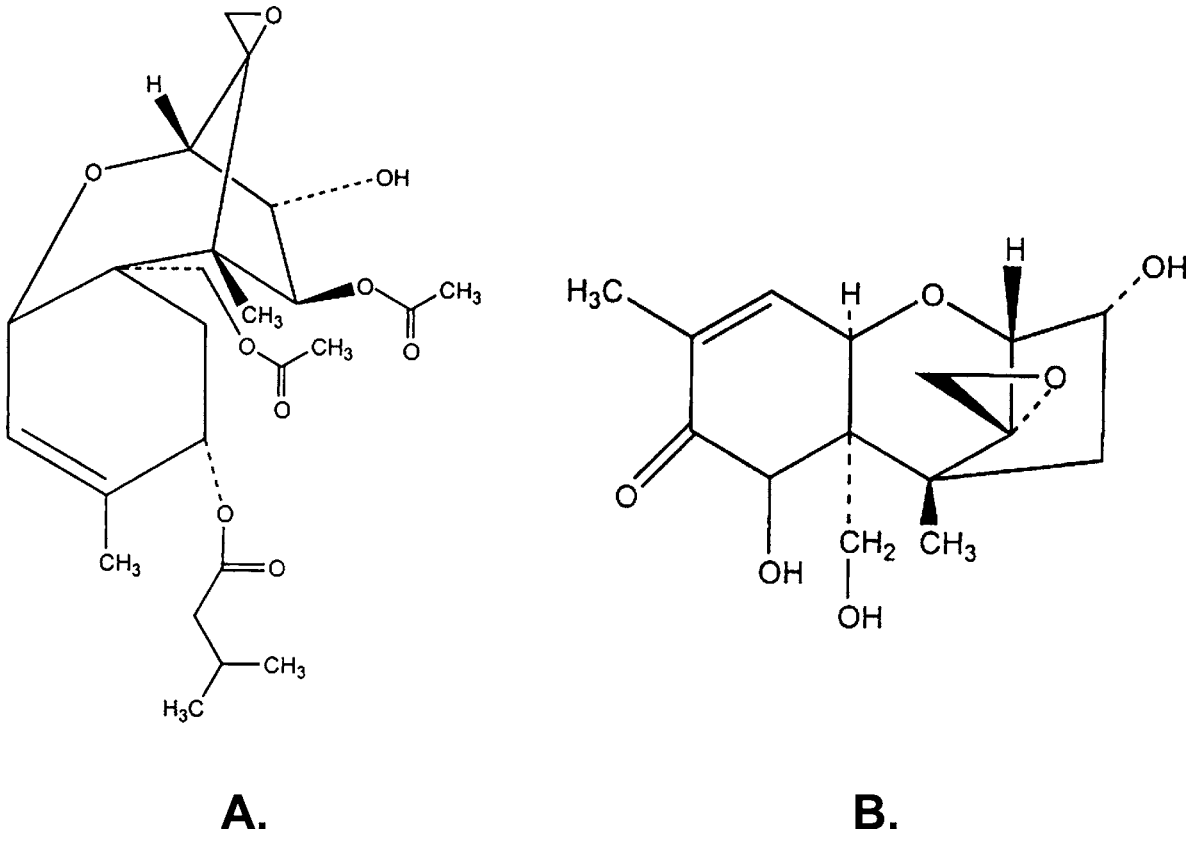


FIGURE 7 (A) T-2 toxin; (B) deoxynivalenol. (Modified and reprinted from reference 6 with permission.)

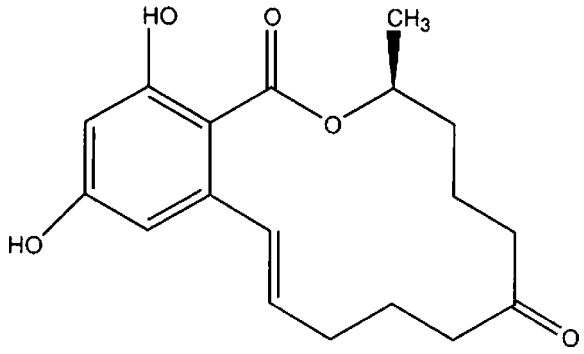


FIGURE 8 Zearalenone. (Reprinted from reference 6 with permission.)

Tables 2 and 3 list the mycotoxins discussed within this chapter, their common food substrates, and the agricultural animals most commonly affected.

It is estimated that one-quarter of the world's crops are contaminated to some extent with mycotoxins (49), which will vary from year to year based on environmental factors. For example, aflatoxin contamination is more abundant when crops are weakened by drought conditions. Contamination can occur at any point, ranging from the crop in the field, through storage and shipping. The economic consequences of mycotoxin contamination are immense, and mycotoxins pose a higher chronic dietary risk than synthetic contaminants, plant toxins, food additives, or pesticide residues (40).

Since it would be impossible to prevent all mycotoxin contamination, methods of monitoring food intended for human and animal consumption must be established. Aflatoxins strongly fluoresce under UV light and can be easily identified in food products, and immunoassays can detect the presence of citrinin. However, the chemical diversity of the mycotoxin group requires that each be separated from its substrate and studied by a unique assay. The reader is referred to the relevant chapters in *Mycotoxins in Agriculture and Food Safety* (83) for a comprehensive review of analytical protocols.

The regulatory guidelines for the limits of mycotoxins in food and feed differ from country to country but have been summarized in a series of compendia published by the Food and Agriculture Organization of the United Nations (23).

Bioterrorism

With the alarming health hazards posed by mycotoxins and the rising level of fear worldwide, concern about the possible use of mycotoxins in bioterrorism has emerged. In 1986, Ciegler proposed the use of mycotoxins as a new class of chemical weapons (12). However, the choice of aflatoxin as a weapon of mass destruction is odd at best, since the effects (liver cancer) of aflatoxicosis are too long-term to be effective during war.

Trichothecenes are much more suited for warfare than aflatoxins because they act immediately upon contact, and several milligrams can be lethal. Of historical note is the "Yellow Rain" incident of 1981 (65). The United States had accused the Soviet Union of using nivalenol, DON, and T-2 as biological weapons against Hmong tribesmen in Kampuchea and Laos. However, it was later concluded that this "yellow rain" was not a weapon but simply the excreta of swarms of wild Asian honeybees (59).

The use of mycotoxins in bioterrorism has been assessed by several investigators in recent years (6, 7, 31, 48, 66, 69, 75, 81, 88). Aflatoxins and trichothecenes have been recently tested for combined toxicity and were found to have additive effects in most cases. The combination of these two mycotoxins resulted in a synergistic effect in a human bronchial epithelial cell line (53). Interestingly, it has been shown that chlorine dioxide may be effective in the detoxification of trichothecenes roridin A and verrucarin A (84) in the case of widespread exposure. In general, it is accepted that while the presence of mycotoxin weapons may cause terror, the actual use of such weapons would not be effective or reliable in a time of war.

SBS

SBS is a loosely defined condition that applies to indoor environments suspected of causing a variety of health complaints for occupants. The buildings usually have many problems, including water damage; improper heating, ventilation, and air conditioning systems; poor construction; and bacterial, fungal, and/or insect infestations. Symptoms may include eye, nose, and throat irritation, fatigue, headache, lack of concentration, frequent respiratory tract infections, shortness of breath, dizziness, and nausea (8).

Mycotoxins are just one of the many factors considered as possible contributors to SBS. Several mycotoxin-producing

TABLE 3 Mycotoxins and animal outbreaks

Toxin	Cattle	Poultry	Swine	Horses	Sheep
Aflatoxin	×	×	×		
Citrinin					
Ergot alkaloid					
Fumonisin		×			
Ochratoxin		×	×		
Patulin					
Trichothecene	×		×		
Zearalenone	×			×	×

TABLE 2 Mycotoxins and their common food substrates

Toxin	Barley	Coffee beans	Corn	Fruits	Oats	Peanuts	Rice	Rye	Wheat
Aflatoxin			×			×	×		×
Citrinin	×		×		×		×	×	×
Ergot alkaloid	×				×			×	×
Fumonisin			×						
Ochratoxin	×	×			×			×	×
Patulin				×					
Trichothecene			×						
Zearalenone			×						

fungi have been implicated in SBS, including *Alternaria, Aspergillus, Cladosporium,* and *Penicillium* spp. One of the most widely investigated species is *S. chartarum* (for a comprehensive review, see Kuhn and Ghannoum [38]).

However, it has not been definitively proven that occupants develop illnesses either from these "sick" buildings or from the molds present inside them. Several recent reviews of SBS conclude that (i) there is no evidence that mold presents an imminent threat to life for healthy members of the general population in typical exposures, (ii) indoor airborne microorganisms are only weakly correlated with human disease and a definite causal relationship has not been established, and (iii) the concentration of mold contamination required to create infective doses is improbable and inconsistent with the reported spore concentrations in buildings (2, 14, 38).

A further argument against mycotoxins as the cause of SBS symptoms is the fact that mycotoxins are not volatile, and therefore, widespread exposure is unlikely. Moreover, the conditions conducive for mold growth are not necessarily the same as for mycotoxin production, and fungi differ in the ability to produce mycotoxins. To conclude that the presence of mold found within a "sick building" is indeed associated with symptoms suffered by occupants, the following must be established: (i) existence of the mold presenting a potential health risk in the indoor environment, (ii) mode of transmission—the source colony must be in direct contact or close proximity for contact with spores and related by-products—and (iii) portal of entry—the mold must be absorbed into the body through inhalation, ingestion, or skin absorption. The mere presence of a fungus in an indoor environment is not sufficient evidence to establish a cause-effect relationship and does not necessarily prove that mycotoxin is also present.

CONCLUSION

There are several classes of mycotoxins, produced by a wide range of fungal species, which pose both human and agricultural economic and health problems. There exists a continuing need for objective studies using relevant animal models and proper epidemiological techniques to describe the causative effects of mycotoxins and to develop antidotes, as well as remediation steps for "sick buildings," as more mycotoxins are identified.

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Lacazia, Pythium, and *Rhinosporidium*

LEONEL MENDOZA

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In the past 100 years, the microbial pathogens described in this section have been classified as fungal and/or parafungal protistan pathogens (1, 2, 8, 17). Based on their apparent epidemiological connection with water, they were at one point also placed in a new category of hydrophilic infectious agents (1). But based on taxonomic and other morphological characteristics, these three anomalous species were not well understood (1, 17). This frustrating fact fueled a strong controversy that has only recently been solved with the advent of molecular methodologies (19, 20). Despite the recent finding that both *Pythium insidiosum* and *Rhinosporidium seeberi* are protistan pathogens, they are still studied by medical mycologists continuing a historical tradition. Table 1 summarizes the most prominent features of the pathogens covered in this section.

LACAZIA LOBOI TABORDA, TABORDA, AND McGINNIS 1999

Taxonomy

Jorge de Oliveira Lobo first described lacaziosis in a Brazilian patient from the state of Pernambuco (18). He stated that the causative agent was very similar to the parasitic stages of organisms of the genera *Blastomyces* and *Paracoccidioides*. Earliest attempts to isolate this pathogen in culture met with failure, and soon it was clear that this pathogen resists culture (17). This fact started a great controversy on its epidemiological and taxonomic affinities, reflected in the numerous names by which this pathogen has been known. Taborda et al. in 1999 (35) suggested the binomial *Lacazia loboi* to end more than 70 years of taxonomic ambiguity. Recent phylogenetic studies placed *L. loboi* near *Paracoccidioides brasiliensis* (13, 19), a finding that validated Taborda et al.'s proposal (35). Thus, *L. loboi* is currently classified as an ascomycete fungus in the order Onygenales and the family Ajellomycetaceae.

Description of the Agent

Because *L. loboi* is intractable to culture, what we know about this pathogen is based on the morphological features observed in its parasitic stage. Its in vivo phenotype is characterized by the development of unicellular, thick-walled, yeast-like cells that can be found forming one, two, or more branches in chains of three or more cells characteristically connected by short tubules (Fig. 1). The yeast-like cells of *L. loboi* measure 5 to 12 μm in diameter and can be easily observed with most stains. Interestingly, *L. loboi*'s in vivo phenotype is very similar to that of the parasitic stage of *Paracoccidioides brasiliensis,* which led Lacaz et al. (17) to classify this pathogen in the genus *Paracoccidioides*. Electron microscopic analysis of this pathogen showed thick, chitinous, yeast-like cell walls and amorphous cytoplasmic contents. Approximately 60% of the yeast-like cells observed lacked a defined cytoplasmic region, a finding in agreement with results of viability studies.

Epidemiology and Transmission

Lacaziosis (41) or Jorge Lobo's (lobomycosis) disease is known to occur only in patients inhabiting the tropical areas of the Americas (17). In addition to the many human cases, cases of the disease in species of dolphins around the coasts of South America, Florida, and the Gulf of Mexico have been reported (24, 34). Although the majority of patients with lacaziosis have been from Brazil, cases from Mexico, Central America, Colombia, Suriname, Venezuela, and other nearby countries have also been recorded (17). Two European human patients apparently acquired the infection after contact with an infected bottle-nosed dolphin in an aquarium. In addition, American and Canadian patients were recently reported to have developed the infection after visiting or working in areas where the disease is endemic (6, 9).

It is believed that the infection is acquired through small, traumatic skin lesions after contact with the pathogen in aquatic ecological niches in nature. The report of dolphins with lacaziosis tends to support this tenet. However, the pathogen's real ecological niche is unknown. The disease seems to occur in apparently healthy hosts. It is quite possible that *L. loboi* cells possess hyphae with propagules (perhaps conidia) similar to those of *Paracoccidioides brasiliensis* that may make contact with the hosts through trauma, thus causing lacaziosis. The transmission of the disease from one patient to another is rare, but some cases of autoinoculation and accidental infection by physicians in contact with infected patients (17) or with infected dolphins (34) have been reported. In addition, the disease can be experimentally reproduced in mice (4).

Clinical Significance

The infections caused by *L. loboi* are observed rarely in the tropical areas of the Americas. Recent reports estimate that some 500 human cases, mostly in males with cutaneous

TABLE 1 Taxonomical, epidemiological, clinical, and mycological features of the hydrophilic microbes *L. loboi, P. insidiosum*, and *R. seeberi*

Pathogen	Taxonomy/phylogeny	Epidemiology	Clinical manifestations of infection/in vivo form	In vitro form
L. loboi	Ascomycete fungus located among the dimorphic Onygenales	Restricted to South America; cases of infection occur in dolphins; acquired by trauma; hydrophilic	Parakeloidal skin lesions/ yeast-like cells in chains connected by small tubules	Uncultivated
P. insidiosum	Kingdom Stramenopila (Protista), class Oomycetes (Peronosporomycetes); better known as the aquatic fungi	Found in tropical, subtropical, and temperate areas; acquired in aquatic or terrestrial environments by trauma; hydrophilic	Cutaneous, intestinal, and vascular infection disseminated by metastasis/ coenocytic hyphae exhibiting Splendore-Hoeppli phenomenon	Flat colonies without aerial mycelia; coenocytic hyphae without fruiting bodies; biflagellate zoospores in water cultures
R. seeberi	Kingdom Protista, class Mesomycetozoea; located at the animal-fungal divergence	Found on all continents except Australia; infection is endemic in India and Sri Lanka; acquired by trauma; hydrophilic	Polypoidal mucosal and skin lesions/spherical sporangia, with or without endospores at different stages of development	Uncultivated

lesions, have been diagnosed so far in the areas where the disease is endemic. The disease occurs in apparently normal patients inhabiting such areas. A genetic predisposition theory was abandoned after the finding that the number of cases in a Brazilian tribe with a high occurrence of the disease dramatically decreased when the tribe was moved to a new location. One common problem in the areas where the disease is endemic is that the patients with the disease do not seek medical attention until years later, when the lesions have increased in size and spread to other skin areas. One explanation may be that the affected population is usually poor, with no access to health care, and that the lesions are rarely painful.

Frequently affected areas are arms, ears, back, chest, face, and lower limbs. Some investigators believe that the distribution of the lesions on cooler areas of human skin and on dolphins may indicate that the pathogen does not tolerate a temperature of 37°C well. Initially, a single small (0.5 to 1.0 cm in diameter), smooth, parakeloidal skin lesion develops. Some patients complain of slight pruritus at this stage. The infection is not life threatening and usually evolves very slowly, sometimes over 20 or more years. Usually, when the patients seek medical attention they have already developed more than one lesion. In the chronic phase, lesions are polymorphic. At least five clinical manifestations are recognized, including the typical parakeloidal lesion and four other forms: infiltrative, gummatous, ulcerated, and verrucous. However, Lacaz et al. (17) pointed out that two or more forms, including a macular form, may be found in one patient. The differential diagnosis includes chromoblastomycosis, leprosy, neoplasias, paraccocidioidomycosis, and similar skin conditions.

Collection, Transport, and Storage of Specimens

The guidelines for the collection, processing, storage, and examination of specimens are provided in chapter 116.

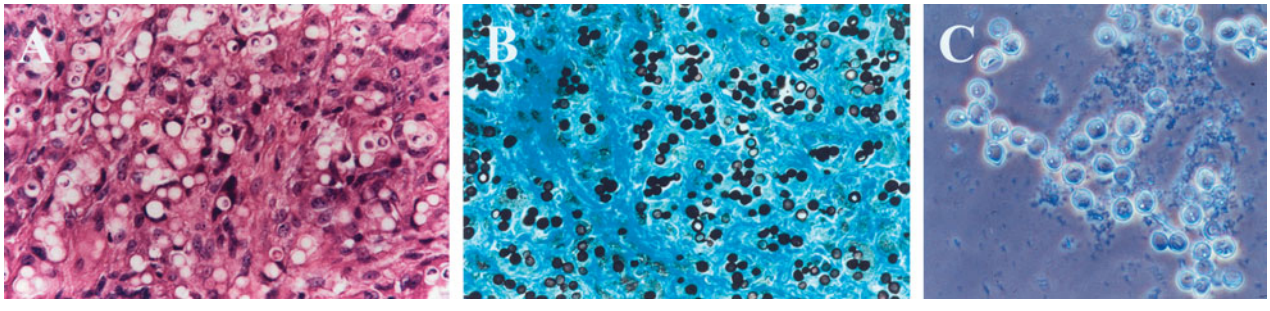


FIGURE 1 *L. loboi*. Histological sections stained with H&E (A) and silver stain (B) show the typical morphological features found in patients with cutaneous lacaziosis. The yeast-like cells of *L. loboi* are poorly stained by H&E and are observed as empty, round structures surrounded by an area of granulomatous reaction. The presence of abundant yeast-like cells in chains is the main feature in silver-stained sections (B). (C) Numerous yeast-like cells of *L. loboi* in chains connected with slender tubules and containing small dancing bodies in their cytoplasm. Magnification for all panels, ×40. Panels A and B are courtesy of P. S. Rosa.

The clinical specimens collected in cases of lacaziosis or rhinosporidiosis are mostly biopsy tissues from the infected sites. Since *L. loboi* resists culture, the clinical specimens from the infected areas are usually fixed upon collection in formaldehyde to be histopathologically evaluated later.

Direct Examination

Microscopy

In contrast to the systemic dissemination of *Paracoccidioides brasiliensis* infections, *L. loboi* infections are typically confined to the cutaneous and subcutaneous tissues. The lesions are characterized by fibrosis, granulomatous reactions with numerous histiocytes, and giant cells containing several yeast-like cells of *L. loboi*. Areas of necrosis and the presence of other inflammatory cells have been reported. When hematoxylin and eosin (H&E) stain is applied, the yeast-like cells arranged in linear chains are not well stained and appear as empty spaces with thick cell walls (Fig. 1A). Special fungal stains such as Gomori methenamine silver (GMS) and periodic acid-Schiff (PAS) are used for the histopathological diagnosis of lacaziosis. Under GMS staining, *L. loboi* yeast-like cells are dark or have the appearance of "empty" cells (Fig. 1B). The yeast-like cells are in chains of two or more cells and form branches, similar to those yeast cells observed in the infected tissue of patients with paracoccidioidomycosis. The cells are typically connected with small tubules, a feature also observed using other fungal stains, including PAS.

For patients with lacaziosis, the biopsy specimens should be cut into pieces of 2 to 5 mm in diameter. One or more pieces are then placed with 1 or 2 drops of 10% KOH on a glass slide with a coverslip. The samples should be heated without boiling and kept for about 15 min at room temperature before microscopic evaluation. In 10% KOH, *L. loboi* yeast-like cells appear in great quantities (Fig. 1C). Cells 2.0 to 12.0 μm in diameter can be found as single yeast-like cells or as chains connected by short tubules of three or more cells. Several moving protoplasmic granules can also be detected within some of the yeast-like cells (Fig. 1C).

Nucleic Acid Detection Techniques

So far, there are no commercially available nucleic acid probes for the detection of this pathogen in clinical samples.

Isolation Procedures, Identification, Typing, and Serologic Tests

Claims that *L. loboi* has been isolated in pure culture have not yet been validated. Most of the recovered organisms have proven to be fungal contaminants or *Paracoccidioides brasiliensis* (17). One major obstacle to developing serological assays for lacaziosis has been the lack of cultures. Thus, most of the serological approaches have been carried out by using the parasitic forms in infected tissues as antigens or by using cultures of closely related pathogens (41). The identification of the etiologic agent is based on the clinical features and the phenotypic characteristics on wet-mount preparations and/or histopathological analyses.

Antimicrobial Susceptibility

Although antifungal therapy has cured some cases of lacaziosis, *L. loboi* is well known for its resistance to antimicrobial drugs, including most antifungals (17). This fact has left clinicians with only one choice: surgery. Due to the pathogen's intractability to culture, susceptibility testing is not possible.

PYTHIUM INSIDIOSUM DECOCK, MENDOZA, PADHYE, AJELLO, AND KAUFMAN 1987

Taxonomy

Although infections caused by *P. insidiosum* were first reported more than 150 years ago in Indonesian equines (8), successful isolation of the pathogen in pure culture was possible only at the beginning of the 20th century. Sixty years later, a similar organism was isolated and the name *Hyphomyces destruens* was introduced. The finding that a strain from New Guinea developed zoospores suggested that this pathogen was an organism in the genus *Pythium*. More recently, deCock et al. (8) introduced the epithet *Pythium insidiosum*. Some dogs were believed to be infected with a new species of *Lagenidium* (10). However, this report is still awaiting verification. Recent molecular studies (28, 31) showed that the strains of *P. insidiosum* cluster phylogenetically according to their geographic distributions. Thus, it is likely that *P. insidiosum* may embrace a phylogenetic complex of closely related species or perhaps a complex of different strains belonging to a single species, *P. insidiosum*, including the *Lagenidium*-like strains described by Grooters (10).

Description of the Agent

Unlike the other microbes described in this section, *P. insidiosum* can be readily isolated on most mycological media (8). This pathogen develops white and submerged colonies with a characteristic radiation pattern and few to no aerial hyphae (Fig. 2A). The hyphae, with perpendicular lateral branches measuring 4 to 10 μm in diameter, possess few cross septa. Zoosporogenesis is possible only in water cultures containing various ions, including Ca^{2+} (8, 22). The structures in which the zoospores develop are termed sporangia.

The zoospores form by progressive cleavage and mature inside 20- to 60-μm-diameter sporangia. Once fully formed, the biflagellate zoospores mechanically break the sporangial wall, swim, and then encyst. Due to their motility, it has been proposed that the zoospores may be the infectious units. The zoospores are reniform, and two unequal flagella arise from inside a lateral groove. Upon encystment, the zoospores lose their flagella and become spherical. Under the right conditions, the encysted zoospores develop a germ tube and form long filaments (7, 22). *P. insidiosum* oogonia (teleomorphic stage) have been rarely observed and are believed to represent the resistant stage.

Epidemiology and Transmission

Infections caused by this hydrophilic pathogen have been recorded in tropical, subtropical, and some temperate areas of the world. In the Americas, pythiosis is common in tropical Central, North, and South America, with most cases reported in Brazil, Colombia, Costa Rica, the United States, and Venezuela (8, 22). In the United States, the infections are more prevalent in animals and humans inhabiting southern states such as Alabama, Georgia, Florida, Louisiana, Mississippi, North Carolina, South Carolina, and Texas. However, cases of the disease have also been reported in northern states, including Illinois, Indiana, Kansas, New Jersey, Missouri, Tennessee, and Virginia, and even in those as far north as Wisconsin and New York. Unusual cases of the disease in California dogs have also been reported. Pythiosis has also been reported in Asia (Japan, India, Indonesia, Pacific islands, South Korea, and Thailand), Australia, New Guinea, and New Zealand. The tropical areas of Africa are

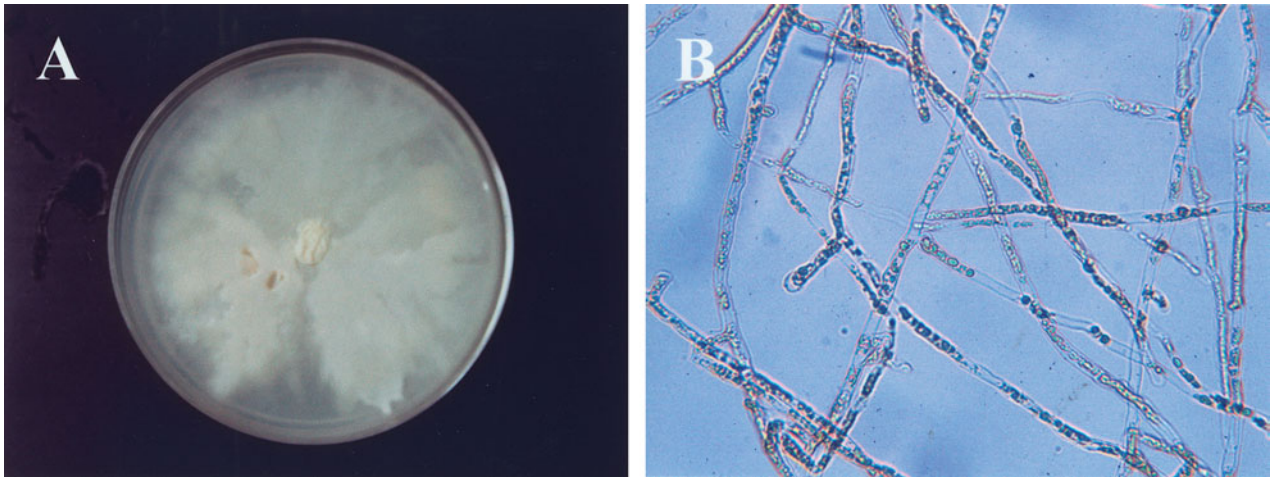


FIGURE 2 *P. insidiosum*. (A) Colony of *P. insidiosum* on Sabouraud dextrose agar. *P. insidiosum* produces cream to white submerged colonies at room temperature and 37°C. (B) Sparsely septate hyphae found on agar plates. No fruiting bodies can be found on dry cultures. Panel B magnification, ×40.

ideal for pythiosis, however; only recently, a case in a dog with a cutaneous form was reported in the northwest African country of Mali, suggesting that the disease is more prevalent on that continent (28).

Until recently, the infections caused by *P. insidiosum* were considered exotic. The disease was believed to be restricted to animals in the tropical regions, with rare to no occurrence in other geographical areas. However, in the last 10 years the number of pythiosis cases has increased on all continents (8, 36). The finding that humans (29, 36, 40, 43), dogs (12, 38), and other animals can be infected indicates that previously pythiosis was erroneously diagnosed as other fungal infections. In general, it is well known that most cases of pythiosis occur in apparently healthy humans and animals. In Thailand, however, the disease in humans is associated with thalassemia or occurs in patients with similar blood disorders (29). Interestingly, no such association has so far been reported in other geographical areas.

The infection is acquired after *P. insidiosum* propagules enter the skin or the intestinal tract through traumatic lesions (22, 38). The infection is believed to be acquired from wet environments; however, cases of pythiosis in the absence of water suggest that *P. insidiosum* can cause infection through contact with resting spores in terrestrial environments. The transmission of *P. insidiosum* from one patient to another (human or animal) has not yet been recorded. Only rabbits seem to be susceptible to experimental inoculation (25).

Clinical Significance

The clinical manifestations of pythiosis vary according to the infection site. Most patients with the disease state that they have had a skin injury prior to the infection. The lesions caused by *P. insidiosum* in humans can be classified as superficial infections (keratitis); a cutaneous and subcutaneous form, including orbital pythiosis; and a vascular form that usually leads to systemic infection and death. Keratitis caused by *P. insidiosum* is similar to that caused by fungi and other etiologic agents (3, 26, 42). It begins with a trauma on the superficial layers of the eye, followed by the development of conjunctivitis, photophobia, corneal ulcers, and hypopyon. The cutaneous and subcutaneous infection is characterized by the formation of granulomatous plaques and/or ulcerated swellings that remain localized. Once the pathogen has reached the subcutaneous tissues, itchy papules may develop. The orbital form of subcutaneous pythiosis is rare and has been observed mainly in children in Australia and the United States (39). Vascular pythiosis starts with a traumatic lesion, usually on the lower extremities, followed by dissemination of the pathogen to the nearby arteries (29, 36). Initially, the infected skin shows signs of dry gangrene, and in some patients, the formation of painful necrotic ulcers has also been reported (36).

The clinical symptoms are claudication of the affected limb, local ischemia, swelling, pain, and the absence of the dorsalis pedis pulse. As the infection progresses, ascending arteritis, with the formation of thrombi and aneurisms of the large arteries, is the main feature. If not treated, *P. insidiosum* infection may spread through the arteries and reach the iliac and renal arteries and abdominal aorta, causing disseminated pythiosis. This form of pythiosis is more common in Thailand among patients with thalassemia, and it is usually fatal (36, 40, 43). Human pythiosis should be differentiated from subcutaneous tuberculosis, cutaneous leishmaniasis, arteriosclerosis, diabetes mellitus, mycotic keratitis, subcutaneous zygomycosis, and other mycoses associated with filamentous fungi.

Collection, Transport, and Storage of Specimens

The clinical specimens required for proper diagnosis of pythiosis vary according to the infected hosts and the clinical form (22, 36). For instance, for patients with keratitis, swabs or scrapings of the affected eyes should be collected. The swabs have to be immediately transported at room temperature in tubes with high humidity (or tubes containing small quantities of sterile distilled water) to the laboratory and should be processed upon arrival. Dryness of clinical specimens from keratitis patients may prevent the visualization and culture of the hyphal elements of *P. insidiosum*. The collected eye scrapings should be placed in small aseptic petri dishes and immediately transported at room temperature to the laboratory for processing. Although some studies indicate that transportation or storage at 4°C of specimens

from patients suspected of having pythiosis does not interfere with the isolation of the pathogen in the laboratory, others report a considerable reduction in the number of positive cultures from samples stored at 4°C (22). Biopsy specimens or samples from necropsies are usually collected in sterile distilled water and transported at room temperature to the laboratory. Specimens collected from patients with cutaneous, subcutaneous, vascular, and disseminated pythiosis can also be placed in tubes containing sterile distilled water plus 100 U of penicillin/ml and 0.25 mg of streptomycin/ml or 0.4 mg of chloramphenicol/ml. If these antibiotics are not available, the use of sterile distilled water is encouraged.

Direct Examination

Microscopy

Clinical specimens of *P. insidiosum* in 10% KOH characteristically show the presence of long, 4.0- to 9.0-μm-diameter, hyaline, sparsely septated hyphal structures (Fig. 3D). Some hyphal elements may have 90°-angle lateral branches, a typical feature of this oomycete. In histopathological preparations stained with H&E, *P. insidiosum* appears as short or long hyaline coenocytic hyphae 6.0 to 10 μm in diameter. The fact that in vivo structures of *P. insidiosum* are detectable under H&E staining has been used to differentiate this oomycete from the hyphal elements developed by members of the order Entomophthorales. In infected tissues, *P. insidiosum* triggers an eosinophilic granuloma with giant cells, mast cells, and other inflammatory cells (Fig. 3A and B). The hyphal elements of *P. insidiosum* are found in the centers of microabscesses with numerous eosinophils that usually degranulate over the organism's hyphae in the Splendore-Hoeppli phenomenon (Fig. 3B). The activation of an eosinophilic inflammatory response with the Splendore-Hoeppli phenomenon is a feature that *P. insidiosum* has in common with the zygomycetes in the order Entomophthorales, from which it has to be differentiated. Although most experts in the diagnosis of pythiosis agree that it is difficult to distinguish between the hyphal structures of *P. insidiosum* and those of the entomophthorales, features such as the poor staining capabilities with H&E, the ribbon type morphology, and the bigger size of the zygomycetes' hyphal filaments sometimes help to distinguish these fungi from *P. insidiosum*. *P. insidiosum*'s hyphal elements are well stained by GMS and appear as short or long, sparsely septate, tubular dark structures (Fig. 3C). Transversely, poorly stained (with GMS or PAS) sectioned hyphae can also be found as ring-shaped bodies.

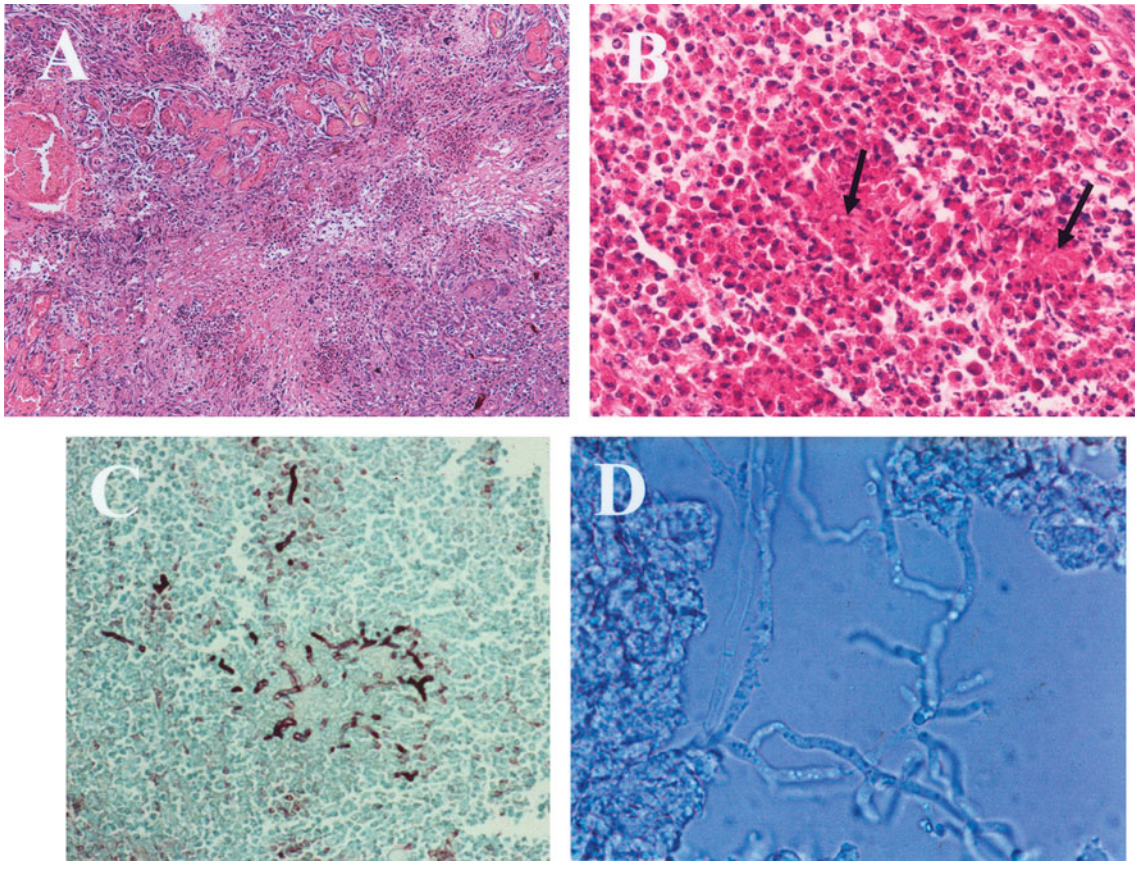


FIGURE 3 *P. insidiosum*. (A) Low-magnification (×10) tissue section showing several eosinophilic microabscesses. (B) Numerous eosinophils around the unstained hyphal elements of *P. insidiosum* (arrows; Splendore-Hoeppli phenomenon), a typical feature in pythiosis. Magnification, ×40. (C) Silver stain showing the typical hypha features of *P. insidiosum*. Magnification, ×40. Panel courtesy of M. G. Rinaldi. (D) 10% KOH wet-mount preparation. Note sparsely septate slender hyaline hyphae with branches at 90° angles. Magnification, ×40.

Nucleic Acid Detection Techniques

The first molecular approach for the diagnosis of *P. insidiosum* infection from clinical specimens was carried out with a patient with keratitis (3). The hyphal elements present in the specimen were identified by sequencing part of the 18S small-unit rRNA gene by using the NS1 and NS2 and the internal transcribed spacer universal primers. This approach has been successful with clinical specimens (3, 40). Groosters and Gee (11) introduced a PCR technique for the identification of *P. insidiosum* from cultures and from clinical specimens. A set of primers (PI-1 and PI-2) that amplified 105 bp of the internal transcribed spacer 1 of *P. insidiosum* were tested. These primers have been used by several laboratories (40) and were entirely specific when tested against several filamentous fungi. Schurko et al. (30) recently introduced a dot blot hybridization technique by constructing a 530-bp species-specific DNA probe for the detection of *P. insidiosum*. This DNA probe specifically binds to the intergenetic spacer 1 of this pathogen, and it did not hybridize with the genomic DNA of 23 other *Pythium* species, that of *Lagenidium gigantum,* and the genomic DNA of several pathogenic fungi, including the zygomycetes *Conidiobolus coronatus* and *Basidiobolus ranarum*. This probe may be ideal for the detection of *P. insidiosum* from environmental samples and for the specific diagnosis of pythiosis from clinical specimens containing susceptible species.

Isolation Procedures, Identification, Typing, and Serologic Tests

In contrast with the other hydrophilic pathogens covered in this chapter, *P. insidiosum* can be cultured on various media. The most common media used for the isolation of *P. insidiosum* are 2% Sabouraud dextrose agar or broth with or without antibiotics (see "Collection, Transport, and Storage of Specimens"), blood agar, corn meal agar (Difco), potato dextrose agar, and nutritive agar (Difco). Biopsied or necropsied tissues and kunkers from patients suspected of having pythiosis are usually cut into small fragments of 2.0 to 5.0 mm in diameter, placed into tubes with sterile distilled water, and vigorously washed two to three times before plating. The small fragments are then physically pushed into the agar, and the plates are incubated at 25 and 37°C for two or more days. The relative humidity of the incubator should be enhanced by placing a beaker of water inside the chamber. Specimens that have been transported for more than 24 h can also be inoculated into tubes containing broth and then incubated at 37°C. Usually, cottony colonies surrounding the clinical specimens are detectable after 24 to 48 h of incubation. In solid media, *P. insidiosum* develops only sparsely septate hyphae (Fig. 2B). The appearance of *P. insidiosum* on solid media is described above.

The accurate identification of *P. insidiosum* in the laboratory can be established only if the isolate develops the characteristic oogonia (teleomorphic stage) on culture plates. The formation of oogonia, however, is extremely rare, which further complicates the final identification of this pathogen in the clinical setting. Based on the fact that *P. insidiosum* is the only pathogen that develops zoospores in water, some laboratories have been using this characteristic for identification (7, 40). This approach, however, does not guarantee that the species involved in that particular case is indeed *P. insidiosum*. The identification of this pathogen by using molecular methods with culture and clinical specimens is recommended. A drawback to this approach is that only a few laboratories possess the necessary capabilities. Currently, there are no typing systems available for the identification of *P. insidiosum* in the clinical setting.

Early serological tests showed the presence of anti-*P. insidiosum* immunoglobulins in the sera of infected hosts and, thus, the importance of these immunoglobulins in the diagnosis of pythiosis. The most common assays for pythiosis are agglutination, enzyme-linked immunosorbent assays, immunodiffusion (ID), and Western blotting. In addition, a histochemical assay and an immunofluoresence assay have been used for the specific detection of *P. insidiosum* in the infected tissues. ID proved to be a very specific test, with several precipitin bands, but it was too insensitive and yielded many false negatives, especially for humans and dogs with pythiosis (16). To overcome this drawback, an enzyme-linked immunosorbent assay and a Western blotting method were later introduced (12, 16, 21, 23, 40). These assays are extremely sensitive and specific in detecting anti-*P. insidiosum* immunoglobulin G. Recently, an agglutination test was used for the rapid diagnosis of pythiosis. Although it proved to be a good screening test, it had a high rate of false positives and false negatives. The most important immunohistological tests for the specific identification of the hyphal elements of *P. insidiosum* in infected tissues have been the peroxidase and the immunofluorescence tests (5). Because the hyphal structures of *P. insidiosum* are difficult to differentiate from those of fungi, especially those of the zygomycetes, these assays have been of paramount importance for the accurate identification of this oomycete in the absence of culture. Most of these assays are available through reference laboratories for pythiosis in the United States and in other countries such as Brazil and Thailand.

Antimicrobial Susceptibility

P. insidiosum, like the other oomycetes, does not possess ergosterol in its cytoplasmic membrane. Despite this obvious counterindication, amphotericin B and other antifungal drugs that target ergosterol have been used with mixed results. For instance, two boys with orbital pythiosis were successfully treated with amphotericin B in Australia (39). However, the same antifungal did not have an effect in humans from Thailand with the disease (29, 43). Recently, susceptibility testing of a strain isolated from a boy in Tennessee with orbital pythiosis showed the strain to be sensitive to terbinafine and itraconazole (32). Although this combination was successfully used in this case, the same combination was less effective when tested in other humans and animals. The inconsistent results obtained with most antifungal drugs for the treatment of pythiosis in humans and animals have led to the use of unconventional treatments such as immunotherapy (43).

RHINOSPORIDIUM SEEBERI (WERNICKE 1903; MINCHIN AND FANTHAN 1903) SEEBER 1912, EMEND. ASHWORTH 1923

Taxonomy

The first two cases of rhinosporidiosis were reported in 1900 by Guillermo Rodolfo Seeber in his M.D. thesis in Argentina (1, 2). He stated that in 1896 he had found two patients with nasal polyps containing an organism similar to that reported by Posadas in 1892 (coccidioidomycosis). He also mentioned that an unpublished case of a nasal polyp, previously studied by Malbran in 1892, showed a spherical microbe with morphological features identical to those of the parasite reported by Seeber. A nasal case of rhinosporidiosis observed by O'Kinealy in 1903 in India was later studied in detail by Minchin and Fanthan in 1905 (2). These investigators suggested the binomial *Rhinosporidium kinealyi* to identify this strain. Seeber in 1912 introduced the name *Rhinosporidium seeberi* and called

attention to its priority over *R. kinealyi*. Ashworth in 1923 stated that the genus *Rhinosporidium* proposed by Minchin and Fanthan should be adopted and that, based on the description of Seeber (2) and the name *Coccidioides seeberia* reintroduced by Belou, the binomial *R. seeberi* had priority.

The frustrating fact that this pathogen has not been cultured has led some investigators to extreme hypotheses. The suggestion that *R. seeberi* is in the cyanobacterium genus *Microcystis* is the most recent in a long list of similar views. The placement of *R. seeberi* among the mesomycetozoeans came as a surprise. The class comprises orphan aquatic fish and amphibian parasites with spherical forms strikingly similar to that of *R. seeberi* (19, 20). By using internal transcribed spacers, it was recently found that *R. seeberi* may include several species-specific strains that may well represent new species (33).

Description of the Agent

This anomalous pathogen has resisted culture; thus, its morphological features are known only through in vivo microscopic and ultramicroscopic studies (1, 2). In infected tissues, *R. seeberi* has a complex parasitic cell cycle. It appears as multiple spherical structures known as sporangia (cysts in most mesomycetozoeans) in different stages of development (Fig. 4A and B). Its in vivo cell cycle starts with the release of hundreds of oval or spherical endospores, 7 to 15 μm in diameter, from a pore developed only in mature sporangia. The endospores increase in size and progressively develop from juvenile (10 to 100 μm) to intermediate (100 to 150 μm) and then to mature (150 to more than 450 μm) size. The endospores are released from the mature sporangia, and the in vivo cycle is somehow reinitiated (Fig. 4C). Although the diameter of the sporangium has been used to identify its in vivo stages, the mature sporangium stage differs from other stages by the presence of endospores.

Epidemiology and Transmission

Rhinosporidiosis usually occurs in most tropical and subtropical areas of the world except Australia (1). Although the infection was first recognized in Argentina, India and

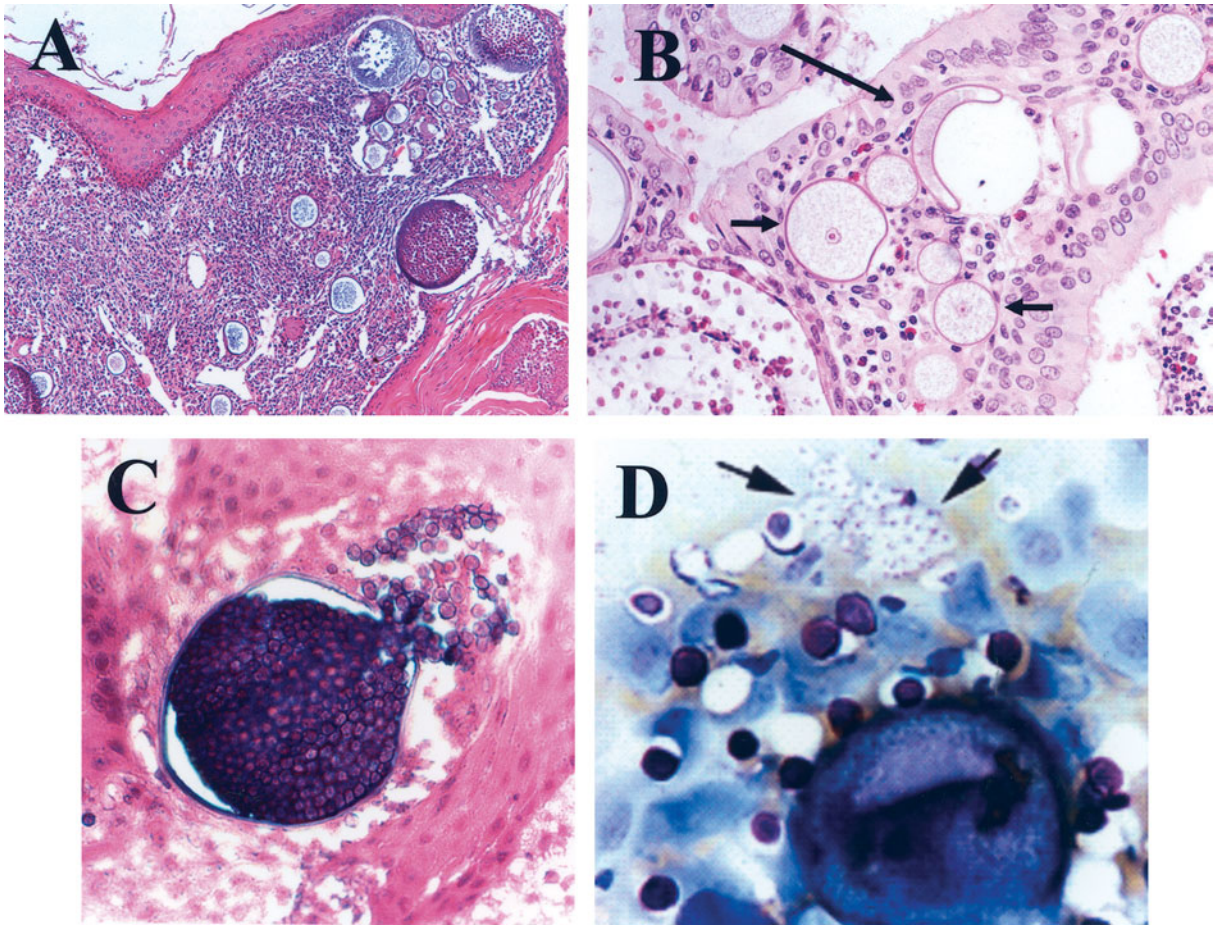


FIGURE 4 *R. seeberi*. H&E-stained mature sporangia with endospores and numerous juvenile sporangia of different sizes are shown in panels A (magnification, ×10) and B (magnification, ×30). A collapsed sporangium in U shape and juvenile sporangia with prominent nuclei and nucleoli are shown in panel B (long and short arrows, respectively). Panel C (magnification, ×30) shows mature sporangia releasing endospores through a cell wall pore. Panel D shows Wright-Giemsa impression smears from a dog with nasal rhinosporidiosis. An immature collapsed sporangium is shown in the lower section of the panel (magnification, ×70). Numerous endospores surrounded by a clear halo are shown in the upper section of panel D (arrows). Panel D is courtesy of William A. Meier.

Sri Lanka show the highest occurrences of the disease (37). Rhinosporidiosis occurs sporadically in other geographical areas such as the Americas, Africa, Europe, and Asian countries including the Middle East. Since some cases of rhinosporidiosis occur in dry areas, especially after sand storms, the hydrophilic nature of this pathogen has been long questioned (1, 2, 37). Based on accounts from patients with the disease, it is believed that rhinosporidiosis is acquired through contact with aquatic environments contaminated with *R. seeberi,* but the precise mechanism of infection from natural sources is unknown.

The finding linking *R. seeberi* to aquatic pathogens of fish and amphibians (14, 19, 20) tends to confirm its hydrophilic nature. Most probably, this pathogen evolved from an aquatic niche, in which it still can be found, and adapted to terrestrial environments by the development of resistant spores. This is a very likely scenario since the formation of zoospores, as in the other members of the dermocystida among which *R. seeberi* is phylogenetically located, has not been established (1, 2, 19, 37). The disease tends to occur as single cases, but two outbreaks of rhinosporidiosis in humans in Serbia (27) and in swans in Florida (15) have also been recorded. The resistant spores present in water and soil may gain entry through small cutaneous or mucocutaneous wounds and establish the infection. Although the disease occurs in apparently normal hosts, some investigators have suggested associations with special occupational and social conditions (1, 37). Little is known about the predisposing factors leading to the disease. The disease has not been induced in experimental animals, and transmission from one host to another has yet to be reported.

Clinical Significance

The most common clinical form is the formation of painless polyps usually located on mucosal areas of the nose, eyes, larynges, genitalia, and rectum. Multicentric skin lesions have also been recorded (37). The disease is not life threatening, but it can cause breathing difficulties when the polyps obstruct the nose or laryngeal passages. Rhinorrhea and bleeding are common on polyps located in the nose. The slow-growing polipoidal masses are usually found as single or multiple, pedunculate, sessile red lesions that bleed easily. Pruritus of the affected areas is also common. The differential diagnosis includes bacterial and fungal infections, neoplasias, and other similar mucosal and skin conditions.

Collection, Transport, and Storage of Specimens

The guidelines for the collection, processing, storage, and examination of specimens are provided in chapter 116. The clinical specimens collected in cases of rhinosporidiosis are usually biopsy tissues from the infected sites. Since *R. seeberi* resists culture, the clinical specimens from the infected areas are usually fixed upon collection in formaldehyde to be histopathologically evaluated later. Nonetheless, fresh samples should also be examined in the laboratory to rule out other etiologic agents and to confirm the histopathological findings. In these cases, the biopsy specimens have to be aseptically collected and transported immediately to the laboratory. For samples collected far from the laboratory, cooling (−80 to 4°C) of collected specimens for shipping or storage purposes may be necessary.

Direct Examination

Microscopy

Wet-mount preparations of clinical specimens from cases of rhinosporidiosis usually show the presence of mature and immature spherical sporangia and numerous endospores. Mature sporangia with endospores have thin cell walls and may measure more than 400 μm in diameter, whereas juvenile and intermediate sporangia are smaller and may have thicker cell walls. In fresh specimens treated only with water, the release of the endospores from mature sporangia has been reported. A purification system to study *R. seeberi* phenotypes was recently proposed (1). The presence of *R. seeberi* sporangia and endospores can also be found on smears stained with Giemsa or Gram stains (Fig. 4D). The spherical unstained elements of *R. seeberi* develop autofluorescence when viewed with a fluorescence microscope.

The parasitic spherical structures of this mesomycetozoean pathogen stain very well with H&E, but they also stain with GMS and PAS, a feature used by many in the past to suggest a link with the members of the fungus kingdom. Biopsy tissue from polypoidal lesions stained with H&E is characterized by the presence of numerous sporangia at different stages of development (Fig. 4A and B). Hyperplasia of the mucous membranes and/or skin with fibrovascular and fibromyxomatous connective tissue containing numerous sporangia is the main feature of the infection. Inflammatory infiltrates of lymphocytes, neutrophils, plasma cells, and, more rarely, giant cells and eosinophils are usually observed in cases of nasal, ocular, and skin infections. The juvenile and intermediary sporangia possess a central nucleus with a prominent nucleolus (Fig. 4B). The collapse of immature sporangia may cause the formation of U-shaped structures. Mature sporangia possessing several thousand endospores are usually found near the mucous epithelium, where they are transported from the internal infected areas by a transepidermal elimination phenomenon (Fig. 4A and C) (37). The mature sporangia's cell wall is usually thin, and the enclosed endospores contain clusters of eosinophilic spherical structures. The endospores have a mucoid capsule that does not stain in H&E preparations. The presence of a pore on the sporangial wall is also observed, depending on the plane of the sectioned tissue (Fig. 4C).

Nucleic Acid Detection Techniques

Currently there are no available DNA-based techniques for the detection of *R. seeberi*.

Isolation Procedures, Identification, Typing, and Serologic Tests

Despite numerous reports claiming that *R. seeberi* has been isolated in pure culture, such claims have not yet been validated. Most of the organisms recovered in cases of rhinosporidiosis have proven to be fungal or bacterial contaminants (1, 37). Because *R. seeberi* has not yet been cultured, the identification of this pathogen is based on its phenotypic characteristics on wet-mount preparations and/or histopathological analyses. The morphological characteristics of *R. seeberi* in infected tissues are almost pathognomonic. Nonetheless, the pathogen's epidemiological features and the clinical signs of disease should be taken into consideration for the final diagnosis of infection. Morphologically, the parasitic stages of *Coccidioides immitis* and *Coccidioides posadasii* mimic the *R. seeberi* sporangia with endospores. However, epidemiological, clinical, and phylogenetic features and the fact that *Coccidioides* spp. can be readily cultured categorically separate these pathogens from *R. seeberi*.

Although early investigators did not find *R. seeberi* antibodies in the sera of infected hosts by using endospores or sporangia as antigens, others suggest that the reason for the failure was the use of insensitive assays such as ID. Recently,

an immunoelectron microscopic approach showed for the first time that anti-*R. seeberi* antibodies against a specific antigen, detected only in mature sporangia, are present in the sera of patients with rhinosporidiosis (19). Despite these efforts, there are no available serological diagnostic assays for rhinosporidiosis in the clinical setting.

Antimicrobial Susceptibility

R. seeberi is resistant to most antifungal drugs (1, 37). Because this pathogen cannot be isolated in culture, susceptibility testing is not possible. Treatment is carried out by surgical removal of the infected tissues.

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ANTIFUNGAL AGENTS AND SUSCEPTIBILITY TEST METHODS

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Fluconazole disk susceptibility test (J. Jorgensen, University of Texas).

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Antifungal Agents

SEVTAP ARIKAN AND JOHN H. REX

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Fungi are the causative agents of various superficial and systemic infections in humans. Although superficial infections are of clinical significance in both immunocompetent and immunocompromised hosts, invasive infections tend to threaten particularly the latter. In addition to the common clinically relevant fungal species, a relatively diverse spectrum of fungi are now isolated in clinical mycology laboratories. There is a great demand for novel agents with reduced toxicities and widened spectrums of activity, and the development of antifungal therapy is now enjoying a period of rapid growth.

In this chapter, currently available topical and systemic antifungal agents (Table 1) are reviewed. The mechanism of action, spectrum of in vitro activity, pharmacology, clinical uses, and adverse effects of each are discussed, and comparative activities of the major systemic antifungal agents against well-known groups of fungi are summarized (Table 2). Novel agents that are on the horizon are also briefly reviewed (Table 3).

CURRENTLY AVAILABLE ANTIFUNGAL AGENTS FOR SYSTEMIC USE

Polyenes

The polyene group of antifungal agents includes amphotericin B and nystatin. Amphotericin B and its lipid formulations are used for the treatment of systemic fungal infections and thus will be reviewed in this section. Historically, nystatin has been solely a topical polyene and is discussed in this chapter. A liposomal formulation of nystatin has also been developed, but clinical development appears to have ceased.

Amphotericin B and Its Lipid Formulations

Amphotericin B is an amphoteric compound possessing a hydrophilic polyhydroxyl chain along one side and a lipophilic polyene hydrocarbon chain on the other. Its solubility in water is poor. The commercially available parenteral amphotericin B formulation is amphotericin B deoxycholate (Fungizone; Bristol-Myers Squibb Pharmaceuticals). Due primarily to the nephrotoxic nature of amphotericin B, lipid formulations have been developed and are now in clinical use. These lipid formulations are liposomal amphotericin B (AmBisome; Gilead Sciences), amphotericin B lipid complex (Abelcet; Enzon Pharmaceuticals), and amphotericin B colloidal dispersion (Amphocil or Amphotec; Sequus Pharmaceuticals) (12). Topical formulations of amphotericin B (cream, lotion, and ointment) are also available to be used in the treatment of cutaneous candidiasis.

Mechanism of Action

Similar to the other polyenes, amphotericin B kills the fungal cell via binding primarily to ergosterol, the major sterol found in the fungal cellular membrane. This binding destroys the osmotic integrity of the membrane, followed by leakage of intracellular potassium, magnesium, sugars, and metabolites and finally cellular death (99). Lipid formulations of amphotericin B act via the same mechanism. It has also been suggested that oxidative damage due to amphotericin B may contribute to the drug's activity against *Candida* species (140). However, an antioxidant effect of amphotericin B has been observed more recently, and thus the relevance of pro-oxidant antifungal activity remains unclear (106).

Spectrum of Activity

Amphotericin B is active against a wide variety of pathogenic fungi, including most strains of *Candida*, *Cryptococcus neoformans*, *Aspergillus* spp., *Mucor* spp., *Blastomyces dermatitidis*, *Coccidioides immitis*, *Histoplasma capsulatum,* and *Paracoccidioides brasiliensis*. However, reduced susceptibility and potential resistance to amphotericin B may be associated with some species or strains belonging to these genera, such as *Candida guilliermondii*, *Candida lusitaniae*, *Candida krusei*, and *Aspergillus terreus*. A new sibling species of *Aspergillus fumigatus*, *Aspergillus lentulus*, which is phenotypically similar to *A. fumigatus* but less susceptible to amphotericin B and other antifungal drugs, has been recently identified (17). *Fonsecaea pedrosoi* may also exhibit reduced susceptibility to amphotericin B. The susceptibilities of *Trichosporon and Fusarium* species and *Pseudallescheria boydii* and *Sporothrix schenckii* isolates to amphotericin B vary (141).

Pharmacology

Amphotericin B is not absorbed well orally, and parenteral (intravenous) administration is required. It is distributed in many tissues and organs of the body, including the lungs, spleen, and kidneys. However, penetration into cerebrospinal fluid is poor, and intrathecal administration is required for the treatment of some central nervous system infections (61).

TABLE 1 Systemic and topical antifungal agents in use

Chemical class	Antifungal agent	Formulation(s)[a]
Allylamine	Naftifine	T
	Terbinafine	O, T
Antimetabolite	Flucytosine	O
Azole (imidazole)	Bifonazole	T
	Butoconazole	T
	Clotrimazole	T
	Econazole	T
	Ketoconazole	O, T
	Miconazole	T
	Oxiconazole	T
	Sulconazole	T
	Terconazole	T
	Tioconazole	T
Azole (triazole)	Fluconazole	O, IV
	Itraconazole	O, IV, OS
	Voriconazole	O, IV
Echinocandin	Caspofungin	IV
	Micafungin	IV
Polyene	Amphotericin B	IV, T
	Liposomal amphotericin B	IV
	Amphotericin B lipid complex	IV
	Amphotericin B colloidal dispersion	IV
	Nystatin	T, OS
Other	Amorolfine	T
	Butenafine HCl	T
	Ciclopirox olamine	T
	Griseofulvin	O
	Haloprogin	T
	Tolnaftate	T
	Undecylenate	T

[a] IV, intravenous; O, oral; OS, oral suspension; T, topical.

Clinical Uses

Amphotericin B is indicated primarily in invasive candidiasis, cryptococcosis, blastomycosis, histoplasmosis, coccidioidomycosis, sporotrichosis, and paracoccidioidomycosis and in empirical therapy of febrile neutropenic patients refractory to treatment with antibacterial agents (8, 108). Based on its superior efficacy and safety compared to amphotericin B, voriconazole is now the drug of choice for the primary treatment of invasive aspergillosis (71).

Lipid amphotericin B formulations have enhanced in vivo activity due not only to their improved tolerability but also to a possible change in drug targeting and enhanced delivery to the site of infection. These formulations are indicated primarily as salvage therapy for patients with poor renal function, poor response to amphotericin B deoxycholate, or poor tolerability of amphotericin B deoxycholate (6, 57, 72). Based on the superior safety profiles of these formulations, their clinical use as suitable alternatives to amphotericin B deoxycholate is considered appropriate for the primary treatment of invasive fungal infections (108).

Adverse Effects

The major adverse effect of amphotericin B is nephrotoxicity. Acute side effects (fever, chills, myalgias, hypotension, and bronchospasm) and thrombophlebitis may also be observed during amphotericin B therapy. The major advantages of lipid formulations of amphotericin B compared to the conventional amphotericin B formulation are the significantly reduced side effects, particularly nephrotoxicity (12).

Azoles

The azole group of antifungal agents includes imidazoles and triazoles. These differ with respect to their chemical structures. Azoles are fungistatic in general and exhibit partial growth inhibition of fungal cells (45). Fungicidal activity is observed for some novel triazoles with some fungi. A good example is the fungicidal activity of voriconazole against *Aspergillus* (55).

Mechanism of action. All azole compounds act via the inhibition of cytochrome P450 14α-demethylase (also known as $P45014_{DM}$ and lanosterol demethylase), which functions in the synthesis of ergosterol from lanosterol. The inhibition of ergosterol synthesis results in the termination of fungal cell membrane synthesis (138).

Imidazoles

Imidazoles contain an azole ring with two nitrogens. Although several imidazoles are in clinical use for topical antifungal therapy and will be discussed elsewhere in this chapter, ketoconazole is the only imidazole used for the treatment of systemic fungal infections.

Ketoconazole

Ketoconazole, a lipophilic antifungal agent (Nizoral; Janssen Pharmaceutica), has both oral and topical (cream and solution) formulations to be used in the treatment of systemic mycoses, dermatophytosis, and cutaneous candidiasis (138).

Spectrum of activity. Ketoconazole has favorable in vitro activity against *H. capsulatum*, *C. immitis*, *B. dermatitidis*, *P. brasiliensis,* and *P. boydii*. Although ketoconazole is also active against *Candida* spp. and *C. neoformans,* this activity remains limited compared to those of triazoles in clinical use (fluconazole, itraconazole, and voriconazole). Cross-resistance to ketoconazole in a subset of *Candida* isolates resistant to other azoles is likely (103). The activity of ketoconazole is low against *Scedosporium prolificans* and variable against *Scedosporium apiospermum* (36). Ketoconazole has in vitro activity against *Trichophyton rubrum* (51) and *Malassezia* spp. (67). *Aspergillus* spp., *Fusarium* spp., and zygomycetes are resistant to ketoconazole (35).

Pharmacology. Ketoconazole is a highly lipophilic antifungal agent provided in an oral formulation. Its absorption following oral administration is optimal in a gastric acid environment. Ketoconazole binds to proteins to a great extent (>99%). Due to its lipophilic nature, it has high affinity for and attains considerably high concentrations in fatty tissues and purulent exudates (138).

Clinical uses. Due to its toxic potential, particularly hepatotoxicity, gastric toxicity, and endocrinological adverse effects, and the availability of less toxic, more efficacious alternatives, ketoconazole is now little used (42). It is a second-line drug for the treatment of nonmeningeal true systemic (endemic) mycoses due to *H. capsulatum*, *C. immitis*, *B. dermatitidis,* and *P. brasiliensis* and also infections due to *P. boydii* and *S. schenckii* (92).

TABLE 2 Spectrums and extents of activity of commonly used systemic antifungal agents[a]

Organism	Activity[b] of antifungal agent:					
	AMB	K	F	I	V	C
Aspergillus spp.	+++	0	0	++	+++	++
B. dermatitidis	+++	++	+	+++	+++	++
C. immitis	+++	++	+++	+++	+++	++
C. neoformans	+++	+	+++	++	+++	0
Candida spp.						
C. albicans	+++	++	+++	+++	+++	+++
C. glabrata	++	+	++	++	++	+++
C. guilliermondii	++	+	+++	+++	+++	++
C. krusei	++	+	0	++	++	+++
C. lusitaniae	++	++	+++	+++	+++	+++
C. parapsilosis	+++	++	+++	+++	+++	++
C. tropicalis	+++	++	+++	+++	+++	+++
Fusarium spp.	++	0	0	+	++	0
H. capsulatum	+++	++	++	+++	+++	++
Mucor, *Rhizopus*, and other fungi belonging to the class *Zygomycetes*	+++	0	0	0	0	+
P. brasiliensis	+++	+++	++	+++	+++	++

[a] This table is a general overview for comparison of the activities of some systemic drugs against various fungi. Readers are recommended to refer to the text for more detailed information or data at the species or strain level.

[b] In some cases, the meaning of reduced in vitro susceptibility is far from certain (e.g., the echinocandins). 0, no meaningful activity; +, occasional activity, ++, moderate activity but resistance is noted; +++, reliable activity with occasional resistance. AMB, amphotericin B; K, ketoconazole; F, fluconazole; I, itraconazole; V, voriconazole; C, caspofungin.

Adverse effects. As just noted, ketaconazole may cause serious side effects. Hepatotoxicity, decreases in testosterone and cortisol levels, nausea, vomiting, photosensitivity, pruritis, and rashes may be observed during ketoconazole therapy (142).

Triazoles

Triazoles contain three nitrogens in their azole ring. Fluconazole, itraconazole, and voriconazole are the triazoles currently in use for systemic fungal infections.

Fluconazole

Fluconazole (Diflucan; Pfizer Pharmaceuticals) is extensively used, particularly in the treatment of candidiasis. Its availability in both oral and intravenous formulations; significantly reduced rates of hepatic, gastric, and endocrinological toxic reactions; and the ability to penetrate cerebrospinal fluid are its advantages (35).

Spectrum of activity. Fluconazole is active against most *Candida* spp. and *C. neoformans*. Among the *Candida* spp., *C. krusei* is intrinsically resistant to fluconazole (95). The fluconazole susceptibilities of *Candida glabrata* strains, on the other hand, vary widely. Although some *C. glabrata* isolates may be dose-dependently susceptible to fluconazole, as many as 15% of the *C. glabrata* strains may exhibit true resistance (120). MICs of fluconazole for strains of *Candida tropicalis, Candida norvegensis, Candida dubliniensis,* and *Candida inconspicua* may occasionally be high (18, 122). In addition, fluconazole-resistant strains of any *Candida* spp. may occasionally be observed. Acquired resistance to fluconazole even among *Candida albicans* isolates may occur following long-term fluconazole prophylaxis, particularly in patients with AIDS (130). MICs of fluconazole for isolates of *Blastoschizomyces capitatus* may be high (38). Fluconazole has limited activity against *B. dermatitidis* (31), and resistance may develop when it is used to treat histoplasmosis (158). It has practically no activity against the molds.

Pharmacology. Fluconazole is a water-soluble compound, and its absorption following oral administration is excellent. It is available in oral and intravenous formulations. The absorption of fluconazole is not affected by gastric acidity or the fatty content of the food with which it is ingested. Following administration, it is distributed to almost all organs and tissues. Significantly, fluconazole readily penetrates cerebrospinal fluid as well due to its water solubility (75).

Clinical uses. Fluconazole is used in the treatment of candidiasis, cryptococcosis, and coccidioidomycosis. It is a second-line antifungal agent in the treatment of histoplasmosis, blastomycosis, and sporotrichosis (138). Fluconazole is widely used for both prophylaxis and chronic suppressive therapy in high-risk patients, particularly in transplant recipients (81) and AIDS patients who have recovered from cryptococcal meningitis (94).

Adverse effects. Side effects and toxic reactions due to fluconazole therapy are rare. Allergic reactions, angioedema (1), thrombocytopenia (2), and alopecia (111) may be observed. Reversible adrenal insufficiency has been reported in critically ill patients with multiple organ failures during high-dose fluconazole therapy (5).

Itraconazole

Itraconazole is manufactured by Janssen Pharmaceutica, and its trade name is Sporanox.

Spectrum of activity. Itraconazole is active against a wide variety of clinically relevant fungi: *Candida* spp. (including some, but not all, of the fluconazole-resistant *C. krusei* and

TABLE 3 Novel antifungal agents in development

Generic name(s) (trade name; company)	Notes
Novel Triazoles	
Posaconazole, Sch-56592 (Noxafil; Schering-Plough Pharmaceuticals)	Currently in phase III trials; active in vitro against *Candida* spp., *C. neoformans*, *Aspergillus* spp., *Rhizopus*, *S. apiospermum*, *H. capsulatum*, *C. immitis*, *B. dermatitidis*, dermatophytes, and dematiaceous fungi (47, 53, 104); available data from salvage therapy of invasive zygomycosis are noteworthy (60); approved by European Commission for treatment of serious invasive fungal infections in adults who remain refractory or intolerant to commonly used antifungal agents
Ravuconazole, BMS-207147, ER-30346 (no trade name announced; Bristol-Myers Squibb)	Currently in phase II–III trials; active in vitro against *Candida* spp. (less active against dose-dependently fluconazole-susceptible and fluconazole-resistant isolates than against fluconazole-susceptible ones), *C. neoformans*, *Trichosporon* spp., *A. fumigatus*, *Rhizopus*, *S. apiospermum*, dermatophytes, and dematiaceous fungi (52, 64, 68, 110, 123–125); no detailed human data yet reported
Albaconazole, UR-9825 (no trade name announced; Uriach & Company)	Currently in phase I–II trials; active in vitro against *Candida* spp., *C. neoformans, Malasezzia, Aspergillus, Paecilomyces, S. apiospermum, S. prolificans*, and dermatophytes (29, 30, 50); no human data yet available
Sordarin and azasordarin derivatives	
Sordarins: GM237354 (lead compound), GM193663, GM211676, GM222712; Azasordarins: GW471558 (lead compound), GW506540, GW531920, GW560849 (no trade names announced; Diversa Corporation)	Not yet in clinical studies; active in vitro against *Candida* spp., *C. neoformans*, *Trichosporon* spp., *Pneumocystis carinii*, *P. boydii*, and *Cladosporium carrionii* (46)
Chitin synthase inhibitor	
Nikkomycin Z (no trade name announced)	Not yet in clinical studies; active in vitro against *A. fumigatus*, *H. capsulatum* (some isolates), and *C. immitis* (34, 58)
Candida isoleucyl-tRNA synthetase inhibitor	
Icofungipen, (BAY-10–8888, PLD-118 (no trade name announced; Pliva)	Currently in phase II trials; active in vitro and in vivo against *Candida* spp., including azole-resistant isolates (117); active in treatment of oropharyngeal candidiasis in HIV-positive patients, although dosage regimen tested did not appear to be as effective as fluconazole[a]

[a] Source: N. H. Brockmeyer, M. Ruhnke, K. Oreskovic, and J. Peterson, *Abstr. 44th Intersci. Conf. Antimicrob. Agents Chemother.,* abstr. M-1036, 2004.

C. glabrata isolates), *C. neoformans, Aspergillus* spp., dermatophytes, *P. boydii, Paecilomyces* spp., *C. immitis, H. capsulatum, B. dermatitidis, P. brasiliensis, Penicillium marneffei,* and *S. schenckii* (4, 138, 146). Its activity against *Aspergillus* is the most significant advantage of itraconazole over fluconazole. However, strains of *A. fumigatus* and *Aspergillus nidulans* that are resistant to itraconazole have been reported (37, 144, 150). MICs of itraconazole for isolates of *A. lentulus* are relatively high (17). Itraconazole has no activity against the agents of zygomycosis (148) and *Scopulariopsis* spp. (3) and has limited activity against *Fusarium* (10).

Pharmacology. Itraconazole is available in oral capsule, oral solution (in cyclodextrin), and intravenous (in hydroxy-propyl-beta-cyclodextrin) formulations. Oral absorption of itraconazole is influenced by food and gastric acidity. The intravenous formulation provides consistent and predictable bioavailability and rapidly achieves optimal levels in blood. Itraconazole is lipophilic in nature, resulting in poor penetration into cerebrospinal fluid and high concentrations in purulent exudates and fatty tissues (15).

Clinical uses. Itraconazole has been used in the treatment of candidiasis, including a number of cases unresponsive to other azoles (131), aspergillosis (33, 41), dermatomycosis (24), onychomycosis (9), chromoblastomycosis (23), true systemic (endemic) mycoses (coccidioidomycosis, histoplasmosis, blastomycosis, and paracoccidioidomycosis) (92), and sporotrichosis (112). It is also effective in the treatment of cryptococcosis (41). Itraconazole was found to be more effective than fluconazole and was associated with fewer invasive fungal infections than fluconazole when it was used for long-term prophylaxis after allogeneic hematopoietic stem cell transplantation (HSCT) (161).

Adverse effects. Side effects due to itraconazole therapy are similar to those due to fluconazole therapy. Gastrointestinal side effects are more common with itraconazole than with fluconazole (161). Hepatotoxicity is rarely noted (138). Hypokalemia, edema, and hypertension may be observed, particularly at high doses (137).

Voriconazole

Voriconazole is a triazole structurally related to fluconazole and manufactured by Pfizer Pharmaceuticals. Its trade name is Vfend, and it is available in oral and intravenous formulations.

Spectrum of activity. Voriconazole has a wide spectrum of antifungal activity. The spectrum covers *Candida* spp. (including some fluconazole-resistant isolates), *C. neoformans, Trichosporon* spp., *B. capitatus, Aspergillus* spp. (including itraconazole-resistant *A. fumigatus* and amphotericin B-resistant *A. terreus*), *Fusarium* spp., *S. apiospermum, S. prolificans, B. dermatitidis, H. capsulatum, C. immitis, P. marneffei,* and dermatophytes (10, 79, 93). Due to azole cross-resistance, a significant number of fluconazole-resistant *Candida* isolates are also resistant to voriconazole as well as ketoconazole and itraconazole (135).

The antifungal activity of voriconazole is mostly fungicidal for filamentous fungi, including *Aspergillus, Fusarium,* and

Scedosporium (55). Voriconazole was found to be fungicidal against *C. krusei,* although it had no or minimal fungicidal activity against *C. albicans* and *C. tropicalis* (132).

Voriconazole has no or limited activity against zygomycetes, *S. schenchkii* (82), and a significant number of *Rhodotorula* strains, particularly *Rhodotorula muciloginosa* (59).

Pharmacology. Voriconazole exhibits good oral bioavailability. It distributes widely into tissues, including those of the central nervous system. Most (80%) of the drug is eliminated by the hepatic route (113). It has nonlinear, dose-dependent pharmacokinetics. Maximum concentrations in sera and total-exposure (area-under-the-curve) values demonstrate notable intersubject variability following oral voriconazole administration (79, 126).

Clinical uses. Voriconazole is indicated for the primary treatment of invasive aspergillosis (71), salvage therapy of infections due to *S. apiospermum* and *Fusarium* spp., and the treatment of cases with esophageal candidiasis and non-neutropenic candidemia (80). Successful treatment of *Saccharomyces boulardii* sepsis (28) and cerebral blastomycosis (16) with voriconazole has also been reported. Favorable activity of voriconazole in infections due to dematiaceous fungi has been observed, but the clinical experience is limited (129).

Voriconazole has no meaningful activity against zygomycetes. Cases of invasive zygomycosis during prophylactic, empirical, or therapeutic use of voriconazole have been reported (76, 86, 139).

Adverse effects. Transient visual disturbances (30%) and hepatic enzyme elevations (25) are the most commonly observed adverse effects of voriconazole. Demyelinating peripheral neuropathy (145), rashes, photosensitivity reactions (127), non-concentration-dependent QT interval prolongation, and nonsustained, polymorphic ventricular tachycardia (7) have also been observed. Hallucinations, hypoglycemia, electrolyte disturbance, and pneumonitis related possibly to high voriconazole concentrations have been reported, and measurement of plasma voriconazole concentrations is recommended to optimize voriconazole dosages (27).

Antimetabolites

Flucytosine

Flucytosine (5-flurocytosine; 4-amino-5-fluoro-2-pyrimidone [Ancobon; Ancotil; Valeant Pharmaceuticals]) is a fluorinated pyrimidine and the only available antifungal agent acting as an antimetabolite. It is available in an oral formulation. The intravenous formulation of flucytosine is not broadly available anymore.

Mechanism of Action

Flucytosine exerts its antifungal activity by disrupting pyrimidine metabolism and thus the synthesis of DNA, RNA, and proteins in the fungal cell. Following its entry into the fungal cell with the help of the permease enzyme, flucytosine is converted to 5-fluorouracil (5FU). 5FU is phosphorylated to form 5-fluorouridylic acid. 5-Fluorouridylic acid is further phosphorylated and is incorporated into RNA. This incorporation results in the inhibition of protein synthesis. The conversion of 5FU to 5-fluorodeoxyuridine monophosphate, on the other hand, causes the inhibition of the thymidylate synthase enzyme, which functions in fungal DNA synthesis and nuclear division (56).

Spectrum of Activity

Flucytosine has a narrow spectrum of activity, including *Candida* spp., *C. neoformans,* and the dematiaceous fungi causing chromoblastomycosis (96). It also exhibits favorable in vitro activity against *Rhodotorula* spp. (59). Primary resistance or decreased susceptibility to flucytosine in many fungal species, including *Candida* strains and *C. neoformans,* is not uncommon (21, 32, 155, 159). Moreover, resistance may also develop during flucytosine monotherapy (73).

Pharmacology

Flucytosine achieves high levels in sera and body fluids and readily penetrates into cerebrospinal fluid and other body fluids (153).

Clinical Efficacy

Due to the potential risk of primary or easily emerging flucytosine resistance, flucytosine monotherapy should be avoided (150). Flucytosine is most commonly used in combination with amphotericin B. Flucytosine-fluconazole combination therapy (97) and triple therapy including amphotericin B, fluconazole, and flucytosine (44) also appear to have promising implications for the treatment of cryptococcal and *Candida* meningitis. The most common indication for amphotericin B-flucytosine combination therapy is cryptococcal meningitis, where the drugs have been shown to act synergistically (20, 151). This combination has also been effective in the treatment of *Candida* meningitis (49).

Adverse Effects

Bone marrow depression, hepatotoxicity, and gastrointestinal intolerance are the major adverse effects of flucytosine. Flucytosine is cleared by the kidneys, and concomitant use of nephrotoxic drugs such as amphotericin B must be approached carefully as flucytosine may accumulate to toxic levels in the blood if renal dysfunction develops (153).

Allylamines

The allylamine group of antifungal agents includes terbinafine and naftifine. Terbinafine has oral and topical formulations, but naftifine has only topical preparations and will be discussed elsewhere in this chapter.

Mechanism of Action

Allylamines inhibit ergosterol biosynthesis via the inhibition of the squalene epoxidase enzyme. This effect finally causes fungal cell death primarily due to the increased membrane permeability mediated by the accumulation of high concentrations of squalene, but not due to ergosterol deficiency (56).

Terbinafine

Terbinafine is a widely used lipophilic antifungal agent.

Spectrum of Activity

Terbinafine is active against dermatophytes (65), *Malassezia furfur* (118), various *Candida* spp. (134), *Aspergillus* spp. (136), *S. schenckii* (78), *P. marneffei* (98), *C. neoformans* (134), *Trichosporon* spp., and *Blastoschizomyces* (133). Primary terbinafine resistance and development of resistance to terbinafine after prolonged exposure have been shown to be rare among isolates of *T. rubrum* (102, 107). In combination with azoles, terbinafine may exhibit synergistic effects against some *Candida* strains that are less susceptible to azoles (19, 115).

Pharmacology

Terbinafine is available in oral and topical (cream) formulations (Lamisil; Sandoz Pharmaceuticals). It achieves high concentrations in skin, nail, and fatty tissues (143).

Clinical Uses

Due to its high rate of efficacy, low rate of toxicity, and ability to achieve clinical success after a relatively short course of therapy compared to other agents, terbinafine is often the drug of choice today in the treatment of various dermatomycoses (22). The latter property of terbinafine is particularly significant for the treatment of onychomycosis, which requires longer therapeutic courses with other agents (9). Terbinafine is as effective as or more effective than its alternative, itraconazole (39), and also fluconazole (69) in the treatment of onychomycosis. Recent data indicate that fluconazole-resistant *Candida* infections may respond to fluconazole-terbinafine combination therapy (54). Similarly, the combination of voriconazole and terbinafine has proven to be efficacious in some cases with infections due to *S. prolificans* (74). Terbinafine therapy has also been efficacious in some patients with sporotrichosis, aspergillosis, chromoblastomycosis, and leishmaniasis (48, 116).

Adverse Effects

Terbinafine is well tolerated with a low incidence of adverse reactions, such as those involving the gastrointestinal system and the skin (66) and rarely observed reversible agranulocytosis (105).

Echinocandins

Echinocandins are lipopeptides with a broad spectrum of antifungal activity. Several years after the development of the first echinocandin, cilofungin, which caused serious toxicity in systemic use, systemically applicable new echinocandins have recently been developed. These are anidulafungin (previously known as VER-002, V-echinocandin, and LY303366; Eraxis; Pfizer Pharmaceuticals), caspofungin (previously known as L-743,872 and MK-0991; Cancidas; Merck Research Laboratories), and micafungin (previously known as FK463; Funguard in Japan and Mycamine in the United States; Astellas Pharma Inc.).

Mechanism of Action

Echinocandins inhibit glucan synthesis and thus cause cessation of fungal cell wall synthesis (40, 149).

Spectrum of Activity

Echinocandins are in general active against various *Candida* and *Aspergillus* spp. (13, 91, 121). Their favorable activity against azole- and amphotericin B-resistant as well as azole- and amphotericin B-susceptible *Candida* strains is one of the major advantages of echinocandin use in clinical practice (13, 119). Echinocandins exert fungicidal activity against various *Candida* species. Their activity against *Aspergillus,* on the other hand, is very disruptive to the apical and subapical branching cells of the hyphae and does not appear to be fungicidal for the whole fungus (11, 14, 26). Thus, the anti-*Aspergillus* activity of echinocandins seems beneficial mostly in decreasing the angioinvasive potential of the organism (119).

Among yeast isolates, the activity of echinocandins is more limited against *Candida parapsilosis, C. guilliermondii,* and *Trichosporon* spp. (13, 147). Although there is evidence of favorable in vivo activity of caspofungin against the *Candida* species with relatively higher caspofungin MICs, the data remain limited (K. Bartizal, M. Motyl, P. Hicks, C. A. Sable, M. Dinubile, and N. Kartsonis, *Abstr. 42nd Intersci. Conf. Antimicrob. Agents Chemother.,* abstr. M-1240, 2002). Emergence of resistance to caspofungin and azoles following treatment with caspofungin and fluconazole has been reported in a patient with prosthetic valve endocarditis (101).

Echinocandins have practically no or very limited activity against *C. neoformans*, *Fusarium* spp., and zygomycetes (11, 47, 62, 152).

Pharmacology

In general, echinocandins exhibit favorable, dose-dependent linear pharmacokinetic properties (63). Oral bioavailability is limited, and only intravenous formulations are available or are currently under development.

Clinical Uses

Data on the in vivo efficacy of anidulafungin in human infections remain limited and await further investigations. Available data suggest favorable efficacy for esophageal candidiasis, candidemia, and aspergillosis (89, 90, 119).

Caspofungin is licensed and indicated for the treatment of oropharyngeal and esophageal candidiasis (including those cases due to fluconazole-resistant isolates), candidemia, intra-abdominal abscess, peritonitis, and pleural space infections due to *Candida;* for empirical therapy for febrile neutropenic patients unresponsive to antibacterial therapy; and for salvage therapy for patients with invasive aspergillosis who have been refractory or intolerant to amphotericin B, lipid amphotericin B formulations, and/or itraconazole (109, 154, 156; http://www.cancidas.com).

Micafungin has been found to be efficacious in the treatment of esophageal candidiasis, candidemia, and invasive aspergillosis (43, 62, 85) and in antifungal prophylaxis for HSCT recipients and recipients of livers from living donors. Micafungin appears to be superior to fluconazole for antifungal prophylaxis in HSCT recipients (77, 84). Its use for prophylaxis for *Candida* infections in patients undergoing HSCT and for the treatment of esophageal candidiasis was approved by the U.S. Food and Drug Administration.

Adverse Effects

Echinocandins are in general safe therapeutic agents with minimal toxicity. No serious side effects have so far been reported. Fever, nausea, vomiting, rashes, and infusion-related side effects may be observed during echinocandin therapy (83).

Other Antifungal Agents for Systemic Use

Griseofulvin

Griseofulvin has been available over several decades since its isolation from *Penicillium*.

Mechanism of Action

The antifungal activity of griseofulvin is via the inhibition of fungal mitosis. It disrupts the mitotic spindle through interaction with polymerized microtubules.

Spectrum of Activity

Griseofulvin exhibits activity against dermatophytes (157). However, as reported so far, griseofulvin MICs are higher than those of terbinafine and itraconazole when the drugs are tested against *T. rubrum*. *Trichophyton mentagrophytes* isolates are less susceptible to griseofulvin than *T. rubrum* in vitro (87). The activity of voriconazole against dermatophytes is also superior to that of griseofulvin (160).

Pharmacology

Griseofulvin is practically insoluble in water and is deposited primarily in keratin precursor cells. It has oral formulations in microcrystalline and ultramicrocrystalline forms. Ingestion with a heavy meal and reduction in particle size enhance the absorption of griseofulvin.

Clinical Uses

Griseofulvin is used in the treatment of dermatomycosis. However, due to the current availability of less toxic, more efficacious agents, such as terbinafine and itraconazole (70), it is now a second-line drug in the treatment of dermatomycosis.

Adverse Effects

Adverse effects of griseofulvin are mild in general. Nausea, diarrhea, headache, skin eruptions, photosensitivity, hepatotoxicity, and neurological side effects may be observed (88, 100).

CURRENTLY AVAILABLE ANTIFUNGAL AGENTS FOR TOPICAL USE

Topical antifungal agents are used in the treatment of superficial cutaneous mycoses caused by dermatophytes and *Candida* as well as vulvovaginal and oropharyngeal candidiasis. There are various topical preparations in use that consist of different classes of compounds with antifungal activity (Table 1). These preparations include cream, lotion, nail lacquer, ointment, powder, and solution and spray forms which are applicable for cutaneous lesions and onychomycosis. Oral suspensions may be used for the treatment of patients with localized oropharyngeal candidiasis. Vaginal suppository, troche, and tablet forms are available for the topical treatment of vulvovaginal candidiasis (114, 128, 138).

The preference for topical or systemic therapy depends primarily on the extent and nature of the infection and the immune status of the host. For instance, onychomycosis and tinea capitis usually require systemic therapy, and cutaneous lesions due to dermatophytes or *Candida* may well respond to topical treatment. Oropharyngeal candidiasis may be treated topically. However, systemic agents are preferred for the treatment of esophageal candidiasis.

NOVEL ANTIFUNGAL AGENTS IN DEVELOPMENT

Among the antifungal agents in development are novel triazoles (posaconazole, ravuconazole, and albaconazole), nikkomycin Z, sordarin and azasordarin derivatives, icofungipen, and anidulafungin. Anidulafungin is discussed above under "Echinocandins," and the other novel antifungal agents are summarized in Table 3. These agents remain promising for future antifungal therapy.

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Mechanisms of Resistance to Antifungal Agents

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The use of antifungal drugs has accelerated in the last few decades because new and more effective antifungals have become available and because of a dramatic increase in the number of fungal infections. The currently available antifungal agents and new drugs under development are summarized in chapter 129. The rise in fungal infections in the last 20 years is the result of several factors usually associated with immune dysfunction. The frequency of patients with immune dysfunction has grown dramatically because of the AIDS epidemic, increased bone marrow transplants (BMT) and solid organ transplants, and aggressive cancer chemotherapy. Hospital-acquired fungal infections are also emerging as significant problems. With the increase in fungal infections, there has been a dramatic rise in the use of antifungals for the treatment of both systemic and localized fungal infections. It is not surprising that the expanded use of antifungal drugs has accelerated the development of resistance to these compounds. This chapter represents an updated version of the chapter in the previous edition of this Manual (28). Detailed reviews of the background of antifungal drug resistance have been presented previously (23, 29–30) and should be consulted for background. Primary references published before 2003 can be found in these reviews.

Classes of antifungal drugs include polyenes, azoles, allylamines, 5-flucytosine (5FC), and echinocandins. The mechanisms of action for these antifungal drugs are described in detail in chapter 129. The azoles, such as fluconazole, and the allylamines, such as terbinafine, target ergosterol biosynthesis. The polyenes, such as amphotericin B (AmB), bind to ergosterol in the plasma membrane. 5FC inhibits pyrimidine metabolism and DNA synthesis. The echinocandins target glucan synthetase, inhibiting cell wall synthesis and stability.

CLINICAL COMPONENTS OF ANTIFUNGAL DRUG RESISTANCE

Clinically, infections that are recalcitrant to treatment with these classes of antifungal drugs are considered resistant. A recalcitrant infection can be the result of a variety of factors associated with the host, the drug, and the fungus (summarized in Table 1).

Host Factors

The single factor that is most important in the resolution of a fungal infection is the immune status of the host. The antifungal drugs and the host immune system work together in controlling the infection. Infections in patients with nonfunctional immune systems are more recalcitrant to treatment, as the drug must combat the infection without the additive effect of the immune system. The site of the infection (systemic or localized to skin, oral mucosa, vaginal mucosa, eye, brain, etc.) contributes to clinical resistance since the site may be protected or inaccessible to drug therapy. The severity of the infection can also contribute to clinical resistance, as larger concentrations of fungal cells require higher concentrations of drug and/or longer treatments. The presence of foreign objects, including catheters, dentures, and artificial heart valves, can also contribute to a recalcitrant infection, as the fungus can attach to and invade these objects, creating a source of constant inoculation and protection from drug therapy. Finally, the patient's adherence to prescribed therapy regimens is extremely important for effective treatment. Poor adherence to a drug regimen reduces the effectiveness of the drug and fails to resolve the infection. Poor adherence also contributes substantially to the development of antifungal drug resistance, as intermittent or low-dose therapies commonly allow organisms that are incrementally more resistant to persist and become the dominant strain.

Drug Characteristics

The characteristics of the antifungal drug are also factors in the treatment of a recalcitrant infection. Fungistatic drugs such as the azoles do not kill the cells and are thus more likely to allow cells to develop resistance than a fungicidal drug such as AmB. The absorption, distribution, and metabolism of the drug also contribute to the overall effectiveness at the site of infection. As discussed above, the dosing of the antifungal drug, including the quantity, frequency, schedule, and cumulative dose, can also contribute to resolution of a fungal infection. Finally, other drug therapies administered to the patient can alter the effectiveness of the antifungal drugs.

Fungal Factors

Several factors of the fungus affect the treatment of the infection. First and foremost, the species and strain clearly determine which drugs are effective. Second, the cell type can alter drug efficacy. Fungi exist in a variety of cell types or morphologies, including yeasts (blastospores), hyphae, chlamydospores, conidia, and mycelia, each of which can have a specific susceptibility to antifungal drugs. For example, *Candida albicans*

TABLE 1 Factors that contribute to clinical antifungal drug resistance

Host factors	Drug factors	Fungal factors
Immune status	Fungistatic nature of drug	Cell types
Site of infection	Dosing	Morphology
Severity of infection	Frequency	Cell states
Presence of foreign materials	Quantity	Serotypes
Poor adherence to drug regimen	Cumulative dose	Biofilms
	Pharmacokinetics	Genomic stability of strain
	Absorption	Size of population
	Distribution	Population "bottlenecks"
	Metabolism	Strain MIC
	Drug-drug interactions	

exists in yeast and hyphal forms; and azole drugs can interfere with hyphal production at 0.1 μg/ml, 1/10 the therapeutic concentration of the drug in vivo. However, resistant strains of *C. albicans* form hyphae in the presence of high concentrations of azole drugs. In addition to morphology, several fungi including *C. albicans* exist in different switch phenotypes or cell states and exist in different serotypes determined by cell surface characteristics. These cell states and serotypes vary in susceptibility to antifungal drugs. An important cell state that has received considerable attention in recent years is fungal biofilms, in which cells grow as a plaque or mat on a surface. Fungal biofilms are known to have altered antifungal susceptibilities (2).

The genome stability of the fungus can also affect antifungal susceptibility. The best example is the development of resistance in *Candida* with different ploidy (copies of the genome). The development of resistance is a common occurrence in haploid *Candida glabrata* and infrequent in diploid *C. albicans,* where mutations in both copies may be needed to have an effect. In addition, a larger number of organisms in severe infections increases the chance that a random mutation can occur, resulting in a resistant fungal cell in the infection.

Population bottlenecks may dramatically alter the susceptibility pattern of the cells in an infection. For example, an infected catheter is removed and replaced with a new catheter in a patient with a systemic infection who is receiving antifungal drugs. The remaining fungal cells in the bloodstream could colonize the new catheter, and those cells may be more resistant due to exposure to antifungal drugs. Such a shift in the population structure is known as a population bottleneck and can clearly have effects on the development of resistance.

Level of Susceptibility

The final fungal factor that has an effect on a recalcitrant infection is the level of susceptibility to antifungal drugs of the fungal isolate, usually measured as the MIC, using standardized CLSI (Clinical and Laboratory Standards Institute, previously known as NCCLS) protocols. The specifics of the protocol, clinical breakpoints, and other factors associated with the standardized method are discussed in chapter 131 by Espinel-Ingroff. The standardized CLSI method has been used to demonstrate the existence of true, clinically relevant fungal resistance and the extent of the resistance in various patient populations.

COMPLICATIONS OF SUSCEPTIBILITY TESTING

Clinical Breakpoints

The CLSI protocol determines levels of susceptibility in vitro. These MICs can then be used to predict resistance in vivo by defining clinical breakpoints, the MIC values above which a strain is clinically resistant to drug treatment. It is important to note that these breakpoints are specific for each drug and fungal species. Thus, clinical isolates of *C. albicans* are resistant to fluconazole if the MIC is ≥ 64 μg/ml and resistant to itraconazole if the MIC is ≥1.0 μg/ml (21). Similar breakpoints for the MIC to fluconazole are being tested for *Cryptococcus neoformans* (1), and recently, a clinical breakpoint for voriconazole has been defined (4 μg/ml), although some believe that this number was set too high. While breakpoints for ketoconazole and clotrimazole have not been determined for *C. albicans,* it is likely that those breakpoints are similar to the 1.0-μg/ml breakpoint used for itraconazole, as the MIC values for clinical isolates to all three drugs have the same relative distributions.

The difference between MIC and clinical breakpoints has created some confusion. A clinical breakpoint is defined as the MIC at which a strain behaves as a resistant strain. The clinical breakpoint must be set for every drug. The problem with clinical interpretations is that strains with MICs to itraconazole or other azoles above 1.0 μg/ml are frequently and incorrectly regarded as susceptible, since their MIC is below the 64-μg/ml value that is the clinical breakpoint for the most common azole, fluconazole. It is important to remember that MICs are numbers determined in vitro, and clinical significance can be determined only using clinical breakpoints to correctly interpret these numerical results. In addition, it is vitally important that antifungal pharmacokinetics and pharmacodynamics be taken into account when dealing with clinical resistance.

90-60 Rule

A very important consideration regarding MIC interpretation is the 90-60 rule (20). Essentially, this rule states that infections will respond to therapy about 90% of the time if the infectious agent is judged to be susceptible in vitro, while infections will respond to therapy about 60% of the time if the infectious agent is judged to be resistant in vitro, even with the correct interpretation of clinical breakpoints. This rule appears to be applicable to most, if not all, microbial infections: fungi, bacteria, and parasites. It implies that patients with susceptible strains will respond 90% of the time and patients with a fluconazole-resistant strain of *C. albicans* will respond to fluconazole 60% of the time. The most likely reasons for successful treatment of a resistant strain or failure to treat a susceptible strain are found in Table 1. The 90-60 rule is important when comparing drug therapies. Case reports or studies of salvage therapy do not necessarily imply that one drug is effective against a strain that is resistant to another drug; the successful treatment may be part of the 60% success rate that is true even for drugs to which the strain is resistant in vitro.

The factors that contribute to a recalcitrant or resistant clinical infection have been described above and are summarized in Table 1. The rest of this chapter focuses on the resistance of fungal isolates, as determined by their MICs.

INTRINSIC AND ACQUIRED RESISTANCE

There are two types of resistance: intrinsic resistance, which is an inherited characteristic of a species or strain, and acquired resistance, which occurs when a previously susceptible isolate develops a resistant phenotype, usually as a result of prolonged treatment with antifungals. The ideal antifungal drug would be effective against all fungi, both yeast and molds. However, with each of the current antifungal drugs, there are genera and species that are intrinsically resistant. Fungal genera and species that are intrinsically resistant to antifungal drugs are summarized in chapter 131. In certain genera, there are some species that are susceptible and certain species that are resistant to specific antifungals. These unique species are briefly discussed below.

Intrinsic Azole Resistance

Candida krusei is intrinsically resistant to azoles. In addition, many strains of *C. glabrata* are intrinsically resistant or susceptible dose dependent. Other strains can quickly acquire resistance during therapy. *C. krusei* and *C. glabrata* are increasing in frequency in oral and systemic candidiasis in patient populations that use azole drugs for treatment or prophylaxis. In addition, the frequency of vaginitis caused by these azole-resistant *Candida* species is increasing, although these strains respond to azole therapy for vaginitis.

AmB and 5FC Resistance

Resistance to AmB is a common problem with the yeasts *C. krusei* and *Candida lusitaniae* and the molds *Aspergillus terreus* and *Scedosporium* sp. In some of these species, many but not all of the strains are resistant. These fungal infections are not common, and there is no indication that the frequency of these species has increased in patient populations exposed to large doses of AmB. Intrinsic and acquired resistance to 5FC is relatively common in many fungi. Because this is a common phenomenon, 5FC monotherapy is rarely used to treat fungal infections.

Acquired Azole Resistance

Acquired azole resistance was rare in the 1980s, when azoles were used primarily to treat patients with chronic mucocutaneous candidiasis. However, in the 1990s, azole resistance became a significant problem in *C. albicans* isolates from AIDS patients. Oral candidiasis is the most common opportunistic infection in human immunodeficiency virus (HIV)-infected patients, occurring in over 90% of all patients. Azole drugs were commonly prescribed at relatively low doses for long-term prophylactic therapy that was administered intermittently. Not surprisingly, azole resistance in oral candidiasis became a significant problem. Resistant strains of *C. albicans* were found in 20 to 33% of symptomatic oral candidiasis patients with HIV and in up to 14% of asymptomatic patients with HIV. In recent years, most HIV-infected patients in developed countries have been receiving highly active antiretroviral therapy, which reduces the frequency of most opportunistic infections including oral candidiasis, thus eliminating the need for azole prophylaxis and reducing issues of azole resistance. At this time, the frequency of antifungal resistance in strains from patients receiving highly active antiretroviral therapy is not known. Children with HIV infection are particularly susceptible to oral candidiasis and receive substantial clotrimazole prophylaxis. A survey demonstrated that up to 10% of oral isolates from these children are resistant to clotrimazole and cross-resistant to other azoles and require AmB for treatment. Unlike oral isolates, vaginal isolates of *C. albicans* do not increase their resistance to azoles, even after long-term exposure. In addition to *C. albicans, Candida dubliniensis* is a recently described, closely related species that is very difficult to distinguish from *C. albicans,* but which has the ability to develop increased resistance to azole drugs.

Acquired resistance has best been described in several matched sets of azole-susceptible and azole-resistant isolates from the same strain of *C. albicans,* usually obtained from oral candidiasis in AIDS patients or systemic infections in BMT patients (28). Azole resistance in a patient is most likely the result of a commensal strain that is previously resistant or that develops resistance, although it is possible that a resistant strain can be acquired by nosocomial transmission or intimate contact.

Acquired azole resistance has also developed in isolates of *C. neoformans* from AIDS patients who have been on prophylactic azole therapy to prevent recurrence of cryptococcal meningitis and in isolates of *Aspergillus fumigatus* from patients who have received repeated treatment with itraconazole.

Azoles are commonly used in surgical wards to prevent systemic candidiasis, as nonprescription drugs to treat fungal infections of the skin including athlete's foot, and in agriculture. Epidemiological studies of azole resistance have not been undertaken in these settings.

Acquired Polyene Resistance

Acquired resistance to fungicidal AmB is rare; it occurs mostly in yeasts from cancer patients who have received repeated doses of AmB to treat recalcitrant or recurring systemic fungal infections. The MICs to AmB of yeasts from cancer patients are elevated compared to the MICs of colonizing isolates from controls. Case reports concerning acquired resistance to AmB appear to be increasing. Intrinsic AmB resistance in *C. lusitaniae* may be related to cell switching between two cell states and to deletion/mutation of *ERG6* (31).

Change to a More Resistant Species or Strain

There are clear differences in the antifungal resistance of different species, as discussed above. There are also clear differences between strains within a species due to randomly occurring genetic differences. These small differences in strain susceptibility result in a distribution of MICs for strains within a species. Intrinsically resistant species and strains may be present in individuals by chance as commensal colonizations. If infection or disease develops, the infection will already be resistant to treatment with antifungal therapy.

Resistant species and strains may also be acquired by nosocomial transmission or intimate contact. The frequency at which fungal strains move from person to person has been investigated to some extent with *C. albicans,* where replacement rates are usually under 50%, although the sensitivity of the techniques used to differentiate strains can have a significant impact on determining if strains are identical, similar, or unrelated (26). Transmission of a strain either orally or sexually between patients has been documented, as has transfer of resistant strains (30).

MOLECULAR MECHANISMS OF AZOLE DRUG RESISTANCE

Most molecular analyses of antifungal drug resistance have focused on *C. albicans* and the related yeasts *C. glabrata* and *C. krusei.* Some work has been done with *C. neoformans* and

with *A. fumigatus*. This section concentrates on the mechanisms identified in *C. albicans*, with appropriate discussion of mechanisms identified in other fungi.

Matched Sets

The molecular analysis of antifungal drug resistance in *C. albicans* is complicated by the clonal nature of this diploid yeast. *C. albicans* grows vegetatively with limited sexual reproduction, as documented by the lack of Hardy-Weinberg equilibrium and by balanced lethal experiments. Recent evidence has demonstrated that *C. albicans* has mating loci similar to those of *Saccharomyces cerevisiae*, and "mating" of two diploid cells into a tetraploid cell can be detected under controlled circumstances. Still, mating in *C. albicans* is limited and most strains have diverged by acquiring mutations asexually, resulting in strains with considerable differences in their genomes. Genetic or expression differences identified between unmatched susceptible and resistant isolates could be the causes of resistance or could simply be the result of strain divergence. Thus, it is important to determine the relatedness of a susceptible isolate and resistant isolate before comparing them by molecular analyses.

Strain differences can be detected by several molecular techniques including restriction fragment length polymorphisms, chromosome karyotyping, and random amplification of polymorphic DNA. A recent review summarized the important considerations in each of these analyses (26). Perhaps the most appropriate method for strain typing is restriction fragment length polymorphism analysis with the repetitive marker Ca3, which can be used to detect even minor differences between clinical isolates.

Alterations in Ergosterol Biosynthesis

The target enzyme of the azole antifungals is lanosterol demethylase, the product of the *ERG11* gene (Erg11p). A defective Erg11p does not remove methyl groups at the 14α carbon of ergosterol precursors, resulting in an accumulation of 14α-methyl sterols, including a diol, 14α-methyl-ergosta-8,24(28)-dien-3β,6α-diol. The 14α-methyl sterols alter the fluidity and function of the fungal plasma membrane, and the resulting cells are more susceptible to oxygen-dependent microbicidal systems of the host. The diol also accumulates in these cells. This diol is fungicidal in *S. cerevisiae*, though its effect in *C. albicans* is not clear. Frequently, fungal cells escape the effects of 14α-methyl sterols and the diol with a mutation in the sterol desaturase, the product of the *ERG3* gene.

Alterations in *ERG11*

Several alterations in *ERG11* have been associated with resistance in *C. albicans*, including (i) point mutations in the coding regions, (ii) overexpression of the gene, (iii) gene amplification (which leads to overexpression), and (iv) gene conversion or mitotic recombination.

Point mutations within the coding region of *ERG11* that are present in a resistant isolate and which are not present in the susceptible isolate of the same strain have been identified by several researchers (for a review, see reference 17). That review surveyed point mutations within *ERG11*, cataloguing 29 amino acid substitutions occurring a total of 98 times in 53 sequences. The 29 substitutions were distributed throughout the sequence but clustered in three regions of the gene, suggesting three hot spots of mutation. These mutations are predicted to interfere with several regions of the gene, including the active site, the channel leading to the active site, and the flexible helix that interacts with and covers the channel. Of these 29 substitutions, six have been experimentally linked to resistance. These include Y132H, D278E, S405F, F449L, G464S, and R467K (letters represent amino acids; the wild-type amino acid precedes the numerical position in the protein sequence, and the mutant amino acid follows). Fifteen other substitutions were found in resistant isolates but require further analysis. Eight substitutions were found in both susceptible and resistant isolates, suggesting that these substitutions are not associated with resistance and are most likely the result of allelic differences. The most common amino acid substitutions that have been detected are D116E, K128T, E266D, and G464S. The first three substitutions are commonly found in both susceptible and resistant isolates; only G464S is experimentally associated with resistance. Silent mutations (no amino acid substitution) have also been identified in the *ERG11* gene. These silent mutations are most likely the result of allelic variation and are unlikely to have an effect on azole susceptibility. The R467K and Y132H mutations have been studied for their effect on resistance by expressing the gene in *S. cerevisiae*, where the mutations alter the azole susceptibility of the enzyme.

Overexpression of the *ERG11* gene should also contribute to resistance, as increased amounts of protein require increased amounts of drug. *ERG11* overexpression has been identified in several clinical isolates, although the level of overexpression has usually been less than fivefold. The effect of overexpression alone has not been determined, as these clinical isolates usually have additional alterations such as point mutations within the *ERG11* gene.

Overexpression can be the result of localized gene amplification. In *C. albicans*, gene amplification has not been associated with gene overexpression, although it is not commonly tested. Gene amplification has been identified in an azole-resistant clinical isolate of *C. glabrata*, although this isolate demonstrated other resistance mechanisms as well. The gene amplification was due to a chromosome duplication that was lost when the strain was grown in the absence of drug, although partial resistance was maintained.

Two groups have documented changes in *ERG11* allelic sequences associated with resistance. In both cases, it appears that a mutation occurred in one allele of *ERG11*. Random gene conversion or mitotic recombination then appears to have created strains in which both alleles of the *ERG11* gene contained the mutation. These homozygous strains may then have been selected under drug pressure, as the cells express only a resistant Erg11p. These gene conversions or mitotic recombinations are detected because silent allelic differences in and around the *ERG11* gene are eliminated as resistance develops.

Resistant clinical isolates usually contain mutated but functional *ERG11* genes. In *C. albicans* and *C. glabrata*, deletions of the *ERG11* genes have been studied for their effect on susceptibility and enhanced oxidative killing by the host. Given an increased susceptibility to oxidative killing, it is unlikely that clinical isolates would have a nonfunctional *ERG11* gene.

The *ERG11* gene appears to be important for the intrinsic resistance of *C. krusei*. The Erg11p activities in *C. krusei* cell extracts are 24- to 46-fold less susceptible to fluconazole than extracts from *C. albicans*. Alterations in drug accumulation were not observed in this study but were observed in a separate study. The intrinsic resistance of *C. krusei* is likely to be due to both reduced enzyme susceptibility and reduced drug accumulation (see below). Alterations in the *ERG11* gene have been demonstrated in other fungal species, including *A. fumigatus* and *C. neoformans*.

Additional Alterations in Ergosterol Synthesis

Using the current *C. albicans* genome sequence, most of the genes in the ergosterol pathway have been annotated or can be identified by homology searching with the *S. cerevisiae* homologs. In addition, the *ERG11* gene has been cloned from the resistant species *C. glabrata* and *C. krusei,* as well as *Candida tropicalis*. Several other genes in the ergosterol pathway have been analyzed for their effects on drug susceptibility. However, changes in other *ERG* genes have not been demonstrated in resistant clinical isolates.

The *ERG11* and *ERG3* genes have been deleted in *C. glabrata,* and their viability and drug susceptibilities have been tested. The growth phenotypes for these deletions are similar to those described for *S. cerevisiae*. However, the drug susceptibilities differ between *C. glabrata* and *S. cerevisiae,* suggesting that sterol control and antifungal resistance are not regulated in the same manner in the two species and that regulation in *C. albicans* might be different still. The deletion analysis included an analysis of mRNA in which the *ERG11* gene was overexpressed in the *ERG3* deletion and vice versa, suggesting transcriptional control of ergosterol biosynthesis, perhaps by negative feedback from ergosterol.

Molecular and genetic studies in *S. cerevisiae* indicate that mutations in *ERG3* alone can be sufficient to create an azole-resistant phenotype. Molecular and biochemical analysis of *C. albicans* suggests that *ERG3* mutations are associated with resistance in several clinical isolates, although reintroduction of a functional *ERG3* gene in one strain restored ergosterol synthesis but did not alter susceptibility. The *ERG3* mutations should prevent the production of the diol that may cause growth arrest, as is the case with *S. cerevisiae*.

In *C. neoformans,* the azoles appear to inhibit other gene products in the ergosterol pathway in addition to the inhibition of *ERG11*. The drugs appear to act directly or indirectly to inhibit the C-4 sterol demethylase complex *(ERG25, ERG26,* and *ERG27)*. Inhibition of this complex reduces the production of the growth-arresting diol, without alterations in *ERG3*. Azole-resistant strains of *C. neoformans* may also have defects in *ERG3* and *ERG2*. Itraconazole appears to have an additional inhibitory effect on the product of the *ERG24* gene, at least in *C. neoformans* and *Histoplasma capsulatum,* where itraconazole has an increased effectiveness compared to other azoles. Finally, it appears that the azoles also have activity against the *ERG5* gene product in *S. cerevisiae,* although the effect of this activity on azole susceptibility is unknown for any yeast.

Drug Import and Metabolism

To date, studies on azole import, modification, activation, or degradation have not been reported. Drug import studies require careful analysis of drug accumulation in short time points with minimum drug efflux, which are conditions that have not been applied in the medically important fungi. It has been reported that azoles are not metabolized in *C. albicans,* although this has not been studied in other species or in resistant isolates of *C. albicans.*

Drug Accumulation

Drug accumulation within a fungal cell is the result of a balance between drug import, drug efflux, and drug metabolism. Drug accumulation has been studied primarily in *C. albicans,* where radioactively labeled azoles and the fluorescent dye rhodamine 123 have been used to show drug accumulation within the cells. Drug accumulation has also been observed in *C. glabrata, C. krusei, C. dubliniensis, C. neoformans,* and *A. fumigatus*. This accumulation was saturable, suggesting an import or efflux mechanism other than diffusion. This azole accumulation is dependent on energy, cell viability, pH, and temperature. Drug accumulation is also dependent on the sterol composition of the cell membrane, as modified membranes lacking ergosterol are less fluid and have altered permeability, reducing azole accumulation. Intracellular drug levels are reduced in resistant clinical isolates compared to matched susceptible controls, and several studies have shown that this reduced accumulation is energy dependent.

Two Types of Efflux Pumps

Two classes of efflux pumps, the ATP binding cassette transporters (ABCT) and the Major Facilitators (MF), are involved in the movement of small molecules across the plasma membrane of the cells in both directions. Specific members of each class of pump have been associated with drug resistance in one or more systems. The ABCT pumps are associated with active efflux of small molecules that are toxic to the cells. These small molecules tend to be hydrophobic or lipophilic, as are the azole drugs. The MF pumps are also associated with relatively hydrophobic molecules such as tetracycline.

The complete *S. cerevisiae* genome includes 30 ABCT and 28 MF pumps. While not all of the 58 pumps function as drug pumps and not all are located to the plasma membrane, there are a significant number of pumps that can be involved in drug efflux. The *C. albicans* genome sequence contains a similar number of efflux pumps.

ABCT Pumps

The ABCT pumps consist of two membrane-spanning domains that assemble into a channel in the plasma membrane and two nucleotide binding domains that bind and use ATP as an energy source for the pump. The nucleotide binding domains contain an ABC, which is a conserved motif among proteins that use ATP as an energy source. The ABCT pumps are usually grouped into six gene families based on homology, and members of several of these families have been associated with resistance to a variety of compounds. However, in *C. albicans,* azole drug resistance has only been associated with the *Candida* drug resistance *(CDR)* gene family, which is homologous to the pleiotrophic drug resistance *(PDR)* gene family of *S. cerevisiae*. Ten *CDR* genes have been identified and annotated; only four *(CDR1* to *CDR4)* have been analyzed in detail.

The *CDR1* gene of *C. albicans* was first identified because it complements the *PDR5* gene deletion of *S. cerevisiae*. The *PDR5* mutant was hypersusceptible to cycloheximide, chloramphenicol, azole drugs, and other compounds that are substrates for this pump. Complementation with the *CDR1* gene of *C. albicans* restored normal susceptibility. Overexpression of *CDR1* has been demonstrated in a variety of resistant and susceptible clinical isolates from oral and systemic disease. Both of the alleles of the *CDR1* gene have been experimentally deleted in *C. albicans*. The resulting strain is hypersusceptible to azoles, terbinafine, amorolfine (another ergosterol biosynthesis inhibitor), and several metabolic inhibitors, although there was no change in susceptibility to AmB or 5FC.

CDR2 was also isolated by complementation of the *PDR5* mutant of *S. cerevisiae*. *CDR2* is closely related to *CDR1,* and like *CDR1,* expression of this gene in *S. cerevisiae* increases resistance to azoles, other sterol biosynthetic drugs, and metabolic inhibitors. In *C. albicans,* the gene disruption alone does not alter drug susceptibility, but the double gene disruption, *CDR1* and *CDR2,* was more hypersusceptible than the single *CDR1* gene disruption. Increased mRNA

levels of *CDR2* have been detected in several resistant clinical isolates; most of these also have increased mRNA levels of *CDR1*. The coregulation of *CDR1* and *CDR2* suggests that the genes are both transcriptionally regulated by the same or similar factors and those factors are involved in clinical resistance.

CDR3 and *CDR4* have also been identified in *C. albicans* by homology. Expression of *CDR3* has been documented only in opaque cells of strain WO-1. This strain undergoes phenotypic switching between white cells and opaque cells, which are the mating-competent form of *C. albicans*. Expression of *CDR4* is detected by Northern blot analysis in most cells, but expression does not correlate with azole resistance. The gene disruption is not hypersusceptible to azoles. Six additional *CDR* genes have been identified in the nearly complete *C. albicans* genome sequence, although these genes have not been further characterized.

ABCT pumps have been identified in *C. krusei, C. glabrata, C. tropicalis, C. dubliniensis, C. neoformans, A. fumigatus, Aspergillus nidulans,* and *Aspergillus flavus*. Gene disruptions of pump genes in *C. glabrata* are hypersusceptible to azoles, and upregulation of the pump genes is associated with the development of resistance at a high frequency in *C. glabrata*.

MF Pumps

The MF pumps also consist of two membrane-spanning domains that assemble into a channel, but the MF pumps do not have nucleotide binding domains. Instead these pumps use the proton gradient of the membrane as an energy source. The MF pumps commonly transport molecules by antiport: protons enter the cell, and small molecules are removed from the cell. Two MF pump genes have been characterized in *C. albicans*.

The *MDR1* gene or *Ben-R* gene was cloned as a DNA fragment from *C. albicans* that confers resistance to a variety of drugs in *S. cerevisiae*. The gene disruption in *C. albicans* increased susceptibilities to some of these drugs and had reduced virulence in an animal model of systemic candidiasis. Increased mRNA levels of the *MDR1* gene have been correlated with azole resistance in several clinical isolates of *C. albicans*. The *MDR1* gene shows specificity between azole drugs. When expressed in *S. cerevisiae,* cells are resistant to fluconazole but not to ketoconazole or itraconazole. The *MDR1* gene has been disrupted in azole-resistant clinical isolates overexpressing *MDR1*. The resulting disruptants had an increased susceptibility to azole drugs, the first demonstration that *MDR1* is in fact a cause of azole resistance.

The second MF gene, *FLU1,* was identified as a DNA fragment from *C. albicans* that complemented the *PDR5* gene of *S. cerevisiae* (see above), increasing fluconazole and cycloheximide resistance. Overexpression of the *FLU1* gene has not been documented in any resistant clinical isolates. *MDR1*-related genes have also been shown to be associated with resistance in *C. dubliniensis* and *C. tropicalis*.

Transcriptional Regulation of Resistance Genes

Increased mRNA levels of resistance genes including *ERG11, CDR1, CDR2,* and *MDR1* are usually the result of increased expression, which is usually not the result of gene amplification but of alterations in the promoter activity of the gene.

The *ERG11* gene appears to be coordinately regulated with other genes in the *ERG* pathway by a transcription factor containing a Zn_2Cys_6 binding motif encoded by *UPC2*. *UPC2* is one member of a large family of fungus-specific transcription regulators. It appears that the *UPC2* gene contains a transcription activation domain at the N terminus and a transmembrane segment at the C terminus. Activation may occur by cleavage of the protein, releasing the N terminus from the endoplasmic reticulum membrane and allowing the transcription activation domain to enter the nucleus and activate genes. This is based on the sterol response element binding protein model for cholesterol regulation in mammalian cells (3). Deletion of *UPC2* in *C. albicans* results in cells hypersusceptible to all drugs associated with the ergosterol pathway, as well as drugs associated with cell wall stability (16, 25). The deletion also affects other aspects of sterol metabolism and is important for azole regulation of the ergosterol pathway, suggesting that *UPC2* is a master regulator of sterols.

To date, both *CDR1* and *CDR2* appear to be regulated together, although there are a small number of clinical isolates that overexpress only one or the other gene, suggesting that these isolates may contain promoter mutations. Regions of the *CDR1* and *CDR2* promoters have been identified that are important for regulation, including the Drug Response Element that is important for azole induction of the pumps (9). Two other elements, Steroid Response Elements 1 and 2, are responsible for steroid induction of the pump genes but not other drugs (13). A Negative Response Element which binds to an as-yet-unidentified 55-kDa protein has also been identified (10).

Several transcription factors that regulate expression of *CDR1* and *CDR2* have been identified. The factor that recognizes the Drug Response Elements is known as *TAC1,* another member of the Zn_2Cys_6 transcription factor family (6). *TAC1* was first identified by genetic linkage to the mating locus in *C. albicans* (see below). A second transcription factor known to regulate *CDR1* and *CDR2* was identified by a one-hybrid genetic screen in *S. cerevisiae*. This transcription factor, *NDT80,* is related to the *S. cerevisiae NDT80* gene that is involved in controlling meiosis. The promoter regions of *CDR1* and *CDR2* that are recognized by *NDT80* are not yet known. Two additional factors, *FCR1* and *FCR3,* which may regulate azole resistance, have been identified in *C. albicans* based on their complementation of *S. cerevisiae PDR1* and *PDR3,* transcription factors known to regulate efflux pumps in *S. cerevisiae*. *FCR1* encodes another Zn_2Cys_6 transcription factor, while *FCR3* encodes a member of the bZip transcription factor family. It is not known if *FCR1* and *FCR3* regulate resistance through the efflux pumps or other pathways.

The transcriptional regulation of the MF family of genes has not been studied in detail. The *C. albicans* gene *CAP1* is a homolog of the *S. cerevisiae* gene *yAP1,* which regulates the response of fungal cells to oxidative stress and other stress conditions, in part by inducing MF pump genes related to *C. albicans MDR1*. Recently, regulation of *C. albicans MDR1* has been shown to act through the *CAP1* recognition site found in the *MDR1* promoter (11). This element is responsible for induction of the *MDR1* pump by many compounds related to stress but does not respond to azole drugs. However, the study also identified other regions of the *MDR1* promoter that increase expression in response to other drugs. In azole-resistant clinical isolates, it has been shown that *C. albicans MDR1* overexpression is the result of altered transcription factors. However, the factor(s) has not yet been identified.

Microarrays and Genetic Screens

Transcription profiling using microarrays has recently been used to identify other genes associated with resistance (for examples, see references 12 and 22). Many of the identified genes are upregulated either with *CDR1* and *CDR2* or with *MDR1.* This suggests that some of these additional genes might be coregulated by the transcription factors described

above. In each of these analyses, the genes are correlated with resistance but have yet to demonstrate a role in resistance.

Genetic screens have also been used in both *C. albicans* and *C. glabrata* to identify additional genes associated with resistance. A screen in *C. albicans* identified the *C. albicans CKA2* gene, encoding casein kinase II, suggesting a role for kinase signaling in resistance (4). A screen in *C. glabrata* (5, 15) identified genes encoding efflux pumps and transporters as expected from the molecular mechanisms described above, and Ca^{2+} channel regulators and genes involved in mitochondrial function, as expected from the phenotypes described below.

Summary

The resistance mechanisms described to date are summarized in Fig. 1. In a susceptible cell, azole drugs enter the cell by an unknown mechanism and target lanosterol demethylase, Erg11p, which is part of the ergosterol biosynthesis pathway. The *CDR* pumps and the *MDR1* pump are expressed at low levels in susceptible cells, at certain times in the growth of the cells. In a resistant cell, the interaction between the azoles and Erg11p can be altered by mutation or overexpression of the *ERG11* gene. Alterations in other enzymes in ergosterol biosynthesis can also affect azole susceptibility.

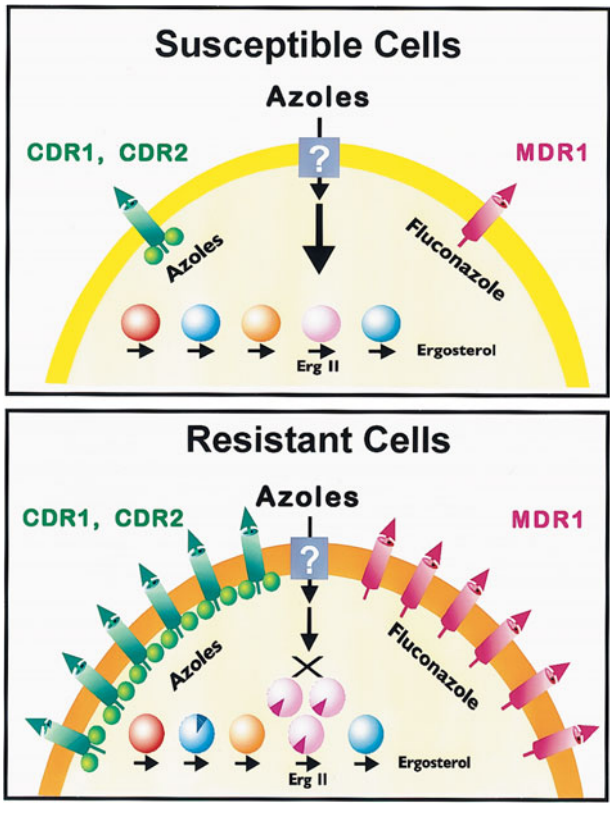


FIGURE 1 Molecular mechanisms of azole resistance. See text for details. Gene products shown in the figure include Erg11p (pink sphere), the *CDR* efflux pumps (green tubes) with ABC domains (green spheres), the *MDR* efflux pump (red tubes), and other ergosterol biosynthetic enzymes (spheres of assorted colors). Point mutations are shown as dark slices in pink and blue spheres. (Figure taken from White [27] with permission.)

Both the *CDR* gene family and the *MDR1* pump can be overexpressed, reducing the amount of drug within the cell. The *CDR* gene family appears to promote efflux for all azoles tested, while the MF pump *MDR1* appears to be specific for fluconazole and voriconazole.

Resistance Is a Composite

Resistance in *C. albicans* clinical isolates is not usually the result of a single alteration. Instead, several alterations appear to contribute to the generation of a resistant cell. Each alteration allows the corresponding isolate to outgrow the other cells in the population, becoming fixed as the major strain. Subsequent alterations allow the strain to gradually develop a highly resistant phenotype, the result of several alterations, each of which increases the resistance of the cell only slightly. This has been well documented in a series of 17 sequential isolates from a single patient (30). All of the isolates were of the same strain, although early in the infection, a substrain appears to have become the major strain. After substrain selection, the *MDR1* gene was overexpressed, correlating with a significant increase in resistance. Later in the infection, isolates acquired an R467K mutation in *ERG11,* so that the Erg11p enzyme became more resistant to azole and the cells had an increased azole resistance. At the same time, the *ERG11* gene was overexpressed, and allelic differences were eliminated, suggesting that a gene conversion or mitotic recombination copied the R467K mutation to both alleles. Even later in the infection, the *CDR1* and *CDR2* genes were overexpressed, again correlating with a significant rise in azole resistance. The timing and the order in which each of these changes occurred is unlikely to have been critical to the development of resistance, but the sum of each of these alterations is a resistant phenotype.

Unidentified Mechanisms

While significant progress has been made in the characterization of molecular mechanisms associated with azole resistance, most of the resistant isolates characterized are oral isolates from AIDS patients with oral candidiasis. When a series of systemic isolates was characterized, some isolates were identified with the same molecular mechanisms of resistance. However, a significant number of azole-resistant systemic isolates showed no alterations in the target enzyme or efflux pumps. It is likely that these strains have alterations in drug import or processing or other, as-yet-unidentified mechanisms.

Resistance in Perspective

Several generalizations concerning resistance can be made based on the work from the last 10 years. First, the predominant molecular mechanism of azole drug resistance in oral isolates is overexpression of efflux pumps, especially *CDR1* and *CDR2*. However, resistance mechanisms in systemic isolates include as-yet-unidentified factors. Unlike resistance in parasite and mammalian systems, drug resistance in the fungi is not normally associated with gene amplification. Fungi in general have elaborate systems for the regulation of gene transcription, and thus, gene amplification is not necessary to alter gene expression. Unlike studies in prokaryotes, studies with fungi have not yet identified any plasmids or other episomes containing resistance markers. Finally, there is no documented situation in which the resistance markers from one isolate were transferred to another isolate, although this situation has not been investigated in detail.

TABLE 2 Cellular phenotypes associated with resistance

Cellular phenotype	Characterization by MIC[a]
Homozygosity at *MTL* locus	Associated with resistant MIC
Trailing	Resistant MIC
Calcineurin effects	No change in standard MIC
Heterogeneous resistance	Susceptible MIC
High-frequency azole resistance	Susceptible → resistant MIC
Inducible resistance	Susceptible → resistant MIC
Transient resistance	Resistant → susceptible MIC
Stable resistance	Consistent resistant MIC
Cross-resistance	Resistant MIC for many drugs
Altered fitness	No change in standard MIC

[a] Standard MIC_{80} at 48 h.

PHENOTYPES ASSOCIATED WITH AZOLE RESISTANCE

MIC susceptibility testing is important in determining a strain's basic response to antifungal drugs. However, in the last several years, several phenotypes associated with resistance have been identified (Table 2) which do not fit simply into the standard susceptible/resistant definitions of MIC testing and which do not appear to be directly the result of the molecular mechanisms described above. Understanding these phenotypes will give additional insight into how a fungal cell responds to antifungal drugs.

Mating Type

As mentioned above, *C. albicans* has two mating types, a and α, defined by the gene set at the *MTL* (mnemonic for mating-type-like) locus. Most strains are diploid a/α. However, strains that are homozygous at the *MTL* locus do exist (a/a and α/α). These homozygous strains are able to undergo phenotypic switching between two cell states, white and opaque. Opaque cells are the mating-competent forms of *C. albicans* that can undergo fusion to form tetraploids. A correlation has been identified between homozygosity at the *MTL* locus and azole resistance. All *MTL* homozygotes are not resistant, and all azole-resistant isolates are not homozygous at the *MTL* locus, but there is an increased frequency of *MTL* homozygotes in azole-resistant strains. This correlation between azole resistance and *MTL* homozygosity has several possible explanations. The transcription factor *TAC1,* which regulates expression of the *CDR1* and *CDR2* efflux pump genes, is genetically linked to the *MTL* locus (6). It is also possible that a mating reaction creates genetic variation, and drug pressure may then select for strains with new genetic compositions. As 50% of the progeny of a mating reaction should be *MTL* homozygotes, then the correlation between azole resistance and *MTL* homozygosity may be the link to mating and genetic variability.

Trailing

Standard susceptibility testing monitors growth at 48 h, and the MIC is defined as the concentration of drug that inhibits growth by 80% (growth is less than 20% of control). Some fungal isolates appear susceptible at 24 h of growth but resistant at 48 h of growth. This difference between the 24- and 48-h growth is known as trailing. Trailing growth at 48 h is usually the result of growth inhibition between 50 and 80%, while growth of true resistant strains is usually similar to the no-drug control. Trailing is a result of residual growth, which is growth of cells above the MIC because the azole drugs are fungistatic, rather than fungicidal. Clinically, infections with these trailing isolates are usually treatable with azole drugs at standard drug concentrations. Consistent with this, trailing isolates behave as susceptible isolates in animal models of candidiasis. It has been observed that trailing can be reduced in acidic media. This may have consequences in the treatment of vaginal isolates in cases where the vaginal pH is more acidic than blood. Under those conditions, trailing would be reduced, which is consistent with the observation that resistance has not been observed in vaginal yeast infections.

Tolerance

Strains that show significant residual growth, or trailing, show drug tolerance, which is associated with fungistatic drugs. Azole drug tolerance is lost in the presence of cyclosporine A, which inhibits fungal calcineurin, an important factor in Ca^{2+} regulation. Cyclosporine A eliminates trailing and drug tolerance, essentially making a fungistatic drug fungicidal. However, cyclosporine does not alter the MIC of the isolate, just its residual growth above the MIC. The use of cyclosporine A with azole drugs decreases the fungal load in animal models, suggesting that the drug combination may have important clinical significance.

Heterogeneous Antifungal Resistance

Heterogeneous resistance is an unusual phenomenon in which a small percentage of the cells in a population are resistant. Only 1 to 5% of isolates in a collection exhibit heterogeneous resistance. When a strain capable of heterogeneous resistance is spread on an agar plate containing drug, colonies form at a low frequency (1 in 10 to 1 in 100,000). The important observation is that these colonies are not 100% resistant; they contain only a small percentage of resistant cells. If the colony is respread on another plate, the same low proportion of resistant cells is observed. The resistant phenotype is not genetically fixed, but the ability of a small percentage to be resistant is fixed in these strains. This phenomenon is called heterogeneous resistance, and it has been observed in bacteria including *Staphylococcus* and *Enterococcus*. Heterogeneous resistance has been observed for azoles in *C. albicans* and *C. neoformans*. The genetic mechanisms of heterogeneous resistance have not been identified in any organism, and this heterogeneous resistance is a significant problem because the strains that show heterogeneous resistance are susceptible by the standard CLSI method for determining MICs.

High-Frequency Azole Resistance

A resistance phenotype that resembles heterogeneous resistance is known as high-frequency azole resistance (HFAR). HFAR isolates are able to form colonies at low frequencies (1 in 10 to 1 in 10,000) on agar containing azole drugs. However, unlike heterogeneous resistant colonies, these HFAR colonies retain their resistance even when grown in the absence of drug. HFAR colonies selected for fluconazole resistance are cross-resistant to all azoles and hypersusceptible to AmB. HFAR has been observed in *C. albicans, C. glabrata,* and even *S. cerevisiae,* although it has not been observed in *C. neoformans*. HFAR in *C. glabrata* has been associated with loss of mitochondrial function. This is consistent with the observation that *C. glabrata* petite mutants (cells lacking mitochondrial DNA) are azole resistant and with the observation that genes with mitochondrial function are identified in a screen for azole resistance (14). It is unlikely that fungal cells without mitochondrial function would survive in an animal model of infection, but these observations provide information on the mechanism behind HFAR.

Inducible Azole Resistance

Most susceptible strains of *C. albicans* cannot be induced in vitro to form resistance, perhaps because *C. albicans* is diploid and point mutations have no effect unless they are dominant or codominant mutations. However, there are a small number of strains in which resistance can be induced. It is possible that these strains have preexisting alterations in genes associated with drug resistance, although the mechanisms allowing the induction of resistance have not been studied. In one set of induced strains, the induction of resistance is associated with an increased expression of the efflux pumps *CDR1* and *CDR2*.

Transient Resistance

Resistance mutations are usually genetically stable and persist in cells in the absence of drug pressure. However, there are reversible mechanisms by which cells can be induced to be resistant in the presence of drug, including gene activation and repression. If resistance is the result of a reversible cell state, then resistance will persist in the presence of drug, and the cells will revert to a susceptible phenotype in the absence of drug. Such inducible resistance has been documented in vitro and in vivo.

Clinical isolates of *C. albicans* from BMT patients have been shown to have transient azole resistance, and some strains are predisposed to induction. When characterized, this transient resistance is associated with increased expression of the *CDR* efflux pumps, while the loss of resistance is associated with reduced expression of the same pumps.

Stable Resistance

Mutations occur randomly in a strain, including alterations that render the strain resistant. Such mutations might include point mutations in the *ERG11*, in the promoters of efflux pumps, or in the transcription factors that regulate those pumps. When these resistance mutations occur during an infection, the mutation would allow those cells to selectively outgrow the other cells in the population under drug pressure. Genetically fixed mutations should render the cell stably resistant to drugs. When these strains are grown in the absence of drugs, the resistant phenotype will persist if the isolates are relatively fit compared to a susceptible version of the strain. In general, stable and fit resistant isolates are obtained from patients who have received long-term antifungal treatment, such as AIDS patients.

Cross-Resistance

Based on the molecular mechanisms outlined above, it is expected that clinical isolates resistant to one azole such as fluconazole should be cross-resistant to other azoles. Point mutations in the target enzyme Erg11p may alter the active site or another part of the enzyme such that one or more azoles are unable to inhibit the enzyme. Overexpression of the *CDR* efflux pumps appears to render cells resistant to most, if not all azoles, while overexpression of the *MDR1* efflux pump does appear to be specific for fluconazole and voriconazole (30). This is consistent with MIC testing for azole-resistant clinical isolates. The *CDR* pumps alter susceptibility to fluconazole, voriconazole, itraconazole, and ketoconazole, as well as nystatin, terbinafine, and amorolfine. They do not have an effect on AmB or 5FC or two other antifungals, nikkomycin or griseofulvin. The *MDR1* efflux pump has an effect on fluconazole and to some extent voriconazole but does not have an effect on the other drugs mentioned above.

Fitness

Alterations in the drug resistance genes described above may affect the overall fitness of a strain, such that it might be less fit in the absence of drug than its susceptible counterparts. Lack of fitness may compromise the virulence of a strain. In one study, fitness was assessed by testing the virulence of matched susceptible and resistant isolates; there was no obvious correlation. Subsequent in vitro studies have been informative (7, 8). When an inducible susceptible isolate was grown in the presence of drug, it developed resistance. The developing resistant population was initially less fit than the susceptible parent when grown in the absence of drug. However, fitness improved under continued drug pressure until the resistant strain was as fit as the parent susceptible strain in the absence of drug. This fitness appears to be independent of the molecular mechanisms of resistance.

Phenotypes and MIC

In this section, several phenotypes associated with resistance have been described. These phenotypes are not usually detected with standard MIC susceptibility testing (Table 2). Even trailing will not be detected unless the investigator measures growth at both 24 and 48 h. There is no progression through these phenotypes during the development of resistance. One does not follow from the other. And strains can exhibit one or more of these phenotypes. The molecular basis has not been determined for most of these phenotypes. Understanding these phenotypes is important, in addition to MIC characterizations, to determine how a strain will behave in a patient.

MOLECULAR MECHANISMS OF NON-AZOLE DRUG RESISTANCE

Resistance to 5FC

Up to 10% of *C. albicans* clinical isolates have intrinsic resistance to 5FC, and 30% of the susceptible isolates develop acquired resistance during drug therapy. Resistance can be associated with mutations in any of the following: cytosine deaminase that converts 5FC to 5-fluorouracil, uracil phosphoribosyltransferase that is important for nucleic acid synthesis, or either of two purine-cytosine permeases. Recently, mutations in one of the purine-cytosine permeases of *C. lusitaniae* were shown to result in cross-resistance to 5FC and fluconazole, suggesting that fluconazole import may be related to 5FC import (18).

C. albicans can be divided into five clades, or groupings, based on molecular markers such as the repetitive marker Ca3 (see above). Recently, *C. albicans* 5FC resistance has been shown to be specific to clade 1 and linked to mutation in the uracil phosphoribosyltransferase gene *FUR1* (19) . In haploid *C. neoformans,* 5FC resistance can result from a single mutation at either of two specific gene loci, *FCY1* and *FCY2*.

Polyene Resistance

AmB-resistant clinical isolates have been identified in *C. tropicalis* and have been constructed in *C. albicans, C. neoformans, A. nidulans,* and *Aspergillus fennelliae* either by mutagenesis or serial passage in the presence of increasing amounts of drug. Most isolates have greatly reduced levels of ergosterol in their plasma membranes, decreasing the binding ability of AmB. Sterol analysis suggests that these isolates are defective in *ERG2* or *ERG3,* although definitive molecular analyses have not been performed. Alterations in the structure of the membrane or the sterol/phospholipid ratio may

also be associated with AmB resistance. Acquired resistance has also been described in cases where environmental isolates of *C. neoformans* were passaged in a mouse model of cryptococcosis. The passaged isolates became more resistant to AmB in the absence of drug, suggesting that resistance levels can be altered by interactions with the host.

Caspofungin Resistance

The echinocandin caspofungin has recently become clinically available, so caspofungin-resistant clinical isolates are limited in number. In vitro, resistance is usually generated by mutation in genes encoding the target enzyme, glucan synthetase, and in proteins that interact with the target. There is some indication in both *C. albicans* and *A. fumigatus* that strains overexpressing *CDR2* may have increased resistance to caspofungin (24), although this change in susceptibility is not detected by MIC analysis.

CLINICAL IMPLICATIONS AND FUTURE DIRECTIONS

Dosing

There have been no clinical studies to determine the optimal dosing of antifungal drugs to prevent resistance. In general, azole drugs should not be used prophylactically in the prevention of oral or vaginal candidiasis. However, the azoles are clearly important in the prevention of systemic infections and in the prevention of recurrent cryptococcal meningitis infections. Based on drug resistance in a variety of organisms, continuous treatment with a large dose of drug for the minimum time period required to cure the infection will have the least risk for the development of resistance.

Detection

The CLSI susceptibility test is satisfactory for determining the MICs of most yeasts and filamentous fungi. Given the multiple molecular mechanisms of resistance presented in Fig. 1 and the real possibility that there exist other mechanisms of resistance which have not been identified, a simple molecular test for resistance would be difficult to develop.

Drug Development

There is always a need for new antifungal agents that are effective against strains and species that show resistance to current therapies. New azole drugs, such as voriconazole, ravuconazole, and posaconazole, need to be carefully monitored for their effect on strains that are resistant to fluconazole.

The multiple efflux pumps in fungal genomes and the broad substrate specificity of each of these pumps suggest that strains or species with high levels of efflux may be resistant to many classes of drug. Strategies to interfere with the pumps or inhibit pump activity are currently the focus of drug development in several industry and biotechnology settings.

Drug combinations are likely to become more important in future antifungal drug strategies. AmB is currently used in combination with 5FC and with azole drugs. Azoles in combination with 5FC or with terbinafine have been discussed and tested. With the development of new antifungal drugs, such as the cell wall inhibitors (echinocandins such as caspofungin by Merck), the possible drug combinations increase dramatically.

The best way to improve the treatment success in a patient is to improve their immune status. Strategies using cytokines have been tested in the treatment of fungal disease, but further work is clearly required before such strategies become clinically applicable.

An increased understanding of antifungal drug resistance should allow for the development of new diagnostic strategies to identify resistant clinical isolates in a patient, new treatment strategies to treat these resistant infections, and new prevention strategies that would forestall the development of antifungal drug resistance in these patient populations. One fact is clear: as strategies emerge to treat resistant fungal infections, the fungal cells will continue to evolve new mechanisms of antifungal drug resistance.

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Susceptibility Test Methods: Yeasts and Filamentous Fungi

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The introduction of modern patient management technologies and therapies has resulted in a rapidly expanding number of chemically induced immunosuppressed patients who are highly susceptible to severe fungal infections. Fungi are also important nosocomial pathogens causing severe morbidity and mortality in hospitalized patients with a combination of a variety of risk factors and immunosuppression (40, 46, 85, 88). Systemic infections caused by non-*albicans Candida* spp., *Aspergillus* spp., and other filamentous fungi (molds) (1, 30, 46) are being reported more frequently. As fungal infections became an important public health problem and resistance to established antifungal agents began to emerge (21, 24, 47, 51, 55, 93), pharmaceutical companies developed new agents with either a broader spectrum or different targets of activity.

Twelve agents have been approved for the treatment of systemic fungal diseases. Conventional amphotericin B (1956; E. R. Squibb & Sons) and its lipid formulations are polyene macrolide antibiotics (50). Resistance to amphotericin B has assumed clinical importance, particularly with certain species, such as *Aspergillus terreus* and other emerging fungal pathogens (55, 93). Amphotericin B is unstable when subjected to heat, light, or acid pH.

Flucytosine (5-FC; Hoffmann-LaRoche Inc.) is a water-soluble, stable, fungistatic antifungal compound used orally in the treatment of systemic infections caused by susceptible pathogenic or opportunistic yeasts and other fungi; development of resistance during treatment is common (23).

Miconazole, ketoconazole, fluconazole, itraconazole, and voriconazole have fungistatic activity against a variety of fungi; voriconazole has fungicidal activity in vitro and in vivo efficacy against *Aspergillus* spp. and other opportunistic molds (27, 36, 49, 67, 81). There are two additional triazoles of note: posaconazole (SCH 56592; Schering-Plough Research Institute, Kenilworth, N.J.), which has recently been approved, and ravuconazole (BMS 207147; Bristol-Myers Squibb, Wallingford, Conn.), which is under clinical evaluation. These two triazoles are non-water soluble and also have a broad spectrum of in vitro and in vivo activity against some yeasts and molds (22, 25, 44, 78, 81). Posaconazole has activity against some zygomycetes (19, 91).

The echinocandins caspofungin (MK-0991; Merck & Co., Inc.), micafungin (SK; Astellas Pharma US Inc., Deerfield, Ill.), anidulafungin (LY 303366, V-echinocandin; Pfizer), and aminocandin (Indevus Pharmaceuticals, Inc.) are inhibitors of 1,3-β-D-glucan synthesis, which leads to the lysis of growing fungal cells. Caspofungin is water-soluble, while aminocandin, anidulafungin, and micafungin lack water solubility. The echinocandins are active in vitro and in vivo against all yeasts except *Cryptococcus neoformans* and *Trichosporon* spp., and against some molds (25, 29, 59, 62, 66, 82, 95). On the other hand, the nikkomycin class inhibits chitin synthase in the fungal cell wall (51).

The mechanisms of resistance to antifungal agents and more detailed information on new and established agents are found elsewhere (chapters 129 and 130).

ANTIFUNGAL SUSCEPTIBILITY TESTING

Rationale

Ideally, in vitro susceptibility tests (i) provide a reliable measure of the relative activities of two or more antifungal agents, (ii) correlate with in vivo activity and predict the likely outcome of therapy, (iii) provide a means with which to monitor the development of resistance among a normally susceptible population of organisms, and (iv) predict the therapeutic potential of newly discovered investigational agents (40). The development of the Clinical and Laboratory Standards Institute (CLSI; formerly the National Committee for Clinical Laboratory Standards [NCCLS]) reference method M27-A2 (13) has improved the reproducibility of in vitro antifungal susceptibility data and facilitated the establishment of interpretive breakpoints for the triazoles fluconazole and itraconazole and more recently for voriconazole (13, 16, 87). Based on historical data and the pharmacokinetics of 5-FC, interpretive breakpoints for 5-FC and *Candida* spp. also have been established (13). Some correlation has been suggested between amphotericin B MIC results obtained by nonstandardized methods and clinical outcome. Unfortunately, most M27-A amphotericin B MICs for yeasts are within a very narrow range (0.25 to 1 μg/ml) (13), precluding a clear discrimination between susceptible and potentially resistant isolates. Also, data are not available for the evaluation of an optimal procedure for the detection of amphotericin B resistance.

With the use of both established and investigational agents has come the recognition of resistance to one or more antifungal agents in selected isolates. As a result, clinical laboratories are now being asked to assume a greater role in the selection and monitoring of antifungal chemotherapy. The methods that have been more frequently applied to

TABLE 1 Methods used for antifungal susceptibility testing

Test method	Means of endpoint determination
Broth macrodilution (yeasts)	Visual comparison of turbidity with turbidity of 80% inhibition standard (1:5 growth control dilution) (M27-A2 document)
Broth microdilution (yeasts)	Visual comparison of growth (≥50% inhibition) with that of growth control (M27-A document)
Colorimetric microdilution (yeasts [YeastOne] and molds)	Visual observation of color change
Spectrophotometric microdilution (yeasts)	Turbidimetric MIC determination by spectrophotometer
Macro- and microdilution (filamentous fungi)	Visual comparison of growth (100% inhibition) with that of growth control (M38-A document)
Agar macrodilution (yeasts and molds, standard dishes)	Visual
Agar diffusion (yeasts and molds)	
Disk (yeasts)	Zone diameter (visual)
Antifungal strip (E test)	Ellipse of inhibition (visual)

antifungal susceptibility testing are listed in Table 1. The CLSI (formerly NCCLS) Subcommittee on Antifungal Susceptibility Testing has developed reference methods for broth macro- and microdilution susceptibility testing of yeasts (CLSI M27-A2 document) and molds (CLSI M38-A document) and more recently a disk diffusion method for yeasts (CLSI M44-A document) (Tables 2 to 8) (13–15).

In vitro antifungal susceptibility testing is influenced by a number of technical variables, including inoculum size and preparation, medium formulation and pH, duration and temperature of incubation, and the criterion used for MIC endpoint determination. In addition, antifungal susceptibility testing is complicated by problems unique to fungi, such as slow growth rates (relative to bacteria) and the ability of

TABLE 2 CLSI (formerly NCCLS M27-A2 document) broth dilution guidelines for antifungal susceptibility testing of yeasts[a]

Parameter	Description
Broth medium	RPMI 1640 broth buffered with MOPS buffer (0.165 M) and 0.2% dextrose to a pH of 7.0 at 25°C
Medium modifications	(i) Yeast nitrogen base broth (pH 7.0) with MOPS provides better growth for *C. neoformans* and (ii) RPMI-1640 with 2% dextrose
Inoculum preparation	From 24-h (*Candida* spp.) or 48-h (*C. neoformans*) cultures on Sabouraud dextrose agar
Stock inoculum suspension	Adjusted by spectrophotometer at 530 nm to match the turbidity of a 0.5 McFarland standard (1×10^6 to 5×10^6 CFU/ml)
Test inoculum	1:2,000 (macrodilution) or 1:1,000 (microdilution) dilutions with medium of the stock inoculum suspension; inoculum size after inoculation, 0.5×10^3 to 2.5×10^3 CFU/ml (both methods)
Drug dilutions	Additive 10× (macrodilution) or 2× (microdilution) twofold drug dilutions with medium (fluconazole, caspofungin, and 5-FC) or 100× with solvent (amphotericin B, other azoles, anidulafungin, and micafungin)
Drug dilution ranges	
5-FC and flucytosine	0.12–64 μg/ml
Other drugs	0.03–16 μg/ml
Methods	
Macrodilution	0.9 ml of diluted test inoculum plus 0.1 ml of 10× drug concentration
Microdilution	100 μl of diluted test inoculum plus 100 μl of 2× drug concentration
Growth control(s)	
Macrodilution	0.9 ml of diluted inoculum plus 0.1 of drug-free medium (or plus 2% of solvent)
Microdilution	100 μl of diluted inoculum plus 100 μl of drug-free medium (or plus 2% of solvent)
MIC by visual examination	
Amphotericin B, macro- and microdilution	Lowest drug concentration that prevents any discernible growth
5-FC, azoles, caspofungin, and other echinocandins	(i) Lowest drug concentration that shows prominent (~50%) growth inhibition (microdilution) and (ii) lowest drug concentration that matches an 80% inhibition standard (macrodilution)
Reading modification[b]	*C. neoformans* MICs determined spectrophotometrically at 492 nm after 48 h of incubation

[a] Data from references 13 and 65.
[b] M27 document includes this reading modification when using the broth microdilution method with yeast nitrogen base broth and a 10^4-CFU/ml inoculum.

TABLE 3 MIC ranges for QC and reference isolates for CLSI broth macrodilution and microdilution methods[a]

QC or reference isolates	Antifungal agent	MIC range (μg/ml)		
		48 h, macrodilution	24 h, microdilution	48 h, microdilution
QC isolates				
Candida parapsilosis ATCC 22019	Amphotericin B	0.25–1.0	0.25–2.0	0.5–4.0
	Fluconazole	2.0–8.0	0.5–4.0	1.0–4.0
	Itraconazole	0.06–0.25	0.12–0.5	0.12–0.5
	Ketoconazole	0.06–0.25	0.03–0.25	0.06–0.5
	Posaconazole	NA	0.06–0.25	0.06–0.25
	Ravuconazole	NA	0.016–0.12	0.03–0.25
	Voriconazole	NA	0.016–0.12	0.03–0.25
	5-FC	0.12–0.5	0.06–0.25	0.12–0.5
	Caspofungin	NA	0.25–1.0	0.5–4.0
	Anidulafungin	NA	0.25–2.0	0.5–2.0
Candida krusei ATCC 6258	Amphotericin B	0.5–2.0	0.5–2.0	1.0–4.0
	Fluconazole	16–64	8.0–64	16–128
	Itraconazole	0.12–0.5	0.12–1.0	0.25–1.0
	Ketoconazole	0.12–0.5	0.12–1.0	0.25–1.0
	Posaconazole	NA	0.06–0.5	0.12–1.0
	Ravuconazole	NA	0.06–0.5	0.25–1.0
	Voriconazole	NA	0.06–0.5	0.12–1.0
	5-FC	4.0–16	4.0–16	8.0–32
	Caspofungin	NA	0.12–1.0	0.25–1.0
	Anidulafungin	NA	0.03–0.12	0.03–0.12
Paecilomyces variotti ATCC MYA-3630	Amphotericin B	NA	NA	1.0–4.0
	Itraconazole	NA	NA	0.06–0.5
	Posaconazole	NA	NA	0.03–0.25
	Voriconazole	NA	NA	0.015–0.12
	Ravuconazole	NA	NA	0.5–4.0
Reference isolates				
Aspergillus flavus ATCC 204304	Amphotericin B	NA	NA	0.5–4.0
	Itraconazole	NA	NA	0.25–0.5
	Posaconazole	NA	NA	0.06–0.5
	Voriconazole	NA	NA	0.5–4
Aspergillus flavus ATCC MYA-3631	Amphotericin B	NA	NA	1.0–8.0
	Posaconazole	NA	NA	0.12–1.0
	Voriconazole	NA	NA	0.5–2.0
Aspergillus fumigatus ATCC MYA-3626	Amphotericin B	NA	NA	0.5–4.0
	Posaconazole	NA	NA	0.25–2.0
	Voriconazole	NA	NA	0.25–1.0
Aspergillus fumigatus ATCC MYA-3627	Amphotericin B	NA	NA	0.5–4.0
	Posaconazole	NA	NA	>16
	Voriconazole	NA	NA	0.25–1.0
Aspergillus terreus ATCC MYA-3633	Amphotericin B	NA	NA	2.0–8.0
	Voriconazole	NA	NA	0.25–1.0
Fusarium moniliforme ATCC MYA-3629	Amphotericin B	NA	NA	2.0–8.0
	Itraconazole	NA	NA	>16
	Posaconazole	NA	NA	0.5–2.0
	Voriconazole	NA	NA	1.0–4.0
Scedosporium apiospermum ATCC MYA-3635	Amphotericin B	NA	NA	4.0–16
	Posaconazole	NA	NA	1.0–4.0
	Voriconazole	NA	NA	0.5–2.0
Trichophyton mentagrophytes MRL 1957	Ciclopirox	NA	NA	0.5–2.0
	Griseofulvin	NA	NA	0.12–0.5
	Itraconazole	NA	NA	0.03–0.25
	Posaconazole	NA	NA	0.03–0.25
	Terbinafine	NA	NA	0.002–0.008
	Voriconazole	NA	NA	0.03–0.25
T. rubrum MRL 666	Ciclopirox	NA	NA	0.5–2.0
	Fluconazole	NA	NA	0.5–4.0
	Voriconazole	NA	NA	0.008–0.06

[a] Data from references 8, 33, and 52 and from the CLSI Subcommittee Meeting Minutes, January 2006. NA, not available for yeasts; not applicable for molds.

TABLE 4 Zone diameters for QC isolates for CLSI disk diffusion method[a]

Antifungal agent	Disk content (μg)	Zone diameter (mm)			
		C. albicans ATCC 90028	*C. parapsilosis* ATCC 22019	*C. tropicalis* ATCC 750	*C. krusei* ATCC 6258
Fluconazole	25	28–39	33–33	26–37	—[b]
Voriconazole	1	31–42	28–37	—[b]	16–25
Posaconazole	5	24–34	25–36	23–33	23–31

[a] Data from references 7, 15, and 70 and from the CLSI Subcommittee Meeting Minutes, January 2006.
[b] —, zone diameters not established due to lack of interlaboratory reproducibility.

certain fungi (dimorphic) to grow either as a unicellular yeast form that produces blastoconidia or as a hyphal or filamentous fungal form that may produce conidia or sporangiospores, depending on pH, temperature, and medium composition. Finally, the basic properties of the antifungal agents themselves, such as solubility, chemical stability, modes of action, and the tendency to produce partial inhibition of growth over a wide range of concentrations above the MIC, must be taken into account.

Standardized Broth Dilution Methods for Yeasts

As a result of several collaborative studies (8, 39, 43, 72), the M27-A2 document (approved standard, second edition) is available for testing yeast fungi such as *Candida* spp. and *C. neoformans* by both macrodilution and microdilution broth formats (13); breakpoints for fluconazole, itraconazole, voriconazole, and 5-FC versus *Candida* spp. have been established (see Table 6). Other relationships between results obtained with the reference method and patient response have not been established, e.g., among others, for *C. neoformans* and other yeasts and yeast-like organisms or other agents.

Microdilution

The broth microdilution test (Table 2) has become the most widely used technique for antifungal susceptibility testing; this approach is described in the M27-A2 document (13) and also below. Although the antifungal broth macrodilution test was the first method proposed by the CLSI Subcommittee, this test is cumbersome for use in the clinical laboratory. The microdilution test provides consistent MIC results, and interlaboratory agreement of the microdilution MICs can be higher than that of the macrodilution MICs for some drugs (39).

Standard Medium

The test medium recommended by the Subcommittee is the RPMI 1640 (RPMI) with L-glutamine and a pH indicator and without sodium bicarbonate broth (04-525Y from BioWhittaker, Walkersville, Md., and American Biorganics, Inc., Niagara Falls, N.Y.; and R-6504, from Sigma Chemical Co., St. Louis, Mo.). The medium should be buffered to a pH of 7.0 at 25°C with MOPS (morpholinepropanesulfonic acid; final molarity at pH 7.0, 0.165). The RPMI medium is suitable for testing most fungi (13, 14, 35, 39, 43), but it may not be adequate to support the growth of some strains of *C. neoformans* or to determine amphotericin B MICs. RPMI medium containing 2% dextrose (13, 18) and yeast nitrogen base broth (2, 89) may enhance the growth of yeasts, facilitating the determination of MICs. However, 2% dextrose does not have a significant impact on the growth density at 24 h and it could falsely elevate the MICs at 48 h (64) and produce lower MICs at 24 h. The European Committee on Antibiotic Susceptibility Testing (EUCAST) method for testing fermentative yeasts has been proposed in Europe (to be described later), which includes the use of this modified RPMI medium (18).

Drug Stock Solutions

Antifungal standards can be obtained directly from the drug manufacturers or from the U.S. Pharmacopeia (Rockville, Md.). Clinical intravenous or oral preparations should not be used (13, 14). Antifungal stock solutions should be prepared at concentrations at least 10 times the highest concentration to be tested (e.g., 1,280 μg/ml for fluconazole and 5-FC).

TABLE 5 CLSI M44-A document guidelines for antifungal disk diffusion susceptibility testing of *Candida* spp.[a]

Paramenter	Description
Agar medium	Mueller-Hinton agar + 2% dextrose and 0.5 μg of methylene blue dye/ml
Inoculum preparation	From 24-h cultures on Sabouraud dextrose agar as described in Table 2 for broth micro- and macrodilution methods
Test medium	Stock inoculum suspension, adjusted by spectrophotometer at 530 nm to match the turbidity of a 0.5 McFarland standard: 1×10^6 to 5×10^6 CFU/ml
Disk contents	Fluconazole, 25 μg; voriconazole, 1 μg; posaconazole, 5 μg
Incubation conditions	20 to 24 h at 35°C
Reading zone diameter	To the nearest whole millimeter at the point at which there is prominent reduction in growth. Pinpoint microcolonies at the zone edge or large colonies within the zone should be ignored.

[a] Data from reference 15.

TABLE 6 Interpretive MIC breakpoints and corresponding zone diameters for in vitro susceptibility testing of *Candida* species[a]

Antifungal agent	MIC breakpoint, in μg/ml (zone diameter, in mm)			
	Susceptible	Susceptible–dose dependent	Intermediate	Resistant
Fluconazole	≤8 (≤19)	16–32 (15–18)		≥64 (≤14)
Voriconazole	≤1 (≥17)	2 (14–16)		≥4 (≤13)
Itraconazole	≤0.125 (NA)	0.25–0.5 (NA)		≥1 (NA)
Flucytosine (5-FC)	≤4 (NA)		8–16 (NA)	≥32 (NA)

[a] Data from references 13, 15–17, 87. For fluconazole and voriconazole, these guidelines are based on mucosal candidiasis and for fluconazole on limited data for invasive *Candida* infections. For itraconazole, the data are based on mucosal infections only. For fluconazole, susceptibility for S-DD MICs depends on achieving the maximal possible blood level, >400 mg/dose. (*C. krusei* is intrinsically resistant to fluconazole.) For itraconazole, a concentration in plasma of >0.5 μg/ml may be required for optimal response. For flucytosine, the clinical significance of intermediate MICs is still uncertain. NA, not available.

Solutions of standard powders of 5-FC (Hoffmann-LaRoche), fluconazole (Pfizer Pharmaceuticals), caspofungin (Merck Co.), or any other water-soluble agent are prepared in distilled water. For testing non-water-soluble agents, sufficient drug standard should be weighed to prepare a solution of 1,600 μg/ml. These standard substances may be solubilized in dimethyl sulfoxide, polyethylene glycol (posaconazole), or dimethylformamide. The actual amount to be weighed must be adjusted according to the specific biological activity of each standard. Amphotericin B solutions must be protected from light, and drug stock solutions prepared with solvents should be allowed to stand for 30 min before use.

The sterile stock solutions may be stored in small volumes in sterile polypropylene or polyethylene vials carefully sealed at −60°C (preferably) or below for 6 months or more without significant loss of activity, but never at a temperature higher than −20°C. Vials should be removed as needed and used on the same day. Any unused drug should be discarded at the end of the day. The use of quality control (QC) strains (8, 13, 33, 52), such as those listed in Table 3, will help in evaluating drug activity.

Preparation of Inocula

Inocula should be prepared by the spectrophotometric method (13, 72) as outlined in Table 2. The yeasts are grown on plates of Sabouraud agar (Emmons 11589 Sabouraud dextrose agar from BBL; or modified 0747 Sabouraud agar from Difco) at 35°C and subcultured at least twice to ensure purity and viability. The inoculum suspension is prepared by picking five colonies, each at least 1 mm in diameter, from 24-h-old cultures of *Candida* spp. or 48-h-old cultures of *C. neoformans* and suspending the material in 5 ml of sterile 0.85% NaCl. The turbidity of the cell suspension measured at 530 nm is adjusted with sterile saline to match the transmittance produced by a 0.5 McFarland barium sulfate standard. This procedure produces a cell suspension containing 1×10^6 to 5×10^6 CFU/ml, which is then diluted 1:1,000 with RPMI medium to provide the 2× test inoculum (1×10^3 to 5×10^3 CFU/ml). The 2× inoculum is diluted 1:1 when the wells are inoculated to achieve the desired final inoculum size (0.5×10^3 to 2.5×10^3 CFU/ml).

TABLE 7 Optical density ranges and mean inoculum sizes for filamentous fungi[a]

Fungus	OD range	Mean inoculum size (10^6 CFU/ml)
Aspergillus spp.	0.09–0.11	1.6
Bipolaris spp.	0.2–0.4	0.6
Dactylaria constricta	0.15–017	1.1
Cladophialophora bantiana	0.15–0.17	1.1
Fusarium spp.	0.15–0.17	3.0
Paecilomyces lilacinus	0.09–0.13	2.1
Scedosporium apisospermum	0.15–0.17	1.0
Rhizopus arrhizus	0.15–0.17	1.3
Scedosporium prolificans	0.15–0.17	0.8
Sporothrix schenckii	0.09–0.11	2

[a] Data from multicenter studies (14, 34, 35, 37, 38). Optical densities at 530 nm.

Drug Dilutions and Performance of Microdilution Test for Yeasts

For drugs dissolved in solvents other than water (e.g., the polyenes, itraconazole, new triazoles, and anidulafungin), intermediate test drug dilutions are prepared from stock solutions to be 100 times the strength of the final drug concentration, with 100% solvent (e.g., dimethyl sulfoxide) used as a diluent according to the standard additive twofold drug dilution schema (13) (e.g., 1,600 to 3 μg/ml for amphotericin B, anidulafungin, aminocandin, and all azoles except fluconazole). This procedure prevents precipitation of agents with low solubility in aqueous media. Despite this procedure, itraconazole and some other agents do not remain completely solubilized upon dilution into aqueous media. Thus, at high concentrations, a certain amount of turbidity is encountered and may interfere with the interpretation of MIC endpoints. For water-soluble drugs (e.g., fluconazole and caspofungin), drug dilutions are prepared from the stock to be 10 times the final test drug concentrations directly in RPMI medium according to the additive, twofold drug dilution schema (13) (e.g., 640 to 1.2 μg/ml for fluconazole and 5-FC). The 10× and 100× drug concentrations should be diluted 1:5 and 1:50, respectively, with RPMI to achieve the 2× drug concentrations needed for the microdilution test; after the inoculation step the drug concentrations are 16 to 0.03 μg/ml for amphotericin B, echinocandins, and triazoles and 64 to 0.12 μg/ml for fluconazole and 5-FC.

The broth microdilution test is performed by using sterile, disposable, multiwell microdilution plates (96 U-shaped wells) (e.g., Dynatech Laboratories, Inc., Alexandria, Va.). A multichannel pipette (or a large dispensing instrument for 96-well trays) is used to dispense the 2× drug concentrations in 100-μl volumes into the wells of rows 1 to 10 of the microdilution plates. Row 1 contains the highest drug

TABLE 8 CLSI M38-A document guidelines for broth dilution antifungal susceptibility testing of filamentous fungi[a]

Parameter	Description
Medium for conidial growth	Potato dextrose, 35°C (7 days); *Fusarium* spp. may need 30°C incubation for the last 4 days
Inoculum morphology	Conidia or sporangiospores
Recommended OD ranges	For *Aspergillus* and for *R. arrhizus*, 0.09–0.11; for *Fusarium* and *S. apiospermum* 0.15–0.17; stock inoculum suspensions, 0.4×10^6 to 5×10^6 CFU/ml
Inoculum concentration (final)	0.4×10^4 to 5×10^4 CFU/ml or 1:50 dilution of stock suspension (*S. apiospermum*, 2:50)
Test medium	RPMI 1640 for the yeasts (pH, 7.0 ± 0.1)
Format	Microdilution assay; total volume/well, 200 μl
Drug concentration	Amphotericin B and itraconazole, 0.01–8.0 μg/ml
Incubation conditions	For *R. arrhizus,* 24 h; for *C. bantiana, D. constricta, S. prolificans,* and *S. apiospermum,* 72 h; for *Aspergillus* spp., *Bipolaris* spp., *Exserohilum* spp., *Fusarium* spp., *P. lilacinus, T. longibrachiatum, S. schenckii,* and *W. dermatitidis,* 48 h
Endpoint determination, visual	Growth relative to that for positive growth control, absence of growth
Colorimetric (Alamar Blue)	For amphotericin B, first blue well or without color change; for azoles, first blue well or slightly purple well

[a] Data from references 14, 34, 35, 37, and 38.

concentration (either 64 or 16 μg/ml), and row 10 contains the lowest drug concentration (either 0.12 or 0.03 μg/ml). Microdilution trays can be stored at −60°C for 3 to 6 months. Each well is inoculated on the day of the test with 100 μl of the corresponding 2× inoculum, which brings the drug dilutions and inoculum densities to the final test concentrations (final volume in each well, 200 μl). The growth control wells contain 100 μl of sterile drug-free medium (for water-soluble agents) or 100 μl of sterile drug-free medium with 2% solvent (for non-water-soluble agents) and are inoculated with 100 μl of the corresponding 2× inoculum. The QC yeasts are tested in the same manner as the other isolates and are included each time an isolate is tested. Row 11 of the microdilution plate can be used for the sterility control (drug-free medium only).

Incubation and Determination of Microdilution MICs for Yeasts

The microdilution plates are incubated at 35°C for 46 to 50 h *(Candida)* and 70 to 74 h *(C. neoformans)* in ambient air. The determination of MIC endpoints is a critical step in antifungal susceptibility testing, especially with the azoles (for yeasts) and echinocandins (for yeasts and molds). The growth in each well is compared with that in the growth control (drug-free) well with the aid of a reading mirror (e.g., Cooke Engineering Co., Alexandria, Va.). The MIC for amphotericin B is defined as the lowest concentration at which complete absence of growth (optically clear) is observed.

The partial inhibition or trailing that is observed with 5-FC, the azoles, and most of the new and investigational agents precludes the determination of well-defined endpoints and creates a great deal of variability in interpretation. Therefore, the MIC of 5-FC and the azoles is defined as the lowest concentration at which prominent growth inhibition is observed (~50% or less of the growth in the growth control well) (Table 2). Agitation of the microdilution trays (3) (see below) is highly recommended prior to MIC determination; this step facilitates the visual estimate of prominent growth inhibition. In most instances when testing *Candida* spp., azole MICs may be read following incubation for 24 h. The shorter incubation minimizes much of the difficulty with trailing and may improve the clinical correlation slightly (32, 88). Another means to overcome the presence of trailing is the cumbersome measurement of ergosterol production in cells exposed to selected concentrations of fluconazole (6). Trailing growth is also a problem when reading MICs of the echinocandins for some *Candida* spp. It appears that a 50% inhibition endpoint after 24 h of incubation may be the best approach for the echinocandins when testing yeasts (65); however, the optimal testing conditions for this class of agents are still under investigation.

Macrodilution

Broth macrodilution tests are adequate for the testing of all antifungal agents and are suitable for small laboratories in which the volume of these tests is low. Only the steps and testing conditions that are relevant to the macrodilution test are discussed in detail here (Table 2). Each intermediate drug concentration solution is further diluted (1:10) in RPMI medium to obtain 10 times the final strength (e.g., 160 to 0.3 μg/ml). This step reduces the final solvent concentration to 10%. The 10× drug dilutions are dispensed in 0.1-ml volumes into round-bottom, snap-cap, sterile polystyrene tubes (12 by 75 mm; e.g., Falcon 2054; Becton Dickinson Labware, Lincoln Park, N.J.); these tubes can be stored at −60°C for 3 to 6 months. On the day of the test, each tube is inoculated with a 0.9-ml volume of the corresponding diluted yeast inoculum suspension. This step brings the drug dilutions to the final test drug concentrations mentioned above and the corresponding solvent to 1% in each MIC tube. The stock inoculum suspensions are prepared and adjusted as described above for the microdilution test and are then diluted 1:2,000 with RPMI to provide an inoculum of 0.5×10^3 to 2.5×10^3 CFU/ml. The growth control tube(s) is inoculated with a 0.9-ml volume(s) of the inoculum suspension(s) and a 0.1-ml volume(s) of drug-free medium with 1% of the corresponding solvent. The QC yeasts (Table 3) are tested in the same manner as the other isolates and are included each time an isolate is tested. In addition, 1 ml of uninoculated drug-free medium (for water-soluble agents) or drug-free medium with 1% of the corresponding solvent is included as a sterility control.

Incubation and Determination of Macrodilution MICs for Yeasts

The MIC tubes are incubated at 35°C without agitation for 46 to 50 h *(Candida)* and 70 to 74 h *(C. neoformans)* in ambient air; the turbidity or growth in each tube is visually

graded. For amphotericin B, the MIC is read as the lowest concentration that prevents any discernible growth. For azoles and 5-FC, the MIC is defined as the lowest drug concentration that reduces growth by 80% relative to that of the growth control (Table 2) (13). The amount of trailing or partial inhibition can be estimated by diluting the drug-free growth control 1:5 (0.2 ml of growth control plus 0.8 ml of medium) with medium, which provides an 80% inhibition standard tube for comparisons with each MIC tube (13, 39). This approach provides a convenient and direct method of establishing a specific turbidity endpoint for each isolate that more precisely reflects an 80% inhibition.

Quality Control for Yeast Testing

QC of MIC tests is essential to good laboratory practice. *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 have been selected as the QC strains according to the CLSI guidelines for such selection. Table 3 summarizes the expected MIC ranges of 10 antifungal agents for these two QC isolates in both macro- (48-h ranges only) and microdilution (both 24- and 48-h ranges) testing (CLSI M27-S2) (16). Each new batch of medium and lot of macrodilution tubes and microdilution trays should be checked with one of the two QC strains to determine if the MICs are within these ranges (8, 16, 52). In addition, the overall performance of the test system should be monitored by testing either or both QC isolates each day on which a test is performed for each drug. Details regarding corrective measures when the MICs for the QC isolates are not within the expected ranges are found in the M27-A2 document (13).

Expected Results

Most (about 95%) clinical yeast isolates are inhibited by ≤8.0 μg/ml of 5-FC (79, 84); however, isolates of *C. neoformans* for which MICs are ≥16 may be recovered from patients during treatment with 5-FC. *Candida* isolates for which 5-FC MICs are ≥32 μg/ml and ≤4 μg/ml are considered resistant and susceptible, respectively (Table 5) (13). Amphotericin B MICs determined by the microdilution M27-A method for 94% of clinical yeast isolates are ≤1.0 μg/ml and >2 μg/ml for the other 6% (13). An amphotericin B MIC of 2.0 μg/ml (or greater) suggests probable clinical resistance, since this MIC only approximates the concentrations achievable in serum at high doses of amphotericin B (≥1 mg/kg of body weight per day) and exceeds that achievable in cerebrospinal fluid. The difference in amphotericin B MICs for susceptible and potentially resistant isolates is probably very small (see above), so caution should be exercised in the interpretation of results. Although it has been suggested that antibiotic medium 3 provides reliable detection of resistant isolates, lot-to-lot variability has been documented (57). In addition, this medium did not improve the detection of potentially amphotericin B-resistant isolates recovered from patients with candidemia, who had failed amphotericin B therapy (microbiologic failure) (63).

Ketoconazole is fungistatic for most yeast isolates, with MICs of 0.03 to 16 μg/ml by the M27-A2 microdilution method. Fluconazole inhibits the majority (~90%) of *Candida* spp. and *C. neoformans* at concentrations of ≤8 μg/ml; however, MICs for *Candida glabrata* and *C. krusei* generally are 4 to 16 μg/ml and 16 to ≥64 μg/ml, respectively (81). Itraconazole is generally quite active in vitro, with MICs of 0.01 to 1.0 μg/ml or less for most yeast isolates, except for *C. glabrata* (0.06 to 8 μg/ml) and *C. krusei* (0.5 to 2 μg/ml). Overall, ≥99% of isolates of *Candida* spp. and *C. neoformans* are inhibited by ≤1 μg/ml of posaconazole, ravuconazole, and voriconazole, but high voriconazole MICs have been reported for *Sporobolomyces salmonicolor*, *Rhodotorula rubra*, and for some *Candida albicans* and *C. glabrata* isolates (25, 36, 44, 81). MICs of the echinocandins are usually ≤2 μg/ml for most *Candida* isolates, except for *Candida guilliermondii* and some *C. parapsilosis* isolates (22, 25, 29, 62, 82); trailing is observed with these species.

Broth-Based Alternative Approaches for Yeasts

Although the CLSI methods for in vitro susceptibility testing were essential for standardization and to improve interlaboratory reproducibility, they may not be the best methods for testing all organisms or for routine use in clinical laboratories. Modifications of reference methods offer promise as alternative approaches that may better serve clinical laboratory needs; some of these methods are described below.

Colorimetric Methods

A novel alternative to the standard method of visually grading turbidity is the use of colorimetric indicators or fluorescent dyes for the determination of MIC endpoints. Commercial (Sensititre YeastOne and FUNGITEST) and noncommercial (tetrazolium salt methods and substrate uptake indicators) procedures have been adapted for antifungal susceptibility testing (20, 40, 41, 69, 73). The agreement of colorimetric MICs with reference macrodilution and microdilution MICs has been quite good (>90%). However, itraconazole MICs determined by the colorimetric method have been reported to be higher than those determined by the CLSI methods, and agreement has been low with fluconazole for *C. glabrata* and *C. tropicalis* (40). The Sensititre YeastOne method (Trek Diagnostic Systems Inc., Westlake, Ohio), which incorporates Alamar Blue as the oxidation-reduction colorimetric indicator (fungal growth changes the indicator from blue to pink), has obtained FDA clearance for testing fluconazole, itraconazole, and 5-FC; azole and 5-FC MICs should be read after 24 h, and amphotericin B should be read after 48 h (40); the MIC is the first well that does not show a color change from blue (amphotericin B and other drugs) or purple (azoles and 5-FC) to pink. This method minimizes the trailing effect of azoles.

Spectrophotometric Methods

Microdilution methods allow the determination of endpoints with automated plate reading and yield MIC values that are more accurate and objective than those by other tests. Agitation of microtiter plates (3) at 50 rpm for at least 5 min by use of a microdilution tray shaker before spectrophotometric MIC determination is essential to obtain homogeneous cell suspensions in the wells and hence a more precise MIC determination. However, the inability to obtain homogeneous cell suspensions with some yeasts demands manual mixing of the cells in each well by using a micropipette tip. The spectrophotometric method allows the determination of different levels (%) of growth inhibition (GI), e.g., a GI_{50} corresponds to the first well that shows a 50% decrease in optical density (OD) relative to the OD in the drug-free growth control well. Turbidimetric growth inhibition levels of 50% (GI_{50}) with the azoles and 5-FC and of 90% (GI_{90}) with amphotericin B (530- to 550-nm wavelength) have provided the most accurate values (25, 40, 64, 89).

Flow Cytometry

Flow cytometric methods also have been adapted for antifungal susceptibility testing by introducing DNA-binding vital dyes into the culture to detect fungal cell damage after exposure to an antifungal agent (11, 40, 97). MICs determined

by this approach have been comparable to those obtained by the M27-A2 methods (11, 97). Although these methods produce faster results (4 to 6 h), the need for a flow cytometer for MIC determination would preclude their use in small laboratories.

Standardized Disk Diffusion Method for Yeasts

Worldwide, the most commonly used technique for antibacterial susceptibility testing is the disk diffusion test, which yields a quantitative result (zones of inhibition) and a qualitative interpretive category (e.g., susceptible or resistant). Agar disk diffusion testing is a simple, flexible, and cost-effective alternative to broth dilution testing. Disk diffusion testing of antifungal agents has been slow to develop despite the fact that early studies with fluconazole disks showed promise for testing *Candida* spp. The CLSI Subcommittee on Antifungal Susceptibility Testing has developed a disk diffusion method (Table 5) for testing *Candida* spp. with fluconazole and voriconazole (M44-A document) (15). One significant advantage of the M44-A disk diffusion method is that results can be obtained after 20 to 24 h of incubation. A comparison of results of fluconazole disk diffusion testing from 54 laboratories (2,949 isolates of *Candida* spp.) with results from a central reference laboratory showed an overall categorical agreement of 90.4% with only 0.4% very major (false-susceptible) errors (74). It is expected that disk diffusion testing will be expanded to include other azoles and additional classes of antifungal agents (58). Recently, the M44 disk test method has been shown to be a useful approach for determining the susceptibility of *C. neoformans* to fluconazole (83).

Zone interpretive criteria (Table 6) have been approved for fluconazole and more recently for voriconazole (CLSI M44-S1) (15, 17).

Standard Medium

The CLSI M44-A method uses Mueller-Hinton agar supplemented with 2% dextrose and 0.5 μg/ml methylene blue (15). The increased dextrose and the methylene blue supplementation improve yeast growth and provide sharper zones surrounding the fluconazole, posaconazole, and voriconazole disks (15); in addition, this medium is readily available and has shown acceptable batch-to-batch reproducibility. The pH of the medium should be 7.2 to 7.4 at room temperature after gelling, and the surface of the agar should be moist, but moisture droplets on the agar surface or on the petri dish cover should not be present (15). The medium can be prepared and poured with the two supplements, or the supplements can be added to commercially prepared Mueller-Hinton agar plates; the latter enables the use of the routine agar plates from the bacteriology laboratory. A detailed instruction for the preparation of the agar plates is found in the M44-A document (15).

Preparation of Inocula

The M44-A method employs an inoculum suspension adjusted to the turbidity of a 0.5 McFarland by the spectrophotometer, as described above for broth dilution standard methods (Tables 2 and 5) (13, 15).

Performance of Disk Diffusion Method for Yeasts

The agar plates are inoculated within 15 min after adjusting the inoculum suspension as follows (Table 5). Briefly, a sterile cotton swab is dipped into the undiluted inoculum suspension, rotated several times, and pressed firmly against the inside wall of the tube above the fluid level to remove excess fluid. The entire dried agar surface is evenly streaked in three different directions, swabbing the rim of the plate as the final step. The lid of the plate should be left ajar to allow the agar surface to dry for no more than 15 min. Fluconazole (25 μg), posaconazole (5 μg), and voriconazole (1 μg) disks are dispensed onto the inoculated agar surface. Disks must be pressed down to ensure complete contact with the agar and distributed evenly so they are not closer than 24 mm from center to center. After the disks are placed, they cannot be moved because drug diffusion is almost instantaneous. Plates should be incubated within 15 min after disks have been disposed (15).

Incubation and Determination of Disk Diffusion Zone Diameters for Yeasts

After 20 to 24 h of incubation at 35°C, the resulting inhibition zones should be uniformly circular and a confluent lawn of growth should be present. The plates are read above a black, nonreflecting background illuminated with reflected light (15). The zone diameters surrounding the 25-μg fluconazole disks, 5-μg posaconazole disks, and 1-μg voriconazole disks are measured to the nearest whole millimeter at the point at which there is prominent reduction in growth. Pinpoint microcolonies at the zone edge or large colonies within a zone are ignored. If growth is insufficient, the plates should be read at 48 h (Table 5) (15).

Quality Control for Antifungal Susceptibility Disk Testing of Yeasts

QC zone diameter limits have been defined for fluconazole, posaconazole, and voriconazole when testing *Candida* spp. (Table 4) (7, 15, 70; CLSI Subcommittee Minutes, January 2006 meeting).

Agar-Based Alternative Approaches for Yeasts

NeoSensitabs Tablets

A commercial agar diffusion test from Rosco Diagnostica (A/S Rosco, Taastrup, Denmark) is available in Europe for antifungal susceptibility testing of yeasts. Tablets of established and some of the new antifungal agents (e.g., voriconazole, caspofungin, and posaconazole) are available. Preliminary comparisons with both M27-A2 and M44-A methods have provided promising results (A. Espinel-Ingroff, E. Cantón, D. L. Gibbs, and A.Wang, *15th Eur. Congr. Clin. Microbiol. Infect. Dis.*, abstr. P-1736, 2005).

E Test

The E test (AB Biodisk, Solna, Sweden) is based on the diffusion of a stable concentration gradient of an antimicrobial agent from a plastic strip onto an agar medium. E-test strips for amphotericin B, fluconazole, 5-FC, ketoconazole, itraconazole, voriconazole, and caspofungin are commercially available (AB Biodisk). However, for clinical use FDA has approved only fluconazole, itraconazole, and 5-FC strips. Agreement of E-test and reference MICs has been species and medium dependent; low agreement has been reported for *C. glabrata*, *C. tropicalis*, and *C. neoformans* (40). The medium that provides the best performance for E-test MICs is solidified RPMI medium supplemented with 2% dextrose, and reading requires expertise and close adherence to the manufacturer's instructions. The E test may be useful in testing yeasts suspected of being potentially resistant to amphotericin B (68, 71, 96). Amphotericin B MICs of ≥0.38 μg/ml determined by E test for *Candida* spp. have been associated with therapeutic failure in patients treated with amphotericin B for candidemia (12). This method has

been evaluated for testing the susceptibilities of *Candida* spp. to new triazoles and caspofungin (76, 77).

Fungicidal Activity

Standard testing parameters are not available for the evaluation of the fungicidal activity of antifungal agents. The determination of minimum fungicidal concentrations (MFCs) requires the subculturing onto an agar medium of fixed volumes from each MIC tube or well that shows complete inhibition of growth. The criteria for MFC determination vary in different publications, and the MFC has been described as the lowest drug concentration resulting in either no growth or three to five colonies (9, 86). It has been reported that amphotericin B MFCs can be better predictors of microbiological failure than MICs in candidemia (63) and trichosporonosis (94). The clinical relevance and the development of standard guidelines for MFC determination need to be addressed.

EUCAST Method

Subsequent to the development of the CLSI M27-A method for broth microdilution testing of yeasts, a similar method has been developed under the auspices of the Subcommittee on Antifungal Susceptibility Testing (AFST) of the EUCAST (18). This method is similar to the M27-A2 method (Table 2) but employs a higher inoculum (10^5 CFU/ml), RPMI medium supplemented with additional dextrose (2%), and spectrophotometric readings of MIC endpoints following incubation at 35 to 37°C for 24 h. Although good intralaboratory reproducibility of the EUCAST method as well as good agreement between EUCAST and CLSI microdilution methods has been documented, CLSI breakpoints should not be used to interpret EUCAST MICs; the latter MICs are consistently lower than reference values, and falsely susceptible results could be reported (31). However, such global collaboration in standardization of an antimicrobial susceptibility testing method is rare and marks a very positive step in the improvement of antifungal susceptibility testing worldwide.

Standardized Broth Dilution Methods for Molds

Although the number of serious infections caused by the filamentous fungi is lower than the number of yeast infections, antifungal susceptibility testing of these opportunistic pathogens may be important in guiding the selection of antifungal agents for the treatment of invasive disease. On the basis of results from multicenter studies (34, 35, 37, 38), the CLSI Subcommittee has developed both broth micro- and macrodilution methods for testing molds that are more frequently associated with invasive infections (14). These optimal testing conditions are described below (Tables 3, 7, and 8). The other steps are similar to the ones described for broth dilution methods for yeasts (see above).

Microdilution

Standard Medium

The test medium is the same MOPS-buffered standard RPMI recommended for yeast testing (13, 14).

Drug Stock Solutions

Drug stock solutions are prepared as described above for yeast testing (13) and in the CLSI M38-A document (14).

Preparation of Inocula

Since nongerminated conidia are easier to prepare and standardize, this is the method of inoculum preparation described in the M38-A document (14). A comparison of germinated versus nongerminated conidial suspensions has demonstrated that MICs and MFCs of amphotericin B, itraconazole, posaconazole, ravuconazole, and voriconazole for *Aspergillus* spp. are similar or within 2 dilutions with both types of inocula (40).

The inoculum for each isolate is prepared by first growing the mold on potato dextrose agar slants (Remel, Lenexa, Kans.) for 7 days at 35°C. A conidial suspension is prepared by flooding each slant with approximately 1 ml of sterile 0.85% saline. The resulting mixture is withdrawn, and the heavy particles are allowed to settle for 3 to 5 min. The upper homogeneous suspension, containing the mixture of nongerminated conidia or sporangiospores and hyphal fragments, is mixed for 15 s with a vortex. The turbidity of the mixed suspension is measured by using a spectrophotometer at 530 nm and adjusted to a specific final OD range (Table 7) for each species tested (14, 37, 38). Stock inoculum suspensions are diluted 1:50 in medium to obtain 2× the strength needed for the test.

Drug Dilutions and Performance of Microdilution Test for Molds

Drug dilutions are prepared and dispensed in sterile, disposable, multiwell microdilution trays, as described above for yeast testing. On the day of the test, each well is inoculated with 100-μl volumes of the 1:50 conidial or sporangiospore suspensions.

Incubation and Determination of Broth Microdilution MICs for Molds

All microdilution trays are incubated at 35°C, and MICs are determined as described in the M38-A document (14) and as recommended for some uncommon molds (37): (i) at 24 h for *Rhizopus arrhizus;* (ii) at 48 h for *Aspergillus* spp., *Bipolaris* spp., *Fusarium* spp., *Paecilomyces lilacinus, Sporothrix schenckii*, *Trichoderma longibrachiatum*, and *Wangiella dermatitidis;* and (iii) at 72 h for *Pseudallescheria boydii (Scedosporium apiospermum)*, *Cladophialophora bantiana*, *Dactylaria constricta*, and *Scedosporium prolificans*. The MIC endpoint criterion for molds is the lowest drug concentration that shows complete growth inhibition when testing amphotericin B, itraconazole, voriconazole, and posaconazole (14). Experience with both established and investigational agents has demonstrated that MICs for other opportunistic and slower growing filamentous fungi can be determined (sufficient or heavy growth present in the growth control) after 48 to 96 h of incubation. Testing of the dimorphic fungi may require 5 to 7 days of incubation (25, 40).

Macrodilution

Good agreement between results obtained by both micro- and macrodilution methods for molds has been documented (38). Some laboratories would rather perform broth macrodilution testing because of safety concerns and low testing volume. Macrodilution testing conditions are described in the CLSI M38-A document (14). Briefly, inoculum stock suspensions and drug dilutions are prepared as for the microdilution test. The 100-fold drug dilutions should be diluted 1:10 with RPMI to achieve 10 times the strength needed for the macrodilution test. The stock inoculum suspensions are diluted 1:100 with medium to obtain 0.4×10^4 to 5×10^4 CFU/ml. The 10× drug concentrations are dispensed into 12- by 75-mm sterile tubes in 0.1 ml volumes. Each tube is inoculated on the day of the test with 0.9 ml of the corresponding suspension.

Incubation and Determination of Broth Macrodilution MICs for Molds

Tubes are incubated at 35°C without agitation and observed for the presence or absence of visible growth. The MICs are determined as described above for the microdilution method for molds.

Quality Control for Mold Testing

Either one of the QC yeast organisms or the QC *Paecilomyces variotii* ATCC MYA-3630 isolate may be tested in the same manner as the other mold isolates or as described above for yeasts and should be included each time an isolate is evaluated with any antifungal agent. In addition, other molds have been selected as reference isolates (Table 3) (33).

Expected Results

Amphotericin B MICs determined by the M38-A microdilution method are ≤1.0 μg/ml for most mold species. High amphotericin B MICs (>2 μg/ml) have been reported for *P. lilacinus*, most *S. apiospermum* isolates, *S. prolificans,* and some isolates of *Alternaria* spp., *Aspergillus* spp., *Fusarium* spp., *Penicillium marneffei*, *Phialophora* spp., and *S. schenckii* (27, 29, 40). MICs of itraconazole are also usually ≤2.0 μg/ml; the exceptions are its high MICs for *Aspergillus ustus* (some isolates), *Fusarium solani*, *F. oxysporum*, *P. lilacinus*, *S. schenckii*, *S. prolificans*, *T. longibrachiatum,* and some isolates of *R. arrhizus* (27, 29, 40). Similar results are obtained with the other triazoles, but good in vitro activity against the Zygomycetes has been reported with posaconazole (19, 91).

Broth Microdilution Method for Dermatophytes

The M38-A broth microdilution method has also been successfully adapted, with minor modifications, to the testing of dermatophytes (45). These modifications include the use of oatmeal agar for inoculum preparation when testing *Trichophyton rubrum* in order to induce conidium formation and 4 to 5 days of incubation at 35°C for MIC determination (80% growth inhibition endpoints). Two isolates of *Trichophyton* spp. have been selected as reference strains by the Subcommittee for testing this group of fungi (Table 3) (CLSI Subcommittee Minutes, January 2006).

Broth-Based Alternative Approaches for Molds

Evaluation of Morphologic Changes

The trailing growth is not a problem when testing investigational or established azoles against most molds. However, when testing the echinocandins, most *Aspergillus* isolates show trailing growth and conventional MIC determination could categorize these trailing isolates as resistant to caspofungin. A more careful examination of the microdilution wells reveals the presence of compact, round microcolonies. Under microscopic examination, these microcolonies correspond to significant morphologic alterations. The hyphae grow abnormally as short, highly branched filaments with swollen germ tubes. Kurtz et al. (53) defined the concentration of drug producing these morphologic changes as the minimum effective concentration (MEC) to distinguish it from conventional MICs. A multicenter study has demonstrated that caspofungin MECs were reliable endpoints in 14 of 17 laboratories (65) and in another study in the eight laboratories that participated in the study when evaluating anidulafungin against a variety of mold species (A. Espinel-Ingroff, M. Ghannoum, E. Manavathy, L. Ostrosky-Zeichner, M. Pfaller, M. Rinaldi, W. Schell, and T. Walsh, *Abstr. 45th Intersci. Conf. Antimicrob. Agents Chemother.*, abstr. M–1605, 2005). Because conflicting results were reported from three laboratories, caution and further refinement of this testing approach are needed.

MICs/MECs of the echinocandins are usually <1.0 μg/ml for *Aspergillus* spp., but higher for other molds (MICs >8 μg/ml). However, MICs of <1.0 μg/ml have been reported for some isolates of the dimorphic fungi *Phialophora* spp. and *S. apiospermum* (29).

Colorimetric Methods

The YeastOne method has also been evaluated for molds, and it appears to be applicable for such testing (10, 56, 60). The measurement of metabolic activity by reading the color change (OD) produced when tetrazolium salts (yellow) are cleaved to their formazan derivative (purple), using a microtiter plate spectrophotometer, has also been evaluated for molds (48, 61). Again, further evaluations, including interlaboratory studies, are needed with more isolates and species.

Agar-Based Alternative Approaches for Filamentous Fungi

As for yeasts, agar-based methods have been applied to susceptibility testing of molds, including agar dilution, disk diffusion, and E-test methods and semisolid agar (54).

Agar Dilution Methods

Agar dilution methods involve the preparation of 10× double dilutions of the agent, which are incorporated into molten agar. Drug-containing plates are inoculated with suspensions of the organism being tested. Since standard methods are not available, the size of the inoculum varies among the different studies.

Disk Diffusion Method

Disk diffusion methodology has been evaluated for caspofungin (5) and voriconazole (90). The reproducibility of this methodology is under investigation by the CLSI Subcommittee for testing caspofungin, amphotericin B, and the triazoles.

E Test

This convenient method also has been adapted in several studies for testing mold pathogens (26, 28, 42, 60, 75, 80, 90, 92). Since the trailing effect is not a major problem for azole testing against most molds, E-test inhibition ellipses are usually sharp and MICs are easily interpreted. Overall, comparisons of E-test and M38-A methods have demonstrated better agreement when testing the triazoles (>90%) than amphotericin B (>80%). Amphotericin B E-test MICs for *A. flavus*, *S. apiospermum*, and *S. prolificans* are usually higher than reference values, especially after 48 h of incubation (26). The reliability of the E-test method and the clinical relevance of its MICs for molds should be addressed.

Fungicidal Activity

A CLSI study has demonstrated that laboratories may reliably perform MFC testing (37) and time-kill curves. In contrast to what is observed with yeasts, the azoles appear to have a certain degree of fungicidal activity as mentioned above for a variety of common and rare opportunistic mold pathogens. MFCs of 0.2 to 4 μg/ml have been reported with the triazoles for various mold species (25, 27, 36). However, standardization of this procedure is needed to reliably assess the potential value of the MFC endpoint in patient management. These standardization efforts should include the correlation of in vitro results with the clearing of target

organs of the infecting organism in animal models and the further clinical relevance of MFC data.

CLINICAL RELEVANCE

In order to be useful clinically, in vitro susceptibility testing should reliably predict the in vivo response to therapy in human infections. However, factors related to the drug, the host immune response and/or the status of the current underlying disease, proper patient management, the infecting organism, and the interactions of the organism with both the host and the therapeutic agent appear to have more value than the MIC as predictors of clinical outcome (87). Because so many factors can influence the process of antifungal therapy for an infection caused by a presumably susceptible isolate, a low MIC does not necessarily predict clinical success. However, in vitro resistance may be able to identify, among a population of susceptible strains, those isolates that are less likely to respond to a specific antifungal regimen. In order to appreciate the clinical value of the in vitro antifungal testing result, one must understand that the predictor value of in vitro susceptibility in bacterial infections has been accurately summarized as the "90-60 rule" (88). Infections due to susceptible isolates respond to therapy ~90% of the time, whereas infections due to resistant isolates respond ~60% of the time. There is now a considerable body of data indicating that standardized antifungal susceptibility testing (CLSI M27-A2) for *Candida* spp. and some triazoles provides results that have a predictive utility consistent with the "90-60 rule."

Interpretive MIC breakpoints (Table 6) have been established for fluconazole, itraconazole, and voriconazole following correlation with clinical data (oropharyngeal candidiasis [fluconazole and itraconazole], candidemia in nonneutropenic patients [fluconazole and voriconazole], and other infections [voriconazole] [13, 17, 87]). Isolates inhibited by ≥64 μg of fluconazole per ml and ≥1 μg of itraconazole per ml are considered resistant to these agents, whereas isolates inhibited by ≤8 μg of fluconazole per ml and ≤0.12 μg of itraconazole per ml are considered susceptible. In addition, fluconazole MICs of 16 to 32 μg/ml and itraconazole MICs of 0.25 to 0.5 μg/ml have been designated susceptible–dose dependent (S-DD). For fluconazole, this novel designation encompasses isolates in which susceptibility is dependent on achievable peak levels in serum of 40 to 60 μg/ml at fluconazole dosages of 800 mg/day, versus the expected peak levels of ≤30 μg/ml at lower dosages. The pharmacodynamic parameter that predicts efficacy for fluconazole is ~25 (area under the concentration-time curve [AUC]/MIC). For itraconazole, an MIC within the S-DD range indicates the need for plasma concentrations of >0.5 μg/ml for an optimal response. However, host factors, rather than azole resistance, can be responsible for clinical failure. Recently, a susceptible breakpoint of ≤1 μg/ml, S-DD of 2 μg/ml, and a resistant breakpoint of ≥4 μg/ml were established for voriconazole and *Candida* spp. (16) (Table 6). Isolates for which the voriconazole MIC is ≥4 μg/ml (resistant endpoint) are mostly *C. glabrata* (93%) and represent a concentration of voriconazole that cannot be maintained with the currently recommended doses (200 to 300 mg administered orally or an intravenous dose of 4 mg/kg every 12 h); as a consequence, patients infected with these isolates are less likely to respond clinically to voriconazole therapy (<60% response). Therefore, the 60% clinical response rate at the voriconazole-resistant breakpoint is consistent with the 90-60 rule. In support of the susceptible breakpoint of ≤1 μg/ml, plasma concentrations of ≥1 μg/ml are determined throughout the dosing interval with the recommended doses; the 1-μg/ml MIC encompasses 99% of all clinical isolates of *Candida*. Pharmacokinetics and pharmacodynamic parameters indicate that a 24-h free drug AUC/MIC ratio of 20 is predictive of efficacy and recommended doses would produce free drug AUCs of ~20 μg·h/ml (4). Therefore, it can be predicted that current recommended dosing regimens could be used successfully to treat patients infected with isolates for which voriconazole MICs are ≤1 μg/ml as determined by the reference method.

Lack of response to fluconazole therapy has been documented in a few cases of fluconazole resistance in invasive candidal infections (24). In cryptococcal infections, fluconazole MICs of >16 μg/ml have correlated with clinical failure to fluconazole maintenance therapy (2).

Very few correlations of in vitro results with in vivo response have been reported for mold infections (30). Clinical failure with amphotericin B has been associated with MICs of >0.8 μg/ml for *Aspergillus fumigatus*, *Fusarium* spp., and *S. prolificans* infections (30); both primary resistance and acquired resistance have been demonstrated. Clinical failures of itraconazole and amphotericin B in the treatment of aspergillosis have been correlated with MICs of >8 μg/ml and >2 μg/ml, respectively (21, 55).

SUMMARY AND CONCLUSIONS

A great deal of progress has been achieved in the field of antifungal susceptibility testing since 1982. Standardized broth macrodilution and microdilution methods are available for testing molds and yeasts, as is the 24-h disk diffusion methodology to test fluconazole and voriconazole against *Candida* spp. More suitable and promising modifications of these methods for use in clinical microbiology laboratories are also available. It must be emphasized that a critical next step in establishing the clinical usefulness of standardized procedures will be to determine the relationship between test results and patient responses to therapy in other clinical settings and for other organism-drug combinations. Additional studies are needed for the establishment of standard methodology for testing the echinocandins versus both yeasts and molds.

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PARASITOLOGY

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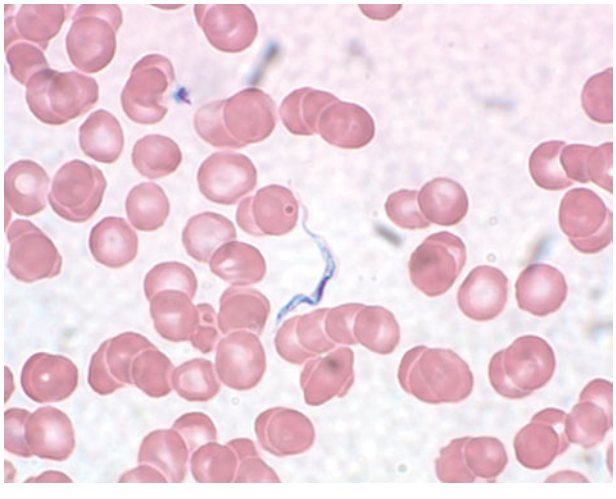
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Taxonomy and Classification of Human Parasites

FRANCIS E. G. COX

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The term "parasite" is traditionally applied to several disparate groups of eukaryotic organisms, mainly protozoa and helminths (embracing nematode, cestode, trematode, and acanthocephalan worms) but also some arthropods, annelids, and molluscs. Medical parasitology is concerned with the 200 or so species of helminth worms and about 80 species of protozoa that infect humans. Many of these are rare and accidental parasites, but about 100 species are commonly found in humans; a small number of these are responsible for a disproportionate number of important diseases, particularly in the tropics, and these inevitably receive the most attention. This chapter is concerned with the classification of the parasites that are commonly encountered in humans, together with a number that are rarer but are occasionally encountered.

Taxonomy is the science of establishing and defining systematic groups of organisms in a hierarchical manner that reflects the evolutionary past and present relationships between the groups. The basic unit is the species, a grouping in which, among higher organisms, all members are morphologically similar and able to interbreed successfully. The taxonomic groups of higher organisms are largely based on morphological criteria reinforced, wherever possible, by palaeontological evidence of past relationships. The classification of the multicellular helminth worms, which have well-defined morphological characters, clear affinities with free-living species, and characteristic life cycles involving sexual reproduction, is based on sound taxonomic principles, but the single-celled protozoa have always constituted an enigmatic group and have been difficult to classify. This subject will be discussed further below.

In his 1758 classification of living things, Carl Linnaeus recognized two kingdoms, Animalia and Plantae. By the middle of the 19th century, scientists had added a third category to include organisms that we now regard as protozoa, and the concept of three kingdoms, Animalia, Plantae, and Protista (= protozoa) emerged. This was later expanded to four kingdoms, Animalia, Plantae, Protoctista (= Protista = Protozoa), and Mychota (prokaryote organisms) (9). When the fungi were separated from the plant kingdom, a fifth kingdom, Fungi, was created, and this formed the basis of what became known as the widely accepted five-kingdom classification, Prokaryota, Animalia, Plantae, Fungi, and Protista (19, 26).

Over the past 30 years, these five kingdoms have been subjects of controversy and have undergone a number of modifications, often on a somewhat arbitrary basis. Gradually, however, a new consensus has emerged based mainly on advances in our understanding of the biochemistry and molecular biology of lower organisms. Comparisons of small subunit ribosomal RNA (SSU rRNA) and protein sequences (2, 16, 25) have made it possible to arrange organisms within groups based on evolutionary distances but not necessarily in a hierarchical way. The identification of certain organelles found in eukaryotic cells with their prokaryote origins, however, has now made it possible to organize all living organisms within a realistic and evolutionarily sound overall scheme (3), which is adopted here. The most important aspect of this classification is that the major elements in it are compatible with more traditional classifications. Essentially there are now six kingdoms, Bacteria, Protozoa, Animalia, Fungi, Plantae, and Chromista. This classification establishes the Protozoa as the basal eukaryotic kingdom and has necessitated the redistribution of some taxa, including some parasites of humans. The six-kingdom classification reestablishes the validity of the kingdom Protozoa and brings the protozoa and helminths under the same rules whereby all species are named according to the guidelines set out in the International Code of Zoological Nomenclature (http://www.iczn.org). From a practical point of view, this confers stability in the naming of species and ensures priority for the name originally given while permitting corrections or amendments when necessary. In this chapter, therefore, some of the generic and specific names are different from those in the previous edition of this Manual, but all such changes have had to be justified on grounds of new discoveries and interpretations. The International Code is, however, not a panacea for all taxonomic problems, and the correct specific name of the species of *Giardia* that infects humans, *G. lamblia* as used here or *G. duodenalis* or *G. intestinalis* as used elsewhere, is still unresolved.

CLASSIFICATION OF THE PROTOZOA

There are estimated to be over 200,000 named species of single-celled eukaryotic organisms (10), of which only about 10,000, some 5%, are parasitic; thus, any system of classification must primarily satisfy zoologists and protozoologists

working with free-living protozoa. Traditionally, the phylum Protozoa embraced four great groups of single-celled organisms, recognized on the basis of their mode of locomotion: Rhizopoda or Sarcodina (amoebae, moving by pseudopodia), Mastigophora (flagellates, moving by flagella), Ciliophora or Ciliata (ciliates, moving by cilia), and Sporozoa (sporozoans, spore-forming protozoans without any obvious means of locomotion). Rapid developments in our understanding of the protozoa during the 1960s and 1970s spawned new and ever more complex classifications, but by the beginning of the 1990s a number of protozoologists (12, 24) had decided to revert to what was essentially the traditional four "groups": the flagellated protozoa, the amoeboid protozoa, the ciliated protozoa, and the sporozoans (including the microsporidians and myxosporidians). This approach had the merit of simplicity and clear links with the traditional classification but was merely a classification of convenience and did not stand up to rigorous analysis at either the evolutionary or molecular level. Further attempts to resolve this problem resulted in an "interim user friendly" classification (10) that served its purpose well until more rational and natural classifications emerged.

Over the past decade or so, developments in molecular biology have given us a clearer understanding of phylogenetic relationships involving particular groups of single-celled eukaryotic organisms, but unfortunately, this has once again resulted in a plethora of classifications. It now seems unlikely that protozoologists, particularly those working with free-living organisms, will ever come up with a scheme of classification acceptable to everyone (22). These apparent vacillations have irritated parasitologists, most of whom are concerned with fewer than 0.05% of all species and simply require a stable framework within which to classify parasites, particularly those of medical and veterinary importance.

The classification outlined below is based on that developed over many years by Cavalier-Smith (3–7). It embraces traditional and novel elements, meets the requirements of parasitologists, and bridges the gap between those working with free-living protozoa and those working with parasitic protozoa. The main advantage of this classification is that it is compatible with traditional and molecular classifications and with the "interim classification" (10) that has found much favor. It is not appropriate here to go into any details of the rationale behind this classification, which is discussed in more detail elsewhere (13), but there are several important features with respect to traditional classifications and the interim classification that must be mentioned. The myxosporidians, formerly classified among the sporozoans (but which do not affect humans), are now clearly recognized as metazoans and placed in the kingdom Animalia. Similarly, the microsporidians, some of which do infect humans, particularly immunocompromised individuals, are now thought to be more closely related to fungi than to protozoa and should now be classified with the Fungi (7, 18). The taxonomic position of *Blastocystis* has always been enigmatic, and this genus has been shuffled between the Fungi and Protozoa but now seems to have to come to rest in the kingdom Chromista (3, 6). The kingdom Chromista was created in 1989 to accommodate a number of plant-like organisms, mainly algae, that were originally chimeras between eukaryotic biflagellate hosts and symbiotic red algae but which had lost their chloroplasts over evolutionary time yet still retain elements of their red algal ancestry (3). Phylogenetic trees based on 18S rRNA and other molecular evidence now place *Blastocystis* firmly within this group, which lies close to the sporozoans and ciliates (6). With the removal of these groups, the Protozoa becomes a kingdom with 13 phyla, of which 7 contain parasites that infect humans. The phylum Amoebozoa, containing the amoebae, is equivalent to the old Rhizopoda (Sarcodina), and the flagellates belonging to the former Mastigophora are now distributed among four phyla, Metamonada, Parabasalia, Percolozoa, and Euglenozoa. Two phyla remain unchanged, the Ciliophora, containing the ciliates, and the Sporozoa. The status of the phylum Sporozoa requires some more explanation. Sporozoa is the traditional name applied to a large group of sexually reproducing, spore-forming protozoans with recognizably similar morphology at the electron microscope level and comparable life cycles. In the 1970s, a new parasite genus, *Perkinsus,* containing parasites of marine molluscs and quite different from any of the other sporozoans, was allocated to this group. The phylum Sporozoa was downgraded to a class, and the name of the phylum was changed to Apicomplexa in order to incorporate two classes, Sporozoea and Perkinsea (11). Not all parasitologists liked this change, but the word Apicomplexa has gradually crept into the literature. It is now clear, however, that *Perkinsus* spp. do not belong with the sporozoans and should be classified close to, or with, the dinoflagellates (15, 23); therefore, the taxon Apicomplexa is redundant and should not be used. References to Apicomplexa or apicomplexans should now revert to Sporozoa or sporozoans, the original names. One final point concerns the microsporidians. Microsporidians have traditionally been considered to belong with the protozoa sensu stricto, and parasitologists have been reluctant to part with this group, whereas mycologists have been equally reluctant to accept it. It therefore seems sensible, at least for the time being, to retain the microsporidians alongside the Protozoa while acknowledging that they are, in fact, fungi.

OUTLINE CLASSIFICATION OF EUKARYOTIC PARASITES

Table 1 shows an outline classification of parasites; its sole purpose is to provide a taxonomically sound and convenient set of pigeonholes to which the various parasitic organisms can be assigned. The classification of all living organisms, and the Protozoa in particular, is discussed in more detail elsewhere (3–6), as are the classifications of the parasitic protozoa (13) and the parasitic helminth worms (17) of humans. Elsewhere there are full annotated lists of the parasitic protozoa, helminths, and arthropods of humans (1) and parasitic helminths (8); comprehensive coverage of all human parasites (14); and additional information about helminth worms (20, 21). The levels of the taxa used for the protozoa and helminths are different, and this is deliberate. Protozoa are classified as far as orders because this is the lowest taxonomic level normally used by parasitologists. Helminth worms are usually classified as far as superfamilies and families, taxa rarely used by protozoologists. For simplicity and clarity, orders are omitted in the lists of nematodes. As well as the formal taxa, common names, such as flagellates, have been added. The most important parasites, either because of their capacity to cause disease or because they are very common and therefore likely to be featured in textbooks, are given in bold.

I thank Tom Cavalier-Smith, Department of Zoology, University of Oxford, for helpful discussions while I was writing the previous version of this chapter.

TABLE 1 Outline classification of parasites that infect humans[a]

KINGDOM PROTOZOA
- Subkingdom 1 Archezoa
  - Phylum Metamonada
    - Class Trepomonadea (intestinal flagellates)
      - Order Diplomonadida ***Giardia lamblia***[b]
      - Order Enteromonadida *Enteromonas hominis*
    - Class Retortamonadea
      - Order Retortamonadida *Chilomastix mesnili, Retortamonas intestinalis*
  - Phylum Parabasala (flagellates)
    - Class Trichomonadea (intestinal and related flagellates)
      - Order Trichomonadida ***Dientamoeba fragilis, Trichomonas vaginalis,*** *T. tenax, Pentatrichomonas hominis*
- Subkingdom 2 Neozoa
  - Infrakingdom 1 Discicristata
    - Phylum Percolozoa (flagellates)
      - Class Heterolobosea (flagellated amoebae)
        - Order Schizopyrenida ***Naegleria fowleri***
    - Phylum Euglenozoa (flagellates)
      - Class Kinetoplastidea (kinetoplastid flagellates)
        - Order Trypanosomatida ***Leishmania donovani, L. infantum, L. major, L. tropica, L. braziliensis, L. mexicana,*** *L. aethiopica, L. amazonensis,* L. colombiensis,* L. garnhami,* L. guyanensis, L. lainsoni,* L. naiffi,* L. panamensis, L. peruviana, L. pifanoi,* L. shawi,** ***Trypanosoma cruzi, T. brucei gambiense,***[c] ***T. brucei rhodesiense,***[c] *T. rangeli*
  - Infrakingdom 2 Sarcomastigota
    - Phylum Amoebozoa (amoebae)
      - Subphylum Lobosa
        - Class Amoebaea (amoebae)
          - Order Acanthopodida ***Acanthamoeba castellanii,*** *A. culbertsoni,* A. hatchetti,* A. polyphaga,** ***Balamuthia mandrillaris***
      - Subphylum Conosa
        - Class Entamoebidea (intestinal amoebae)
          - Order Euamoebida ***Entamoeba histolytica, E. coli, E. dispar, E hartmanni,*** *E. gingivalis, E. moshkovskii,* E. chattoni,* E. polecki,* Endolimax nana, Iodamoeba bütschlii*
  - Infrakingdom 3 Alveolata
    - Phylum Sporozoa (sporozoans)
      - Class Coccidea
        - Order Eimeriida ***Cryptosporidium parvum,*** *C. hominis, Cryptosporidium* spp.,* ***Toxoplasma gondii, Cyclospora cayetanensis,*** *Isospora belli,* Sarcocystis hominis, S. lindemanni,* S. suihominis*
        - Order Piroplasmida ***Babesia microti,*** *B. divergens,* B. gibsoni,* Babesia* spp.[d]
        - Order Haemosporida ***Plasmodium falciparum, P. malariae, P. ovale, P. vivax***
    - Phylum Ciliophora (ciliates)
      - Class Litostomatea
        - Order Vestibulifera *Balantidium coli*

KINGDOM CHROMISTA
- Subkingdom Chromobiota
  - Phylum Bigyra
    - Class Blastocystea
      - ***Blastocystis hominis***

KINGDOM FUNGI
- Phylum Microspora (microsporidians)
  - Class Microsporea[e] ***Encephalitozoon cuniculi,*** *E. hellem,** ***E. intestinalis,*** ***Enterocytozoon bieneusi,*** *Brachiola algerae,* B. connori,* B. vesicularum,* M. africanum,* Microsporidium ceylonensis,* Nosema ocularum,* Pleistophora ronneafiei,* Trachipleistophora hominis,* T. anthropophthera,* Vittaforma corneae*

KINGDOM ANIMALIA
- Subkingdom 1 Radiata
- Subkingdom 2 Myxozoa
- Subkingdom 3 Bilateria
  - Infrakingdom 1 Ecdysozoa
    - Phylum Nemathelminthes (Infraphylum Nematoda: roundworms)
      - Class Adenophorea (Asphasmidea)
        - Superfamily Trichinelloidea
          - Family Trichinellidae ***Trichinella spiralis,*** *T. britovi,* T. murrelli,* T. nativa,* T. nelsoni,* T. papuae,* T. pseudospiralis,* T. zimbabwensis**
          - Family Trichuridae ***Trichuris trichiura,*** *T. suis,* T. vulpis,* Calodium hepaticum,* Capillaria philippinensis,*[f] *C. aerophilus,**[f] *C. hepatica,**[f] *C. plica**[f]

(Continued on next page)

TABLE 1 Outline classification of parasites that infect humans[a] *(Continued)*

KINGDOM ANIMALIA *(continued)*
- Class Secernentea (Phasmidea)
  - Superfamily Ancylostomatoidea
    - Family Ancylostomatidae ***Ancylostoma duodenale,*** *A. brazilienese,*[+] *A. caninum,*[+] *A. ceylanicum,* ***Necator americanus***
  - Superfamily Ascaridoidea
    - Family Ascarididae ***Ascaris lumbrioides,*** *A. suum,** *Toxocara canis,*[+] *T. cati,*[+] *Parascaris equorum,*[+] *Lagochilascaris minor,*[+] *Baylisascaris procyonis*[+]
    - Family Anisakidae *Anisakis physeteris,*[+] *A. simplex,*[+] *Phocanema decipiens*[+]
  - Superfamily Dracunculoidea
    - Family Dracunculidae ***Dracunculus medinensis***
  - Superfamily Filarioidea
    - Family Onchocercidae ***Brugia malayi,*** *Brugia timori, B. beaveri,** *B. guyanensis,** ***Loa loa, Wuchereria bancrofti, Onchocerca volvulus,*** *Dipetalonema* spp.,* *Dirofilaria immitis, D. repens, D. striata,** *D. tenuis,** *D. ursi,** *Mansonella ozzardi, M. perstans, M. semiclarum, M. streptocerca, M. rodhaini*[+]
  - Superfamily Gnathostomatoidea
    - Family Gnathostomatidae ***Gnathostoma spinigerum,***[+] G. binucleatum,[+] Gnathostoma spp.[+]
  - Superfamily Metastrongyloidea
    - Family Metastrongylidae *Metastrongylus elongatus**
    - Family Angiostrongylidae *Parastrongylus cantonensis, P. costaricensis*
  - Superfamily Oxyuroidea
    - Family Oxyuridae ***Enterobius vermicularis,*** *E. gregorii*
  - Superfamily Physalopteroidea
    - Family Physalopteridae *Physaloptera caucasica**
  - Superfamily Rhabditoidea
    - Family Strongyloididae, ***Strongyloides fuelleborni, S. stercoralis,*** *Strongyloides* spp.[+]
  - Superfamily Spiruroidea
    - Family Gongylonematidae *Gongylonema pulchrum**
  - Superfamily Strongyloidea
    - Family Chabertiidae, *Oesophagostomum bifurcum, Oesophagostomum* spp.,* *Ternidens deminutus*
    - Family Sygamidae *Mammomonogamus laryngeus**
  - Superfamily Thelazioidea
    - Family Thelaziidae *Thelazia californiensis,** *Thelazia callipaeda**
  - Superfamily Trichostrongyloidea
    - Family Trichostrongylidae, *Trichostrongylus axei,** *T. brevis,** *T. capricola, T. colubriformis, T. orientalis, T. probolurus, T. skrjabini, T. vitrinus, Trichostrongylus* spp.,* *Haemonchus contortus**
    - Family Dioctophymatidae *Dioctophyme renale*

Infrakingdom 2 Platyzoa
- Phylum Acanthognatha (thorny- or spiny-headed worms)
  - Class Archiacanthocephala (= Archaeacanthocephala)
    - Order Moniliformida
      - Family Moniliformidae *Moniliformis moniliformis**
    - Order Oligacanthorhynchida
      - Family Oligacanthorhynchidae *Macracanthorhynchus hirudinaceus,** *M. ingens**
- Phylum Platyhelminthes
  - Class Digenea (Trematoda, flukes)
    - Order Strigeida
      - Family Diplostomidae *Alaria alata,*[+] *A. americana,*[+] *Diplostomum spathaceum*[+]
      - Family Schistosomatidae ***Schistosoma haematobium, S. japonicum, S. mansoni, S. mekongi, S. intercalatum,*** *S. bovis,*[+] *S. malayensis,*[+] *S. mattheei, Schistosoma* spp.,[+] *Austrobilharzia terrigalensis,*[+] *Bilharziella polonica,*[+] *Gigantobilharzia sturniae,*[+] *G. huttoni,*[+] *Heterobilharzia americana,*[+] *Microbilharzia variglandis,*[+] *Orientobilharzia turkestanica,*[+] *Trichobilharzia* spp.[+]
      - Family Clinostomatidae *Clinostomum complanatum*[+]
    - Order Echinostomatida
      - Family Echinostomatidae *Echinochasmus* spp.,* *Echinoparyphium recurvatum,** *Echinostoma echinatum, E. hortense,** *E. ilocanum,** *Echinostoma* spp.,* *Hypoderaeum conoideum*
      - Family Fasciolidae ***Fasciola hepatica, Fasciolopsis buski,*** *Fasciola gigantica*
      - Family Gastrodiscidae ***Gastrodiscoides hominis,*** *Watsonius watsoni**
    - Order Plagiorchiida
      - Family Dicrocoeliidae ***Dicrocoelium dendriticum,*** *Eurytrema pancreaticum**
      - Family Heterophyidae *Apophallus donicus,** *Centrocestus cuspidatus,** *C. formosanus,** *Cryptocotyle lingua,** *Heterophyes heterophyes, Heterophyes* spp.,* *Haplorchis* spp.,* *Metagonimus yokogawai, M. minutus,** *M. takahashi,** *Stellantchasmus falcatus,** *Stichodora* spp.*
      - Family Opisthorchidae ***Clonorchis sinensis, Opisthorchis felineus, O. viverrini,*** *O. guayaquilensis,** *Metorchis* spp.*

(Continued on next page)

TABLE 1 *(Continued)*

KINGDOM ANIMALIA *(continued)*
- Family Lecithodendriidae *Phaneropsolus bonnei, Prosthodendrium molenkampi*
- Family Paragonimidae ***Paragonimus westermani,*** *P. africanus, P. heterotremus, P. mexicanus,* P. miyazakii, P. ohirai,* P. pulmonalis, P. skrjabini, P. uterobilateralis, Paragonimus* spp.*
- Family Plagiorchiidae *Plagiorchis* spp.*
- Family Troglotrematidae *Nanophyetus salmincola**
- Family Achillurbainiidae *Achillurbainia* spp+

Class Cestoidea (Cestoda, tapeworms)
- Order Pseudophyllidea
  - Family Diphyllobothriidae ***Diphyllobothrium latum,*** *D. dalliae, D. klebanovskii, D. pacificum, Diphylobothrium* spp.,+ *Diplogonoporus grandis, Diplogonoporus* spp.,* *Ligula intestinalis,* Schistocephalus solidus,* Spirometra* spp.*
- Order Cyclophyllidea
  - Family Anoplocephalidae *Bertiella mucronata,* B. studeri,* Inermicapsifer cubensis, I. madagascariensis, Mathevotaenia symmetrica**
  - Family Davaineidae *Buginetta alouettae,* Raillietina celebensis,* R. demerariensis**
  - Family Dipylidiidae *Dipylidium caninum*
  - Family Hymenolepididae ***Hymenolepis* (= *Rodentolepis*) *nana,***[g] *Hymenolepis diminuta*
  - Family Mesocestoidae *Mesocestoides* spp.*
  - Family Taeniidae ***Taenia saginata, T. solium,*** *Taenia* spp.,* ***Echinococcus granulosus,*** *E. multilocularis, Echinococcus* spp.,* *Multiceps* spp.*

Subkingdom 4 Mesozoa (no parasites of humans)

[a] Parasites in bold type are the most important species. Symbols: *, rare or very rare parasites of humans; +, accidental infections with larval forms and in which the parasite fails to develop to its adult stage.

[b] There is no agreement about the name of this organism, which is also known as *Giardia duodenalis* or *G. intestinalis*.

[c] *Trypanosoma brucei gambiense* and *T. brucei rhodesiense* are subspecies but are often referred to as species *T. gambiense* and *T. rhodesiense*, respectively.

[d] Increasing numbers of *Babesia microti*-like isolates have been identified, mainly in the northeastern and midwestern United States but also elsewhere; they have not yet been given specific status and are currently known by their codes, e.g., WA-1, CA-1, MO-1, and EU-1.

[e] The classification of the microsporidians is in a state of flux pending agreement as to how they should be classified with the fungi, so these organisms are not assigned to any class or order in this classification.

[f] Zoologists have assigned the various species of *Capillaria*, including those found in humans, to other genera *(Calodium*, *Eucoelus*, *Pearsonema*, *Paracapillaria,* and *Crossicapillaria)*, but as these genera have largely been ignored by medical parasitologists, and in order to avoid confusion, they are not listed here.

[g] Zoologists have assigned this worm, commonly known as *Hymenolepis nana,* to the genus *Rodentolepis* (17), but this change has not been widely accepted by parasitologists working with human parasites.

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Specimen Collection, Transport, and Processing: Parasitology

PETER DEPLAZES, LYNNE S. GARCIA, AND ROBYN Y. SHIMIZU

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Routine diagnostic parasitology generally includes laboratory procedures that are designed to detect organisms within clinical specimens by using morphologic criteria rather than culture, biochemical tools, and/or physical growth characteristics (Table 1). Parasite identification is frequently based on light microscopic analysis of concentrated and/or stained preparations. Small organisms often require high magnification such as oil immersion (×1,000) (ranging from 1.5 μm [microsporidia] upwards). Electron microscopy for species characterization of organisms has been replaced in recent years mainly by molecular methods (PCR and sequence analyses of selected genes). Furthermore, new commercial test kits designed especially for detection of antigens (e.g., coproantigens of *Giardia*, *Cryptosporidium,* and *Entamoeba histolytica* [Table 2] or circulating *Plasmodium* antigens [Table 3]) have expanded the methodical repertoire. In addition to those methods for direct parasite detection (morphology, antigens, and DNA), methods for indirect detection of parasite infections demonstrating specific antibodies directed to a variety of native or recombinant parasite antigens have been developed and partially commercialized (Table 4). Diagnostic techniques are available to detect a large range of protozoan and helminth species. They are generally characterized by a variety of methods depending on clinical specimens and the biology of the parasites. An important precondition for reliable diagnostic results is the proper collection, processing, and examination of clinical specimens. Based on the biology of the parasites and the procedural features of the tests, multiple specimens must often be submitted and examined before the suspected organism(s) is found and its identity is confirmed.

SPECIMEN COLLECTION AND TRANSPORT

Various collection methods are available for specimens suspected of containing parasites or parasitic elements (Tables 5, 6, 7, and 8) (3, 9, 10, 18, 21, 25, 31). When collection methods are selected, the decision should be based on a thorough understanding of the value and limitations of each. The final laboratory results are based on parasite recovery and identification and will depend on the initial handling of the specimen. Unless the appropriate specimens are properly collected and processed, these infections may not be detected. Therefore, specimen rejection criteria have become much more important for all diagnostic microbiology procedures. Diagnostic laboratory results based on improperly collected specimens may require inappropriate expenditures of time and supplies and may also mislead the physician. As a part of any continuous quality improvement program for the laboratory, the generation of test results must begin with stringent criteria for specimen acceptance or rejection.

All fresh specimens should be handled carefully, since each specimen represents a potential source of infectious material. Safety precautions should include proper labeling of fixatives; specific areas designated for specimen handling (biological safety cabinets may be necessary under certain circumstances, such as parasite cultures); proper containers for centrifugation; acceptable discard policies; appropriate policies of no eating, drinking, or smoking within the working areas; and, if applicable, correct techniques for organism culture and/or animal inoculation. Universal Precautions as outlined by the Occupational Safety and Health Act must be followed when applicable, particularly when blood and other body fluids are being handled (7).

Collection of Stool (Fresh)

Collection of stool for parasite detection should always be performed before barium is used for radiological examination. Stool specimens containing the opaque, chalky sulfate suspension of barium are unacceptable for examination, and intestinal protozoa may be undetectable for 5 to 10 days after barium is given to the patient. Certain substances and medications also interfere with the detection of intestinal protozoa; these include mineral oil, bismuth, antibiotics (metronidazole, tetracyclines), antimalarial agents, and nonabsorbable antidiarrheal preparations. After administration of any of these compounds, parasites may not be recovered for a week to several weeks. Therefore, specimen collection should be delayed for 5 to 10 days or at least 2 weeks after barium or antibiotics, respectively, are administered (9, 10, 20, 24). Some laboratories add the following comment to their negative reports: "Certain antibiotics such as metronidazole or tetracycline may interfere with the recovery of intestinal parasites, particularly the protozoa."

Fecal specimens should be collected in clean, wide-mouth containers; often a waxed cardboard or plastic container with a tight-fitting lid is selected for this purpose. The specimens should not be contaminated with water or urine because water may contain free-living organisms that can be mistaken for human parasites and urine may destroy motile organisms. Stool specimen containers should be placed in

TABLE 1 Body sites and possible parasites recovered[a]

Site	Parasites
Blood	
Erythrocytes	*Plasmodium* spp., *Babesia* spp.
Leukocytes	*Leishmania* spp., *Toxoplasma gondii*
Whole blood/plasma	*Trypanosoma* spp., microfilariae
Bone marrow	*Leishmania* spp., *Trypanosoma cruzi, Plasmodium* spp.
Central nervous system	*Taenia solium* (cysticerci), *Echinococcus* spp., *Naegleria fowleri*, *Acanthamoeba* spp., *Balamuthia mandrillaris, Toxoplasma gondii,* microsporidia, *Trypanosoma* spp.
Cutaneous ulcers	*Leishmania* spp., *Acanthamoeba* spp.
Intestinal tract	*Entamoeba histolytica, Entamoeba dispar, Entamoeba coli, Entamoeba hartmanni, Endolimax nana, Iodamoeba bütschlii, Blastocystis hominis*, *Giardia, Chilomastix mesnili, Dientamoeba fragilis, Pentatrichomonas hominis, Balantidium coli, Cryptosporidium* spp., *Cyclospora cayetanensis, Isospora belli, Enterocytozoon bieneusi, Encephalitozoon* spp., *Ascaris lumbricoides, Enterobius vermicularis,* hookworm, *Strongyloides stercoralis, Trichuris trichiura, Hymenolepis nana, Hymenolepis diminuta, Taenia saginata, Taenia solium, Diphyllobothrium latum, Clonorchis sinensis (Opisthorchis), Paragonimus* spp., *Schistosoma* spp., *Fasciolopsis buski, Fasciola hepatica, Metagonimus yokogawai, Heterophyes heterophyes*
Liver, spleen	*Echinococcus* spp., *Entamoeba histolytica, Leishmania* spp., microsporidia, *Capillaria hepatica*
Lungs	*Cryptosporidium* spp.,[b] *Echinococcus* spp., *Paragonimus* spp., *Toxoplasma gondii,* helminth larvae
Muscle	*Trichinella* spp., *Taenia solium* (cysticerci), *Onchocerca volvulus* (nodules), *Trypanosoma cruzi,* microsporidia
Skin	*Leishmania* spp., *Onchocerca volvulus,* microfilariae
Urogenital system	*Trichomonas vaginalis, Schistosoma* spp., microsporidia, microfilariae
Eyes	*Acanthamoeba* spp., *Toxoplasma gondii, Loa loa,* microsporidia, *Thelazia* spp.

[a] Parasites include trophozoites, cysts, oocysts, spores, adults, larvae, eggs, and amastigote and trypomastigote stages. This table does not include every possible parasite that could be found in a particular body site. However, the most likely organisms have been listed.

[b] Disseminated in severely immunosuppressed individuals.

plastic bags when transported to the laboratory for testing. Specimens should be identified with the following information: patient's name and identification number, physician's name, and the date and time the specimen was collected. The specimen must also be accompanied by a request form indicating which laboratory procedures should be performed. The presumptive diagnosis or relevant travel history information is helpful and should accompany the test request. In some situations, it may be necessary to contact the physician for additional patient history.

In the past, it has been recommended that a normal examination for stool parasites before therapy include three specimens. Three specimens, collected as outlined above, have also been recommended for posttherapy examinations. However, a patient who has received treatment for a protozoan infection should be checked 3 to 4 weeks after therapy, and those treated for *Taenia* infections should be checked 5 to 6 weeks after therapy. In many cases, the posttherapy specimens will not be collected, often as a cost containment measure; if the patient becomes symptomatic again, additional specimens can be submitted (18, 21).

Although some recommend collection of only one or two specimens, there are differences of opinion regarding this approach. It has also been suggested that three specimens be pooled and examined as a single specimen; again, this approach is somewhat controversial. However, physicians should be aware that the probability of detecting clinically relevant parasites in a single stool specimen may be as low as 50 to 60% but is >95% if three samples are examined (21).

If a series of three specimens is collected, they should be submitted on separate days; if possible, the specimens should be submitted every other day; otherwise, the series of three specimens should be submitted within no more than 10 days. If a series of six specimens is requested, the specimens should also be collected on separate days or within no more than 14 days. Many organisms, particularly the intestinal protozoa, do not appear in the stool in consistent numbers on a daily basis, and the series of three specimens is considered the

TABLE 2 Commercially available kits for immunodetection of parasitic organisms or antigens in stool samples

Organism and kit name[a]	Manufacturer and/or distributor	Type of test[b]	Comments[c]
Cryptosporidium spp.			The tests detect *C. hominis,* different *C. parvum* genotypes, and other species depending on intensity of the infection
ProSpecT Cryptosporidium Microplate Assay	Remel	EIA	Can be used with fresh, frozen, or formalin-preserved stool; http://www.remel.com
Xpect *Cryptosporidium* Kit	Remel	Cartridge, IC	
Cryptosporidium Antigen ELISA	Medical Chemical	EIA	Contact manufacturer; http://www.med-chem.com
Crypto-CELISA	Cellabs	EIA	Can be used with fresh, frozen, or formalin-preserved stool; http://www.cellabs.com
Crypto Cel	Cellabs	DFA	
Cryptosporidium Test	TechLab	EIA	http://www.techlabinc.com
Cryptosporidium Test	Wampole	EIA	http://www.wampolelabs.com
Entamoeba histolytica			These tests will differentiate between between *E. histolytica* and *E. dispar*
Entamoeba histolytica II Test	TechLab	EIA	Requires fresh or frozen stool
Entamoeba CELISA Path	Cellabs	EIA	Contact manufacturer
Entamoeba histolytica Test	Wampole	EIA	Contact manufacturer
Entamoeba histolytica/*E. dispar* Group			These tests *will not* differentiate *E. histolytica* from *E. dispar*
ProSpectT *Entamoeba histolytica* Microplate Assay	Remel	EIA	
Giardia lamblia			
ProSpecT *Giardia* Microplate Assay	Remel	EIA	Different EIA formats – contact manufacturer
GiardEIA	Antibodies, Inc.	EIA	http://www.antibodiesinc.com
Giardia Antigen ELISA	Medical Chemical	EIA	Contact manufacturer
Giardia-CELISA	Cellabs	DFA	Contact manufacturer
Giardia-Cel	Cellabs	EIA	
Giardia	Wampole	EIA	See TechLab
SIMPLE-READ *Giardia*	Medical Chemical	Cartridge IC	Can be used with fresh, frozen, or formalin-preserved stool
Xpect *Giardia*	Remel	Cartridge IC	Can be used with fresh, frozen, or formalin-preserved stool
Giardia II	TechLab	EIA	
Giardia Test	Wampole	EIA	
Combination tests: *Cryptosporidium* and *Giardia*			
ProSpecT Giardia/Cryptosporidium Microplate Assay	Remel	EIA	Can be used with fresh, frozen, or formalin-preserved stool
MERIFLUOR Cryptosporidium/Giardia	Meridian Bioscience	DFA	
Cryptosporidium/Giardia DFA	Medical Chemical	DFA	Contact manufacturer
Crypto/Giardia-Cel	Cellabs	DFA	Contact manufacturer
ColorPAC Giardia/*Cryptosporidium* Rapid Assay	Becton-Dickinson	Cartridge IC	Can be used with fresh, frozen, or formalin-preserved stool; http://www.bd.com
ImmunoCard STAT! *Cryptosporidium/Giardia*	Meridian Bioscience	Cartridge IC	Can be used with fresh, frozen, or formalin-preserved stool; http://www.meridianbioscience.com
Xpect Giardia/Cryptosporidium	Remel	Cartridge IC	Can be used with fresh, frozen, or formalin-preserved stool
Combination tests: *Cryptosporidium, Giardia,* and *Entamoeba*			
Triage Parasite Panel	Biosite Diagnostics, Inc.	Cartridge device, lateral flow	Requires fresh or frozen stool; combination test with *Giardia* and *E. histolytica*/*E. dispar* group; will not differentiate between *E. histolytica* and *E. dispar;* http://www.biosite.com

[a] A number of the kits are manufactured by a single manufacturer but labeled under different company names; consequently, some of the data for sensitivity and specificity may be identical to those of kits produced under another name or by another company.
[b] EIA, enzyme immunoassay; DFA, direct fluorescent antibody; IC, immunochromatography.
[c] URLs are given only the first time the company name appears in the table.

TABLE 3 Commercially available test kits for immuno- or molecular detection of parasitic organisms or antigens in serum, plasma, blood, or vaginal discharge[a]

Manufacturer	Test	Company websites	Usable specimen
Malaria			
ACON Laboratories	Malaria P.f. Rapid Test	http://www.aconlabs.com	Whole blood
AmeriTek, Inc.	One Step Malaria Test	http://www.ameritek.org	Whole blood
Binax	NOW Malaria P.f./P.v.	http://www.binax.com	Whole blood
Bio-Quant, Inc.	One Step Malaria Test	http://www.bio-quant.com	Whole blood
Core Diagnostics Ltd.	CORE Malaria (Pf, Pf/Pv, Pan Pf)	http://www.corediag.com	Whole blood
Cortez Diagnostics, Inc.	OneStep RapiCard InstaTest	http://www.rapidtest.com	Whole blood
DiaMed SA	OptiMAL Rapid Malaria Test, Optimal –IT Rapid Malaria Test	http://diamed.com	Whole blood
Genix Technology	Malaria (P. fal.) Ag	http://www.genixtech.com	Whole blood
MP Biomedicals Asia Pacific Pte. Ltd.	ASSURE Malaria P.f. Rapid Test	http://www.genelabs.com.sg	Whole blood
Global eMed, LLC	Smart Strip Malaria Test	http://www.globalemed.com	Whole blood
International Immuno-Diagnostics	One-Step Malaria (PF/PV)	http://www.intlimmunodiagnostics.com	Whole blood
Kat-Medical	KAT-Quick Malaria test	http://www.katmedical.com	Whole blood
Mega Diagnostics, Inc.	MegaKwik Malaria (Pf) Card Test	http://www.mega-dx.com	Whole blood
Orchid Biomedical Systems	Paracheck Pf	http://www.tulipgroup.com/Orchid	Plasma, whole blood
Premier Medical Corporation	First Response Malaria P.f / P.v Antigen Strips	http://www.premiermedcorp.com	Whole blood
Princeton BioMeditech Corporation	BioSign Malaria	http://www.pbmc.com	Whole blood
SPAN Diagnostics	ParaHIT f	http://www.span.co.in	Whole blood
Standard Diagnostics Inc.	Malaria P.f/P.v Antigen Test	http://www.standardia.com	Whole blood, serum
Trinity Biotech	Rapid Uni-Gold Malaria (P.f.)	http://www.trinitybiotech.com	Whole blood
Vencor International Inc.	DxStrip Malaria Combo	http://www.vencorinternational.com	Whole blood
Filariasis			
Cellabs Pty Ltd.	CELISA	http://www.cellabs.com.au	Whole blood, serum
Binax Inc.	NOW Filariasis Test	http://www.binax.com	Whole blood, plasma, serum
Trichomonas vaginalis			
Genzyme Diagnostics	OSOM Trichomonas Rapid Test	http://www.genzymediagnostics.com	Genital swab
Xenotope Diagnostics Inc.	XenoStrip-Tv rapid test	http://www.xenotope.com	Genital swab
Thermo Electron Corp.	Acceava Trichomonas	http://www.thermo.com	Genital swab
Kalon Biological Limited	Kalon Tvaginalis latex kit	http://www.kalonbio.co.uk	Genital swab
Chemicon LTD	Light Diagnostics T. vaginalis DFA Kit	http://www.chemicon.com	Genital swab
Becton-Dickinson "DNA Probe"	BD Affirm VP III	http://www.bd.com	Genital swab

[a] For detailed information, see www.rapid-diagnostics.org and references 4, 6, 8, and 32.

minimum for an adequate examination. Multiple specimens from the same patient should not be submitted on the same day. One possible exception would be a patient who has severe, watery diarrhea, in whom any organisms present might be missed because of a tremendous dilution factor related to fluid loss. These specimens should be accepted only after consultation with the physician. It is also not recommended for the three specimens to be submitted one each day for three consecutive days; however, use of this collection time frame would not be cause enough to reject the specimens.

To evaluate patients who are at risk for giardiasis, the negative predictive value of some of the immunoassays on a single stool specimen is not sufficiently high to exclude the possibility of a *Giardia lamblia* infection. In cases where the clinical suspicion for *G. lamblia* infection is moderate or high and the first assay yields a negative result, testing of a second specimen may be recommended (17).

Fresh specimens are mandatory for the recovery of motile trophozoites (amebae, flagellates, or ciliates). The protozoan trophozoite stage is normally found in cases of diarrhea; the intestinal tract contents are moving through the system too rapidly for cyst formation to occur. Once the stool specimen is passed from the body, trophozoites do not encyst but may disintegrate if not examined or preserved within a short time after passage. However, most helminth eggs and larvae, coccidian oocysts, and microsporidian spores will survive for extended periods. Liquid specimens should be examined within 30 min of passage, not 30 min from the time they reach the laboratory. If this general time recommendation of 30 min is not possible, the specimen should be placed in one of the available fixatives (9). The current College of American Pathologists checklist requires that "the microscopic examination of fresh (unpreserved) liquid stools should include a wet mount of fresh specimen by direct technique." Soft (semiformed) specimens may have a mixture of

TABLE 4 Commercially available kits or antigens for immunodetection of specific serum antibodies

Disease (organism)	Manufacturer and/or distributor[b]	Type of test[c]	Comments
Protozoa			
Toxoplasmosis *(Toxoplasma gondii)*[a]			
Amebiasis *(Entamoeba histolytica)*	Chemicon	EIA	Early extraintestinal infections may be missed (follow-ups recommended); infections with *E. dispar* do not induce detectable antibodies.
	IVD Research	EIA	
	bioMérieux	IFT	
Chagas' disease *(Trypanosoma cruzi)*	IVD Research	EIA	
	Chemicon	EIA	
	Hemagen	EIA	
	Diagnostics	EIA	
	InBios	Rapid	
Helminths			
Cysticercosis *(Taenia solium)*	IVD Research	EIA	Cross-reactions in cases of other helminthic infections (especially echinococcosis) may occur.
	Chemicon	EIA	
	Immunetics	IB	
Schistosomiasis (*Schistosoma* spp.)	IVD Research	EIA	
Cystic echinococcosis *(Echinococcus granulosus)*	IVD Research	EIA	Not species specific. Cases of alveolar echinococcosis will cross-react. False-positive reactions may occur with patients suffering from other helminthic infections.
	Immunetics	IB	
	Bordier Affinity Products	EIA	
Alveolar echinococcosis *(E. multilocularis)*	Bordier Affinity Products	EIA	Screening test for alveolar echinococcosis; cases of cystic echinococcosis may cross-react.
Strongyloidiasis *(Strongyloides stercoralis)*	IVD Research	EIA	Cross-reactions in patients with other helminth infections may occur.
	Bordier Affinity Products	EIA	
Toxocariasis *(Toxocara canis)*	IVD Research	EIA	
	Bordier Affinity Products	EIA	
Trichinellosis *(Trichinella spiralis)*	IVD Research	EIA	Numerous cross-reactions with other helminth infections may occur.
Filariasis	Bordier Affinity Products	EIA	

[a] Serological tests for the detection of *Toxoplasma* infections are given in chapter 138.

[b] Bordier Affinity Products, Chatanerie 2, CH-1023, Crissier, Switzerland (http://www.bordier.ch); Chemicon, 28835 Single Oak Dr., Temecula, CA 92590 (http://www.chemicon.com); Hemagen, 34-40 Bear Hill Rd., Waltham, MA 02154 (http://www.hemagen.com); Immunetics, 380 Green St., Cambridge, MA 02139 (http://www.immunetics.com); InBios, 562 1st Ave. South, Suite 600, Seattle, WA 98104 (http://www.inbios.com); IVD Research, 5909 Sea Lion Place, Suite D, Carlsbad, CA 92008 (http://ivd@ivdresearch.com).

[c] Abbreviations: EIA, enzyme immunoassay; IFT, immunofluorescence test; IB, immunoblot; Rapid, rapid immunochromatographic (some are dipstick, cartridge, or other rapid test formats).

protozoan trophozoites and cysts and should be examined within 1 h of passage; again, if this time frame is not possible, preservatives should be used. Immediate examination of formed specimens is not as critical; in fact, if the specimen is examined any time within 24 h after passage, the protozoan cysts should still be intact.

Collection of Stool (Preserved)

If there are delays from the time of specimen passage until examination in the laboratory, the use of stool preservatives should be considered. To preserve protozoan morphology and to prevent the continued development of some helminth eggs and larvae, the stool specimens can be placed in preservative either immediately after passage (by the patient using a collection kit) or once the specimen is received by the laboratory. There are several fixatives available; these include formalin, sodium acetate-acetic acid-formalin (SAF), Schaudinn's fluid, and polyvinyl alcohol (PVA) (Table 8). Regardless of the fixative selected, adequate mixing of the specimen and preservative is mandatory. Specimens preserved in stool fixatives should be stored at room temperature.

When selecting an appropriate fixative, keep in mind that a permanent stained smear is mandatory for a complete examination for parasites (9, 10, 20, 24, 27). You may also want to use some of the newer immunoassay procedures or molecular diagnostic methods (PCR); make sure that the fixative you are using is compatible with the kit or the method you have selected. It is also important to remember that disposal regulations for compounds containing mercury are becoming stricter; each laboratory will have to check applicable regulations to help determine fixative options.

Formalin

Formalin is an all-purpose fixative that is appropriate for helminth eggs and larvae and for protozoan cysts. Two concentrations are commonly used: 5%, which is recommended for preservation of protozoan cysts, and 10%, which is recommended for helminth eggs and larvae. Although 5% is often recommended for all-purpose use, most commercial manufacturers provide 10%, which is more likely to kill all helminth eggs. To help maintain organism morphology, the formalin can be buffered with sodium phosphate buffers, i.e., neutral formalin. Selection of specific formalin formulations

TABLE 5 Specimen preparation and procedures, recommended stain(s), relevant parasites, and additional information

Body site	Procedures[a] and specimens	Recommended methods and relevant parasites[a]	Additional information
Blood	Microscopy[b]: thin and thick blood films. Fresh blood (preferred) or EDTA-blood (fill EDTA tube completely with blood, then mix).	Giemsa stain (all blood parasites); hematoxylin-based stain (sheathed microfilariae). For malaria, thick and thin blood films are definitely recommended and should be prepared within 30 to 60 min of blood collection via venipuncture (other tests may be used as well).	Most drawings and descriptions of blood parasites are based on Giemsa-stained blood films. Although Wright's stain (or Wright-Giemsa combination stain) will work, stippling in malaria may not be visible and the organisms' colors will not match the descriptions. However, with other stains (those listed above, in addition to some of the "quick" blood stains), the organisms should be detectable on the blood films.
	Concentration methods: EDTA blood	Buffy coat, fresh blood films for detection of moving microfilariae or trypanosomes. QBC, a screening method for blood parasites (hematocrit tube contains acridine orange) has been used for malaria, *Babesia,* trypanosomes, and microfilariae. It is usually impossible to identify malaria organisms to the species level; requires high levels of training.	The use of blood collected with anticoagulant (rather than fresh) has direct relevance to the morphology of malaria organisms seen in peripheral blood films. If the blood smears are prepared after more than 1 h, stippling may not be visible, even if the correct pH buffers are used. Also, if blood is kept at room temperature (with the stopper removed), the male microgametocyte may exflagellate and fertilize the female macrogametocyte, and development continues within the tube of blood (as it would in the mosquito host). The ookinete may actually resemble a *Plasmodium falciparum* gametocyte.
	Antigen detection: EDTA-blood for malaria, serum or plasma for circulating antigens (hemolysed blood can interact in some tests)	Commercial test kits for malaria and some microfilariae (see Table 3)	
	PCR: EDTA blood, ethanol fixed or unfixed thin and thick blood films, coagulated blood, possible with hemolyzed or frozen blood samples	Sensitivity not higher than thick films for *Plasmodium* spp., much more sensitive for *Leishmania* (peripheral blood is used from immunodeficient patients only). Sequencing of PCR product is often used for species or genotype identification.	So far, no commercial tests are available; high laboratory standards are needed (may work with frozen, coagulated, or hemolyzed blood samples).
	Specific antibody detection: serum or plasma, anticoagulated or coagulated blood (hemolyzed blood can cause problems in some tests)	Most commonly used are EIA (many test kits commercially available), EITB (commercially available for some parasites), and IFT.	Many labs are using in-house tests; only a few fully defined antigens are available; sensitivities and specificities of the tests should be documented by the lab.
Bone marrow	Biopsies or aspirates Microscopy: thin and thick films with aspirate collected in EDTA	Giemsa stain (all blood parasites)	*Leishmania* amastigotes are recovered in cells of the reticuloendothelial system; if films are not prepared directly after sample collection, infected cells may disintegrate. Sensitivity of microscopy is low; use only in combination with other methods.
	Cultures: sterile material in EDTA or culture medium	Culture for *Leishmania* (or *Trypanosoma cruzi*)	
	PCR: aspirate in EDTA	PCR for blood parasites including *Leishmania* and *Toxoplasma* and rare other parasites	
Central nervous system	Microscopy: spinal fluid and CSF (wet examination, stained smears), brain biopsy material (touch or squash preparations, stained)	Stains: Giemsa (trypanosomes, *Toxoplasma*); Giemsa, trichrome, or calcofluor (amebae [*Naegleria*-PAM, *Acanthamoeba*- or *Balamuthia*- GAE]); Giemsa, acid-fast, PAS, modified trichrome, silver methenamine (microsporidia) (tissue Gram stains also recommended for microsporidia in	If CSF is received (with no suspect organism suggested), Giemsa would be the best choice; however, modified trichrome or calcofluor is also recommended as a second stain (amebic cysts, microsporidia). If brain biopsy material is received (particularly from an immunocompromised patient), cultivation is recommended for microsporidia isolation and PCR for

(Continued on next page)

TABLE 5 *(Continued)*

Body site	Procedures[a] and specimens	Recommended methods and relevant parasites[a]	Additional information
		routine histological preparations); H&E, routine histology (larval cestodes, *Taenia solium* cysticerci, *Echinococcus* spp.)	identification to the species or genotype level. A small amount of the sample should always be stored frozen for PCR analyses in case the results of the other methods are inconclusive.
	Culture: Sterile aspirate or biopsy material (in physiologic NaCl)	Free-living amebae (exception: *Balamuthia* will not grow in the routine agar/bacterial overlay method), microsporidia, and *Toxoplasma*	
	PCR: Aspirate or biopsy material native, frozen, or fixed in ethanol	Protozoa and helminths, species and genotype characterization	
Cutaneous ulcers	Microscopy: aspirate, biopsy (smears, touch or squash preparations, histological sections)	Giemsa *(Leishmania)*; H&E, routine histology *(Acanthamoeba* spp., *Entamoeba histolytica)*.	Most likely causative parasites would be *Leishmania,* which would stain with Giemsa. PAS could be used to differentiate *Histoplasma capsulatum* from *Leishmania* in tissue.
	Cultures (less common)	*Leishmania*, free-living amebae (often bacterial contaminations)	In the immunocompromised patient, skin ulcers have been documented with amebae as causative agents.
	PCR: aspirate, biopsy material (native, frozen, or fixed in ethanol)	*Leishmania* (species identification), free-living amebae	
Eye	Microscopy: smears, touch or squash preparations; biopsy specimens, scrapings, contact lens, sediment of lens solution	Calcofluor, cyst only (amebae *[Acanthamoeba]*); Giemsa trophozoites, cysts (amebae); modified trichrome (preferred) or silver methenamine stain, PAS, acid-fast, (microsporidial spores), H&E, routine histology (cysticerci, *Loa loa, Toxoplasma*)	Some free-living amebae (most commonly *Acanthamoeba*) have been implicated as a cause of keratitis. Although calcofluor will stain the cyst walls, it will not stain the trophozoites. Therefore, in suspected cases of amebic keratitis, both stains should be used. H&E (routine histology) can be used to detect and confirm cysticercosis. The adult worm of *Loa loa*, when removed from the eye, can be stained with a hematoxylin-based stain (Delafield's) or can be stained and examined by routine histology.
	Culture: native material (see above) in PBS supplemented with antibiotics, if possible, to avoid bacterial growth	Cultures: free-living amebae, *Toxoplasma,* microsporidia	
	PCR: native material in physiological NaCl or PBS, ethanol, or frozen	Free-living amebae, *Toxoplasma,* microsporidia species and genotype identification	Microsporidia confirmation to the species or genotype levels by PCR and sequence analyses; however, the spores could be found by routine light microscopy with modified trichrome, calcofluor, and/or tissue Gram stains.
Intestinal tract	(a) Stool and other intestinal material		
	Microscopy: stool, sigmoidoscopy material, duodenal contents (all fresh or preserved; see Table 4), direct wet smear, concentration methods	Concentration methods: ethyl acetate sedimentation of SAF-fixed stool samples (most protozoa); flotation or combined sedimentation flotation methods (helminth ova); agar- or Baermann-concentration (larvae of *Strongyloides* spp., native stool required)	Stool fixation with formalin or fixatives containing formalin preserves trophozoite morphology, allows prolonged storage (room temperature) and long transportation, and prevents hatching of *Schistosoma* eggs, but makes *Strongyloides* larval concentration impossible and impedes further PCR analyses.
		Direct wet smear (examination of unpreserved fresh material [motile protozoan trophozoites, helminth eggs, protozoan cysts])	Taeniid eggs can be identified morphologically, but require PCR so they can be identified to the species level.
		Stains: trichrome or iron hematoxylin (intestinal protozoa); modified trichrome (microsporidia); modified	Microsporidia: identification to the species or genotype level requires PCR; however, modified trichrome and/or calcofluor stains can be used to confirm the presence of spores.

(Continued on next page)

TABLE 5 Specimen preparation and procedures, recommended stain(s), relevant parasites, and additional information *(Continued)*

Body site	Procedures[a] and specimens	Recommended methods and relevant parasites[a]	Additional information
		acid-fast (*Cryptosporidium, Cyclospora,* and *Isospora)*	
	Anal impression smear	Adhesive cellulose tape, no stain *(Enterobius vermicularis)*	Four to six consecutive negative tapes required to rule out infection with pinworm *(Enterobius vermicularis)*.
	Adult worms or tapeworm segments (proglottids)	Carmine stains (rarely used for adult worms or cestode segments). Proglottids can usually be identified to the genus level *(Taenia, Diphyllobothrium,* and *Hymenolepis)* without using tissue stains.	Worm segments can be stained with special stains. However, after dehydration through alcohols and xylenes (or xylene substitutes), the sexual organs and the branched uterine structure will be visible, allowing identification of the proglottid to the species level.
	Antigen detection: fresh native or frozen material, suitability of fixation is test dependent	Commercial immunoassays (Table 2), e.g., EIA, FA, cartridge formats (*Entamoeba histolytica,* the *Entamoeba histolytica/E. dispar* group, *Giardia, Cryptosporidium* spp.); in-house tests for *T. solium* and *T. saginata*	Coproantigens can be detected in the prepatent period and independently from egg excretion.
	PCR: native fresh or frozen or ethanol-fixed material	No commercial tests available. Primers for genus or species identification of most helminths and protozoa are published.	Due to potential inhibition after DNA extraction from stool samples, concentration or isolation methods may be required prior to DNA extraction. However, new DNA isolation kits facilitate isolation of high-quality DNA from stool. Sequence analyses may be required for species or genotype identification.
	(b) Biopsies Microscopy: fixed for histology or touch or squash preparations for staining PCR: see above	H&E, routine histology (*Entamoeba histolytica, Cryptosporidium, Cyclospora, Isospora belli, Giardia*, microsporidia); less common findings would include *Schistosoma* spp., hookworm, or *Trichuris*	Special stains may be helpful for the identification of microsporidia (tissue Gram stains, silver stains, PAS, and Giemsa) or of cryptosporidia (modified acid-fast stains).
Liver and spleen	Biopsies or aspirates		
	Microscopy: unfixed material in physiological NaCl; fixed for histology	Examination of wet smears for *Entamoeba histolytica* (trophozoites), protoscolices of *Echinococcus* spp. or eggs of *Capillaria hepatica*. Giemsa (*Leishmania,* other protozoa and microsporidia); H&E (routine histology).	There are definite risks associated with punctures (aspirates and/or biopsy) of spleen or liver lesions *(Echinococcus)*. Keep always a small amount of material frozen for PCR.
	Culture: sterile preparation of native material	For *Leishmania* (not common)	
	Animal inoculation: sterile preparation of native material	Intraperitoneal inoculation of *E. multilocularis* cyst material for viability test after long-term chemotherapy	
	PCR: native, frozen, or ethanol fixed	Species or genotype identification (e.g., *Echinococcus* spp.)	
Respiratory tract	Sputum, induced sputum, nasal and sinus discharge, bronchoalveolar lavage fluid, transbronchial aspirate, tracheobronchial aspirate, brush biopsy specimen, open-lung biopsy specimen	Helminth larvae *(Ascaris, Strongyloides),* eggs (*Paragonimus, Capillaria),* or hooklets *(Echinococcus)* can be recovered in unstained respiratory specimens. Stains: Giemsa for many protozoa including *Toxoplasma* tachyzoites, modified acid-fast stains *(Cryptosporidium)*; modified trichrome (microsporidia)	There are immunoassay reagents (FA) available for the diagnosis of pulmonary cryptosporidiosis. Routine histologic procedures allow the identification of any of the helminths or helminth eggs present in the lung. Disseminated toxoplasmosis or microsporidiosis is well documented, with organisms being found in many different respiratory specimens.

(Continued on next page)

TABLE 5 *(Continued)*

Body site	Procedures[a] and specimens	Recommended methods and relevant parasites[a]	Additional information
	Microscopy: unfixed material, treated for smear preparation	Routine histology (H&E; silver methenamine stain, PAS, acid-fast, tissue Gram stains for helminths, protozoa, and microsporidia)	
	PCR: unfixed native, frozen, or fixed in ethanol		
Muscle	Biopsy material Microscopy: unfixed, touch and squash preparations, or fixed for histology and EM	Larvae of *Trichinella* spp. can be identified unstained (species identification with single larvae by PCR). H&E, routine histology (*Trichinella* spp, cysticerci); silver methenamine stain, PAS, acid-fast, tissue Gram stains, EM (rare microsporidia).	If *Trypanosoma cruzi* are present in the striated muscle, the organisms could be identified in routine histology preparations. Modified trichrome and/or calcofluor stains can be used to confirm the presence of microsporidial spores.
	PCR: unfixed or native, frozen, ethanol fixed	Microsporidia identification to the species level requires subsequent sequencing.	
Skin	Aspirates, skin snips, scrapings, biopsy specimens	See cutaneous ulcer (above).	Any of the potential parasites present can be identified by routine histology procedures.
	Microscopy: wet examination, stained smear (or fixed for histology or EM)	Wet preparations (microfilariae), Giemsa stained smears or H&E, routine histology (*Onchocerca volvulus, Dipetalonema streptocerca*, *Dirofilaria repens*, other larvae causing cutaneous larva migrans [zoonotic *Strongyloides* spp., hookworms], *Leishmania*, *Acanthamoeba* spp., *Entamoeba histolytica*, microsporidia, and arthropods [*Sarcoptes* and other mites])	
	PCR: unfixed native or frozen or fixed in ethanol.	Primers for most parasite species are available.	
Amniotic fluid	PCR (and/or culture): native material Animal inoculation (Toxoplasmosis)	PCR-based detection of highly repetitive gene sequences is the method of choice.	Only applicable to confirm suspected prenatal *Toxoplasma* infections
Urogenital system	Vaginal discharge, saline swab, transport swab (no charcoal), air-dried smear for IFT, urethral discharge, prostatic secretions, urine (single unpreserved, 24-h unpreserved, or early-morning specimens)	Giemsa, immunoassay reagents (IFT) *(Trichomonas vaginalis);* Delafield's hematoxylin (microfilariae); modified trichrome (microsporidia); H&E, routine histology PAS, acid-fast, tissue Gram stains, (microsporidia). Direct examination of urine sediment for *Schistosoma haematobium* eggs or microfilariae.	Although *T. vaginalis* is probably the most common parasite identified, there are others to consider, the most recently implicated organisms being in the microsporidia group. Microfilariae could also be recovered and stained. Fixation of urine with formalin prevents hatching of *Schistosoma* eggs.
	Microscopy: wet smears, smears of urine sediment, stained smears Cultivation: vaginal or urethral discharge or swab preparations	Identification and propagation of *T. vaginalis* (commercialized plastic envelope culture systems available); moving trophozoites can be detected under inverted microscope.	Material must be put into culture medium immediately after collection; do not cool or freeze.
	PCR: material unfixed native, frozen, or fixed in ethanol		

[a] EIA, enzyme immunoassay; EM, electron microscopy; EITB, enzyme-linked immunoelectro transfer blot (Western blot); GAE, granulomatous amebic encephalitis; GI, gastrointestinal; H&E, hematoxylin and eosin; IFT, immunofluorescence test; PAM, primary amebic encephalitis; PAS, periodic acid-Schiff stain; QBC, quantitative buffy coat; CSF, cerebrospinal fluid.

[b] Many parasites or parasite stages may be detected in standard histological sections of tissue material. However, species identification is difficult and additional examinations may be required. Usually, these techniques are not considered first-line methods. Additional methods like EM (electron microscopy) are carried out only by specialized laboratories and are not available for standard diagnostic purposes. EM examination for species identification has largely been replaced by PCR assays.

TABLE 6 Fecal specimens for parasites: options for collection and processing[a]

Options	Pros	Cons
Although physicians can order stools for parasitology when deemed appropriate, many laboratories include in their protocols the rejection of stools from inpatients who have been in-house for >3 days.	Patients may become symptomatic with diarrhea after they have been inpatients for a few days; symptoms are usually not attributed to parasitic infections but generally other causes.	There is always the chance that the problem is related to a nosocomial parasitic infection (rare), but *Cryptosporidium, Giardia,* and microsporidia may be possible considerations.
Examination of a single stool (O&P examination). Of organisms present, 40–50% will be found with only a single stool exam; two O&P exams are acceptable, but with three specimens the procedure is more sensitive; any patient remaining symptomatic would require additional testing.	If parasites are diagnosed in the first sample or if the patient becomes asymptomatic after collection of the first stool, subsequent specimens may not be necessary. However, with some intestinal parasitic infections, patients may alternate with constipation and diarrhea.	Diagnosis from examination of a single stool specimen depends on the parasite load in the specimen. In a series of three stool specimens, frequently not all three specimens are positive and/or may be positive for different organisms (may be a cost-effective approach).
Examination of a second stool specimen only after the first one is negative and the patient is still symptomatic.	With additional examinations, yield of protozoa increases (*E. histolytica,* 22.7%; *Giardia,* 11.3%; and *D. fragilis,* 31.1%).	Assumes that the second (or third) stool specimen is collected within the recommended 10-day time frame for a series of stools; protozoa are shed periodically. May be inconvenient for patient.
Examination of a single stool, O&P examination, and an immunoassay (EIA, FA, cartridge format) *(Giardia)*	If the examinations are negative and the patient's symptoms subside, then no further testing is probably required.	Patients may exhibit symptoms (off and on), so it may be difficult to rule out parasitic infections with only a single stool and immunoassay. If the patient remains symptomatic, then even if the *Giardia* immunoassay is negative, other protozoa may be missed *(Cryptosporidium, E. histolytica/ E. dispar, D. fragilis).*
Pooling of three specimens for examination; one concentration and one permanent stain are performed. The laboratory will pool the specimens.	Three specimens are collected over 7–10 days, which may save time and expense.	Procedure not recommended. Decreases strongly the sensitivity of the procedure; organisms present in low numbers may be missed due to the dilution factor.
Permanent stained smears performed, one from each of the three specimens, subsequently pooling of the three specimens for a single concentration on the pooled specimen.	Three specimens are collected over 7–10 days; this would maximize recovery of protozoa in areas where these organisms are most common.	Might miss light helminth infection (eggs, larvae) due to the pooling procedure.
Collection of three stool specimens; samples of stool from all three are placed into a single vial (patient is given a single vial only).	Pooling of the specimens would require only a single vial.	Absolutely not recommended. Lack of sensitivity; proper mixing of specimen and fixative complicates patient collection and depends on patient compliance.
Immunoassays performed only on selected patients[b] (children <5 yr old, children from day care centers, patients with immunodeficiencies, and patients from diarrheal outbreaks) for intestinal protozoa (see Table 7).	Would be more cost-effective than performing immunoassay procedures on all specimens.	The competence and the information needed to group patients are often not available in the laboratory.

[a] See key references 26 and 30.

[b] It's difficult to recognize an early outbreak situation where screening of all specimens for either *Giardia, Cryptosporidium* spp., or both may be relevant. If it appears that an outbreak is in the early stages, then performing the immunoassays on request can be changed to screening all stools.

TABLE 7 Approaches to stool parasitology: test ordering[a]

Patient and/or situation	Test ordered[b]	Follow-up test ordered
Immunocompromised patients with diarrhea Potential waterborne outbreak from (municipal) water supply	*Cryptosporidium* or *Giardia*/*Cryptosporidium* immunoassay[b] (see Table 2)	If immunoassays are negative and symptoms continue, special tests for microsporidia (modified trichrome stain) and coccidia (modified acid-fast stain) and O&P exam should be performed.
Patient with diarrhea (nursery school, day care center, camper, backpacker) Patient with diarrhea and potential waterborne outbreak (resort setting)	*Giardia* or *Giardia*/*Cryptosporidium*[c] immunoassay	If immunoassays are negative and symptoms continue, special tests for microsporidia and coccidia (see above) and O&P exam should be performed.
Patient with diarrhea and relevant travel history Patient with diarrhea who is a past or present resident of a developing country Patient in areas of the United States where parasites other than *Giardia* are found (e.g., in large metropolitan areas)	O&P exam, the *Entamoeba histolytica*/*E. dispar* group immunoassay, confirmation for *E. histolytica*, *Cryptosporidium* or *Giardia*/*Cryptosporidium* immunoassay[c]	If exams are negative and symptoms continue, special tests for coccidia and microsporidia should be performed.
Patient with unexplained eosinophilia	O&P exam with emphasis on helminths (unfixed stool, for sedimentation/flotation techniques); it is very important to make sure that an infection with *Strongyloides stercoralis* has been ruled out–particularly if the patient is immunosuppressed or may become immunosuppressed from therapy, etc. (unfixed stool, Baermann-concentration or agar plate culture).	If the O&P exams are negative, agar plate cultures for *S. stercoralis* are recommended, particularly if the history is suggestive for this infection. The serological detection of specific antibody is an additional diagnostic option.
Patient with diarrhea (suspected food-borne outbreak)	Test for *Cyclospora cayetanensis* (modified acid-fast stain)	If test is negative and symptoms continue, special procedures for microsporidia and other coccidia and O&P exam should be performed.

[a] Modified from reference 10.
[b] Depending on the particular immunoassay kit used, various single or multiple organisms may be included. Selection of a particular kit depends on many variables: clinical relevance, cost, ease of performance, training, personnel availability, number of test orders, training of physician clients, sensitivity, specificity, equipment, time to result, etc. Very few laboratories will handle this type of testing in exactly the same way. Many options are clinically relevant and acceptable for patient care.
[c] More than one stool specimen may need to be tested using an immunoassay in order to rule out an infection with *Giardia;* fecal immunoassays may also be negative in cases with a low parasite load.

is at the user's discretion. Aqueous formalin will permit the examination of the specimen as a wet mount only, a technique much less accurate than a stained smear for the identification of intestinal protozoa.

Protozoan cysts (not trophozoites), coccidian oocysts, helminth eggs, and larvae are well preserved for long periods in 10% aqueous formalin. Hot (60°C) formalin can be used for specimens containing helminth eggs, since in cold formalin some thick-shelled eggs may continue to develop, become infective, and remain viable for long periods. Several grams of fecal material should be thoroughly mixed in 5 or 10% formalin (ratio, 1:10).

Formaldehyde vapor concentrations must be monitored and maintained at concentrations below the following: 8-h time-weighted average, i.e., 0.75 ppm, and 15-min short-term exposure limit, i.e., 2.0 ppm (9). The amount of formaldehyde used in microbiology is quite small; laboratory monitoring values are generally well below the required maximum concentrations. Initial monitoring must be repeated any time there is a change in production, equipment, process, personnel, or control measures which may result in new or additional exposure to formaldehyde. Stool fixatives that contain formaldehyde indicate that fact on the label. A number of single-vial fixative collection systems are now available; although they may not contain formaldehyde, the actual formulas are proprietary.

SAF

Both the concentration and the permanent stained smear can be performed with specimens preserved in SAF, which does not contain mercuric chloride, which is found in Schaudinn's fluid and PVA. It is a liquid fixative, much like the 10% formalin described above. The sediment is used to prepare the permanent smear, and it is frequently recommended that the stool material be placed on an albumin-coated slide to improve adherence to the glass (29, 33).

TABLE 8 Fecal preservatives: pros and cons

Stool preservative	Pros	Cons
Formalin	Good overall fixative for stool concentration. Easy to prepare, long shelf life. Concentrated sediment can be used with different stains[a] but not with all immunoassays (see Table 2).	Does not preserve trophozoites well. Does not adequately preserve organism morphology for a good permanent stained smear; not optimal for all immunoassays; not appropriate for molecular diagnosis (PCR).
SAF	Can be used for concentration and permanent stained smears. Contains no mercury compounds. Easy to prepare, long shelf life. Concentrated sediment can be used with most of the new immunoassay methods and special stains.	Poor adhesive properties; albumin-coated slides recommended. Protozoan morphology better if iron hematoxylin stain is used for permanent stained smears (trichrome is not quite as good). May be a bit more difficult to use; however, this is really not a limiting factor; formalin (see above).
Schaudinn's fixative	Fixative for smears prepared from fresh fecal specimens or samples from the intestinal mucosa surfaces. Provides excellent preservation of protozoan trophozoites and cysts.	Not generally recommended for concentration procedures. Contains mercuric chloride; creates disposal problems. Poor adhesive qualities with liquid or mucoid specimens.
PVA	Can prepare permanent stained smears and perform concentration techniques (less common). Provides excellent preservation of protozoan trophozoites and cysts. Specimens can be shipped to the laboratory for subsequent examination; organism morphology excellent after processing. Suitable for DNA-PCR analysis.	*Trichuris trichiura* eggs and *Giardia* cysts are not concentrated as easily as from formalin-based fixatives. *Strongyloides stercoralis* larval morphology will be poor (better from formalin-based preservation). *Isospora belli* oocysts may not be visible from PVA-preserved material (better from formalin-based preservation). Contains mercury compounds (Schaudinn's fluid). May turn white and gelatinous when it begins to dehydrate or when refrigerated. Difficult to prepare in the laboratory. Specimens containing PVA cannot be used with the immunoassay methods.
Modified PVA	Can prepare permanent stained smears and perform concentration techniques (less common). Many workers prefer the zinc substitutes over those prepared with copper sulfate. Does not contain mercury compounds.	Overall protozoan morphology of trophozoites and cysts is poor when preserved in the copper-sulfate-based fixative, particularly when compared with organisms preserved in mercuric chloride-based fixatives. Zinc-based fixatives appear to be some of the better alternatives. Staining characteristics of protozoa not consistent; some are good, some are poor. Organism identification may be difficult, particularly with small protozoan cysts *(Endolimax nana)*.
Single-vial systems	Can prepare permanent stained smears and perform concentration techniques. Can perform immunoassays—"universal fixative" under development. Do not contain mercury compounds. Unless organism numbers are very low, acceptable organism recovery and identification are possible; additional training may be required to recognize the organisms because the overall morphology will not be comparable to that seen using mercury-based fixatives.	Overall protozoan morphology of trophozoites and cysts is not as good as when compared with organisms preserved with mercuric chloride-based fixatives; similar to modified PVA options. Staining characteristics of protozoa not consistent; some are good, some are poor. Identification of *Endolimax nana* cysts may be difficult. Not all immunoassays can be performed from stool specimens preserved in these fixatives.

[a] Modified acid-fast and modified trichrome stains.

SAF is considered to be a "softer" fixative than mercuric chloride. The organism morphology will not be quite as sharp after staining as will that of organisms originally fixed in solutions containing mercuric chloride. The pairing of SAF-fixed material with iron hematoxylin staining provides better organism morphology than does staining of SAF-fixed material with trichrome (personal observation). Although SAF has a long shelf life and is easy to prepare, the smear preparation technique may be a bit more difficult for less experienced personnel who are not familiar with fecal specimen techniques. Laboratories that have considered using only a single preservative have selected this option. Helminth eggs and larvae, protozoan trophozoites and cysts, coccidian oocysts, and microsporidian spores are preserved using this method.

Schaudinn's Fluid

Schaudinn's fluid (contains mercuric chloride) is used with fresh stool specimens or samples from the intestinal mucosal

surface. Many laboratories that receive specimens from in-house patients (no problem with delivery times) often select this approach. Permanent stained smears are then prepared from fixed material. A concentration technique using Schaudinn's fluid-preserved material is also available but is not widely used (3, 9, 20, 24).

PVA

PVA is a plastic resin that is normally incorporated into Schaudinn's fixative. The PVA powder serves as an adhesive for the stool material; i.e., when the stool-PVA mixture is spread onto the glass slide, it adheres because of the PVA component. Fixation is still accomplished by the Schaudinn's fluid itself. Perhaps the greatest advantage in the use of PVA is that a permanent stained smear can be prepared. Like SAF, PVA fixative solution is recommended as a means of preserving cysts and trophozoites for later examination and permits specimens to be shipped (by regular mail service) from any location in the world to a laboratory for subsequent examination. PVA is particularly useful for liquid specimens and should be used in the ratio of 3 parts PVA to 1 part fecal specimen (5).

Modified PVA

Although preservatives that do not contain mercury compounds have been developed, substitute compounds have not provided the quality of preservation necessary for good protozoan morphology on the permanent stained smear. Copper sulfate has been tried but does not provide results equal to those seen with mercuric chloride (19). Zinc sulfate has proven to be an acceptable mercury substitute and is used with trichrome stain. Although zinc substitutes have become widely available, each manufacturer has a proprietary formula for the fixative (14, 16).

Single-Vial Collection Systems

Several manufacturers now have available single-vial stool collection systems, similar to SAF or modified PVA methods. From the single vial, both the concentration and permanent stained smear can be prepared. It is also possible to perform immunoassays from some of these vials. Ask the manufacturer about all diagnostic capabilities (concentrate, permanent stained smear, immunoassay procedures, molecular analyses) and for specific information indicating that there are no formula components that would interfere with any of the methods. Like the zinc substitutes, these formulas are proprietary (16). The ability to perform immunoassays using fixed specimens will vary depending on the fixative and specific immunoassay being used.

New-Formulation Single-Vial Collection Systems

New formulations continue to be developed, some of which appear to adhere to the slide without the use of albumin or PVA. These formulations can be used to perform concentrations and some of the immunoassays and to prepare permanent stained smears.

Collection of Blood

Depending on the life cycle, there are a number of parasites that may be recovered in a blood specimen, either whole blood, buffy coat preparations, or various types of concentrations (10, 20, 24). These parasites include *Plasmodium, Babesia, Trypanosoma, Leishmania, Toxoplasma,* and microfilariae. Although some organisms may be motile in fresh, whole blood, species identification will normally be accomplished from the examination of permanent stained blood thin and/or thick films. Blood films can be prepared from fresh, whole blood collected with no anticoagulants, anticoagulated blood, or sediment from the various concentration procedures.

Unless it is certain that well-prepared slides will be available, it is necessary to request a tube of fresh blood (EDTA anticoagulant is preferred) and prepare the smears. The tube should be filled with blood to provide the proper blood/anticoagulant ratio. For detection of stippling, the smears should be prepared within 1 h after the specimen is drawn. After that time, stippling may not be visible on stained films; however, the overall organism morphology will still be excellent. Most laboratories routinely use commercially available blood collection tubes; preparation of EDTA collection tubes in-house is neither necessary nor cost-effective.

The time the specimen was drawn should be clearly indicated on the tube of blood and also on the result report. The physician will then be able to correlate the results with any fever pattern or other symptoms that the patient may have. There should also be some comments on the test result report that is sent back to the physician, stating that one negative specimen does not rule out the possibility of a parasitic infection (9, 28).

Collection of Specimens from Other Body Sites

Although clinical specimens for examination can be obtained from many other body sites, these specimens and appropriate diagnostic methods are not as commonly performed as those used for the routine stool specimen or for blood specimens. The majority of specimens from other body sites (Table 1) would be submitted as fresh specimens for further testing.

DIRECT DETECTION BY ROUTINE METHODS

Intestinal Tract Specimens

The most common specimen submitted to the diagnostic laboratory is the stool specimen, and the most commonly performed procedure in parasitology is the ova and parasite examination (O&P), which is composed of three separate protocols: the direct wet mount, the concentration, and the permanent stained smear. The direct wet mount requires fresh stool, is designed to allow the detection of motile protozoan trophozoites, and is examined microscopically at low and high dry magnifications (×100, entire 22- by 22-mm coverslip [larvae, larger helminth eggs]; ×400, one-third to one-half of a 22- by 22-mm coverslip [protozoan cysts and/or trophozoites, smaller helminth eggs]). However, due to potential problems with the lag time between specimen passage and receipt in the laboratory, the direct wet examination has been eliminated from the routine O&P in favor of receipt of specimens collected in stool preservatives; if specimens are received in the laboratory in stool collection preservatives, the direct wet preparation is not performed.

The second part of the O&P is the concentration, which is designed to facilitate the recovery of protozoan cysts, coccidian oocysts, microsporidial spores, and helminth eggs and larvae. Both flotation (zinc sulfate, zinc chloride, and others) and sedimentation methods are available, the most common procedure being the formalin-ethyl acetate sedimentation method (formerly called the formalin-ether method). The concentrated specimen is examined as a wet preparation, with or without iodine, using low and high dry magnifications (×100 and ×400) as indicated for the direct wet smear

examination. It is important to remember that large, heavy helminth eggs (unfertilized *Ascaris* eggs) or eggs that are operculated (trematode and some cestode eggs) will not float optimally when flotation fluids with densities of <1.35 are used; both the surface film and sediment must be examined by this method.

The third part of the O&P is the permanent stained smear, which is designed to facilitate the identification of intestinal protozoa. Several staining methods are available, the two most common being the Wheatley modification of the Gomori tissue trichrome and the iron hematoxylin stains. This part of the O&P examination is critical for the confirmation of suspicious objects seen in the wet examination and identification of protozoa that might not have been seen in the wet preparation. The permanent stained smears are examined using oil immersion objectives (×600 for screening, ×1,000 for final review of ⩾300 oil immersion fields).

Other specimens from the intestinal tract such as duodenal aspirates or drainage, mucus from the Entero-Test Capsule technique (HDC Corp., San Jose, Calif.), and sigmoidoscopy material can also be examined as wet preparations and as permanent stained smears after processing with either trichrome or iron hematoxylin staining. Although not all laboratories will examine these types of specimens, they are included to give some idea of the possibilities for diagnostic testing.

Amniotic Fluid

Methods for the diagnosis of congenital *Toxoplasma* infection are summarized in chapter 138, "Toxoplasma." Amniotic fluid collected under sterile conditions allows both mouse or tissue culture inoculation and molecular diagnosis by PCR. PCR is the recommended test of choice for the detection of *Toxoplasma* in amniotic fluid.

Urogenital Tract Specimens

The identification of *Trichomonas vaginalis* is usually based on the examination of wet preparations of vaginal and urethral discharges and prostatic secretions or urine sediment. Multiple specimens may have to be examined before the organisms are detected. These specimens are diluted with a drop of saline and examined under low power (×100) and reduced illumination for the presence of motile organisms; as the jerky motility begins to diminish, it may be possible to observe the undulating membrane, particularly under high dry power (×400). Culture systems (InPouch TV; BioMed Diagnostics, San Jose, Calif. [http://www.biomed1.com/TV.htm] and Empyrean Diagnostics, Inc., Mountain View, Calif.) that allow direct inoculation, transport, culture, and microscopic examination are available commercially (2, 9). Stained smears such as Papanicolaou or Giemsa can be used but are usually not necessary for the identification of this organism and can be difficult to interpret. If a dry smear is received by the laboratory, it can be fixed with absolute methanol and stained with Giemsa stain; this approach is not optimal, but the smear can be examined and may confirm a positive infection. The number of false-positive and false-negative results reported on the basis of stained smears strongly suggests the value of confirmation by observation of motile organisms from the direct mount, from appropriate culture media, or from direct detection by more sensitive immunological or molecular methods (Table 3) (6).

Examination of urinary sediment may be indicated in certain filarial infections. Administration of the drug diethylcarbamazine (Hetrazan) has been reported to enhance the recovery of microfilariae from the urine. The triple-concentration technique is recommended for the recovery of microfilariae (9). The membrane filtration technique can also be used with urine for the recovery of microfilariae (9). *Schistosoma haematobium* eggs can be concentrated by centrifugation of urine specimens; a membrane filter technique for the egg recovery has also been useful (9). Fresh samples or fixed samples in formalin should be used to prevent hatching of eggs.

Sputum

Although not one of the more common specimens, expectorated sputum may be submitted for examination for parasites. Organisms in sputum that may be detected and may cause pneumonia, pneumonitis, or Loeffler's syndrome include the migrating larval stages of *Ascaris lumbricoides, Strongyloides stercoralis,* and hookworm; the eggs of *Paragonimus* spp.; *Echinococcus granulosus* hooklets; and *Entamoeba histolytica, Entamoeba gingivalis, Trichomonas tenax, Cryptosporidium* spp., and possibly the microsporidia. In a *Paragonimus* infection, the sputum may be viscous and tinged with brownish flecks, which are clusters of eggs ("iron filings"), and may be streaked with blood. Sputum is usually examined as a wet mount (saline or iodine), using low and high dry power (×100 and ×400). The specimen is not concentrated before preparation of the wet mount. If the sputum is thick, an equal amount of 3% sodium hydroxide (NaOH) (or undiluted chlorine bleach) can be added; the specimen is thoroughly mixed and then centrifuged at 500 × *g* for 5 min. NaOH should not be used if one is looking for *Entamoeba* spp. or *T. tenax.* After centrifugation, the supernatant fluid is discarded and the sediment can be examined as a wet mount with saline or iodine. If examination has to be delayed for any reason, the sputum should be fixed in 5 or 10% formalin to preserve helminth eggs or larvae or in PVA fixative to be stained later for protozoa.

Aspirates

The examination of aspirated material for the diagnosis of parasitic infections may be extremely valuable, particularly when routine testing methods have failed to demonstrate the organisms. These specimens should be transported to the laboratory immediately after collection. Aspirates include liquid specimens collected from a variety of sites where organisms might be found. The aspirates most commonly processed in the parasitology laboratory include fine-needle and duodenal aspirates. Fluid specimens collected by bronchoscopy include bronchoalveolar lavage fluid and bronchial washings (23).

Fine-needle aspirates may be submitted for slide preparation, culture, and/or molecular analyses. Aspirates of cysts and abscesses for amebae may require concentration by centrifugation, digestion, microscopic examination for motile organisms in direct preparations, and cultures and microscopic evaluation of stained preparations. Antigen detection and PCR are other possibilities, depending on individual laboratory testing options.

Bone marrow aspirates for *Leishmania* amastigotes, *Trypanosoma cruzi* amastigotes, or *Plasmodium* spp. require Giemsa staining. Examination of these specimens may confirm an infection that has been missed by examination of routine blood films. In certain situations, culture, immunoassays for antigen detection, or PCR will also provide more-sensitive results (22).

Biopsy Specimens

Biopsy specimens are recommended for the detection of tissue parasites (Table 5). The following procedures may be used for this purpose in addition to standard histologic

preparations: impression smears and teased and squash preparations of biopsy tissue from skin, muscle, cornea, intestine, liver, lung, and brain. Tissue to be examined by permanent sections or electron microscopy should be fixed as specified by the laboratories that will process the tissue. In certain cases, a biopsy may be the only means of confirming a suspected parasitic infection. Specimens that are going to be examined as fresh material rather than as tissue sections should be kept moist in saline and submitted to the laboratory immediately.

Detection of parasites in tissue depends in part on specimen collection and on having sufficient material to perform the recommended diagnostic procedures. Biopsy specimens are usually quite small and may not be representative of the diseased tissue. Multiple tissue samples will often improve diagnostic results. To optimize the yield from any tissue specimen, all areas should be examined by as many procedures as possible. Tissues are obtained from invasive procedures, many of which are very expensive and lengthy; consequently, these specimens deserve the most comprehensive procedures possible.

Tissue submitted in a sterile container in sterile saline or on a sterile sponge dampened with saline may be used for cultures or molecular analyses of protozoa after mounts for direct examination or impression smears for staining have been prepared. If cultures for parasites are to be made, sterile slides should be used for smear and mount preparation.

Blood

Depending on the life cycle, a number of parasites may be recovered in a blood specimen, either whole blood, buffy coat preparations, or various types of concentrations (9, 28). Although some organisms may be motile in fresh whole blood, species identification will normally be accomplished from the examination of permanent stained blood films, both thick and thin films. Blood films can be prepared from fresh whole blood collected with no anticoagulants, anticoagulated blood, or sediment from the various concentration procedures. The recommended stain of choice is Giemsa stain; however, the parasites can also be seen on blood films stained with Wright's stain. Delafield's hematoxylin stain is often used to stain the microfilarial sheath; in some cases, Giemsa stain does not provide sufficient stain quality to allow differentiation of the microfilariae.

Thin Blood Films

In any examination of thin blood films for parasitic organisms, the initial screen should be carried out with the low-power objective (10×) of a microscope. Microfilariae may be missed if the entire thin film is not examined. Microfilariae are rarely present in large numbers, and frequently only a few organisms are present in each thin film preparation. Microfilariae are commonly found at the edges of the thin film or at the feathered end of the film because they are carried to these sites during spreading of the blood. The feathered end of the film, where the erythrocytes (RBCs) are drawn out into one single, distinctive layer of cells, should be examined for the presence of malaria parasites and trypanosomes. In these areas, the morphology and size of the infected RBCs are most clearly seen.

Depending on the training and experience of the microscopist, examination of the thin film usually takes 15 to 20 min (≥300 oil immersion fields) for the thin film at a magnification of ×1,000. Although some people use a 50× or 60× oil immersion objective to screen stained blood films, there is some concern that small parasites such as plasmodia, *Babesia* spp., or *Leishmania* spp. may be missed at this lower total magnification (×500 or ×600) compared with the ×1,000 total magnification obtained using the more traditional 100× oil immersion objective. Because people tend to scan blood films at different rates, it is important to examine a minimum number of fields. If something suspicious has been seen in the thick film, the number of fields examined on the thin film is often considerably greater than 300. The request for blood film examination should always be considered a STAT procedure, with all reports (negative as well as positive) being relayed by telephone to the physician as soon as possible. If positive, notification of appropriate governmental agencies (local, state, and federal) should be done within a reasonable time frame in accordance with guidelines and laws. Most county or state public health laboratories require reporting of *Plasmodium*- and *Babesia*-positive cases; the local public health facility should be consulted for a list of reportable parasitic diseases relevant for your location.

Both malaria and *Babesia* infections have been missed with automated differential instruments, and therapy was delayed. Although these instruments are not designed to detect intracellular blood parasites, the inability of the automated systems to discriminate between uninfected RBCs and those infected with parasites may pose serious diagnostic problems (15).

Thick Blood Films

In the preparation of a thick blood film, the highest concentration of blood cells will be in the center of the film. The examination should be performed at low magnification to detect microfilariae more readily. Examination of a thick film usually requires 5 to 10 min (approximately 100 oil immersion fields). The search for malarial organisms and trypanosomes is best done under oil immersion (total magnification, ×1,000). Intact RBCs are frequently seen at the very periphery of the thick film; such cells, if infected, may prove useful in malaria diagnosis since they may demonstrate the characteristic morphology necessary to identify the organisms to the species level.

Blood Stains

For accurate identification of blood parasites, a laboratory should develop proficiency in the use of at least one good staining method. It is better to select one method that will provide reproducible results than to use several on a hit-or-miss basis. Blood films should be stained as soon as possible, since prolonged storage may result in stain retention. Failure to stain positive malarial smears within a month may result in failure to demonstrate typical staining characteristics for individual species.

The most common stains are of two types. Wright's stain has the fixative in combination with the staining solution, so that both fixation and staining occur at the same time; therefore, the thick film must be laked before staining. In Giemsa stain, the fixative and stain are separate; therefore, the thin film must be fixed with absolute methanol before staining.

Buffy Coat Films

Trypanosomes, occasionally *Histoplasma capsulatum* (a fungus with intracellular elements resembling those of *Leishmania* amastigote stages) and, in immunocompromised patients, potentially *Leishmania* spp. (*L. infantum, L. chagasi,* and *L. donovani)* are detected in the peripheral blood. The parasite or fungus will be found in the large mononuclear cells in the buffy coat (a layer of leukocytes resulting from

centrifugation of whole anticoagulated blood). The nuclear material will stain dark red-purple, and the cytoplasm will be light blue (*Leishmania* spp.). *H. capsulatum* will appear as a large dot of nuclear material (dark red-purple) surrounded by a clear halo area. Trypanosomes in the peripheral blood will also concentrate with the buffy coat cells.

Screening Methods

Microhematocrit centrifugation with use of the QBC malaria tube, a glass capillary tube and closely fitting plastic insert (CBC malaria blood tubes; Becton Dickinson, Tropical Disease Diagnostics, Sparks, Md.), has been used for the detection of blood parasites. At the end of centrifugation of 50 to 60 μl of capillary or venous blood (5 min in a QBC centrifuge, 14,387 × *g*), parasites or RBCs containing parasites are concentrated into a small, 1- to 2-mm region near the top of the RBC column and are held close to the wall of the tube by the plastic float, making them readily visible by microscopy. Tubes precoated with acridine orange provide a stain which induces fluorescence in the parasites. This method automatically prepares a concentrated smear, which represents the distance between the float and the walls of the tube. Once the tube is placed into the plastic holder (Para-viewer) and immersion oil is applied to the top of the hematocrit tube (no coverslip is necessary), the tube is examined with a 40× to 60× oil immersion objective (which must have a working distance of 0.3 mm or greater) (9).

Antigen and DNA Detection for Blood Parasites

Several antigen detection tests are available. Many among them are designed for rapid and individual diagnoses (Table 3; see also below). Most of the kits are not available in the United States but have proven to be very useful in other countries. Rapid tests are available to diagnose specifically *P. falciparum* or, on a genus level, *Plasmodium* sp. infections (Table 3). These tests are simple to perform and can be applied during the more time-consuming microscopic identification of the thin and thick blood smears. However, one has to be aware that false-positive and false-negative reactions do occur (4, 8, 32). The general recommendation is to use these tests only in addition to the microscopical examination of thick and thin blood smears. Various PCR-based methods haven been described in the scientific literature. Although the methods are more sensitive than standard microscopical examinations of blood films, they are not routinely used for malaria diagnosis (see below).

Knott Concentration

The Knott concentration procedure is used primarily to detect the presence of microfilariae in the blood, especially when a light infection is suspected. The disadvantage of the procedure is that the microfilariae are killed by the formalin and are therefore not seen as motile organisms.

Membrane Filtration Technique

The membrane filtration technique using Nuclepore filters (25-mm Nuclepore filter [5-μm porosity]) has proved highly efficient in demonstrating filarial infections when microfilaremias are of low density. It has also been successfully used in field surveys (9).

Culture Methods

Very few clinical laboratories offer specific culture techniques for parasites. The methods for in vitro culture are often complex, while quality control is difficult and not really feasible for the routine diagnostic laboratory. In certain institutions, some techniques may be available, particularly where consultative services are provided and for research purposes.

Few parasites can be routinely cultured, and the only procedures that are in general use are for *Entamoeba histolytica, Naegleria fowleri, Acanthamoeba* spp., *Trichomonas vaginalis, Toxoplasma gondii, Trypanosoma cruzi, Encephalitozoon* spp., and the leishmanias. These procedures are usually available only after consultation with the laboratory and on special request. Commercialized tests are available only for *T. vaginalis* (InPouch TV; BioMed Diagnostics [http://www.biomed1.com/TV.htm]).

Animal Inoculation and Xenodiagnosis

Most routine clinical laboratories do not have the animal care facilities necessary to provide animal inoculation capabilities for the diagnosis of parasitic infections. Host specificity for many animal parasite species is well known and will limit the types of animals available for these procedures. In certain suspect infections animal inoculation may be requested and can be very helpful in making the diagnosis, although animal inoculation certainly does not take the place of other, more routine procedures. Mouse inoculation with amniotic fluid has been used in the past for diagnosis of congenital toxoplasmosis; this method, however, has mostly been replaced by PCR. Intraperitoneal inoculation of *Echinococcus multilocularis* metacestode material from surgical resection or from biopsies in mice or gerbils is still the most reliable procedure available for viability testing after long-term chemotherapy.

Xenodiagnosis is a technique that uses the arthropod host as an indicator of infection. Uninfected reduviid bugs are allowed to feed on the blood of a patient who is suspected of having Chagas' disease (*T. cruzi* infection). After 30 to 60 days, feces from the bugs are examined over a 3-month time frame for the presence of developmental stages of the parasite, which are found in the hindgut of the vector. This type of procedure is used primarily in South America for fieldwork, and the appropriate bugs are raised in various laboratories specifically for this purpose.

Antigen Detection

The detection of parasite-specific antigen is indicative of current infection. Immunoassays are generally simple to perform. Some formats allow the processing of large numbers of tests at one time, thereby reducing overall costs; others are specially designed for rapid individual diagnoses (cartridge formats) (Tables 2 and 3). A major disadvantage of antigen detection is that in most cases the method can detect only a single pathogen at one time. Therefore, additional parasitological examinations must be performed to detect other parasitic pathogens. The current commercially available immunoassays for the detection of intestinal protozoa have excellent sensitivity and specificity compared to routine microscopy (1, 11, 12, 13). Specific ordering approaches using both immunoassays and routine O&P examinations are listed in Table 7. Rapid tests for the diagnosis of malaria should be used only in parallel to the examination of thick and thin blood smears.

Parasite DNA Detection

Nucleic acid-based diagnostic tests (particularly PCR with its inherent potential for highly efficient and specific amplification of DNA) have been developed for almost all species of parasites. However, only a few are routinely used in diagnostic settings. The main reason for this minor role of diagnostic

PCR in parasitology is the fact that many parasite stages can be adequately diagnosed using established, more traditional techniques (microscopy, detection of antigens and antibodies, in vitro cultivation) that are generally less expensive than PCR and technically less demanding. Therefore, diagnosis by PCR is of great value in cases where these techniques are insufficient, i.e., in cases where (i) the immune response is not informative (e.g., acute infections, short-term follow-up after therapy, congenital infections); (ii) high sensitivity is needed because of low parasite levels (e.g., cutaneous leishmaniasis); or (iii) morphologically indistinguishable organisms need to be identified (e.g., *E. histolytica*/*E. dispar,* eggs of taeniid tapeworms).

Diagnostic PCR may become more widespread when simple, fully standardized (commercialized) test kits are available and costs are reduced through the implementation of pre- and post-PCR automated techniques. Furthermore, the possibilities to not only detect and identify but also quantify organisms and determine their genotypes by analyzing the diagnostic PCR product extend the diagnostic power of PCR. Indeed, PCR coupled with genetic characterization is already widely being used in parasitology to address questions such as parasite host range and host specificity, ways of transmission, and molecular epidemiology. Such genotyping applications should increase in the future with increasing knowledge of the relationship between genetic variation in parasites and features such as virulence or drug resistance. An important limitation of PCR-based diagnosis is the fact that sensitivity dramatically decreases with material stored for more than 1 day in formalin due to the fragmentation (fragment length of a few hundred base pairs) of the DNA. However, by selecting primers that produce PCR products as short as possible, which is recommended for real-time PCR, sensitivity might be reasonably high. Such tests, however, are not yet widely available in parasitology. Therefore, it seems to be the best choice to avoid formalin fixation if PCR analyses have to be considered.

APPENDIX

Parasite images

Parasites and Parasitological Resources
http://www.biosci.ohio-state.edu/~parasite/home.html

University of Delaware
http://www.udel.edu/medtech/dlehman/medt372/index.html

Parasite Image Library
http://www.dpd.cdc.gov/DPDx/HTML/Image_Library.htm

Protozoa Images
http://www.medicine.cmu.ac.th

Pictorial presentation of parasites and patients
http://curezone.com/ig/igallery.asp?d=\parasites\

University of Georgia College of Agricultural and Environmental Sciences
http://sacs.cpes.peachnet.edu/nemabc/slideset1.htm

University of Cincinnati Clermont College
http://biology.clc.uc.edu/fankhauser/Labs/Microbiology/Prepared_Slides/Micro_Protozoans.htm

Parasitology information

Centers for Disease Control and Prevention
http://www.dpd.cdc.gov/DPDx/

World Health Organization
http://www.who.int

Medical Chemical Corporation
http://www.med-chem.com

Additional parasitology websites

Department of Parasitology, Faculty of Medicine, Chiang Mai University, Thailand
http://www.med.cmu.ac.th/dept/parasite/Default.htm

Biosystematics & the United States, National Parasite Collection Unit
http://www.lpsi.barc.usda.gov/bnpcu/

Medical Parasitology Links
http://www.hanmat.org/links.htm

Oklahoma State University
http://www.cvm.okstate.edu/instruction/kocan/vpar5333/vpar5333.htm

University of California, Davis, Department of Nematology
http://ucdnema.ucdavis.edu/imagemap/nemmap/ENT156HTML/E156charac

University of Edinburgh
http://www.ed.ac.uk/cpb/websites/htm

Medical Parasitology Links
http://www.geocities.com/aaadeel/

Literature

NCBI National Library of Medicine (PubMed)
http://www.ncbi.nim.nih.gov/entrez/query.fcgi

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Reagents, Stains, and Media: Parasitology

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The evaluation of clinical specimens for ova and parasites in the clinical laboratory can involve the use of direct macroscopic examination of the specimen and microscopic examination of fresh and preserved specimens, as well as culture for some parasitic organisms. These examinations necessitate the use of a variety of stains, reagents, and media, the most common of which are discussed in this chapter.

As many parasitic organisms cannot be cultured, microscopic examination is the mainstay of diagnostic parasitology. Examination after proficient staining of fresh and unconcentrated specimens, as well as preserved and/or concentrated specimens with permanent stained preparations, most often provides for a rapid and accurate diagnosis. A variety of reagents and stains are available for these purposes, and each laboratory must decide which ones to use to best serve its patient population. In addition, as most specimens are submitted in fixatives and preservatives because of the inability to perform immediate direct observation, the reader should note the specific interference of polyvinyl alcohol (PVA) and mercury reagents with immunoassays that are commonly used for parasitic diagnosis.

Caution should be taken when using reagents in the parasitology laboratory. Many routinely used compounds can be dangerous if not handled appropriately. For example, formalin and formaldehyde solutions can cause severe skin irritation and if swallowed can cause violent vomiting and diarrhea; mercury compounds are local irritants and systemic poisons that can be absorbed through the skin; phenol is a skin irritant and can affect the central nervous system if one is exposed to large amounts; and xylene can cause serious skin irritation, with extended exposure causing gastrointestinal, neurologic, and tissue damage (5).

REAGENTS

All of the following reagents listed are preservatives and/or fixatives.

Formalin Preparations

■ Formalin

Formalin has always been used in parasitology as an all-purpose preservative and for use in concentration procedures. Formalin alone cannot be used for concentration techniques and permanent stained smears. It is easy to prepare and has a long shelf life. It is most routinely used as a preservative for stool and duodenal aspirate specimens. Formalin works well to preserve the morphologies of helminth ova and larvae and those of protozoan cysts, oocysts, and spores, although it does not preserve protozoan trophozoites well. Although both 5 and 10% solutions of formalin are currently used (5% for the best preservation of protozoan stages and 10% for ova and larvae), the 10% formulation is most widely used in clinical parasitology today. Formalin (10%) is prepared as indicated below:

Formaldehyde (37 to 40% HCHO solution)	100 ml
Saline (0.85% NaCl) OR distilled water	900 ml

Buffered formalin

Formalin buffered with sodium and potassium phosphates can be used to help maintain the morphology of parasites for long-term storage. To make buffered formalin, mix the following dry ingredients together and store in a tightly closed container. Add 0.8 g of this mixture to 1 liter of 10% (or 5%) formalin.

Sodium phosphate, dibasic (Na_2HPO_4)	6.1 g
Potassium phosphate, monobasic (KH_2PO)	0.15 g

■ Merthiolate-iodine-formalin (MIF)

Protozoa and helminth ova and larvae can be distinguished on direct wet mount after stool and duodenal aspirate specimens are fixed in MIF. MIF allows not only for fixation but also for staining of the organisms, although organism morphology is not as good as when other permanent staining compounds are used. This method requires that two stock solutions be combined into a fresh working solution immediately prior to use. In addition, after mixing of specimens with MIF, the mixture must be left undisturbed for 24 h prior to preparation of smears from the bottom two layers of the three layers that will form. Parasitological examination of specimens placed in MIF can be performed for several weeks after preservation, making it very useful for field surveys. MIF is also readily available commercially.

PVA-Containing Preservatives/Fixatives

PVA acts as an adhesive for stool material, allowing the stool to adhere to glass slides. Several modifications are commercially available. The accompanying compound with the PVA, specifically, mercuric chloride, zinc sulfate, or cupric-sulfate, acts as the preservative and allows fixation of protozoan cysts and trophozoites for use with trichrome or iron hematoxylin stains for permanents smears. All PVA-containing preservatives interfere with immunoassays.

■ Mc-PVA

The Mc-PVA fixative uses mercuric chloride as the preservative. Protozoan morphology is best preserved with PVA incorporating mercury compounds. However, due to the toxic nature of mercury compounds and the difficulty in preparation, most laboratories no longer prepare Mc-PVA.

■ ZN-PVA

The ZN-PVA fixative uses zinc sulfate in place of mercury as a preservative and fixative for protozoans. Specimens treated with ZN-PVA may also be stained with trichrome or iron hematoxylin stains for permanent smears.

■ Cu-PVA

The Cu-PVA fixative uses cupric sulfate in place of mercury as a preservative and fixative for protozoans. Specimens treated with Cu-PVA may also be stained with trichrome or iron hematoxylin stains for permanent smears.

Studies have shown that zinc sulfate provides a satisfactory, but not equal, substitute for mercury in the permanent staining procedures and that copper sulfate does not preserve morphology equal to that seen with mercuric chloride (6, 7).

■ Schaudinn's fixative/solution

Schaudinn's solution is a fixative made of mercuric chloride, distilled water, and 95% ethyl alcohol that gives excellent morphological preservation of protozoan organisms. It is used primarily in the preparation of permanent stained smears for parasitological examination from fresh, nonpreserved specimens. Specimens fixed in Schaudinn's solution can be used with either trichrome or iron hematoxylin stains. As with Mc-PVA, due to the toxic nature of mercuric compounds and the difficulty in preparation of this fixative, Schaudinn's solution is not routinely made in the clinical microbiology laboratory. In addition, Schaudinn's fixative is becoming increasingly more difficult to purchase commercially.

■ Sodium acetate-acetic acid-formalin (SAF)

SAF is very similar to formalin in that it is a liquid fixative and contains no mercury. However, unlike formalin, SAF can be used for both concentration techniques and permanent stained smears. The sediment from the concentration procedure is used for both the wet preparation and the permanent stain. Albumin-coated slides allow for better adhesion of the concentrated material to the slide and are recommended for use with SAF. SAF is an acceptable substitute for PVA or Schaudinn's solution for permanent smears stained with either trichrome or iron hematoxylin. SAF is available commercially and has a long shelf life, but it can also readily be made in the laboratory by mixing the reagents indicated below:

Sodium acetate	1.5 g
Glacial acetic acid	2.0 ml
Formaldehyde (37 to 40% HCHO solution)	4.0 ml
Distilled water	92.0 ml

■ Nonmercury, nonformalin fixatives—single-vial systems

There are several nonmercury, nonformalin proprietary fixatives commercially available that can be used for concentration of stool specimens, preparations of permanently stained smears, and fecal immunoassays (check with manufacturers for specific uses). Special stains are also available for use with some of these fixatives.

Table 1 lists several types of preservatives along with their content, specific permanent stained smears that can be performed, and immunoassays for which they can be used, as well as additional comments concerning the fixative.

STAINS

■ Iodine (Lugol's or D'Antoni's)

A solution of iodine can be used when preparing direct or concentrated wet mounts for parasitological examination. These nonspecific dyes allow the differentiation of parasitic cysts from leukocytes, the former of which retain the iodine and appear light brown. Iodine stains can be purchased commercially for use in routine parasitology. These solutions should be stored in dark containers in a dark environment. D'Antoni's iodine preparation has the advantage of use without further dilution, whereas Lugol's iodine must first be diluted into a working solution (1:5 dilution in distilled water) prior to use. Working solutions of both iodine preparations fade with time and should be discarded and replaced when their dark tea color lightens.

Basic procedure

Place 1 drop of iodine on a slide and add to this a small amount of fecal specimen and mix until homogeneous. The iodine stains fecal material immediately, and the timing of this step is not important. Place a coverslip on top of the suspension and view under ×100 magnification. Examine any suspicious material under ×400 magnification.

Acid-Fast Stains (Modified)

■ Modified Kinyoun stain (cold method)

■ Modified Ziehl-Neelsen stain (hot method)

Cryptosporidium parvum, Isospora belli, and *Cyclospora cayetanensis* are parasites that can cause diarrheal disease in humans. All three of these organisms require special staining for detection, and modifications of the classic acid-fast staining techniques used for the detection of mycobacteria are used. The modifications include the use of decolorizing agents less harsh than those used for staining of mycobacteria (see chapter 21). In the modified acid-fast staining procedures, a 1% solution of sulfuric acid (1 ml of sulfuric acid in 99 ml of water) is used as opposed to the 3% solution that is used in the acid-fast bacillus (AFB) Kinyoun staining procedure, and a 5% sulfuric acid solution (5 ml of concentrated sulfuric acid in 95 ml of distilled water) is used as opposed to the 3% HCl solution in 95% ethanol that is used

TABLE 1 Preservatives used in diagnostic parasitology (intestinal tract specimens)

Preservative	Concn	Permanent stained smear	Immunoassays (*Giardia lamblia*, *Cryptosporidium parvum*)	Comments[g]
5 or 10% formalin	Yes	No	Yes	EIA, FA
5 or 10% buffered formalin	Yes	No	Yes	EIA, FA
MIF	Yes	Polychrome IV stain[e]	ND[f]	No published data for immunoassay systems
SAF	Yes	Iron hematoxylin	Yes	EIA, FA
Mc-PVA[a]	Yes	Trichrome or iron hematoxylin	No	PVA interferes with immunoassays
Cu-PVA[b]	Yes	Trichrome or iron hematoxylin	No	PVA interferes with immunoassays
Zn-PVA[c]	Yes	Trichrome or iron hematoxylin	Some, but not all	PVA interferes with immunoassays
Single-vial systems[d]	Yes	Trichrome or iron hematoxylin	Some, but not all	Check with the manufacturer
Schaudinn's (without PVA)[a]	No	Trichrome or iron hematoxylin	No	Mercury interferes with immunoassays

[a] PVA and Schaudinn's fixatives use a mercuric chloride base and are still considered to be the "gold standard" against which all other fixatives are evaluated (organism morphology after permanent staining). Additional fixatives prepared with nonmercuric chloride-based compounds are continuing to be developed and tested.
[b] This modification uses a copper sulfate base rather than mercuric chloride.
[c] This modification uses a zinc base rather than mercuric chloride and apparently works well with both trichrome and iron-hematoxylin stains.
[d] These modifications use a combination of ingredients (including zinc) but are prepared from proprietary formulas. The aim is to provide a fixative that can be used for the fecal concentration, permanent stained smear, and available immunoassays for *G. lamblia*, *C. parvum*, and *Entamoeba histolytica* (or the *E. histolytica*/*E. dispar* group).
[e] This stain can be used in place of trichrome for staining fecal smears preserved with MIF, PVA, or SAF methods. It is available commercially.
[f] ND, no data.
[g] EIA, enzyme immunoassay; FA, fluorescent antibody.

in the AFB Ziehl-Neelsen staining procedure. Application of heat in the modified Ziehl-Neelsen staining procedure facilitates the entry of the carbol fuchsin dye into the organisms to stain them pink or red, whereas phenol replaces heat in the modified Kinyoun staining procedure. Staining reagents for mycobacteria are readily available commercially, and the only preparation that is required for these stains is for the modification of the decolorizing agents. See "Modified safranin stain" in "Other Stains" at the end of this section.

Basic procedure (modified Kinyoun stain)
Specimen (1 or 2 drops) is applied to a slide, allowed to air dry, and fixed with absolute methanol for 1 min. Carbol fuchsin is applied to the slide for 5 min, and then the slide is rinsed with 50% ethanol, followed by a water rinse. Sulfuric acid (1%) decolorizer is added for 2 min, and the slide is rinsed again with water. Methylene blue is added for 1 min, and then the slide is rinsed again with water. The slide is then air dried and examined at ×100 to ×1,000 magnification.

Blood Film Stains

■ Giemsa and Wright's stains

Examination of blood films for parasites includes the use of two common stains, the Giemsa and the Wright's stains, both derivatives of the original Romanowsky stain. These stains are very similar, differing primarily in that no fixative is included in the Giemsa stain and the blood film must be fixed with absolute methanol prior to staining. Although both stains work well with thinly prepared blood films, thick films stained with Wright's stain are reported to be inferior to those stained with Giemsa solution (5, 18). Although stock solutions of these stains can be prepared in the laboratory, the procedure is very cumbersome and involves grinding of powdered stain with methanol and/or glycerin with a mortar and pestle, days to weeks of storage with shaking, and removal of supernatant or filtering prior to use. Alternatively, Giemsa, Wright's, and Wright-Giemsa stains are readily available from commercial suppliers in liquid form and may need only dilution in a buffer solution. Blood films may be stained manually, but many laboratories rely on automated hematological instruments for staining of thin (not thick) blood films with acceptable results. These stains allow the detection of blood parasites including the agent of malaria, microfilariae, and *Leishmania*, *Babesia*, and *Trypanosoma* species.

Basic procedure
Cover the surface of a thin blood film with stain for 1 to 3 min. Add an equal volume of buffered water to the slide and mix by gently blowing on the surface of the slide. Wait 4 to 8 min and flood the slide with buffered water. Allow the slide to dry and examine it under ×1,000 magnification. Thick films must be laked in distilled water for approximately 2 min prior to performance of the staining procedure described above (4). Satisfactorily stained smears show the following characteristics for the Giemsa and Wright's stains: erythrocytes, pale red and light tan, reddish, or buff, respectively; nuclei of leukocytes, purple with pale purple cytoplasm and bright blue with contrasting light cytoplasm, respectively; eosinophilic granules, bright purple-red and bright red, respectively; and neutrophilic granules, deep pink-purple and pink or light purple, respectively.

■ Calcofluor white stain

Calcofluor white, one of a number of optical brighteners, binds to cellulose and chitin and fluoresces best when exposed to long-wavelength UV light. These properties allow for its use in detecting fungi, *Pneumocystis jirovecii*, free-living amebae like *Naegleria*, *Acanthamoeba*, and *Balamuthia*

species, as well as the larvae of *Dirofilaria* species (its cuticle contains chitin). Calcofluor white is available through several commercial suppliers and is also easily made in the laboratory following the manufacturer's recommendations.

Basic procedure
Place the specimen on a slide and allow it to air dry. Fix the slide in methanol for 1 to 2 min, rinse with distilled water, and allow it to air dry. Add 1 or 2 drops of 10% KOH, place 1 or 2 drops of calcofluor white solution on the specimen for 3 min, and view the slide with a fluorescent microscope and barrier filter (refer to summary table) at ×100 to ×400 magnification.

Hematoxylin Stains

■ Delafield's hematoxylin stain

Delafield's hematoxylin stain is used for thin and concentrated blood films for the detection of microfilaria and may show greater detail of the nuclei and sheaths than that shown with Giemsa and Wright's stains. Although the stain is not procedurally difficult to prepare, it does involve aging processes of 1 week followed by 1 month before it can be used. Delafield's hematoxylin stain is not readily available commercially and is used only in specialized circumstances. Preparation of the stain involves dissolving 180 g of aluminum ammonium sulfate in 1 liter of distilled water, heating until dissolved, and cooling (ammonium alum). Hematoxylin crystals (4 g) are dissolved in 25 ml of 95% ethyl alcohol, and the solution is then added to 400 ml of the ammonium alum. The solution is then covered with a cotton plug and exposed to sunlight and air for 1 week, after which it is filtered. To this solution, 100 ml each of glycerin and 95% ethyl alcohol is added. This solution is placed in sunlight for at least 1 month (5).

Basic procedure
Allow the specimen on a slide to air dry. Lake blood films with distilled water for 15 min and fix in absolute methanol for 5 min, followed by air drying. Stain for 10 to 15 min and rinse in water. Air dry, add a coverslip with mounting fluid, and examine the slide under ×1,000 magnification.

■ Iron hematoxylin stain

Iron hematoxylin stains are used for the detection, identification, and enumeration of intestinal protozoan parasites. There are many derivations of the iron hematoxylin stain, all of which can be used with fresh fecal specimens, fixed specimens containing PVA, specimens preserved in Schaudinn's solution, or specimens preserved in SAF to make permanent stained smears. Solutions of hematoxylin-ethanol and ferrous ammonium sulfate-hydrochloric acid are combined in equal parts to make working iron hematoxylin stain, and both solutions are available commercially.

Basic procedure
Place prepared slides in 70% ethanol for 5 min. Place the slide in 70% ethanol with iodine for 5 min if mercury-based fixatives are being used and then again in 70% ethanol for 5 min more (these last two steps are not necessary for non-mercury-based fixatives). Wash the slide in running tap water for 10 min, followed by placement in iron hematoxylin working solution for 5 min. After this staining step, wash again in running tap water for 10 min and then place the slide in the following reagents for 5 min each: 70% ethanol, 95% ethanol, 100% ethanol (two times), xylene (or a substitute) (two times). Add Permount, and add a coverslip. Examine under ×1,000 magnification.

■ Trichrome stain (Wheatley trichrome stain)

The Wheatley trichrome stain, a modification of the Gomori tissue stain, is used for the detection, identification, and enumeration of intestinal protozoan parasites (22). This stain uses chromotrope 2R and light green SF stains to visually distinguish internal elements of protozoan parasitic cysts and trophozoites. Trichrome staining is usually performed on fixed fecal specimens containing PVA or Schaudinn's solution-preserved specimens. MIF- or SAF-preserved specimens may also be stained with the trichrome stain, as well as specimens preserved with single-vial systems. In addition, some proprietary stains are also available that may work better with these and other fixatives to make permanent stained preparations. Trichrome stain can be easily prepared in the laboratory or purchased from a commercial supplier.

Basic procedure
Place a prepared slide in 70% ethanol for 5 min. For mercury-based fixatives only, place the slide in 70% ethanol with iodine for 1 min (fresh specimens) or up to 10 min (for PVA-fixed air-dried specimens). Then place again in 70% ethanol for 5 min (two times). Place in trichrome stain for 10 min, followed by a 1- to 3-s rinse in 90% ethanol with acetic acid. Dip the slide several times in 100% ethanol, and then place it in 100% ethanol for 3 min (two times), followed by xylene for 5 to 10 min (two times). Add Permount, and add a coverslip. Dry overnight or for 1 h at 37°C. Examine under ×1,000 magnification. If 95%/5% alcohol is substituted for 100% ethanol and xylene substitutes are used, it is important to increase the dehydration times for both the alcohol and xylene substitute by at least 5 to 10 min.

Modified Trichrome Stains for Microsporidia

■ Weber green

■ Ryan blue

Modifications of the trichrome stain have been successfully developed for the detection of microsporidia and are commercially available. Smears prepared from fresh or preserved fecal material can be used in this staining procedure. These stains are based on the standard trichrome technique, but a much more concentrated trichrome reagent is used and a longer staining time is incorporated. These changes are necessary because the microsporidia are not easily stained due to the difficulty in penetrating their outer spores. The Weber stain (21) stains the organisms pink with a green background, while the Ryan stain (13) also stains the organisms pink but gives a blue background. Staining at a temperature of 50°C for 10 min (7) or at 37°C for 30 min (3) may provide more intensely staining organisms and a cleaner background.

Basic procedure
Place a prepared smear in absolute methanol for 5 min and air dry prior to placement in trichrome stain for 90 min. Rinse in acid-alcohol (995.5 ml of 90% ethyl alcohol plus 4.5 ml of glacial acetic acid) for 10 s, dip several times in 95% alcohol, and place in 95% alcohol for 5 min. Prior to mounting, the slide is placed in 100% alcohol for 10 min. Examine at ×1,000 magnification. A study evaluating modifications to this procedure can be found in the reference by Kokoskin and colleagues (10).

TABLE 2 Media used for cultivation of parasites

Media	Organism(s)	Comments
Acanthamoeba monoxenic culture	*Acanthamoeba* and *Naegleria* species	• Acanthamoeba medium plus nonnutrient agar overlaid with *Escherichia coli* or *Enterobacter aerogenes* • Used for cerebrospinal fluid, tissue, or soil samples
Balamuth's aqueous egg yolk infusion medium	Intestinal amebae	• Cannot grow *Balamuthia* • *Balamuthia* grows only in cell culture lines
Boeck and Drbohlav's Locke-eff-serum (LSE) medium	Intestinal amebae	• Inspissated egg base slant with serum and rice powder
Buffered charcoal-yeast extract (BCYE) agar	*Acanthamoeba* species	• Some manufacturers' media support growth better than others • Some of these media support growth of trophozoites and/or cysts
Cysteine-peptone-liver-maltose (CPLM) medium	*Trichomonas vaginalis*	• Methylene blue dye is added to aid in the visualization of the organisms
Defined medium for pathogenic *Naegleria* species	*Naegleria fowleri*	• Defined medium
DGM-21A medium	*Acanthamoeba* species	• Defined medium
Diamond's Trypticase-yeast extract-maltose (TYM) complete medium, Diamond's complete medium modified by Klass	*Trichomonas vaginalis*	• TYM contains no antibiotics; a nonselective medium • Klass modification contains penicillin G, streptomycin sulfate, and amphotericin B to inhibit bacterial overgrowth
Evan's modified Tobie's medium	*Leishmania* species and *Trypanosoma cruzi*	• Uses beef extract, defibrinated horse blood, and phenol red as a pH indicator
InPouch TV (Biomed Diagnostics, Inc.)	*Trichomonas vaginalis*	• Commercially available
Lash's casein hydrolysate-serum medium	*Trichomonas vaginalis*	• Contains beef blood serum, which is absent from other media for *T. vaginalis*
LYI-S-2 medium	*Entamoeba histolytica*	• Similar to TYI-S-33
M-11 medium	*Acanthamoeba culbertsoni*	• Defined medium containing 11 amino acids
Modified Columbia agar	*Trichomonas vaginalis*	• Solid agar medium, *T. vaginalis* colonies change pH and appearance of the agar plates
Nelson's medium	*Naegleria fowleri*	• Contains fetal calf serum, which is necessary for the growth of *N. fowleri*
NIH medium	*Leishmania* and *Trypanosoma* species	• Similar to Evan's modified Tobie's medium
Novy-MacNeal-Nicolle (NNN) medium	*Leishmania* and *Trypanosoma* species	• NaCl base agar medium
Novy-MacNeal-Nicolle (NNN) medium with Offutt's modifications	*Leishmania* species	• Contains blood in base medium, differentiating it from NNN medium
4 N (NNNN) media	*Leishmania* and *Trypanosoma* species	• Uses a sugar base • Uses NIH medium overlay
Nonnutrient agar with live or dead bacteria	*Acanthamoeba* species	• Some manufacturers' media support growth better than others
Proteose peptone yeast extract glucose (PPYG) medium	*Acanthamoeba* species	• Basic medium to support growth of *Acanthamoeba* species
Peptone yeast extract glucose (PYG) medium	*Acanthamoeba* species	• Similar to PPYG
SCGYEM medium	*Naegleria fowleri*	• Undefined medium
Schneider's *Drosophila* medium with 30% fetal calf serum	*Leishmania* and *Trypanosoma* species	• Liquid media, less costly than blood-based agars
Trichomonas culture system	*Trichomonas vaginalis*	• Commercially available
Trypticase soy agar with 5% sheep, rabbit, or horse blood	*Acanthamoeba* species	• Some manufacturers' media support growth better than others
TYI-S-33 medium	*Entamoeba histolytica*	• Contains no antibiotics; a nonselective medium
TYSGM-9 medium	*Entamoeba histolytica*	• Contains penicillin G and streptomycin sulfate to inhibit bacterial overgrowth
USAMRU	*Leishmania* species	• Particularly useful in isolation of *Leishmania brasiliensis* complex
Yeager's LIT (liver infusion trypose) mediumgut specimens	*Trypanosoma cruzi*	• Hemin and antibiotics are added to isolate *T. cruzi* from triatoma gut specimens
YI-S medium	*Entamoeba histolytica*	• Similar to TYI-S-33 medium

■ Acid-fast trichrome stain

This staining technique allows for the detection of acid-fast organisms *(C. parvum, I. belli,* and *C. cayetanensis)* and microsporidia from the same smear (9). Smears prepared from fresh or preserved fecal material can be used in this staining procedure. This procedure stains the microsporidial organisms pink and the acid-fast organisms pink to violet on a blue background.

Basic procedure

Place a prepared, air-dried slide in absolute methanol for 5 to 10 min and then again allow it to air dry. Place in carbol fuchsin solution for 10 min before rinsing in tap water. Decolorize with 0.5% acid-alcohol and rinse in tap water. Place in trichrome stain for 30 min at 37°C. Rinse in acid-alcohol for 10 s and then dip the slides several times in 95% alcohol for 10 s. Place the slide in 95% alcohol for 30 s and then allow it to air dry. Examine at ×1,000 magnification.

Other Stains

■ Modified Field's stain

This stain facilitates the identification of *Acanthamoeba* species. The modified Field's stain was evaluated and shown to give very good contrast compared with other stains, such as Wright's, Giemsa, Ziehl-Neelsen, and trichrome stains. For information on preparation and use of the modified Field's stain, the reader is referred to the article by Pirehma and colleagues (12).

■ Modified safranin stain

This staining technique allows for the detection of *Cyclospora* oocysts in formalin-fixed specimens and fecal concentrates. The stain most commonly used in the past for these organisms was the modified acid-fast stain; however, tremendous variations in staining properties can be seen with this stain. The modified safranin stain reportedly uniformly stains oocysts of *Cyclospora*. It has also been shown to be fast, reliable, and easy to perform (20).

Basic procedure

Place a prepared, thin smear of stool on a 60°C slide warmer until dried. Cover smear with a 1% safranin solution and heat in a microwave oven at full power (650 W) for 30 to 60 s. Rinse the smear with tap water for 30 s, counterstain with 1% aqueous methylene blue for 1 min, rinse with tap water, and air dry. Examine at ×1,000 magnification.

MEDIA

Cultures can be performed for only a few parasitic organisms, including *Acanthamoeba* species, *Naegleria fowleri,* intestinal amebae (like *Entamoeba histolytica*), *Trichomonas vaginalis, Leishmania* species, and *Trypanosoma* species (6). However, most clinical diagnostic microbiology laboratories do not provide parasite cultures. Cultures of these organisms are done only at large reference laboratories or research facilities. The exception is *Trichomonas vaginalis,* for which commercial products have been adapted for the routine laboratory. Many of the media listed in Table 2 are not commercially available but are listed for reference. Additional information regarding these media is available in the literature (1, 2, 8, 11, 14, 15, 16, 17, 19).

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Algorithms for Detection and Identification of Parasites

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This chapter discusses various approaches and diagnostic methods currently in use for the diagnosis of parasitic infections. Assuming that clinical specimens have been properly collected and processed according to specific specimen rejection and acceptance criteria, the examination of prepared wet mounts, concentrated specimens, permanent stained smears, blood films, and various culture materials can provide critical information leading to organism identification and confirmation of the suspected cause of clinical disease (4, 5, 12, 13, 15). With the exception of relatively few immunoassay diagnostic kits, the majority of this diagnostic work depends on the knowledge and microscopy skills of the microbiologist. The field of diagnostic parasitology has taken on greater importance during the past few years for a number of reasons. Expanded world travel has increased the potential levels of exposure to a number of infectious agents. It is important to be aware of those organisms commonly found within certain areas of the world and the makeup of the patient population being ser-viced at your institution, particularly if immunocompromised patients are frequently seen as a part of your routine patient population. It is also important for the physician and microbiologist to recognize and understand the efficacy of any diagnostic method for parasite recovery and identification. Specific information on specimen collection and processing can be found in chapter 133.

STOOL SPECIMENS

For review, see the list of options for the collection of fecal specimens in chapter 133, Tables 3 and 6. Algorithms for the processing of stool specimens are presented in Fig. 1 to 3. The procedures that normally comprise the ova and parasite (O&P) examination are provided below and include the direct wet mount in saline, the concentration, and the permanent stained smear (1, 2, 4, 6, 9–11, 13, 16).

Direct Wet Mount in Saline

The purpose of a direct wet mount is to confirm the possibility of infection with certain protozoa and helminths, to assess the worm burden of the patient, and to look for organism motility (Table 1) (4, 6, 11). Any fresh stool specimens that have not been refrigerated and that have been delivered to the laboratory within specified time frames are acceptable for testing; however, it is extremely important to examine liquid or soft stools rather than formed stools. Liquid and soft stools are much more likely to contain motile protozoan trophozoites rather than cysts, which will not demonstrate motility. Low-power examination (magnification, ×100) of the entire coverslip preparation (22 mm by 22 mm) and high dry power examination (magnification, ×400) of at least one-third of the coverslip area are recommended before the preparation is considered negative. Often, results from the direct smear examination should be considered presumptive; however, some organisms (*Giardia lamblia* cysts, *Entamoeba coli* cysts, *Iodamoeba bütschlii* cysts, helminth eggs and larvae, *Isospora belli* oocysts) can be definitively identified. Reports of results obtained by this method should be considered preliminary, with the final report being provided after the results of the concentration wet mount and permanent stained smear are available.

If iodine is added to the preparation for increased contrast, the organisms will be killed and motility will be lost. Specimens that arrive in the laboratory in stool preservatives do not require a direct smear examination; proceed to the concentration and permanent stained smear.

Concentration Wet Mount

The purpose of the concentration method is to separate parasites from fecal debris and to concentrate any parasites present through either sedimentation or flotation (4, 6, 11). The concentration is specifically designed to allow recovery of protozoan cysts, coccidian oocysts, microsporidian spores, and helminth eggs and larvae (Table 2). Any stool specimen that is fresh or preserved is acceptable for testing. Wet mounts prepared from concentrated stool are examined in the same manner as that used for the direct wet mount method. The addition of too much iodine may obscure helminth eggs (the eggs may resemble debris). Often, results from the concentration examination should be considered presumptive; however, some organisms (*G. lamblia* cysts, *E. coli* cysts, *I. bütschlii* cysts, helminth eggs and larvae, *I. belli* oocysts) can be definitively identified. Reports of results obtained by this method should be considered preliminary, with the final report being provided after the results of the permanent stained smear are available.

The formalin-ethyl acetate sedimentation concentration procedure is the most commonly used procedure, and the recommended centrifugation speed and time are 500 × *g* and 10 min, respectively. In this procedure, the use of ether has been replaced with ethyl acetate. However, it is important to

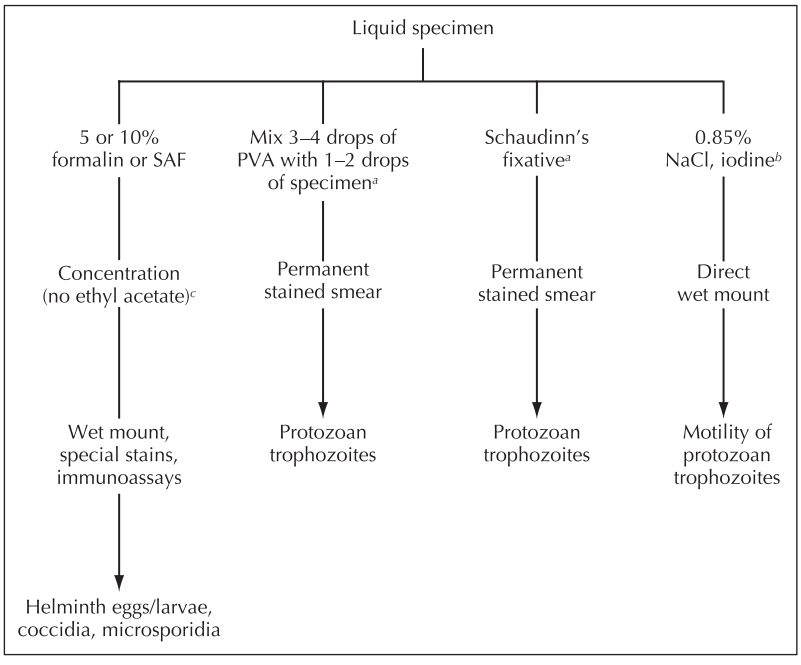


FIGURE 1 Processing liquid stool for O&P examination. [a]Either PVA or Schaudinn's fixative can be used for the preparation of the permanent stained smear. [b]Organism motility will be seen using saline; iodine will kill the organisms, so motility will no longer be visible. [c]The use of ethyl acetate may remove the entire specimen and pull it into the layer of debris that will be discarded (liquid specimen normally contains mucus); centrifuge at 500 × *g* for 10 min (normal centrifugation time), but do not use ethyl acetate in the procedure. In general, laboratories have switched to nonmercury substitutes; the original Schaudinn's fixative contains mercuric chloride. However, in some instances the term "Schaudinn's fixative" is still used to describe the original fixative in a different formulation that is prepared with a copper or zinc base or other proprietary compounds. When selecting fixatives, it is important to know their contents in order to comply with disposal regulations.

remember that ethyl acetate should not be used for liquid specimens or those containing a great deal of mucus. The ethyl acetate may pull the liquid/mucus specimen contents into the debris layer, which will be discarded. The standard zinc sulfate flotation procedure will not detect operculated or heavy eggs; when using this method, both the surface film and sediment should be examined before a negative result is reported.

Permanent Stained Smears

Trichrome, Iron-Hematoxylin, or Iron-Hematoxylin/Carbol Fuchsin

The permanent stained smear provides contrasting colors for both the background debris and the parasites present (Tables 3–5) (4, 6, 11). Permanent stained stool smears are designed to allow examination and recognition of detailed organism morphology under oil immersion magnification (magnification, ×1,000). This method is primarily designed to allow the recovery and identification of the more common intestinal protozoan trophozoites and cysts, excluding the coccidia and microsporidia. Oil immersion examination of a minimum of 300 oil immersion fields is recommended; additional fields may be required if suspect organisms have been seen in the wet mounts. Although review of 300 fields seems like a time-consuming procedure, it takes less time than one would assume. This is particularly true for a trained microscopist in microbiology. Based on their expertise, patient population, and percentage of positive specimens, different laboratories may approach the examination of permanent stained smears by using different guidelines. Some laboratories may use a 60× oil immersion objective for screening purposes (magnification, ×600); however, it is important to examine a sufficient number of fields at a total magnification of ×1,000 before reporting the specimen as negative (no parasites seen).

Modified Acid-Fast Staining

The modified acid-fast staining method is used to provide contrasting colors for both the background debris and the parasites present and to allow examination and recognition of the acid-fast characteristic of the organisms under high dry magnification (magnification, ×400) (4, 16). Organisms that can be identified with this stain are the coccidia *Cryptosporidium* spp., *Cyclospora cayetanensis,* and *I. belli;* it is important to remember that *C. cayetanensis* stains much more acid-fast variable than *Cryptosporidium* spp. Although some microsporidian spores are acid-fast positive, their small size will make recognition very difficult; modified trichrome stains are recommended for the detection of microsporidian spores. Oil immersion examination of a minimum of 300 oil immersion fields is recommended.

Both hot and cold modified acid-fast staining methods are excellent for staining coccidian oocysts. Limitations of

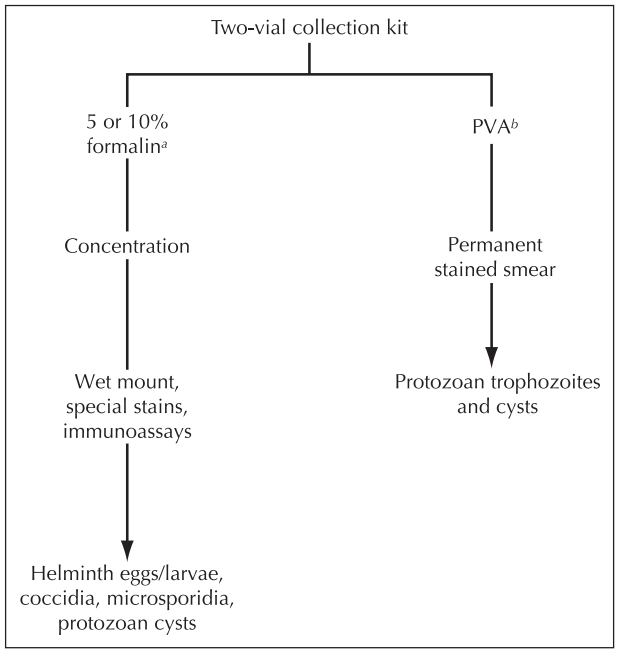


FIGURE 2 Processing preserved stool for O&P examination (two-vial collection kit). [a]The formalin can be buffered or nonbuffered, depending on the laboratory protocol in use. [b]PVA fixative prepared with mercuric chloride provides the best organism preservation. Alternatives are available, including zinc-based PVA, copper sulfate-based PVA, and SAF.

the procedure are generally related to specimen handling, including proper collection and centrifugation speed and time.

Modified Trichrome

Modified trichrome stains were primarily designed to allow recovery and identification of microsporidial spores from centrifuged stool specimens; internal morphology (horizontal and diagonal stripes of the polar tubule) may be seen in some spores under oil immersion (magnification, ×1,000). Any stool specimen that is submitted fresh or preserved in formalin or sodium acetate-acetic acid-formalin (SAF) is acceptable. Oil immersion examination of a minimum of 300 oil immersion fields is recommended. The identification of microsporidial spores may be possible; however, their small size makes recognition difficult, particularly in infections with few organisms in the clinical specimen. Identification to the species level will require electron microscopy or molecular methods such as PCR.

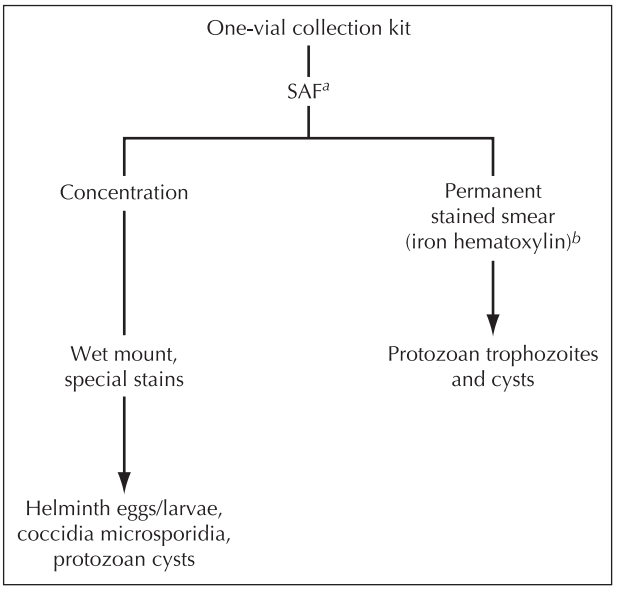


FIGURE 3 Processing preserved stool for O&P examination (one-vial collection kit). [a]There are single-vial collection systems for which the formulas are proprietary; however, many contain zinc sulfate as one of the key ingredients. With the exception of SAF, compatibility of these fixatives with immunoassay reagents has been difficult to achieve. [b]If the iron hematoxylin method containing the carbol fuchsin step is used, the coccidian oocysts will stain pink (*Cryptosporidium* spp., *Cyclospora cayetanensis*, *Isospora belli*). Also, when using SAF, some recommend the use of an adhesive such as egg albumin to "glue" the fecal material onto the slide prior to staining. It is highly recommended that special stains be performed for the detection and identification of the coccidia (modified acid-fast stains) and the microsporidia (modified trichrome stains) from concentrated sediment to enhance organism recovery. Coccidian oocysts of *Isospora belli* can easily be detected in the concentration sediment wet mount; however, unless a very heavy infection is present, *Cryptosporidium* spp. oocysts may not be seen without special modified acid-fast stains. The small size of the microsporidian spores prevents identification without the use of special modified trichrome stains and microscopic examination using the 100× oil immersion objective.

Immunologic Methods

Immunoassay reagents are available commercially for several of the protozoan parasites, including *G. lamblia*, *Cryptosporidium* spp., the *Entamoeba histolytica-Entamoeba dispar* group, and *Entamoeba histolytica* (Table 6). These methods (enzyme immunoassay [EIA], fluorescent-antibody assay, and immunochromatographic assay) are

TABLE 1 Diagnostic characteristics of organisms in wet mounts

Specimen	Protozoa	Helminths
Stool, other specimens from gastrointestinal tract, urogenital system specimens	Size, shape, stage (trophozoite, precyst, cyst, oocyst), motility (fresh specimens only), refractility, cytoplasm inclusions (chromatoidal bars, glycogen vacuoles, axonemes, axostyles, median bodies, sporozoites)	Egg, larvae, or adult; size; internal structure: Egg: embryonated, opercular shoulders, abopercular thickenings or projections, hooklets, polar filaments, spines Larvae: head and tail morphology, digestive tract Adult: nematode, cestode, or trematode

TABLE 2 Identification of helminth eggs[a]

1. Eggs small (~25–40 μm)	
A. Operculate, generally oval (egg <35 μm); *Clonorchis*, operculated shoulders, bile stained, may be an abopercular knob, miracidium present, but difficult to see	*Clonorchis (Opisthorchis)* spp. (Chinese liver fluke) or intestinal flukes *(Heterophyes heterophyes* or *Metagonimus yokogawai)*
B. Thick, radially striated shell (six-hooked oncosphere, individual eggs resemble those of *Taenia* spp.; eggs passed in egg packets containing 6–10 eggs; each egg is 25–40 μm)	*Dipylidium caninum* (dog, cat tapeworm); note: egg packets could measure >150 μm
C. Thick, radially striated shell (six-hooked oncosphere may not be visible in every egg from formalinized fecal specimens) (eggs cannot be identified to species level without special stains) (each egg is 30–47 μm)	*Taenia* spp. (*T. saginata,* beef tapeworm; *T. solium,* pork tapeworm)
D. Thin egg shell, clear space between developing shell and embryo, spherical or subspherical, containing a six-hooked oncosphere; polar filaments (filamentous strands) present between thin egg shell and embryo (each egg is 31–43 μm)	*Hymenolepis nana* (dwarf tapeworm)
2. Eggs medium (~40–100 μm)	
A. Egg barrel-shaped, with clear polar plugs (each egg is 50–54 by 20–23 μm)	*Trichuris trichiura* (whipworm)
B. Egg flattened on one side, may contain larva (each egg is 70–85 by 60–80 μm)	*Enterobius vermicularis* (pinworm)
C. Egg with thick, tuberculated (bumpy) capsule (in decorticate eggs, capsule may be missing) (each egg is 45–75 by 35–50 μm)	*Ascaris lumbricoides* (large roundworm), fertilized eggs
D. Egg bluntly rounded at ends, thin shell (contains developing embryo at 8–16-ball stage of development) (each egg is 56–75 by 36–40 μm)	Hookworm
E. Operculate, operculum break in shell sometimes hard to see, smooth transition from shell to operculum; small "bump" may be seen at abopercular end (each egg is 58–75 by 40–50 μm)	*Diphyllobothrium latum* (broad fish tapeworm)
F. Thin egg shell, clear space between developing shell and embryo, spherical or subspherical, containing a six-hooked oncosphere; no polar filaments (filamentous strands) present between thin egg shell and embryo (each egg is 70–85 by 60–80 μm)	*Hymenolepis diminuta* (rat tapeworm)
3. Eggs large (~100–180 μm)	
A. Egg with opercular shoulders into which the operculum fits (looks like a teapot lid and the flange into which the lid fits), abopercular end somewhat thickened (not always visible) (each egg is 80–120 by 48–60 μm); egg has been described as "urn-shaped"	*Paragonimus* spp. (lung fluke)
B. Egg tapered at one or both ends; long thin shell containing developing embryo (each egg is 73–95 by 40–50 μm)	*Trichostrongylus* spp.
C. Egg with thick, tuberculated (bumpy) capsule (in decorticate eggs, capsule may be missing) (each egg is 85–95 by 43–47 μm)	*Ascaris lumbricoides* (large roundworm), unfertilized eggs
D. Egg spined, ciliated miracidium larva may be seen, lateral spine very short (each egg is 70–100 by 55–65 μm)	*Schistosoma japonicum* (blood fluke, stool), *Schistosoma mekongi* (rounder and smaller than *S. japonicum*); measure 50–65 by 30–55 μm
E. Egg spined, ciliated miracidium larva may be seen, spine terminal (each egg is 112–170 by 40–70 μm)	*Schistosoma haematobium* (blood fluke, urine)
F. Egg spined, ciliated miracidium larva may be seen, spine terminal (each egg is 140–240 by 50–85 μm)	*Schistosoma intercalatum* (blood fluke, stool)
G. Egg spined, ciliated miracidium larva may be seen, large lateral spine (each egg is 114–180 by 45–70 μm)	*Schistosoma mansoni* (blood fluke, stool)
H. Egg >85 μm, operculum break in shell sometimes hard to see; smooth transition from shell to operculum; egg passed in undeveloped stage (each egg is 130–140 by 80–85 μm)	*Fasciolopsis buski* (giant intestinal fluke) or *Fasciola hepatica* (sheep liver fluke) or *Echinostoma* spp.

[a] This table does not include every possible helminth that could be found as a human parasite; however, the most likely helminth infections are included.

designed to detect the antigens of selected organisms; a negative result does not rule out the possibility that these organisms are present in low numbers or that other intestinal parasites are etiologic agents causing disease, including *Dientamoeba fragilis*, the microsporidia, and helminth parasites (4, 7, 8, 18). Immunoassay reagents are currently under development and trial for *D. fragilis* and the microsporidia.

TABLE 3 Diagnostic characteristics of organisms in permanent stained smears

Specimen	Protozoa	Helminths
Stool, other specimens from gastrointestinal tract, urogenital system specimens	Size, shape, stage (trophozoite, precyst, cyst, oocyst, spore)	Egg, larvae, and/or adults may not be identified because of excess stain retention or distortion
	Nuclear arrangement, cytoplasm inclusions (chromatoidal bars, vacuoles, axonemes, axostyles, median bodies, sporozoites, polar tubules)	

ADDITIONAL TECHNIQUES FOR STOOL EXAMINATION

Although the routine O&P examination consisting of the direct wet mount, the concentration, and the permanent stained smear is an excellent procedure recommended for the detection of most intestinal parasites, several other diagnostic techniques are available for the recovery and identification of specific parasitic organisms (4, 6). Most laboratories do not routinely offer all of these techniques, but many can be performed relatively simply and inexpensively. Occasionally, it is necessary to examine stool specimens for the presence of scolices and proglottids of cestodes and adult nematodes and trematodes to confirm the diagnosis and/or for species identification (Table 7). A method for the recovery of these stages is also described in this chapter.

Culture of Larval-Stage Nematodes

Nematode infections giving rise to larval stages that hatch in soil or in tissues may be diagnosed by using fecal culture methods to concentrate the larvae (1, 4, 6, 16). *Strongyloides stercoralis* larvae are the most common larvae found in stool specimens. Depending on the fecal transit time through the intestine and the patient's condition, rhabditiform and filariform larvae may be present. Caution must be exercised when handling larval cultures because infective filariform larvae may be present. If there is a delay in the preservation of the stool specimen, then embryonated ova as well as larvae of hookworm may be present. Culture of feces for larvae is useful for (i) revealing their presence when they are too scanty to be detected by concentration methods; (ii) distinguishing whether the infection is due to *S. stercoralis* or hookworm on the basis of rhabditiform larval morphology by allowing hookworm egg hatching to occur, releasing first-stage larvae; and (iii) allowing the development of larvae into the filariform stage for further differentiation.

Fecal culture methods are especially helpful for the detection of light infections with hookworm, *S. stercoralis,* and *Trichostrongylus* spp. and for the specific identification of parasites. Also, such techniques are useful for obtaining a large number of infective-stage larvae for research purposes. One enhanced recovery method, the agar plate culture for

TABLE 4 Key to identification of intestinal amebae (permanent stained smear)

1. Trophozoites present	2
Cysts present	7
2. Trophozoites >12 μm	3
Trophozoites <12 μm	4
3. Karyosome central, compact; peripheral nuclear chromatin evenly arranged; "clean" cytoplasm	*Entamoeba histolytica*[a]
Karyosome eccentric, spread out; peripheral nuclear chromatin unevenly arranged; "dirty" cytoplasm	*Entamoeba coli*
4. Peripheral nuclear chromatin	5
Other than above	6
5. Karyosome central, compact; peripheral nuclear chromatin evenly arranged; "clean" cytoplasm	*Entamoeba hartmanni*
Karyosome large, blot-like; extensive nuclear variation	*Endolimax nana*
6. No peripheral chromatin, karyosome large, junky cytoplasm	*Iodamoeba bütschlii*
No peripheral chromatin, karyosome variable, clean cytoplasm	*Endolimax nana*
7. Cysts >10 μm (including any shrinkage "halo")	8
Cysts <10 μm (including any shrinkage "halo")	10
8. Single *Entamoeba*-like nucleus with large inclusion mass	*Entamoeba polecki*[b]
Multiple nuclei	9
9. Four *Entamoeba*-like nuclei, chromatoidal bars have smooth, rounded ends	*Entamoeba histolytica*[a]
Five or more *Entamoeba*-like nuclei, chromatoidal bars have sharp, pointed ends	*Entamoeba coli*
10. Single nucleus (may be "basket" nucleus), large glycogen vacuole	*Iodamoeba bütschlii*[c]
Multiple nuclei	11
11. Four *Entamoeba*-like nuclei, chromatoidal bars have smooth, rounded ends (nuclei may also number only two)	*Entamoeba hartmanni*
Four karyosomes, no peripheral chromatin, round to oval shape	*Endolimax nana*

[a] *Entamoeba histolytica* refers to the *Entamoeba histolytica/Entamoeba dispar* group. *E. histolytica* (pathogenic) can be determined by finding red blood cells in the cytoplasm of the trophozoites. Otherwise, on the basis of morphological grounds *E. histolytica* (pathogen) and *E. dispar* (nonpathogen) cannot be differentiated.

[b] It is very difficult to differentiate *Entamoeba polecki* trophozoites from those of the *E. histolytica/E. dispar* group or *E. coli.*

[c] Although some *I. bütschlii* cysts are larger than 10 μm, the majority of cysts measure 9 to 10 μm and the presence of the typical glycogen vacuole ensures correct identification.

TABLE 5 Key to identification of intestinal flagellates

1. Trophozoites present	2
Cysts present	7
2. Pear shaped	3
Shape other than pear	6
3. Two nuclei, sucking disk present	*Giardia lamblia*
One nucleus present	4
4. Costa length of body	*Pentatrichomonas hominis*
No costa	5
5. Cytostome present, >10 μm	*Chilomastix mesnili*
Cytostome present, <10 μm	*Retortamonas intestinalis* or *Enteromonas hominis*
6. Ameba shaped, one or two fragmented nuclei	*Dientamoeba fragilis*
Oval shaped, one nucleus	*Enteromonas hominis*
7. Oval or round cyst	8
Lemon-shaped cyst	9
8. Four nuclei, median bodies, axoneme, >10 μm	*Giardia lamblia*
Two nuclei, no fibrils, <10 μm	*Enteromonas hominis*
9. One nucleus (Shepherd's crook), fibril	*Chilomastix mesnili*
One nucleus, bird's beak fibril	*Retortamonas intestinalis*

Strongyloides, and several culture techniques are described in this chapter. Since these procedures are less common, brief descriptions are included.

Agar Plate Culture for *S. stercoralis*

Agar plate cultures are recommended for the recovery of *S. stercoralis* larvae and tend to be more sensitive than some of the other diagnostic methods (4, 6). Approximately 2 g of stool (diameter, about 1 in.) is placed in the center of the agar plate; the plate is sealed to prevent accidental infections and is held for 2 days at room temperature. As the larvae crawl over the agar, they carry bacteria with them, thus creating visible tracks over the agar. The plates are examined under the microscope for confirmation of the presence of larvae, the surface of the agar is then washed with 10% formalin, and final confirmation of larval identification is made via wet examination of the sediment from the formalin washings.

Baermann Technique

Another method of examining a stool specimen suspected of having small numbers of *Strongyloides* larvae is the use of a modified Baermann apparatus. The Baermann technique uses a funnel apparatus and relies on the principle that active larvae will migrate from a fresh fecal specimen that has been placed on a wire mesh with several layers of gauze, which are in contact with tap water (4). Larvae migrate through the gauze into the water and settle to the bottom of the funnel, where they can be collected and examined. The main difference between this method and the Harada-Mori and petri dish methods is the greater amount of fresh stool used, possibly providing a better chance of larval recovery in a light infection. Besides being used for patient fecal specimens, this technique can be used to examine soil specimens for the presence of larvae. However, the agar plate method is considered to be a more sensitive method for the recovery of *S. stercoralis* larvae.

Harada-Mori Filter Paper Strip Culture

To detect light infections with hookworm, *S. stercoralis*, and *Trichostrongylus* spp., as well as to facilitate specific identification, the Harada-Mori filter paper strip culture technique is very useful (1, 4, 6). The technique requires filter paper (strip cut to fit into a 15-ml test tube) to which fresh fecal material (similar in size to a raisin or a small grape) is added to the middle of the filter paper and a test tube into which the filter paper is inserted. Moisture is provided by adding water to the tube (within a half-inch of the stool, but not covering the stool), which continuously soaks the filter paper by capillary action. Incubation under suitable conditions favors hatching of ova and/or development of larvae. Fecal specimens to be cultured should not be refrigerated, since some parasites are susceptible to cold and may fail to develop after refrigeration. Also, caution must be exercised in handling the filter paper strip itself, since infective *Strongyloides* larvae may migrate upward as well as downward on the paper strip.

Filter Paper/Slant Culture Technique (Petri Dish)

An alternative technique for culturing *Strongyloides* larvae is a filter paper/slant culture on a microscope slide placed in a glass or plastic petri dish (4). As with the techniques described above, sufficient moisture is provided by continuous soaking of filter paper in water. Fresh stool material is

TABLE 6 Commercially available immunoassays for detection of intestinal parasites

Fresh stool		Preserved in 5–10% formalin, SAF, or other single-vial system[a]	
No concentration	Concentration	No concentration	Concentration
EIA	DFA[b]	EIA	DFA[b]
Cryptosporidium spp.	*Cryptosporidium* spp.	*Cryptosporidium* spp.	*Cryptosporidium* spp.
G. lamblia	*G. lamblia*	*G. lamblia*	*G. lamblia*
E. histolytica		Immunochromatographic assay (cartridge format)	
E. histolytica/*E. dispar* group		*Cryptosporidium* spp.	
Immunochromatographic assay (cartridge format)		*G. lamblia*	
Cryptosporidium spp.			
G. lamblia			
E. histolytica/*E. dispar* group			

[a] Some single-vial collection systems may use fixatives that are compatible with immunoassay testing; the specific manufacturer should be consulted.
[b] DFA, direct fluorescent-antibody assay.

TABLE 7 Additional helminth recovery and identification techniques (other than O&P examination)

Organism	Specimen	Procedure
Nematodes		
S. *stercoralis*	Fresh stool, not refrigerated (all organisms	Harada-Mori filter paper strip
Hookworm	can be recovered by any of the procedures	Filter paper/slant culture
Trichostrongylus spp.	indicated in the column to the right)	Charcoal culture
		Baermann test
		Agar plate culture (primarily for *S. stercoralis*)
Hookworm	Fresh stool, refrigeration acceptable	Direct smear (Beaver)
Ascaris lumbricoides		Dilution egg count (Stoll)
Trichuris trichiura		Either method acceptable for estimation of worm burden
Enterobius vermicularis	Scotch tape preparations, paddles, anal swab, other collection devices	Direct microscopic examination
Trematodes		
Schistosoma spp.	Fresh stool, not refrigerated	Egg hatching test
	Fresh urine (24-h and single collection)	Egg viability test
Cestodes		
Tapeworms	Proglottids (gravid in alcohol)	India ink injection
	Stool in 5–10% formalin	Scolex search

placed on filter paper, which is cut to fit the dimensions of a standard (1- by 3-in.) microscope slide. The filter paper is then placed on a slanted glass slide in a glass or plastic petri dish containing water. This technique allows direct examination of the culture system with a dissecting microscope to look for nematode larvae and free-living stages of *S. stercoralis* in the fecal mass or the surrounding water without having to sample the preparation.

Egg Studies and Scolex Search

Estimation of Worm Burdens

The only human parasites for which it is reasonably possible to correlate egg production with adult worm burdens are *Ascaris lumbricoides*, *Trichuris trichiura*, and the hookworms *(Necator americanus* and *Ancylostoma duodenale)*. The specific instances in which information on approximate worm burdens is useful are when one is determining the intensity of infection, deciding on possible chemotherapy, and evaluating the efficacies of the drugs administered. With current therapy, the need for the monitoring of therapy through egg counts is no longer as relevant. Remember that egg counts are estimates; you will obtain count variations regardless of how carefully you follow the procedure. If two or more fecal specimens are being compared, it is best to have the same individual perform the technique with both samples and to do multiple counts. A number of methods have been described (1, 4, 6, 9, 11, 13).

Hatching of Schistosome Eggs

When schistosome eggs are recovered from either urine or stool, they should be carefully examined to determine viability. The presence of living miracidia within the eggs indicates an active infection that may require therapy. The viability of the miracidia can be determined in two ways: (i) the cilia of the flame cells (primitive excretory cells) may be seen on a wet smear by using high dry power and are usually actively moving, and (ii) the miracidia may be released from the eggs by the use of a hatching procedure (4). The eggs usually hatch within several hours when placed in 10 volumes of dechlorinated or spring water (hatching may begin soon after contact with the water). The eggs that are recovered in the urine (24-h specimen collected with no preservatives) are easily obtained from the sediment and can be examined under the microscope to determine viability. A sidearm flask has been recommended, but an Erlenmeyer flask is an acceptable substitute.

Both urine and stool specimens must be collected without preservatives and should not be refrigerated prior to processing. Hatching will not occur until the saline is removed and nonchlorinated water is added. If a stool concentration is performed, use saline throughout the procedure to prevent premature hatching. Make sure that the light is not too close to the side arm or top layer of water in the Erlenmeyer flask. Excess heat will kill the miracidia. The lamp light mimics the sun shining on a water source, and the hatched larvae tend to swim toward the light. The absence of live miracidia does not rule out the presence of schistosome eggs. Nonviable eggs or eggs that failed to hatch are not detected by this method. Microscopic examination of direct or concentrated specimens should be used to demonstrate the presence or absence of eggs. Egg viability can be determined by placing some stool or urine sediment (the same material used for the hatching flask) on a microscope slide. Low-power magnification (×100) can be used to locate the eggs. Individual eggs can be examined with high dry magnification (×400); moving cilia on the flame cells (primitive excretory system) confirm egg viability.

Search for Tapeworm Scolex

Since therapy for the elimination of tapeworms is usually very effective, a search for the tapeworm scolex is rarely requested and is no longer as clinically relevant as previously. However, stool specimens may have to be examined for the presence of scolices and gravid proglottids of cestodes for proper species identification. This procedure requires mixing a small amount of feces with water and straining the mixture through a series of wire screens (graduated from coarse to fine mesh) to look for scolices and proglottids. Remember to use standard precautions and to wear gloves when performing this procedure. The appearance of scolices after therapy is an indication of successful treatment. If the scolex has not been passed, it may still be attached to the mucosa; the parasite is capable of producing more segments from the neck region of the scolex, and the infection continues.

EXAMINATION OF OTHER SPECIMENS FROM THE INTESTINAL TRACT

Examination for Pinworm

A roundworm parasite that has a worldwide distribution and that is commonly found in children is *Enterobius vermicularis*, known as pinworm or seatworm. The adult female worm migrates out of the anus, usually at night, and deposits her eggs on the perianal area. The adult female (8 to 13 mm long) may occasionally be found on the surface of a stool specimen or on the perianal skin. Since the eggs are usually deposited around the anus, they are not commonly found in feces and must be detected by other diagnostic techniques. Diagnosis of pinworm infection is usually based on the recovery of typical eggs, which are described as thick-shelled, football-shaped eggs with one slightly flattened side. Each egg often contains a fully developed embryo and is infective within a few hours after being deposited. Commercial collection devices are available and can be used for specimen collection, similar to the approach with cellulose tape indicated below (4). Some clinicians may treat patients on the basis of clinical symptoms without confirmation of the suspected diagnosis of pinworm infection.

Cellulose Tape Preparations

The most widely used procedure for the diagnosis of pinworm infection is the cellulose tape (adhesive cellophane tape) method (2, 4, 9, 10). Specimens should be obtained in the morning, before the patient bathes or goes to the bathroom. The tape is applied to the anal folds and is then placed sticky side down on a microscope slide for examination. At least four to six consecutive negative slides should be observed before the patient is considered free of infection.

Anal Swabs

The anal swab technique (4) is also available for the detection of pinworm infections; however, most laboratories use the cellulose tape method because it eliminates the necessity of preparing and storing swabs. At least four consecutive negative preparations should be observed before the patient is considered negative; some recommend six consecutive negative preparations.

A paraffin-coated swab should be gently rubbed over the perianal surface and into the folds. Place the swab into the anal opening about 1/4 in. and then replace the swab in the tube. The tube containing the swab is half filled with xylene substitute and is allowed to stand for a few minutes. After centrifugation, the sediment is examined microscopically for the presence of eggs.

Sigmoidoscopy Material

Material obtained from sigmoidoscopy can be helpful in the diagnosis of amebiasis that has not been detected by routine fecal examinations, and the procedure is recommended for this purpose. However, usually a series of at least three routine stool examinations for parasites should be performed for each patient before a sigmoidoscopy examination is done (4).

Material from the mucosal surface should be aspirated or scraped and must not be obtained with cotton-tipped swabs. At least six representative areas of the mucosa should be sampled and examined (six samples, six slides). Usually, the amount of material is limited and should be processed immediately to ensure the best examination possible (Table 8). Three methods of examination can be performed. All three are acceptable; however, depending on the availability of trained personnel, the availability of proper fixation fluids, or the amount of specimen obtained, one or two procedures are recommended. If the amount of material limits the examination to one procedure, the use of polyvinyl alcohol (PVA) fixative is highly recommended for the subsequent preparation of permanent stains.

Although the fecal immunoassays are rarely used for this purpose and are not the tests of choice, if the material is going to be examined by using any of the fluorescent-antibody assay, EIA, or immunochromatographic immunoassay detection kits (*Cryptosporidium* spp. or *G. lamblia*), then 5 or 10% formalin or SAF fixative is recommended. However, immunoassays for the detection of the *Entamoeba histolytica*/*E. dispar* group or confirmation of the pathogen *Entamoeba histolytica* require fresh or frozen stool for testing; preserved stool is not acceptable.

Physicians performing sigmoidoscopy procedures may not realize the importance of selecting the proper fixative for material to be examined for parasites by the concentration and permanent stained smear methods. It is recommended that a parasitology specimen tray (containing Schaudinn's fixative, PVA, and 5 or 10% formalin) be provided or that a trained technologist be available at the time of sigmoidoscopy to prepare the slides.

Direct Saline Mount

If there is no lag time after collection and a microscope is available in the immediate vicinity, some of the material should be examined as a direct saline mount for the presence of motile trophozoites (4). A drop of material is mixed with a drop of 0.85% sodium chloride and is examined under low light intensity for the characteristic movement of amebae. It may take time for the organisms to become acclimated to this type of preparation; hence, motility may not be obvious for several minutes. There will be epithelial cells, macrophages, and possibly polymorphonuclear leukocytes and erythrocytes, which will require a careful examination to reveal amebae.

Since specific identification of protozoan organisms can be difficult when only the direct saline mount is used, this technique should be used only when there is sufficient material left to prepare permanent stained smears.

Permanent Stained Smear

Most of the material obtained at sigmoidoscopy can be smeared (gently) onto a slide and immediately immersed in Schaudinn's fixative (4). These slides can then be stained with trichrome or iron hematoxylin stain and examined for specific cell morphology, either protozoan or otherwise. The procedure and staining times are identical to those for routine fecal smears.

If the material is bloody, contains a lot of mucus, or is a "wet" specimen, a few (no more than 2 or 3) drops of PVA can be mixed with 1 or 2 drops of material directly on the slide, which is allowed to air dry (a 37°C incubator can be used) for at least 2 h before staining. If time permits, the PVA smears should be allowed to dry overnight; they can be routinely stained with trichrome stain and examined as a permanent mount.

Material from sigmoidoscopy can be placed in small amounts of SAF. After fixation for 30 min, the specimen can be centrifuged at 500 × *g* for 10 min and smears from the small amount of sediment can be prepared for permanent staining with iron hematoxylin (trichrome stain would be the second choice). One of the organisms most suspected when sigmoidoscopy is performed is *E. histolytica*, the

TABLE 8 Recovery of parasites from other intestinal tract specimens

Source	Organism	Procedure
Sigmoidoscopy specimens		
Unpreserved	Ameba trophozoites (motility)	Direct wet mount, immunoassay tests[a]
Air-dried smears	Coccidia	Modified acid-fast stain
	Microsporidia	Modified trichrome, optical brighteners, immunoassay tests[b]
Preserved 5–10% formalin or SAF	Helminth eggs and larvae (rare), ameba and flagellate cysts,[c] and trophozoites (SAF only)	Concentration wet mount, immunoassay tests
	Coccidia	Modified acid-fast smear, immunoassay tests
	Microsporidia	Modified trichrome, optical brighteners, immunoassay tests[b]
PVA	Helminth eggs and larvae (rare), ameba and flagellate cysts[c]	Wet mount
	Ameba and flagellate cysts[c] and trophozoites	Permanent stained smear
Schaudinn's fixative	Ameba and flagellate cysts[c] and trophozoites	Permanent stained smear
Duodenal specimens[d]		
Unpreserved	Helminth eggs and larvae, flagellate trophozoites (motility)	Direct wet mount
Entero-Test capsule		
Preserved 5–10% formalin or SAF	Helminth eggs and larvae, flagellate trophozoites	Concentration wet mount, immunoassay tests
		Permanent stained smear
Entero-Test capsule	Coccidia	Modified acid-fast smear, immunoassay tests
	Microsporidia	Modified trichrome, optical brighteners, immunoassay tests[b]
PVA	Flagellate trophozoites	Permanent stained smear
Anal impression smear	Pinworm adult and eggs	No stain, cellulose tape preparation and other collection devices
Adult worm or segments	Helminth adult worms or proglottids	Carmine stain (rarely used), India ink
Tissue biopsy	Helminth eggs, larvae, and adults; protozoan cysts; trophozoites; oocysts; sporozoites; and spores	Touch preparations, squash preparations, permanent stains, histology

[a] Immunoassay tests for the *Entamoeba histolytica/E. dispar* group or *Entamoeba histolytica* require fresh or frozen stool; preserved stool specimens are not acceptable for testing. Immunoassays for *Cryptosporidium* spp. and *Giardia lamblia* are approved for use with stool; immunoassay use for other intestinal tract specimens may or may not be appropriate, depending on specimen source, consistency, and volume.

[b] Some genus-specific immunoassay reagents for the microsporidia are being used on an experimental basis but are not yet available commercially.

[c] Although cysts may be present in stool, sigmoidoscopy specimens are often obtained from patients with severe diarrhea or dysentery. In such cases, the cyst forms are usually absent; trophozoites would be the most likely stage seen, particularly in the case of *Entamoeba histolytica*.

[d] Duodenal specimens are often submitted as aspirates; in such cases the volume may be sufficient to perform concentrations. However, if small amounts of duodenal mucus and/or biopsy material are obtained, squash preparations preserved with Schaudinn's fixative are preferred. This approach may require the use of slides precoated with albumin to facilitate adhesion. Mucus obtained from the Entero-Test capsule string may be treated as a fresh specimen; the string can also be immediately placed in preservative after retrieval, and the mucus can be processed as a permanent stained smear.

morphology of which is normally seen from the permanent stained smear. However, if fresh stool or aspirated material is obtained, then both the permanent stained smear and an antigen detection reagent kit for the detection of the *Entamoeba histolytica/E. dispar* group or confirmation of the pathogen *Entamoeba histolytica* can be used. If enough material is present for only a single procedure, then the permanent stained smear is recommended, particularly if the iron hematoxylin stain (incorporating the carbol fuchsin step) is used (4). This method would enhance the detection of the coccidia, which are modified acid-fast positive. However, this approach is less commonly used for the detection of *Cryptosporidium* spp. or *G. lamblia*, which can be confirmed using special stains and/or fecal immunoassays performed on routine stool specimens rather than sigmoidoscopy material.

Duodenal Contents

Duodenal Drainage

In infections with *G. lamblia* or *S. stercoralis*, routine stool examinations may not reveal the organisms. Duodenal drainage material can be submitted for examination (Table 8).

A fresh, unpreserved specimen should be submitted to the laboratory; the amount may vary from 0.5 ml to several milliliters of fluid. The specimen may be centrifuged (at 500 × *g* for 10 min) and should be examined immediately as a wet mount for motile organisms (iodine may be added later to facilitate identification of any organisms present). If the specimen cannot be completely examined within 2 h after it is taken, any remaining material should be preserved in 5 to 10% formalin.

If the duodenal fluid contains mucus, this is where the organisms tend to be found. Therefore, centrifugation of the specimen is important, and the sedimented mucus should be examined. *Giardia* trophozoites may be caught in mucus strands, and the movement of the flagella on the trophozoites may be the only subtle motility seen for these flagellates. *Strongyloides* larvae will usually be very motile. Immunoassay methods for *Cryptosporidium* spp. and *G. lamblia* can also be used with fresh or formalinized material.

If the amount of duodenal material submitted is very small, rather than using any of the specimen for a wet smear examination, permanent stains can be prepared. This approach provides a more permanent record, and the potential problems with unstained organisms, very minimal motility, and a lower-power examination can be avoided by using oil immersion examination of the stained specimen at ×1,000 magnification.

Duodenal Capsule Technique (Entero-Test)

A method of sampling duodenal contents that eliminates the need for intestinal intubation has been devised and consists of the use of a length of nylon yarn coiled inside a gelatin capsule (4, 6). The yarn protrudes through one end of the capsule, and this end of the line is taped to the side of the patient's face. The capsule is then swallowed, the gelatin dissolves in the stomach, and the weighted string is carried by peristalsis into the duodenum. The weight is released and passes out in the stool when the line is retrieved after a period of 4 h. Bile-stained mucus clinging to the yarn is then scraped off with gloved fingers and is collected in a small petri dish. Usually 4 or 5 drops of material are obtained.

The specimen should be examined immediately as a wet mount for motile organisms. If the specimen cannot be completely examined within an hour after the yarn has been removed, the material should be preserved in 5 to 10% formalin or PVA-mucus smears should be prepared. Organism motility will be similar to that described above for duodenal drainage.

The pH of the terminal end of the yarn should be checked to ensure adequate passage into the duodenum (a very low pH means that it never left the stomach). Also, since the bile duct drains into the intestine at this point, the terminal end of the yarn should be a yellow-green color.

UROGENITAL SPECIMENS

Several parasites may be recovered and identified from urogenital specimens. Although the most common pathogens are probably *Trichomonas vaginalis* and *Schistosoma haematobium*, other organisms such as the microsporidia are becoming much more important (Table 9). Also, the Knott concentration for the recovery of microfilariae can be performed from blood, as can the membrane filtration approach from urine.

Direct Wet Mount

The identification of *T. vaginalis* is usually based on the examination of a wet preparation of vaginal and urethral discharges and prostatic secretions or urine sediment and may require the testing of multiple specimens to confirm the diagnosis. These specimens are diluted with a drop of saline and are examined under low power under reduced illumination for the presence of actively motile organisms. As the jerky motility begins to diminish, it may be possible to observe the undulating membrane, particularly under high dry power (magnification, ×400).

While the membrane filtration technique can be used for the recovery of microfilariae, examination of urinary sediment may be indicated in certain filarial infections. The occurrence of microfilariae in urine has been reported with increasing frequency in *Onchocerca volvulus* infections in Africa.

TABLE 9 Detection of urogenital parasites

Organism	Procedure
Trichomonas vaginalis	Wet mount (motility) Culture Giemsa stain Direct fluorescent antibody DNA probe Latex agglutination EIA Rapid immunochromatographic assay (dipstick)
Schistosoma haematobium	Wet mount (urine sediment) Membrane filtration Tissue section
Microfilariae	Knott concentration Membrane filtration (Nuclepore filter) Giemsa stain Delafield's hematoxylin stain
Microsporidia	Modified trichrome stain[a] Optical brighteners (calcofluor)[a] Immunoassay tests[a,b] Routine histology Electron microscopy

[a] Staining procedures and fluorescent antibody tests for organism detection performed on centrifuged sediment (500 × *g* for 10 min).

[b] Immunoassays are available in the research setting; commercial reagents are not yet available.

Urine is collected in a bottle, this volume is recorded, and thimerosal (1 ml/100 ml of urine) is added. The specimen is placed in a funnel fitted with tubing and a clamp; this preparation is allowed to settle overnight. On the following day, 10 to 20 ml of urine is withdrawn and centrifuged. The supernatant is discarded, and the sediment is resuspended in 0.85% NaCl. This preparation is again centrifuged, and 0.5 to 1.0 ml of the sediment is examined under the microscope for the presence of nonmotile microfilariae. The membrane filtration technique can also be used with urine for the recovery of *Schistosoma haematobium* eggs (4). This approach uses a 25-mm-diameter Nuclepore filter (5-μm porosity) (4, 6).

The use of stained smears is usually not necessary for the identification of *T. vaginalis*. Many times the number of false-positive and false-negative results reported on the basis of stained smears strongly suggests the value of confirmation by observation of motile organisms from the direct mount, from appropriate culture media (4, 6, 13), or from direct detection with immunoassay reagents (4).

Stained smears may be prepared from material obtained from the membrane filtration techniques used for the recovery of microfilariae; Delafield's hematoxylin or Giemsa stain may be used. It is important to remember that Giemsa stain may not adequately stain the sheath, and correct identification of the organisms may require staining with a hematoxylin-based stain.

Some microsporidial infections can also be diagnosed from the examination of urine sediment that has been stained by one of the modified trichrome methods or using optical

brightening agents such as calcofluor (4). Multiple methods are recommended for confirmation of the diagnosis.

Culture

Specimens from women for culture (for *T. vaginalis*) may consist of vaginal exudate collected from the posterior fornix on cotton-tipped applicator sticks or genital secretions collected on polyester sponges. Specimens from men can include semen, urethral samples collected with a polyester sponge, or urine. Urine samples collected from the patient should be the specimen first voided in the morning. It is critical that clinical specimens be inoculated into culture medium as soon as possible after collection (4, 10, 13). Although collection swabs can be used, there are often problems with specimens drying prior to culture; immediate processing is mandatory for maximum organism recovery. Another approach would be to use the plastic envelope methods (*Trichomonas* Culture System [Empyrean Diagnostics, Mountain View, Calif.]; InPouch TV [BIOMED Diagnostics, San Jose, Calif.]), which are simplified techniques for transport and culture (4). The following control strain should be available when using these cultures for clinical specimens: *T. vaginalis* ATCC 30001. Many media for the isolation of *T. vaginalis* are available, and some of these can be purchased commercially and have relatively long shelf lives, particularly the plastic envelope methods.

If no trophozoites are seen after 4 days of incubation, then discard the tubes and report the culture as negative. Results for patient specimens should not be reported as positive unless control cultures are positive. Since culture may take as long as 3 to 4 days and the clinical specimens may contain nonviable organisms, it is recommended that microscopic examination of wet smears be performed as well.

Antigen Detection *(Trichomonas vaginalis)*

The culture method is considered to be the most sensitive for the diagnosis of trichomoniasis; however, due to the time and effort involved, some laboratories have decided to use some of the new immunoassay detection kits (4). The OSOM Trichomonas Rapid Test (Genzyme Diagnostics, Cambridge, Mass.) is an immunochromatographic method for antigen detection using the dipstick format. Results are available within 10 min, and according to the manufacturer, there is a 95% agreement with the reference standard (culture and wet mount).

SPECIMENS FROM OTHER BODY SITES

When routine testing methods have failed to demonstrate the organisms, the examination of aspirated material for the diagnosis of parasitic infections may be extremely valuable (Table 10). Specimens should be transported to the laboratory immediately after collection. Aspirates include liquid specimens collected from a variety of sites as well as fine-needle aspirates and duodenal aspirates. Fine-needle aspirates are often collected by cytopathology staff who process the specimens, or they may be collected and sent to the laboratory directly for slide preparation and/or culture. Fluid specimens collected by bronchoscopy include bronchoalveolar lavage and bronchial washing fluids.

Procedural details for processing sigmoidoscopic aspirates and scrapings for the recovery of *E. histolytica* and techniques for preparation of duodenal aspirate material have been presented earlier in this chapter.

Biopsy specimens are recommended for use in the diagnosis of parasitic infections in tissues (Table 10). In addition to standard histologic preparations, the following can be used: impression smears and teased and squash preparations of biopsy tissue from skin, muscle, cornea, intestine, liver, lung, and brain. Tissue to be examined as permanent sections or by electron microscopy should be fixed as specified by the laboratories that will process the tissue, and in certain cases, testing of a biopsy specimen may be the only means of confirming a suspected parasitic problem. Specimens that are going to be examined as fresh material rather than as tissue sections should be kept moist in saline and submitted to the laboratory immediately.

Detection of parasites in tissue depends on specimen collection and the retrieval of sufficient material for examination. Biopsy specimens are usually quite small and may not be representative of the diseased tissue. Multiple tissue samples will often improve diagnostic results. To optimize the yield from any tissue specimen, examine all areas and use as many procedures as possible. Tissues are obtained from invasive procedures, many of which are very expensive and lengthy; consequently, these specimens deserve the most comprehensive procedures possible.

Tissue submitted in a sterile container on a sterile sponge dampened with saline may be used for cultures of protozoa after mounts for direct examination or impression smears for staining have been prepared. If cultures for parasites are to be made, use sterile slides for smear and mount preparation or inoculate cultures prior to smear preparation.

Bone Marrow

Bone marrow aspirates to be evaluated for *Leishmania* amastigotes, *Trypanosoma cruzi* amastigotes (African trypanosomiasis organisms not relevant), *Plasmodium* spp., or *Toxoplasma gondii* (less common site) require Giemsa staining. However, when a hematoxylin/Giemsa combination stain is used, the organisms will be visible as well. If specimens are going to be processed for culture, it is important to maintain sterility of the specimen prior to inoculation of media for parasitology cultures. After inoculation of appropriate media, the remaining specimen can be processed for smear preparation and staining.

Brain

Generally, when cerebrospinal fluid or brain aspirates or biopsy specimens are received, the most likely parasites would include the free-living amebae, *Acanthamoeba* spp., *Balamuthia mandrillaris*, or *Naegleria fowleri.* The use of nonnutrient agar (with bacterial overlays) cultures is recommended for *Acanthamoeba* spp. and *N. fowleri;* quality control cultures with known positive organisms are recommended as a basis for acceptable interpretation of patient culture results. If free-living ameba cultures are ordered for central nervous system specimens, the agar plates should be incubated at 37°C (room air) (4, 6). Unfortunately, cultures for *B. mandrillaris* are difficult to perform; consultation with CDC is recommended if this infection is suspected. The remaining specimen can then be processed for smear preparation and staining. Although *T. gondii* would also be seen in stained smears, one could also use immunospecific reagents or PCR. Spinal fluid should not be diluted before examination. Impression smears from tissues should be prepared and stained with Giemsa stain. The material is pressed between two slides, with the smear resulting when the slides are pulled apart (one across the other). The smears are allowed to air dry and are then processed like a thin blood film (fixed in absolute methanol and stained with Giemsa stain).

Patients with primary amebic meningoencephalitis are rare, but the examination of spinal fluid may reveal the amebae, usually *Naegleria fowleri*. Unspun sedimented spinal fluid

TABLE 10 Specimen, possible parasite recovered, and appropriate tests (other than intestinal tract)[a]

Body site	Specimen	Possible parasites	Tests
Bone marrow	Aspirate	*Leishmania* spp., *Trypanosoma cruzi*, *Plasmodium* spp., *Toxoplasma gondii*	Giemsa, culture
Brain	Tissue biopsy specimen, cerebrospinal fluid	*Naegleria* spp., *Acanthamoeba* spp.	Giemsa, trichrome, culture
		Balamuthia mandrillaris, *Sappinia diploidea* *Entamoeba histolytica*	Giemsa, trichrome
		Toxoplasma gondii	Giemsa, immunospecific reagent, culture, PCR
		Microsporidia *(Encephalitozoon* spp., *Trachipleistophora antropophtera)*	Modified trichrome, acid-fast stain, Giemsa, optical brightening agent (calcofluor), histology[b] (methenamine silver, PAS[c], tissue Gram stains), electron microscopy
		Taenia solium (cysticerci), *Echinococcus* spp.	Routine histology
Eye	Cornea, conjunctiva, contact lens, lens solutions[d]	Microsporidia (*Encephalitozoon* spp., *Trachipleistophora* spp., *Nosema* spp.)	Acid-fast stain, Giemsa, modified trichrome, methenamine silver, optical brightening agent calcofluor), histology (methenamine silver, PAS, tissue Gram stains), electron microscopy
		Acanthamoeba spp.	Giemsa, trichrome, culture, calcofluor (cysts only)
		Toxoplasma gondii	Giemsa, immunospecific reagent, culture
	Larval or adult worms	*Loa loa, Dipetalonema, Thalezia, Dirofilaria immitis, Gnathostoma* spp., *Toxocara canis, Taenia solium* (cysticerci), *Echinococcus* spp.	Direct examination, routine histology
	Fly larvae, adult lice	Myiasis, lice infestation	Direct examination
Kidney, bladder	Biopsy specimen	Microsporidia *(Encephalitozoon* spp., *Enterocytozoon bieneusi)*	Modified trichrome, acid-fast stain, Giemsa, optical brightening agent (calcofluor), histology (methenamine silver, PAS, tissue Gram stains), electron microscopy
		Schistosoma haematobium	Direct examination
	Adult worm, eggs	*Dioctyphyma renale*	Direct examination
Liver, spleen	Aspirates, biopsy specimen	*Echinococcus* spp.	Wet mount, routine histology
		Clonorchis spp., *Opisthorchis* spp.	Routine histology
		Capillaria hepatica, Toxocara canis, T. cati, *Toxoplasma gondii, Leishmania donovani*	Giemsa, culture
		Cryptosporidium spp.	Modified acid-fast stain, immunospecific reagents
		Microsporidia *(Encephalitozoon* spp., *Enterocytozoon bieneusi)*	Modified trichrome, acid-fast stain, Giemsa, optical brightening agent (calcofluor), histology (methenamine silver, PAS, tissue Gram stains), electron microscopy
		Entamoeba histolytica[e]	Wet mount, trichrome
Lymph node, lymphatics	Aspirates, biopsy specimen	*Toxoplasma gondii, Trypanosoma cruzi, Trypanosoma brucei rhodesiense, T. brucei gambiense*	Direct examination, routine histology, Giemsa, culture
		Microsporidia	Modified trichrome, acid-fast stain, Giemsa, optical brightening agent (calcofluor), histology (methenamine silver, PAS, tissue Gram stains), electron microscopy

(Continued on next page)

TABLE 10 Specimen, possible parasite recovered, and appropriate tests (other than intestinal tract)[a] *(Continued)*

Body site	Specimen	Possible parasites	Tests
		Wuchereria bancrofti, Brugia malayi, Brugia spp.	Thick blood films, concentration, membrane filtration
Lung	Sputum (expectorated or induced), bronchoalveolar lavage, transbronchial aspirates, brush biopsy, specimen open lung biopsy specimen	*Ascaris lumbricoides, Strongyloides stercoralis,* hookworm, *Paragonimus* spp., *Echinococcus granulosus*	Wet mount, routine histology
		Microsporidia *(Encephalitozoon* spp., *Enterocytozoon bieneusi)*	Modified trichrome, acid-fast stain, Giemsa, optical brightening agent (calcofluor), histology (methenamine silver, PAS, tissue Gram stains), electron microscopy
		Toxoplasma gondii	Giemsa, immunospecific reagent, culture
		Cryptosporidium spp.	Modified acid-fast stain, immunospecific reagent
	Saliva	*Entamoeba gingivalis, Trichomonas tenax*	Trichrome
Muscle	Biopsy specimen	*Trichinella* spp.	Wet examination, squash preparation, routine histology
		Microsporidia *(Pleistophora* spp., *Nosema* spp., *Trachipleistophora hominis)*	Modified trichrome, acid-fast stain, Giemsa, optical brightening agent (calcofluor), histology (methenamine silver, PAS, tissue Gram stains), electron microscopy
		Sarcocystis spp., *Baylisascaris procyonis*, *Ancylostoma* spp., *Taenia solium* (cysticerci), *Multiceps* (coenurus), *Echinococcus* spp., Spirometra (spargana), *Onchocerca volvulus* (nodules), *Gnathostoma* spp., *Trypanosoma cruzi*	Routine histology
Nasopharynx, sinus cavities	Scraping, biopsy specimen, aspirates	Microsporidia *(Encephalitozoon* spp., *Enterocytozoon bieneusi, Trachipleistophora hominis)*	Modified trichrome, acid-fast stain, Giemsa, optical brightening agent (calcofluor), histology (methenamine silver, PAS, tissue Gram stains), electron microscopy
		Acanthamoeba spp.	Giemsa, trichrome, culture, calcofluor (cysts only)
		Naegleria spp.	Giemsa, trichrome, culture
		Leishmania spp.	Giemsa, culture
Rectal tissue	Scraping, aspirate, biopsy specimen	*Schistosoma mansoni*, *S. japonicum*	Direct examination
Skin	Skin snips	*Onchocerca volvulus, Mansonella streptocerca*	Giemsa, routine histology
	Scraping, aspirates, biopsy specimen	*Leishmania* spp.	Giemsa, culture, routine histology
		Acanthamoeba spp.	Giemsa, trichrome, culture, calcofluor (cysts only)
		Entamoeba histolytica, *Schistosoma* spp.	Routine histology

[a] This table does not include every possible parasite that could be found in a particular body site. Parasite stages include trophozoites, cysts, oocysts, spores, adults, larvae, eggs, hooklets, amastigotes, and trypomastigotes. Although PCR methods have been used in the research setting for most of the organisms listed in the table, reagents are generally not commercially available.

[b] Routine histology can be used for the detection and identification of many parasites. In some cases, it may be the only means of diagnosis.

[c] PAS, periodic acid-Schiff stain.

[d] Although eye specimens are much preferred, free-living amebae have been cultured from patient contact lenses and lens solutions; we would not reject these specimens. An exception would be *unopened* commercial lens care solutions; these solutions would be rejected.

[e] The examination of abscess aspirates for the presence of *Entamoeba histolytica* trophozoites is an uncommon procedure and not always reliable in diagnosing extraintestinal amebiasis; serologic tests are preferred.

should be placed on a slide, under a coverslip, and observed for motile amebae; smears can also be stained with Trichrome, Wright's, or Giemsa stain. Spinal fluid, exudate, or tissue fragments can be examined by light microscopy or phase-contrast microscopy. Care must be taken not to confuse leukocytes with actual organisms and vice versa. The spinal fluid may appear cloudy or purulent (with or without erythrocytes), with a cell count of from a few hundred to more than 20,000 leukocytes (primarily neutrophils) per ml. Failure to find bacteria in this type of spinal fluid should alert one to the possibility of primary amebic meningoencephalitis; however, false-positive bacterial Gram stains have been reported because of excess debris. Isolation of these organisms from tissues can be done by using special media. When spinal fluid is placed in a counting chamber, organisms that settle to the bottom of the chamber tend to round up and look very much like leukocytes. For this reason, it is better to examine the spinal fluid on a slide directly under a coverslip, not in a counting chamber.

Possible infection with microsporidia (the most likely organism would be *Encephalitozoon* spp.; less common, *Trachipleistophora antropophtera*) should also be considered. Specific methods include modified trichrome, acid-fast, and Giemsa stains; a nonspecific optical brightening agent (calcofluor); routine histology (methenamine silver, periodic acid-Schiff stain [PAS], tissue Gram stains); and electron microscopy. Electron microscopy or immunospecific reagents are required to identify the microsporidia to the genus and species levels.

Helminth parasite stages such as *Taenia solium* cysticerci and hydatid cysts of *Echinococcus* spp. are generally identified through examination of routine histologic slides; however, confirmation of hydatid disease could also be made from the hooklets seen in the hydatid cyst fluid contents.

Eye

Eye specimens include those from the cornea, conjunctiva, contact lens, or contact lens solutions. Although eye specimens are preferred, *Acanthamoeba* spp. have been cultured from patient contact lenses and lens solution. These specimens are acceptable; however, due to risk management issues, the laboratory should not accept unopened commercial lens care solutions. After appropriate media have been inoculated, smears from acceptable specimens should be prepared and stained using Giemsa or trichrome stains. Although *T. gondii* can be seen in stained smears, one can also use an immunospecific reagent or PCR for confirmation. Most individuals who are infected with *T. gondii* do not develop ocular disease; however, two populations are at risk, the immunocompromised patient with HIV and neonates who have been exposed transplacentally to the mother's acute infection.

In addition to the free-living amebae, microsporidia are highly suspect from this body site, and specific methods include modified trichrome, acid-fast, and Giemsa stains; a nonspecific optical brightening agent (calcofluor); routine histology (methenamine silver, PAS, tissue Gram stains); and electron microscopy. Electron microscopy or immunospecific reagents are required to identify the microsporidia to the genus and species levels, which could include *Encephalitozoon* spp., *Trachipleistophora* spp., and *Nosema* spp.

Ocular disease caused by nematodes includes onchocerciasis, loiasis, dirofilariasis, gnathostomiasis, and toxocariasis. Diagnosis of onchocerciasis can be accomplished by confirming the presence of microfilariae in a corneal biopsy specimen or from the microscopic examination of multiple skin snips. The diagnosis of loiasis can be confirmed from the detection of circulating microfilariae of *Loa loa* in the blood or direct examination of the eye for the presence of the adult worm. Ocular disease can occur with the migration of *Dirofilaria immitis* larvae through periorbital or palpebral tissue; however, laboratory diagnosis of this infection is rare. Diagnosis of infections with *Gnathostoma* spp. and *Toxocara canis* is difficult but should be considered for patients with marked eosinophilia and elevated immunoglobulin E.

Eye disease can also be caused by cestodes, including *T. solium* (cysticercosis) and *Echinococcus* spp. (hydatid disease). These infections would generally be identified through direct ophthalmoscopic demonstration of the larval *T. solium*, computed tomography scans for hydatid cysts, or examination of routine histologic slides.

Ocular myiasis and infestations with lice are also possible; laboratory personnel may be asked to identify various fly larvae *(Dermatobia*, *Gasterophilus*, *Oestra*, *Cordylobia*, *Chrysomia*, *Wohlfahrtia*, *Cochliomyia*, and *Hypoderma)* and/or lice *(Pediculus humanus corporis* or *P. humanus capitis,* or *Phthirus pubis)*.

Kidney and Bladder

The kidney serves as the primary site for the adult worm *Dioctyphyma renale.* These worms generally live in the pelvis of the right kidney or in body cavities. Although these infections have been isolated from dogs in many areas of the world, they tend to be uncommon in humans. Infections can be confirmed at autopsy, by observation of the migration of worms from the urethra or discharge of worms from the skin over an abscessed kidney, or by recovery of eggs in the urine. Possible infection with microsporidia (the most likely organism would be *Encephalitozoon* spp.; less common, *Enterocytozoon bieneusi*) should also be considered. Specific methods would include modified trichrome, acid-fast, and Giemsa stains; a nonspecific optical brightening agent (calcofluor); routine histology (methenamine silver, PAS, tissue Gram stains); and electron microscopy. Electron microscopy or immunospecific reagents would be required to identify the microsporidia to the genus and species levels.

Mucosa from the bladder wall may reveal eggs of *Schistosoma haematobium* when they are not being recovered in the urine. The eggs in the bladder wall should be checked for viability by either a hatching technique or microscopic observation of the functioning flame cells within the miracidium larva (4).

Liver and Spleen

Although the liver and spleen can serve as secondary sites for a number of organisms, specific organisms that need to be considered for these organs as primary sites include the following: *Leishmania donovani*, *Toxoplasma gondii*, and *Echinococcus* sp. (spleen and liver) and *Toxocara canis, T. cati, Capillaria hepatica, Clonorchis* spp., *Opisthorchis* spp., *Cryptosporidium* spp., *Entamoeba histolytica*, *Encephalitozoon* spp., and *Encephalitozoon intestinalis* (liver). Typical methods for diagnosis include wet mounts, routine histology, Giemsa stain, culture, modified acid-fast stains, immunospecific reagents, and trichrome stains (routine and modified).

Examination of aspirates from lung or liver abscesses may reveal trophozoites of *E. histolytica;* however, demonstration of the organisms may be difficult (4). Liver aspirate material should be taken from the margin of the abscess rather than the necrotic center. The organisms are often trapped in the viscous pus or debris and do not exhibit typical motility. A minimum of two separate portions of exudate should be removed (more than two are recommended). The first portion of the aspirate, usually yellowish white, rarely contains

organisms. The last portion of the aspirated abscess material is reddish and is more likely to contain amebae. The best material to be examined is that obtained from the actual wall of the abscess. The Amoebiasis Research Unit, Durban, South Africa, has recommended the use of proteolytic enzymes to free the organisms from the aspirate material.

After the addition of the enzyme streptodornase to the thick pus (10 U/ml of pus), the mixture is incubated and shaken. After centrifugation (500 × *g* for 5 min), the sediment may be examined microscopically as wet mounts or may be used to inoculate culture media. Some of the aspirate can be mixed directly with PVA on a slide and examined as a permanent stained smear (4).

Aspiration of cyst material for the diagnosis of hydatid disease is a dangerous procedure and is usually performed only when open surgical techniques are used for cyst removal. Aspirated fluid usually contains hydatid sand (intact and degenerating scolices, hooklets, and calcareous corpuscles). Some older cysts contain material that resembles curded cottage cheese, and the hooklets may be very difficult to see. Some of this material can be diluted with saline or 10% KOH; usually, scolices or daughter cysts will have disintegrated. However, the diagnosis can be made from seeing the hooklets by using high dry power (magnification, ×400). The absence of scolices or hooklets does not rule out the possibility of hydatid disease, since some cysts are sterile and contain no scolices and/or daughter cysts. Histologic examination of the cyst wall should be able to confirm the diagnosis.

Lung

Expectorated Sputum, Induced Sputum

Although it is not one of the more common specimens, expectorated sputum may be submitted for examination for parasites (Table 10). Organisms in sputum that may be detected and that may cause pneumonia, pneumonitis, or Loeffler's syndrome include the migrating larval stages of *Ascaris lumbricoides*, *S. stercoralis*, and hookworm; the eggs of *Paragonimus* spp.; *Echinococcus granulosus* hooklets; the protozoa *E. histolytica* and *Cryptosporidium* spp.; and possibly the microsporidia (4). In a *Paragonimus* infection, the sputum may be viscous and tinged with brownish flecks ("iron filings"), which are clusters of eggs, and may be streaked with blood. Although *Entamoeba gingivalis* and *Trichomonas tenax* may be found in sputum, they are generally indicators of poor oral hygiene and/or periodontal disease, not pulmonary disease.

A sputum specimen should be collected properly so that the laboratory receives a "deep sputum" from the lower respiratory tract for examination rather than a specimen that is primarily saliva from the mouth. If the sputum is not induced, then the patient should receive specific instructions regarding collection.

Care should be taken not to confuse *E. gingivalis*, which may be found in the mouth and saliva, with *E. histolytica*, as this could result in an incorrect suspicion of pulmonary abscess. *E. gingivalis* usually contains ingested polymorphonuclear leukocytes, while *E. histolytica* may contain ingested erythrocytes but not polymorphonuclear leukocytes. *T. tenax* is also found in saliva from the mouth and thus would be an incidental finding and normally not an indication of pulmonary problems.

Direct Wet Mount

Sputum is usually examined as a wet mount (saline or iodine), using low and high dry power (magnifications, ×100 and ×400, respectively). The specimen is not concentrated before preparation of the wet mount. If the sputum is thick, an equal amount of 3% sodium hydroxide (or undiluted chlorine bleach) can be added; the specimen is thoroughly mixed and then centrifuged. However, NaOH will destroy any protozoan trophozoites that might be present. After centrifugation, the supernatant fluid is discarded, and the sediment can be examined as a wet mount with saline or iodine. If examination must be delayed for any reason, the sputum should be fixed in 5 or 10% formalin to preserve helminth eggs or larvae or in PVA fixative to be stained later for protozoa.

Permanent Stained Smears

If *Cryptosporidium* spp. is suspected (rare), then acid-fast or immunoassay techniques normally used for stool specimens can be used (4). Trichrome or iron hematoxylin stains of material may aid in differentiating *E. histolytica* from *E. gingivalis*, and Giemsa stain may better define larvae and juvenile worms.

Bronchoscopy Aspirates

Fluid specimens collected by bronchoscopy may be from lavages or washings, with bronchoalveolar lavages preferred. Specimens are usually concentrated by centrifugation prior to microscopic examination of stained preparations. Organisms that may be detected in such specimens are *Paragonimus* spp., *S. stercoralis*, *T. gondii*, *Cryptosporidium* spp., and the microsporidia.

If *T. gondii* is suspected, Giemsa stain, immunospecific reagents, and/or culture (tissue culture) can be used to confirm the diagnosis. A number of cell lines have been used (human foreskin fibroblast is one example), and most routine cell lines work well for the growth and isolation of this organism.

Lymph Node and Lymphatics

Material from lymph nodes and lymphatics confirm parasitic infections (toxoplasmosis, Chagas' disease, trypanosomiasis, microsporidiosis, filariasis) and should be processed as follows. Fluid material can be examined under low power (magnification, ×100) and high dry power (magnification, ×400) as a wet mount (diluted with saline) for the presence of motile organisms.

Material obtained from lymph nodes should be processed for tissue sectioning and as impression smears that should be processed as thin blood films and stained with Giemsa stain. Appropriate culture media can also be inoculated, again making sure that the specimen has been collected under sterile conditions.

If microsporidia are suspected, modified trichrome stains can be used; calcofluor white and immunoassay methods (currently under development) are also excellent options (4). If *T. gondii* is suspected, Giemsa stain, immunospecific reagents, and/or culture (tissue culture) can be used to confirm the diagnosis.

Specific filarial infections are generally caused by *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia* spp. In most cases, the microfilariae can be recovered and identified through examination of thick blood films, specific concentration sediment, and/or membrane filtration methods (4).

Muscle

Muscle is considered the primary site for the following organisms: *Sarcocystis* spp., microsporidia (*Pleistophora* spp., *Nosema* spp.), *Gnathostoma* spp., *Trichinella* spp. larvae, and cestode larval forms (coenurus/*Taenia* spp., cysticercosis/*T. solium*, sparganum). As a secondary site, *T. cruzi* amastigotes can also

be found in muscle; *Baylisascaris procyonis* and *Ancylostoma* spp. are also possibilities, as are hydatid cysts (*Echinococcus* spp.). In most cases, biopsy specimens processed using routine histologic methods provide the most appropriate specimen for examination and confirmation of the causative agent.

The presumptive diagnosis of trichinosis is often based on patient history: ingestion of raw or rare pork, walrus meat, or bear meat; diarrhea followed by edema and muscle pain; and the presence of eosinophilia. Generally, the suspected food is not available for examination. The diagnosis may be confirmed by finding larval *Trichinella spiralis* in a muscle biopsy specimen. The encapsulated larvae can be seen in fresh muscle if small pieces are pressed between two slides and examined under the microscope (4). Larvae are usually most abundant in the diaphragm, masseter muscle, or tongue and may be recovered from these muscles at necropsy. Routine histologic sections can also be prepared.

Human infection with any of the larval cestodes may present diagnostic problems, and frequently, the larvae are referred for identification after surgical removal. In addition to *E. granulosus* (hydatid disease) and the larval stage of *Taenia solium* (cysticercosis), other larval cestodes occasionally cause human disease. The larval stage of tapeworms of the genus *Multiceps*, a parasite of dogs and wild canids, is called a coenurus and may cause human coenurosis. The coenurus resembles a cysticercus but is larger and has multiple scolices developing from the germinal membrane surrounding the fluid-filled bladder. These larvae occur in extraintestinal locations, including the eye, central nervous system, and muscle.

Human sparganosis is caused by the larval stages of tapeworms of the genus *Spirometra*, which are parasites of various canine and feline hosts; these tapeworms are closely related to the genus *Diphyllobothrium*. Sparganum larvae are elongated, ribbon-like larvae without a bladder and with a slightly expanded anterior end lacking suckers. These larvae are usually found in superficial tissues or nodules, although they may cause ocular sparganosis, a more serious disease.

Finding prominent calcareous corpuscles in the tapeworm tissue frequently supports the diagnosis of larval cestodes; specific identification usually depends on referral to specialists.

Nasopharynx and Sinus Cavities

Organisms that might be found in these body sites include the microsporidia, *Acanthamoeba* spp., and *Naegleria*. Specimens submitted for examination could include scrapings, aspirates, and/or biopsy specimens. The special stains include modified trichrome, acid-fast, and tissue Gram stains. Giemsa, calcofluor, and regular trichrome stains would also be appropriate. If *Naegleria* or *Acanthamoeba* spp. are suspected, culture is highly recommended. In certain cases of mucocutaneous leishmaniasis, *Leishmania* spp. can also be found from these body sites; Giemsa stain is recommended for confirmation for both aspirates and biopsy specimens; cultures are also an option (4).

Rectal Tissue

Often when a patient has an old, chronic infection or a light infection with *Schistosoma mansoni* or *Schistosoma japonicum*, the eggs may not be found in the stool and an examination of the rectal mucosa may reveal the presence of eggs. The fresh tissue should be compressed between two microscope slides and examined under the low power of the microscope (low-intensity light) (4). Critical examination of these eggs should be made to determine whether living miracidia are still found within the egg. Treatment may depend on the viability of the eggs; for this reason, the condition of the eggs should be reported to the physician.

Skin

The use of skin snips is the method of choice for the diagnosis of human filarial infections with *O. volvulus* and *M. streptocerca* (1, 4). Microfilariae of both species occur chiefly in the skin, although *O. volvulus* microfilariae may rarely be found in blood and occasionally in urine. Skin snip specimens should be thick enough to include the outer part of the dermal papillae. With a surgical blade, a small slice may be cut from a skin fold held between the thumb and forefinger, or a slice may be taken from a small "cone" of skin pulled up with a needle. Significant bleeding should not occur, and there should be just a slight oozing of fluid. Corneal-scleral punches (either Holth or Walser type) have been found to be successful in taking skin snips of uniform size and depth and an average weight of 0.8 mg (range, 0.4 to 1.2 mg); this procedure is easy to perform and is painless. It has been demonstrated that in African onchocerciasis, it is preferable to take skin snips from the buttock region (above the iliac crest); in Central American onchocerciasis, the preferred skin snip sites are from the shoulders (over the scapula).

Skin snips are placed immediately in a drop of normal saline or distilled water and are covered so that they will not dry; teasing of the specimen with dissecting needles is not necessary but may facilitate release of the microfilariae. Microfilariae tend to emerge more rapidly in saline; however, in either solution, the microfilariae usually emerge within 30 min to 1 h and can be examined with low-intensity light and the 10× objective of the microscope. To see definitive morphological details of the microfilariae, allow the snip preparation to dry, fix it in absolute methyl alcohol, and stain it with Giemsa stain.

Skin biopsy specimens used for the diagnosis of cutaneous amebiasis (*Entamoeba histolytica, Acanthamoeba* spp.) and cutaneous leishmaniasis should be processed for tissue sectioning and subsequently stained by the hematoxylin-eosin technique.

Although cutaneous disease is an unusual presentation for schistosomiasis, it does occur with skin lesions as the only manifestation. Based on routine histologic examination of skin biopsies, eggs can be found in the cellular infiltrate from within the lesion. When evaluating patients with unusual skin lesions, a complete history may reveal travel to an area where schistosomiasis is endemic.

Material containing intracellular *Leishmania* organisms can be aspirated from below the ulcer bed through the uninvolved skin, not from the surface of the ulcer. The surface of the ulcer must be thoroughly cleaned before specimens are taken; any contamination of the material with bacteria or fungi may prevent recovery of the organism from culture. Aspirated material is placed on a slide and stained with Giemsa stain.

Some prefer to take a punch biopsy through the active margin of the lesion (after cleaning the lesion); good results have also been seen when using dermal scrapings from the bottoms of the ulcers. When microscopic examinations of dermal scrapings of both the ulcer bottom and active margins are combined, the sensitivity of diagnosis may increase to 94% (17). Aspirate culture has been shown to be the most sensitive method for the diagnosis of patients with chronic ulcers. However, any successful culture depends on the prevention of contamination with bacteria and/or fungi; sampling of the ulcer must be done correctly in order to prevent false-negative culture results.

BLOOD

Depending on the life cycle, a number of parasites may be recovered in a blood specimen, either whole-blood or buffy

coat preparations or following concentration by various types of procedures. These parasites include *Plasmodium*, *Babesia*, and *Trypanosoma* species, *Leishmania donovani*, and microfilariae. Although some organisms may be motile in fresh, whole blood, species identification will usually be accomplished by the examination of permanent stained thick and thin blood films. Blood films can be prepared from fresh, whole blood collected with no anticoagulants; anticoagulated blood (EDTA is recommended; heparin is acceptable, but organism morphology is not as good; other anticoagulants are not recommended); or sediment from the various concentration procedures. The recommended stain of choice is Giemsa stain; however, the parasites can also be seen on blood films stained with Wright's stain. Delafield's hematoxylin stain is often used to stain the microfilarial sheath; in some cases, Giemsa stain does not provide sufficient stain quality to allow differentiation of the microfilariae (Table 11). When handling blood, as well as other clinical specimens, universal precautions should be observed (3, 4, 13, 14, 15).

Preparation of Thick and Thin Blood Films

Microfilariae and trypanosomes can be detected in fresh blood by their characteristic shape and motility; however, specific identification of the organisms requires a permanent stain. Two types of blood films are recommended. Thick films allow a larger amount of blood to be examined, which increases the possibility of detecting light infections (4, 10, 14). However, only experienced workers can usually make species identification with a thick film, particularly in the case of malaria, and the morphological characteristics of blood parasites are best seen in thin films.

The accurate examination of thick and thin blood films and identification of parasites depend on the use of absolutely clean, grease-free slides for preparation of all blood films. Old (unscratched) slides should be cleaned first in detergent and then with 70% ethyl alcohol; new slides should also be cleaned with alcohol and allowed to dry before use.

Blood films should be prepared when the patient is admitted or seen in the emergency room or clinic; typical fever patterns are frequently absent, and the patient may not be suspected of having malaria. If malaria remains a possible diagnosis, after the first set of negative smears samples should be taken at intervals of 6 to 8 h for at least three successive days. Another option is to collect blood immediately on admission; if the initial blood films are negative, collect daily specimens for two additional days (ideally between paroxysms if present). Using either collection option, quality patient care depends on the fact that both the physician and laboratory staff know that one negative set of blood films does not eliminate *Plasmodium* spp. as possible etiologic agents.

TABLE 11 Techniques for the recovery of blood parasites (EDTA or heparin)[a]

Organism	Procedure	Stain
Malaria	Thick and thin films	Giemsa, Wright's, Field's, rapid stains
	QBC[b]	Stain not relevant (centrifugation, acridine orange, microscopy)
	ParaSight F[c]	Stain not relevant (HRP-2 immunochromatographic assay)
	Binax Now Malaria[d]	Stain not relevant (HRP-2 and aldolase immunochromatographic assay)
	PATH IC Falciparum malaria IC[e]	Stain not relevant (HRP-2 immunochromatographic assay)
	Malaria Ag-CELISA[f]	Stain not relevant (HRP-2 immunochromatographic assay)
	Rapimal Dipstick[f]	Stain not relevant (HRP-2 immunochromatographic assay)
	Rapimal Cassette[f]	Stain not relevant (HRP-2 immunochromatographic assay)
	OptiMAL[g]	Stain not relevant (pLDH immunochromatographic assay)
Babesia spp.	Thick and thin films	Giemsa, Wright's, Field's, rapid stains
Microfilariae	Thick and thin films, Knott concentration, membrane filtration, gradient centrifugation	Giemsa, Wright's or Delafield's hematoxylin
	QBC	Stain not relevant
	NOW ICT Filariasis	Rapid, tends to be dipstick format
	Filariasis Ag-CELISA	EIA
	TropBio[h]	Rapid, tends to be dipstick format
Trypanosomes	Thick and thin films, buffy coat smears, triple centrifugation, culture	Giemsa, Wright's, Field's, rapid stains
	QBC	Stain not relevant
Leishmaniae	Thick and thin films, buffy coat smears, culture	Giemsa, Wright's, Field's, rapid stains

[a] Molecular techniques are still experimental and are not always available.
[b] QBC-Blood Parasite Detection Method (Becton-Dickinson Tropical Disease Diagnostics, Sparks, Md.).
[c] ParaSight F-Rapid Test for *P. falciparum* malaria (Becton-Dickinson Tropical Disease Diagnostics). This test is not licensed for diagnostic use in the United States. This test is a dipstick format.
[d] Binax Now malaria (all four *Plasmodium* species), Binax Now ICT Filariasis *(Wuchereria bancrofti)* (Binax, Inc., Portland, Maine). These tests are in a dipstick format.
[e] PATH IC Falciparum malaria IC test (PATH, Seattle, Wash.). This test is in a dipstick format.
[f] Filariasis Ag-CELISA *(Wuchereria bancrofti)*, Malaria Ag-CELISA *(P. falciparum)*. Rapimal Dipstick *(P. falciparum)*, Rapimal Cassette *(P. falciparum)* (Cellabs, Brookvale, NSW, Australia).
[g] OptiMAL (differentiates between *P. falciparum* and *P. vivax*) (Flow, Inc., Portland, Oreg.). This test is in a dipstick format.
[h] TropBio (James Cook University Tropical, Queensland, Australia).

Subsequent to a finger stick, the blood should flow freely; blood that has to be "milked" from the finger will be diluted with tissue fluids, which decrease the number of parasites per field. An alternative approach to the fingerstick is collection of fresh blood containing anticoagulant (preferably EDTA) for the preparation of blood films. Ideally, the smears should be prepared within 1 h after the specimen is drawn. After that time, stippling may not be visible on stained films; however, the overall organism morphology may still be acceptable.

The time that the specimen was drawn should be clearly indicated on the tube of blood and also on the result report. The physician will then be able to correlate the results with any symptoms that the patient may have. There should also be some indication on the slip that is sent back to the physician that one negative specimen does not rule out the possibility of a parasitic infection.

Thick Blood Films

To prepare the thick film, place 2 or 3 small drops of capillary blood directly from the fingerstick (no anticoagulant) on an alcohol-cleaned slide. With the corner of another slide and using a circular motion, mix the drops and spread them over an area 2 cm in diameter. Continue stirring for 30 s to prevent the formation of fibrin strands that may obscure the parasites after staining. If blood containing an anticoagulant is used, 1 or 2 drops may be spread over an area about 2 cm in diameter; it is not necessary to continue stirring for 30 s, since there will be no formation of fibrin strands. If too much blood is used or any grease remains on the slide, the blood may flake off during staining. Allow the film to air dry (at room temperature) in a dust-free area. Never apply heat to a thick film, since heat will fix the blood, causing the erythrocytes to remain intact during staining; the result is stain retention and an inability to identify the parasites. However, the thick films can be placed in a 37°C incubator for 10 to 15 min to dry; this seems to work quite well. Remember, do not make the films too thick; one should be able to see newsprint through the wet film prior to drying. After the thick films are thoroughly dry, they can be laked to remove the hemoglobin. To lake the films, place them in buffer solution before staining for 10 min or directly into a dilute, buffered aqueous Giemsa stain. If thick films are to be stained at a later time, they should be laked before storage (4).

Thin Blood Films

The thin blood film is routinely used for specific parasite identification, although the number of organisms per field is much reduced compared with the number in the thick film. The thin film is prepared exactly as one used for a differential count, and a well-prepared film is thick at one end and thin at the other (one layer of evenly distributed erythrocytes with no cell overlap). The thin, feathered end should be at least 2 cm long, and the film should occupy the central area of the slide, with free margins on both sides. Holes in the film indicate the presence of grease on the slide. After the film has air dried (do not apply heat), it may be stained. The necessity for fixation before staining will depend on the stain selected.

Staining Blood Films

For accurate identification of blood parasites, a laboratory should develop proficiency in the use of at least one good staining method (2, 4, 9). Since prolonged storage may result in stain retention, blood films should be stained on the same day or within a few days of collection. If thick blood films are not prepared or received, it is possible to stain one of the thin blood films as a thick film and examine the thick portion of the thin film. During staining the red blood cells will be laked, thus leaving the white blood cells, platelets, and any parasites present.

Wright's stain has the fixative in combination with the staining solution, so that both fixation and staining occur at the same time; therefore, the thick film must be laked before staining. In aqueous Giemsa stain, the fixative and stain are separate; thus, the thin film must be fixed with absolute methanol before staining.

When slides are removed from either type of staining solution, they should be dried in a vertical position. After being air dried, they may be examined under oil immersion by placing the oil directly on the uncovered blood film. If slides are to be stored for a considerable length of time (for teaching or legal purposes), they should be protected with a coverglass by being mounted in a medium such as Permount. Blood films that have been stained with any of the Romanowsky stains and that have been mounted with Permount or other resinous mounting media are susceptible to fading of the basophilic elements and generalized loss of stain intensity. One can add an antioxidant such as 1% (by volume) 2,6-di-*t*-butyl-*p*-cresol (butylated hydroxytoluene; Sigma-Aldrich) to the mounting medium. Without the addition of this antioxidant, mounted stained blood films eventually become pink; stained films protected with this compound generally remain unchanged in color for many years.

Giemsa Stain

Each new lot number of Giemsa stain should be tested for optimal staining times before being used on patient specimens. If the blood cells appear to be adequately stained, the timing and stain dilution should be appropriate to demonstrate the presence of malaria and other parasites. The use of prepared liquid stain or stain prepared from the powder depends on personal preference; there is apparently little difference between the two preparations.

The commercial liquid stain or the stock solution prepared from powder should be diluted by approximately the same amount to prepare the working stain solution (4, 14). Stock Giemsa liquid stain is diluted 1:10 with buffer for both thick and thin blood films, with dilutions ranging from 1:10 up to 1:50. Staining times usually match the dilution factor (e.g., 1:20 for 20 min or 1:50 for 50 min). Some people prefer to use the longer method with more dilute stain for both thick and thin films. The phosphate buffer used to dilute the stock stain should be neutral or slightly alkaline (pH 7.0 to 7.2). A phosphate buffer solution may be used to obtain the right pH. In some laboratories, the pH of tap water may be satisfactory and may be used for the entire staining procedure and the final rinse. Some workers recommend the use of pH 6.8 to emphasize Schüffner's dots.

Giemsa stain colors the blood components as follows: erythrocytes, pale red; nuclei of leukocytes, purple with pale purple cytoplasm; eosinophilic granules, bright purple-red; and neutrophilic granules, deep pink-purple. In malaria parasites, the cytoplasm stains blue and the nuclear material stains red to purple-red. Schüffner's dots and other inclusions in the erythrocytes stain red. The nuclear and cytoplasmic staining characteristics of the other blood parasites such as *Babesia* spp., trypanosomes, and leishmaniae are like those of the malaria parasites. While the sheath of microfilariae may not always stain with Giemsa, the nuclei within the microfilaria itself stain blue to purple.

Wright's Stain

Wright's stain is available in liquid form and also as a powder, which must be dissolved in anhydrous, acetone-free methyl alcohol before use. Since Wright's stain contains

alcohol, the thin blood films do not require fixation before staining. Thick films stained with Wright's stain are usually inferior to those stained with Giemsa solution. Great care should also be taken to avoid excess stain precipitate on the slide during the final rinse. Before staining, thick films must be laked in distilled water (to rupture and remove erythrocytes) and air dried. The staining procedure is the same as that for thin films, but the staining time is usually somewhat longer and must be determined for each batch of stain. Wright's stain colors blood components as follows: erythrocytes, light tan, reddish, or buff; nuclei of leukocytes, bright blue with contrasting light cytoplasm; eosinophilic granules, bright red; and neutrophilic granules, pink or light purple.

In malaria parasites, the cytoplasm stains pale blue and the nuclear material stains red. Schüffner's dots and other inclusions in the erythrocytes usually do not stain or stain very pale with Wright's stain. Nuclear and cytoplasmic staining characteristics of the other blood parasites such as *Babesia* spp., trypanosomes, and leishmaniae stain like those seen in the malaria parasites. While the sheath of microfilariae may not always stain with Wright's stain, the nuclei within the microfilaria itself stain pale to dark blue.

Proper Examination of Thin and Thick Blood Films

In cases where malaria has not been indicated as the suspect organism, the initial screen of the thin blood film should be carried out with the low-power objective of a microscope because microfilariae may be missed if the entire thin film is not examined. Microfilariae are rarely present in large numbers, and frequently, only a few organisms occur in each thin film preparation. Microfilariae are commonly found at the edges of the thin film or at the feathered end of the film because they are carried to these sites during the process of spreading the blood. This approach to thin film examination is particularly important in cases where a suspect organism has not been indicated. The feathered end of the film where the erythrocytes are drawn out into one single, distinctive layer of cells should be examined for the presence of malaria parasites and trypanosomes. In these areas, the morphology and size of the infected erythrocytes are most clearly seen.

In the case of a suspect malaria diagnosis, the request for blood film examination should always be considered a STAT procedure, with all reports (negative as well as positive) being reported by telephone to the physician as soon as possible (4, 14). Examination of the thin film should include viewing of 200 to 300 oil immersion fields at a magnification of ×1,000. Although some people use a 50× or 60× oil immersion objective to screen stained blood films, there is some concern that small parasites such as *Plasmodium* spp., *Babesia* spp., or *Leishmania donovani* may be missed at this smaller total magnification (×500 or ×600), although they are usually detected at the ×1,000 total magnification obtained with the more traditional 100× oil immersion objective. Because people tend to scan blood films at different rates, it is important to examine a minimum number of fields, regardless of the time that it takes to perform this procedure. If something suspicious has been seen in the thick film, often the number of fields examined on the thin film may be considerably more than 200 to 300.

Diagnostic problems with the use of automated differential instruments have been reported (4). Both malaria and *Babesia* infections can be missed with these instruments, and therapy is therefore delayed. Because these instruments are not designed to detect intracellular blood parasites, any reliance on the automated systems for discrimination between uninfected erythrocytes and those infected with parasites may pose serious diagnostic problems, particularly in light infections.

In the preparation of a thick blood film, the greatest concentration of blood cells is in the center of the film. A search for parasitic organisms should be carried out initially at low magnification to detect microfilariae more readily. Examination of a thick film usually requires 5 to 10 min (approximately 100 oil immersion fields). The search for malarial organisms and trypanosomes is best done under oil immersion (total magnification, ×1,000). Close examination of the very periphery of the thick film may reveal intact erythrocytes; such cells, if infected, may prove useful in malaria diagnosis since the characteristic morphology necessary to identify the organisms to the species level is more easily seen.

Immunochromatographic Tests for Malaria

Immunochromatography relies on the migration of liquid across the surface of a nitrocellulose membrane. Using monoclonal antibodies prepared against a malaria antigen target that has been incorporated onto the strip of nitrocellulose, these tests are based on the capture of parasite antigen from peripheral blood. Currently, malaria antigens used for these rapid diagnostic tests are the histidine-rich protein-2 (HRP-2), parasite lactate dehydrogenase (pLDH), and *Plasmodium* aldolase (Table 11). These dipsticks offer the possibility of more rapid, nonmicroscopic methods for malaria diagnosis. The tests are easy to perform and interpret; however, there are a number of questions that remain concerning the relevant uses for this type of testing, especially considering the fact that they are not yet approved by the Food and Drug Administration (FDA).

Sensitivity remains a problem, particularly for nonimmune populations. Parasite densities of >100 parasites/μl (0.002% parasitemia) should be detected, and this is a reasonable target to expect from dipsticks for *P. falciparum* diagnosis. However, this level of sensitivity is at the lower end of the capability of most devices using capture methods for HRP-2 or pLDH. It is probably as good as the sensitivity that clinical laboratory staff in nonspecialized laboratories with limited exposure to malaria cases could expect to provide using microscopy diagnosis. One of the potential benefits would be for inexperienced evening staff personnel for whom the dipstick identification of a life-threatening parasitemia with *P. falciparum* could prevent a missed infection. However, a negative test could not be accepted and would require confirmation by microscopic examination of both thick and thin blood films for the detection of parasitemia below the present threshold of detection by these rapid tests. Although these tests are not yet approved for use in the United States, FDA approval may be possible in the future. The U.S. military has conducted FDA-approved trials because of possible use of the devices in the field.

Concentration Procedures

Buffy Coat Films

L. donovani, trypanosomes, and *Histoplasma capsulatum* (a fungus with intracellular elements resembling those of *L. donovani*) may occasionally be detected in the peripheral blood. The parasite or fungus is found in the large mononuclear cells that are found in the buffy coat (a layer of leukocytes resulting from centrifugation of whole citrated blood). The nuclear material stains dark red-purple, and the cytoplasm is light blue (*L. donovani*). *H. capsulatum* appears as a dot of nuclear material (dark red-purple) surrounded by a clear halo area. Trypanosomes in the peripheral blood also concentrate with the buffy coat cells.

Use alcohol-cleaned slides for preparation of the blood films. A microhematocrit tube can also be used; the tube is carefully scored and snapped at the buffy coat interface, and the leukocytes are prepared as a thin film. The tube can also be examined prior to removal of the buffy coat under low and high dry powers of the microscope. If trypanosomes are present, the motility may be observed in the buffy coat. Microfilaria motility would also be visible.

QBC Microhematocrit Centrifugation Method

Microhematocrit centrifugation with use of the QBC malaria tube (glass capillary tube and closely fitting plastic insert; QBC malaria blood tubes; Becton Dickinson, Tropical Disease Diagnostics, Sparks, Md.) has been used for the detection of blood parasites (4). At the end of centrifugation of 50 to 60 μl of capillary or venous blood (5 min in a QBC centrifuge, 14,387 $\times$ *g*), parasites or erythrocytes containing parasites are concentrated into a small, 1- to 2-mm region near the top of the erythrocyte column and are held close to the wall of the tube by the plastic float, thereby making them readily visible by microscopy. Tubes precoated with acridine orange provide a stain that induces fluorescence in the parasites. This method automatically prepares a concentrated smear that represents the distance between the float and the walls of the tube. Once the tube is placed into the plastic holder (Paraviewer) and immersion oil is applied onto the top of the hematocrit tube (no coverslip is necessary), the tube is examined with a 40$\times$ to 60$\times$ oil immersion objective (it must have a working distance of 0.3 mm or greater).

Although a malaria infection could be detected by this method (which is much more sensitive than the thick or the thin blood smear), appropriate thick and thin blood films need to be examined to accurately identify the species of the organism causing the infection.

Knott Concentration

The Knott concentration procedure is used primarily to detect the presence of microfilariae in the blood, especially when a light infection is suspected (4, 10). The disadvantage of the procedure is that the microfilariae are killed by the formalin and are therefore not seen as motile organisms.

Membrane Filtration Technique

The membrane filtration technique is highly efficient in demonstrating filarial infections when microfilaremias are of low density. This method is unsatisfactory for the isolation of *Mansonella perstans* microfilariae because of their small size. A 3-μm-pore-size filter can be used for recovery of this organism. Other filters with similar pore sizes are not as satisfactory as the Nuclepore filter (4).

Delafield's Hematoxylin

Some of the material that is obtained from the concentration procedures can be allowed to dry as thick and thin films and then stained with Delafield's hematoxylin, which will demonstrate greater nuclear detail as well as the microfilarial sheath, if present. In addition, fresh thick films of blood containing microfilariae can be stained by this hematoxylin technique (4).

Triple-Centrifugation Method for Trypanosomes

The triple-centrifugation procedure may be valuable in demonstrating the presence of trypanosomes in the peripheral blood when the parasitemia is light (4). After repeated centrifugation of the supernatant, the sediment is examined as a wet preparation or is stained as a thin blood film.

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Plasmodium and *Babesia*

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TAXONOMY

The agents of malaria and babesiosis are members of the phylum Apicomplexa, which includes protozoa characterized by the presence of an apical complex consisting of polar rings, conoid micronemes, rhoptries, subpellicular microtubules, and a small plastid related to a red algal chloroplast, the apicoplast. The phylum Apicomplexa is classified within the superphylum Alveolata, which includes apicomplexans, ciliates, and dinoflagellates. Although the exact evolutionary pathway is controversial, there is good evidence that the Alveolata acquired chloroplasts by secondary endosymbiosis of a red alga (21). While some dinoflagellates retain functional chloroplasts and are photosynthetic, in apicomplexans the apicoplast does not photosynthesize but is involved in lipid, heme, and isoprenoid synthesis (22, 81). All members of the phylum Apicomplexa are parasitic. The agents of malaria and babesiosis are classified within the orders Haemospororida and Piroplasmorida, respectively.

PLASMODIUM

Introduction

The genus *Plasmodium* includes at least 172 named species of intraerythrocytic parasites infecting a wide range of mammals, birds, reptiles, and amphibians (45). Four species, *P. falciparum, P. vivax, P. ovale,* and *P. malariae,* commonly infect humans. Rarely, *Plasmodium* species normally infecting nonhuman primates may infect humans. Recently, a large focus of naturally occurring infection of humans with *P. knowlesi,* a species normally infecting long-tailed macaques *(Macaca fascicularis),* was described in Malaysian Borneo (69).

Life Cycle

The *Plasmodium* life cycle (Fig. 1) begins with the injection of sporozoites by the bite of an infected mosquito. Sporozoites move rapidly to the liver, where they invade hepatocytes and undergo exoerythrocytic development for 7 to 10 days, leading to the formation of liver schizonts. Rupture of each liver schizont releases thousands of free merozoites into the peripheral blood, where they invade erythrocytes. In the erythrocyte the early trophozoites (rings) develop into mature trophozoites with an enlarged cytoplasm and an accumulation of hemozoin pigment. Nuclear and cytoplasmic division leads to production of the mature schizont, which ruptures, releasing merozoites and completing the erythrocytic cycle. A fraction of the merozoites undergo sexual differentiation into gametocytes, which undergo fertilization and zygote formation following ingestion with a mosquito blood meal. Fertilized gametes develop into ookinetes, which penetrate the intestinal lining and develop into oocysts on the extraluminal wall of the intestine. Oocysts rupture, releasing sporozoites, which migrate to the salivary glands to complete the life cycle.

There are important species-specific variations in the life cycle. The late trophozoites and schizonts of *P. falciparum* insert an antigenically variable, high-molecular-weight protein in the erythrocyte membrane, which causes the infected erythrocyte to adhere to the vascular endothelium (7). Thus, these stages of the life cycle do not circulate in the peripheral blood. *P. vivax* and *P. ovale* may be associated with relapse from the liver months to years after clearance of the initial blood stage parasitemia. Sporozoites of these species may enter hepatocytes and complete exoerythrocytic stage development normally, or they may arrest development in the liver as hypnozoites, which subsequently develop into mature liver schizonts, leading to relapse of parasitemia months to a few years later.

Epidemiology

Malaria imposes an enormous burden of illness and substantial mortality on the tropical world and in many subtropical regions. There are an estimated 515 (330 to 660) million clinical episodes of malaria due to *Plasmodium falciparum,* the most common cause of malaria (71), and 1.5 to 2.7 million deaths due to malaria each year (89). Transmission depends on the presence and abundance of an effective anopheline mosquito vector and a reservoir of infected humans. Transmission cannot occur at temperatures outside the range from 16 to 33°C or above 2,000 m of altitude, because development in the mosquito cannot take place. The intensity of transmission in any location depends on temperature, rainfall, the abundance and behavior of the local anopheline vector, and the existence of a reservoir of infected humans. The level of endemicity of malaria is classified based on the percentage of children ages 2 to 9 with splenomegaly or patent parasitemia as follows: hypoendemic, 0 to 10%; mesoendemic, 10 to 50%; hyperendemic, 50 to 75%; holoendemic, >75%. In areas where malaria is holoendemic, such as much of sub-Saharan Africa, the most

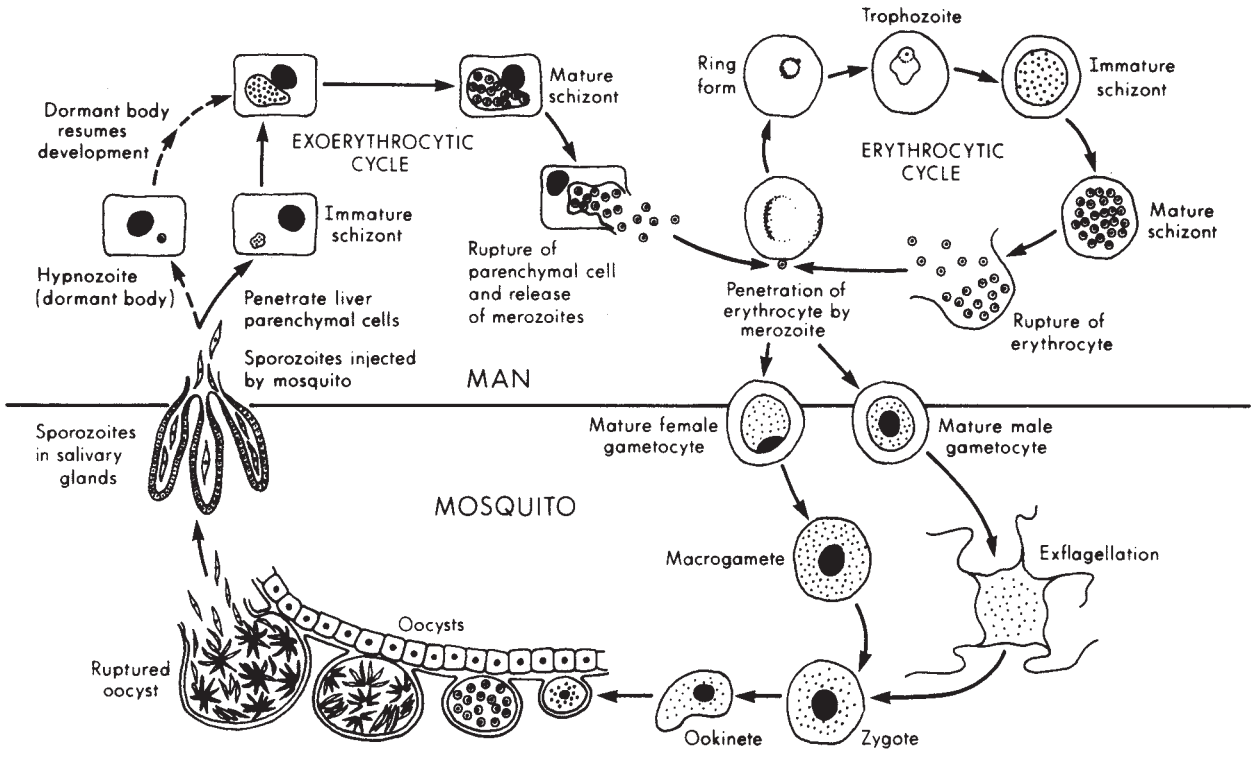


FIGURE 1 Life cycle of the malaria parasite. (Reproduced with permission from Blackwell Publishing [41a].)

severe morbidity and mortality occur in early childhood and the majority of deaths are due to severe anemia. Individuals surviving through early childhood develop partial clinical immunity and are less likely to die from malaria. In areas of lesser endemicity, particularly where transmission is seasonal or unstable, development of clinical immunity is less effective, symptomatic and severe malaria occurs at all ages, and cerebral malaria is a common manifestation of severe infection.

Although malaria was eradicated from North America by the mid-20th century, competent vector species of anopheline mosquito are widely present, and small, focal outbreaks of malaria can occur when a gametocytemic immigrant or traveler serves as a reservoir of infection (9–11, 43, 49, 75).

Clinical Disease

The classic clinical manifestation of malaria is the febrile paroxysm, which begins with a chill and rigors, leading to an abrupt fever lasting 1 to 2 h, and finally resolves with profuse sweating and a return to normal temperature. The typical periodicity of these paroxysms, approximately 48 h in disease caused by *P. falciparum, P. vivax,* and *P. ovale,* and 72 h in that caused by *P. malariae,* is not always seen, particularly in nonimmune individuals. The fever is frequently accompanied or preceded by headache, myalgias, and malaise. Splenomegaly and anemia are common. Gastrointestinal complaints, nausea, vomiting, and diarrhea, which may be bloody, are also not uncommon and should not distract the clinician from the diagnosis of malaria. Infection with *P. falciparum* may be complicated by various forms of severe malaria. Table 1 shows the World Health Organization criteria for severe malaria (88). Severe malaria due to *P. falciparum* may develop rapidly in a patient who initially presents with mild symptoms and a low parasitemia. It is therefore critical to make the diagnosis of *P. falciparum* quickly.

P. vivax and *P. ovale* preferentially invade reticulocytes and therefore only very rarely cause parasitemias greater than 2%. They may relapse from the liver following successful therapy of blood stage infection and must therefore be treated with primaquine to eradicate hypnozoites in the liver. *P. malariae* preferentially infects older erythrocytes and may cause chronic, asymptomatic parasitemia lasting for many years. Proteinuria is a common finding in *P. malariae* infection and may progress to the nephrotic syndrome in children.

Diagnosis

Specimen Collection and Patient History

Because *P. falciparum* infection can progress rapidly to severe malaria, the diagnosis of malaria is an urgent matter and all requests for malaria diagnosis should be handled on a STAT basis. Although it is important that thick smears dry completely before staining, drying can be accomplished at 37°C in 15 to 30 min; laboratory protocols should not specify long periods for drying at room temperature (e.g., 4 h to overnight), as this may unduly delay diagnosis. Ideally, blood for preparation of peripheral smears may be collected by finger prick. If other tests are to be performed, blood may be collected by venipuncture, using EDTA as the anticoagulant. Use of heparin as an anticoagulant may lead to some morphological distortion. Smears should be prepared and stained within an hour of drawing the specimen. Otherwise, confusing alterations in morphology may occur. For example, *P. falciparum* may continue to develop within the stored blood to the mature trophozoite stage, which is normally not

TABLE 1 Classification of severe malaria

Category	Criteria
Cerebral malaria	Unrousable coma or Blantyre Coma Score of 3 or less
	Coma persists for more than 30 min after any seizures have passed
	Normal CSF findings
	No other pathological process accounts for coma
Generalized convulsions	Generalized convulsions lasting more than 30 min
	Generalized convulsions more than twice in 24 h in spite of cooling
Severe anemia	Hemoglobin, <5 mg/dl
Respiratory distress	Presence of any of the following: alar flaring, intercostal retractions, use of accessory muscles of respiration, Kussmaul's respiration
Hypoglycemia	Blood glucose < 40 mg/dl
Circulatory collapse	Systolic blood pressure < 80 mmHg (adults) or < 50 mmHg (children)
Renal failure	Urine output < 12 ml/kg/24 h, or serum creatinine > 3.0 mg/dl
Malarial hemoglobinuria	Hemoglobinuria by urine dipstick in patient with malaria
Other	Hyperparasitemia (>100,000 parasites/μl)
	Jaundice (serum bilirubin, ≥3.0 mg/dl)
	Hyperpyrexia (rectal temperature, ≥40°C)
	Impaired consciousness (rousable)

seen in peripheral smears, leading to misidentification of the species. Diagnostic malaria smears should always be prepared, stained, and read on a STAT basis. However, if a laboratory is to prepare large numbers of slides from a single patient for later teaching purposes, it is acceptable to prepare the smears immediately but to delay staining for up to a few days. Unstained slides cannot be stored indefinitely, however, as the morphology degrades over time. It is therefore not possible to maintain a supply of unstained, malaria-positive slides to use for quality control of the Giemsa stain; adequate staining of the normal blood elements in the smear is a sufficient quality control marker. Blood containing malaria parasites is infectious, and universal precautions should be strictly followed.

Requests for malaria smears should include several important pieces of historical information. If the following historical information is not submitted with the request for malaria smears, the laboratory must be proactive in obtaining the history. (i) The patient's travel history and date of return to or arrival in the United States can suggest the likelihood of infection and the possible species involved. The interval between exposure to an infective bite and development of symptoms is typically 9 to 14 days for *P. falciparum, P. vivax,* and *P. ovale* and up to 40 days for *P. malariae.* Because there may be no primary attack in *P. vivax* or *P. ovale,* if all sporozoites that invaded hepatocytes transformed into hypnozoites, the first symptoms may occur months to as many as 3 years after exposure. There have been extremely rare reports of *P. falciparum* malaria first being detected up to 24 months after exposure (42), but in general, malaria appearing more than 1 month after return from an area of endemicity is relatively unlikely to be due to *P. falciparum,* unless the patient has received antimalarial agents which might have suppressed parasitemia. (ii) A history of prophylaxis or treatment for malaria may result in low parasitemia. Since currently available prophylactic regimens are difficult to comply with, and since drug resistance is common in *P. falciparum,* a history of prophylaxis should never be used to exclude malaria from the differential diagnosis. (iii) A history of transfusions or shared needles may suggest direct person-to-person transmission, although cases of transfusion-related malaria are extremely rare in the United States (53). (iv) A history of malaria in the patient suggests the possibility of relapse or recrudescence. (v) Knowledge of the periodicity of the fever pattern and the time in relation to a paroxysm when the specimen was obtained is helpful because in a regularly periodic *P. falciparum* infection the circulating parasitemia can be very low and difficult to detect between paroxysms. Regardless of the presence or absence of a fever pattern, blood should be taken immediately for an initial malaria smear when the patient presents.

Blood Film Examination

Detection and identification of the organisms are performed by examination of Giemsa-stained thick and thin blood films. Examination of thick films is the "gold standard" for detection of organisms because of the relatively large volume of blood (10 μl) that can be examined directly. Hyposmotic lysis of the erythrocytes during staining with Giemsa stain deposits the free parasites onto a background of red cell ghosts, white cell nuclei, and platelets. Approximately 20-fold more blood is examined in each high-power (1,000×) field examined than in a thin film. Although substantial training and practice are required to develop proficiency at distinguishing the free parasites from platelets, leukocyte granules, and other debris present in thick films, thick-film examination is substantially more sensitive than thin-film examination. Early in infection, or following relapse or partial treatment, patients may be symptomatic with parasitemias low enough that they require thick smear examination for detection. While it is often possible to identify parasites to the species level in thick films, thin films are the gold standard for species identification. Although fewer parasites are present in thin films, methanol fixation prior to staining preserves erythrocyte morphology, allowing evaluation of infected erythrocyte size and the position of organisms within the erythrocytes. In difficult cases, therefore, examination of thin films may be necessary to make a specific identification. Morphology is best preserved in thin areas of the smear near the feathered edge; scanning of the thicker areas of the slide may facilitate detection of rare gametocytes or schizonts but must not replace careful examination of the thick film. Smears should be examined at length under oil immersion; a negative report should not be rendered until 200 oil immersion fields of a thick film have been examined. A single set of negative smears does not

exclude malaria. Additional specimens should be examined at 12-h intervals for the subsequent 36 h.

The morphology of the four species of *Plasmodium* that commonly infect humans in thin films is reviewed in the literature (25) and is demonstrated in Fig. 2 to 6 and summarized in Table 2. The appearance of *P. falciparum* in thick films is demonstrated in Fig. 7. When abundant organisms encompassing several life cycle stages are present for examination, species identification is not difficult. A high parasitemia consisting only of ring forms suggests *P. falciparum*, even if, as is commonly the case, no gametocytes are found. In samples containing only rare early trophozoites, a species identification may not be possible. Ring morphology is quite variable; early trophozoites of *P. falciparum* may mimic early "band" forms of *P. malariae* or early ameboid trophozoites of *P. vivax*. It is important not to exclude *P. falciparum* solely on the basis

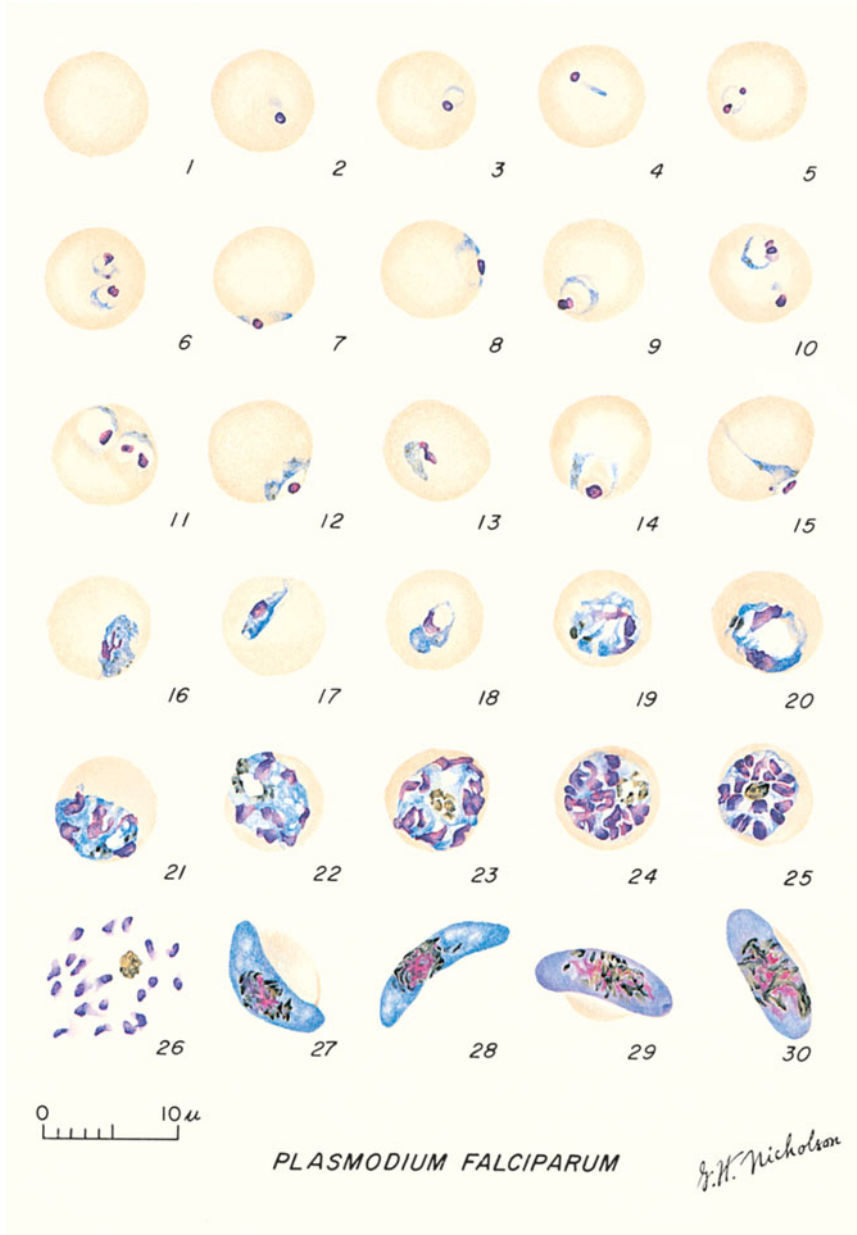


FIGURE 2 *P. falciparum:* morphology of successive developmental stages in Giemsa-stained thin blood smears. The cytoplasm of the rings is normally paler than shown in this figure: color intensity has been increased for purposes of illustration. Rings and gametocytes are found in peripheral blood smears; other stages occur in infected erythrocytes but are sequestered in the capillaries and venules of internal organs and are rarely found in the periphery. Drawings: 1, normal erythrocyte; 2 to 11, young trophozoites (rings); 12 to 15, growing trophozoites; 16 to 18, mature trophozoites; 19 to 22, immature schizonts; 23 to 26, nearly mature and mature schizonts; 27 and 28, mature macrogametocytes: 29 and 30, mature microgametocytes. (Reproduced from reference 13a.)

of ring morphology. Finally, the possibility of mixed infections should be borne in mind; examination of the smear should not be ended prematurely simply because one species of *Plasmodium* has been identified. *P. knowlesi* infections in humans are not morphologically distinguishable from *P. malariae* infections; this difficulty is not serious, however, as *P. knowlesi* is sensitive to chloroquine and does not cause severe malaria in humans.

Although many North American clinicians treat all malaria patients with regimens designed to cover drug-resistant *P. falciparum*, species-specific identification of *Plasmodium* spp. is clinically important for several reasons. *P. falciparum* should be identified because it is both more clinically aggressive and more likely to be multiply drug resistant than the other species. *P. vivax* and *P. ovale* should be identified because radical cure of these infections requires supplemental treatment with primaquine to eliminate dormant hypnozoites in the liver. Primaquine may cause hemolytic anemia in some patients with G6PD deficiency; correct species identification prevents its unnecessary use in *P. falciparum* or *P. malariae* infection.

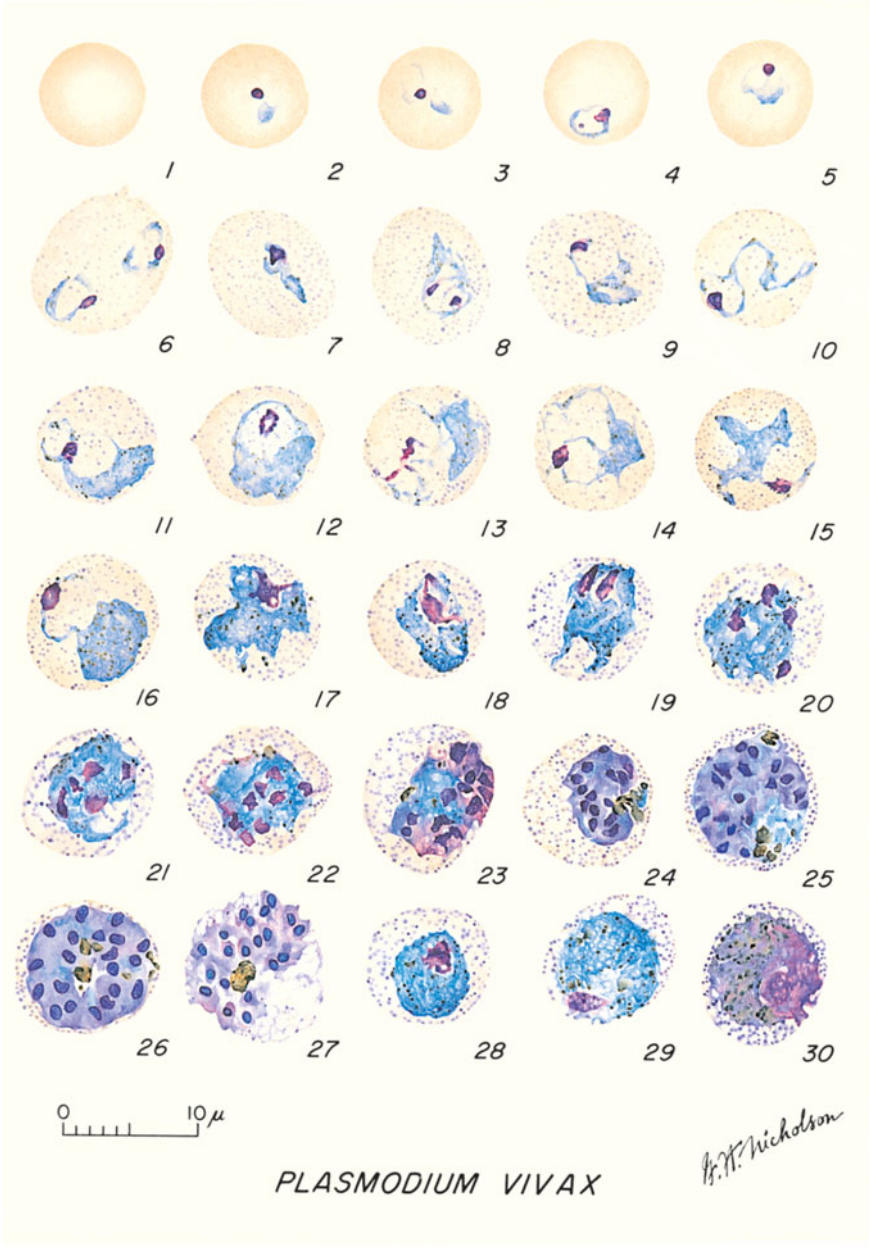


FIGURE 3 *P. vivax:* morphology of successive developmental stages in Giemsa-stained thin blood smears. The cytoplasm of the developing trophozoites and immature schizonts is normally paler than shown in this figure: color intensity has been increased for purposes of illustration. Drawings: 1, normal erythrocyte; 2 to 5, young trophozoites (rings); 6 to 16, growing trophozoites; 17 and 18, mature trophozoites; 19 to 21, young immature schizonts; 22 and 23, older immature schizonts; 24 to 27, nearly mature and mature schizonts; 28 and 29, nearly mature and mature macrogametocytes; 30, mature microgametocyte. (Reproduced from reference 13a.)

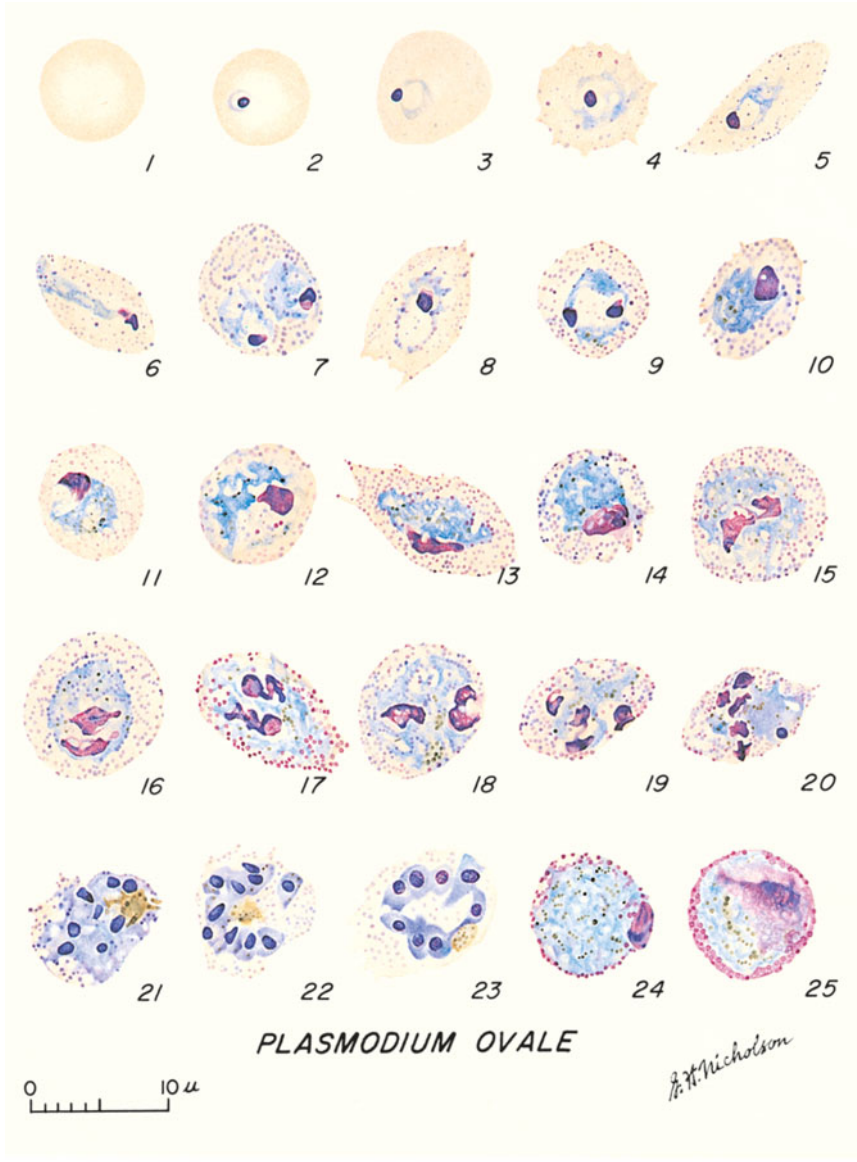


FIGURE 4 *P. ovale:* morphology of successive developmental stages in Giemsa-stained thin blood smears. The Schüffner's dots (James' stippling) are normally larger than shown here. Drawings: 1, normal erythrocyte; 2 to 5, young trophozoites (rings); 6 to 12, growing trophozoites; 13 to 15, mature trophozoites; 16 to 22, immature schizonts; 23, mature schizont; 24, mature macrogametocyte; 25, mature microgametocyte. (Reproduced from reference 13a.)

Alternative Diagnostic Methods

A number of new approaches to detection of malaria parasites have been described, including staining with acridine orange (AO) (63), PCR (4, 20), and antigen detection (14, 68). These and other alternative approaches have been recently reviewed (51) and are summarized in Table 3.

Thin or thick blood films can be stained with AO in place of Giemsa stain. AO is a fluorescent dye that binds to DNA; since mature erythrocytes do not contain DNA, the brightly staining parasite nuclei stand out clearly against a background of uninfected erythrocytes. Nonetheless, particularly in thick films stained with AO, it may be difficult to detect parasite nuclei in the presence of leukocyte nuclei and erythrocytes containing Howell-Jolly bodies. In addition, since it may be difficult to evaluate erythrocyte morphology, species identification can be difficult. The method can be performed with a fluorescence microscope or a standard microscope with an excitation filter (470 to 490 nm) mounted in the transmitted light path and a separate filter (590 nm) mounted in the ocular. A number of comparisons of AO staining to standard malaria microscopy have been recently reviewed (40); sensitivity and specificity were adequate in most studies. The most frequently cited advantage of AO staining was the speed with which slides could be prepared and read. An alternative version of the AO technique, designated Quantitative Buffy Coat, is available, in which whole blood is stained with AO and spun in a microcentrifuge tube; the resulting buffy coat is examined directly within the microcentrifuge tube by using a

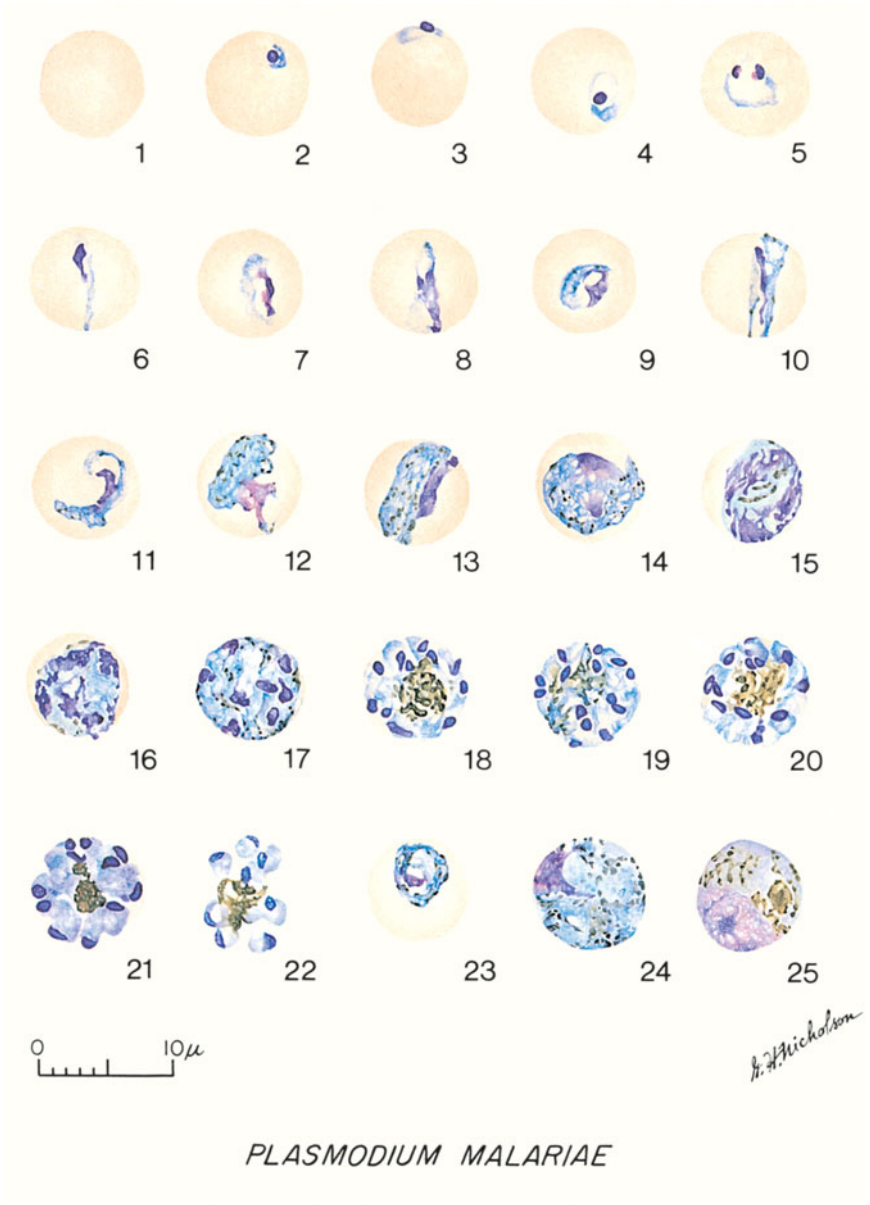


FIGURE 5 *P. malariae:* morphology of successive developmental stages in Giemsa-stained thin blood smears. Drawings: 1, normal erythrocyte; 2 to 5, young trophozoites (rings); 6 to 11, growing trophozoites; 12 and 13, nearly mature and mature trophozoites; 14 to 20, immature schizonts; 21 and 22, mature schizonts; 23, developing gametocyte; 24, mature macrogametocyte; 25, mature microgametocyte. (Reproduced from reference 13a.)

fluorescence microscope fitted with a specialized long-focal-length objective.

The immunochromatographic rapid dipstick antigen detection assays are potentially very useful, both in areas where malaria is endemic and trained laboratory personnel are scarce and in the developed world, where laboratory personnel may be inexperienced in malaria diagnosis. Rapid immunochromatographic methods for malaria diagnosis have been developed based on several *Plasmodium* antigens. Histidine-rich protein 2 (HRP-2) is a *P. falciparum* protein with roles in heme polymerization and actin binding and is localized in the infected erythrocyte cytoplasm. Dipsticks for diagnosis of *P. falciparum* based on antigen detection of HRP-2 have been widely tested and found to have sensitivity and specificity close to that of expert microscopy (6, 44, 77, 78, 86) and probably superior to that of routine microscopy. These HRP-2-based tests are not appropriate for monitoring therapy, as they may remain positive for up to 28 days after successful therapy (36, 68). HRP-2 is unique to *P. falciparum;* immunochromatographic tests based on HRP-2 cannot detect infection due to the other *Plasmodium* spp. Aldolase is an enzyme within the glycolytic pathway and is found in

TABLE 2 Comparative morphology of *Plasmodium* spp. in Giemsa-stained thin smears

Characteristic	*P. falciparum*	*P. vivax*	*P. ovale*	*P. malariae*
Size and shape of infected erythrocytes	Normal size and shape	Enlarged up to twofold; may be oval	Normal to enlarged, frequently oval, may be fimbriated	Small to normal size, normal shape
Stippling (best seen with Giemsa stain pH 7.0–7.2)	Occasional Maurer's dots, less numerous than Schüffner's	Schüffner's dots usually present, except in rings	James' stippling, darker than Schüffner's present in all stages, including rings	Ziemann's dots, rarely seen; requires deliberate overstaining
Stages seen in peripheral blood	Rings and gametocytes	All	All	All
Multiply infected erythrocytes	Common	Occasional	Occasional	Rare
Early trophozoites	Delicate ring, frequently with two small chromatin dots. Often at edge of erythrocyte (appliqué form)	Ring up to 1/3 diameter of erythrocyte; larger chromatin dot than *P. falciparum*	Similar to *P. vivax*	Smaller than *P. vivax;* otherwise similar
Mature trophozoites	Not seen in peripheral blood	Ameboid shape, fine golden-brown pigment	Similar to *P. vivax* except less ameboid, pigment darker brown	Compact cytoplasm, oval, round, or band-shaped, dark brown pigment
Schizonts	Not seen in peripheral blood	12–24 merozoites	8–12 merozoites	6–12 merozoites often radially arranged around central pigment ("daisy-head" schizont)
Gametocytes	Crescent or "banana" shaped	Round to slightly oval	Round to slightly oval	Round to slightly oval
Most characteristic findings	Absence of mature trophozoites and schizonts; normal size of infected erythrocytes; multiple infections; appliqué forms; "banana"-shaped gametocytes	Enlarged infected erythrocytes; Schüffner's dots frequently present; ameboid trophozoite; 12–24 merozoites in each schizont	Normal to enlarged, oval or fimbriated infected erythrocytes; James' stippling may be seen in rings; schizonts with 8–12 merozoites	Normal size of infected erythrocytes; no stippling; "band" trophozoite; "daisy head" schizont with 6–12 merozoites

all species of *Plasmodium*. Monoclonal antibodies raised against *Plasmodium* aldolase recognize aldolase from all *Plasmodium* species which infect humans. Recently, dipsticks combining antibodies against HRP-2 and aldolase which allow detection of *P. falciparum* and non-*P. falciparum* infections have been devised (19, 78). An alternative dipstick test uses monoclonal antibodies to capture parasite lactate dehydrogenase (LDH) (61). *P. falciparum* species-specific and *Plasmodium* genus-specific anti-LDH antibodies distinguish between *P. falciparum* and non-*P. falciparum* infections. Preliminary studies suggest that the LDH-based assay may have useful sensitivity and specificity (24, 37, 56). Both HRP-2- and LDH-based assays lose sensitivity when parasitemias are low, i.e., <50 to 100/mm^3, and no dipstick assay is yet licensed for clinical diagnostic use in the United States. While such assays will not soon replace microscopy, they may shortly become an important adjunct in malaria diagnosis. It should be pointed out that automated hematology instrumentation does not reliably detect malaria parasites. Finally, it is important that negative results obtained by the alternative diagnostics methods be confirmed by repeated thick-film examination. As of 2005, thick-film examination remains the gold standard for malaria diagnosis.

Serology

Indirect fluorescent-antibody tests using antigens prepared from the four species of *Plasmodium* that infect humans have been described (74). However, serologic testing is not a reliable method for identifying the infecting species, because of cross-reactions among the four species, and is not useful in clinical diagnosis of malaria because antibodies may be absent in an acute attack and because their presence may reflect past rather than current infection. It has a role in the investigation of transfusion malaria, where it may be used to determine which of a number of potential donors may have been the source of a transfusion-associated case of malaria, and in epidemiological studies, to determine the prevalence of exposure to malaria in a population. Assistance in serological evaluation of suspected transfusion malaria can be obtained from the Malaria Epidemiology Branch, Division of Parasitic Diseases, Centers for Disease Control and Prevention, Atlanta, Ga. (phone: 770-488-7788).

Interpretation and Reporting of Results

A positive finding of malaria parasites is a critical result, which must be reported immediately to the clinician. The report should include the species identification, if possible. When identification to this level is not possible, an explicit

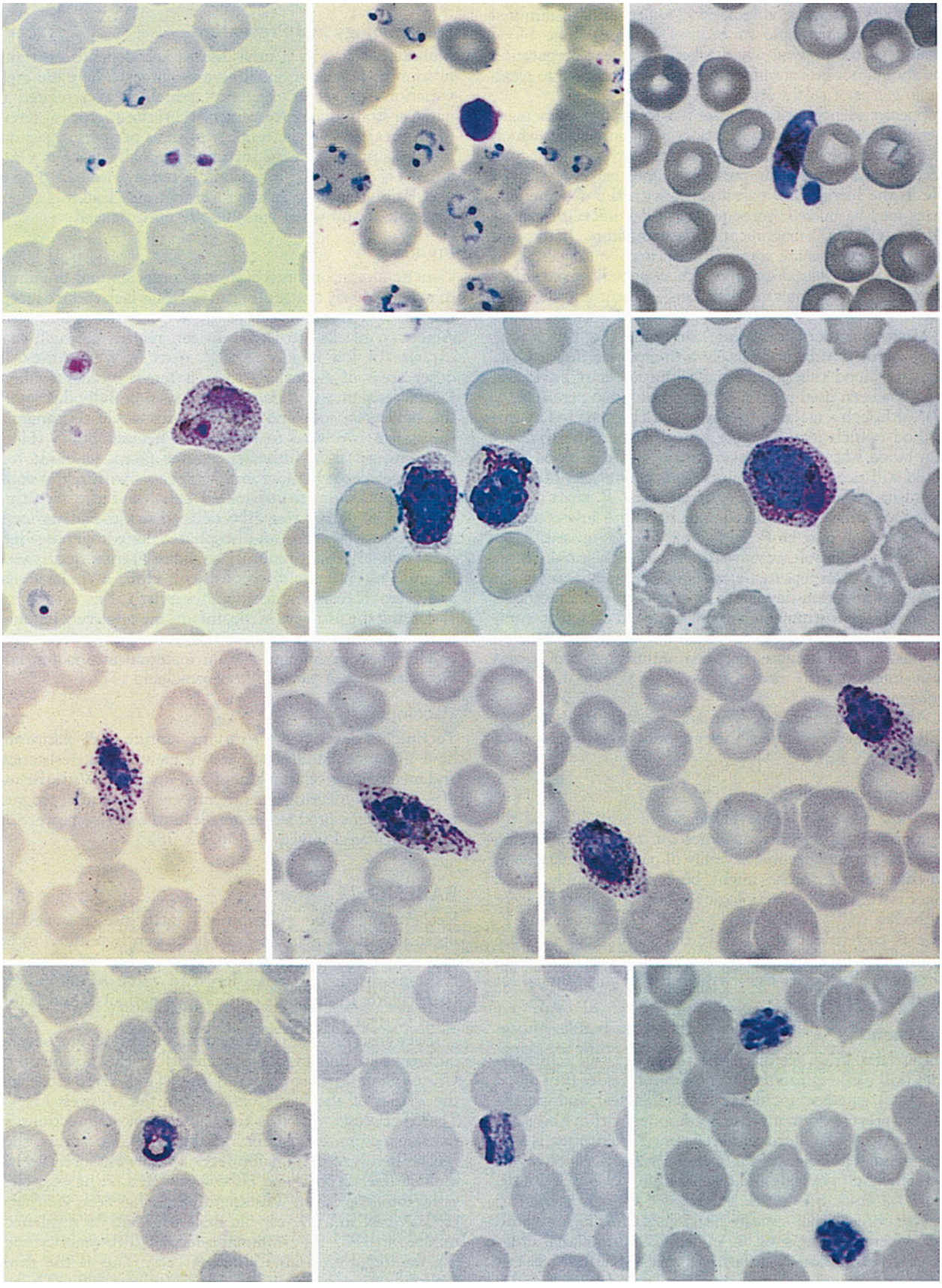


FIGURE 6 Photomicrographs of *Plasmodium* species in thin blood films (Giemsa stain; magnification, ×1,000). First row: *P. falciparum.* (Left) Two ring trophozoites. Note also two platelets overlying erythrocytes. (Center) Abundant rings in erythrocytes of a patient with *P. falciparum* hyperparasitemia. (Right) Banana-shaped gametocyte. Second row: *P. vivax.* (Left) Developing trophozoite in enlarged erythrocyte with Schüffner's dots; ring trophozoite in lower left corner. (Center) Mature and immature schizonts, left to right. (Right) Macrogametocyte. Third row: *P. ovale.* (Left) Developing trophozoite in enlarged, elongated erythrocyte with Schüffner's dots. (Center) Mature schizont. (Right) Schizont in upper right, macrogametocyte in lower left. Fourth row: (Left) Trophozoite in normal-size erythrocyte. (Center) Band trophozoite. (Right) Mature schizonts; note peripheral placement of nuclei around central hemozoin pigment granule. (Photomicrographs from Lawrence Ash; used with permission.)

statement that *P. falciparum* cannot be excluded is advisable. Because patients with high parasitemias (>3 to 5%) require intensive therapy, quantification of parasitemia is useful. This is typically performed after species identification and may be variously expressed as percent parasitemia (parasites/100 erythrocytes), number of parasites per 200 leukocytes, or number of parasites/mm^3. At high parasitemias, the direct counting of parasites and erythrocytes in a thin film will provide an accurate parasitemia. In low parasitemias and, of course, in thick smears, it is necessary to count the number of parasites/200 leukocytes and to calculate the percent parasitemia or the parasite density based on the patient's complete blood count. A negative report may be accompanied by a reminder that a single negative set of smears does not exclude the diagnosis of malaria. Serial malaria smears may be performed to monitor therapy. Parasitemia normally resolves within 2 to 3 days following initiation of treatment with a drug to which the patient's strain is susceptible. Continued parasitemia at day 7 or failure of the parasitemia to decrease by 75% within the first 48 h following treatment is an indication of drug resistance. However, gametocytes may continue to circulate for up to 2 weeks after successful cure, and their presence is not an indication that treatment has failed.

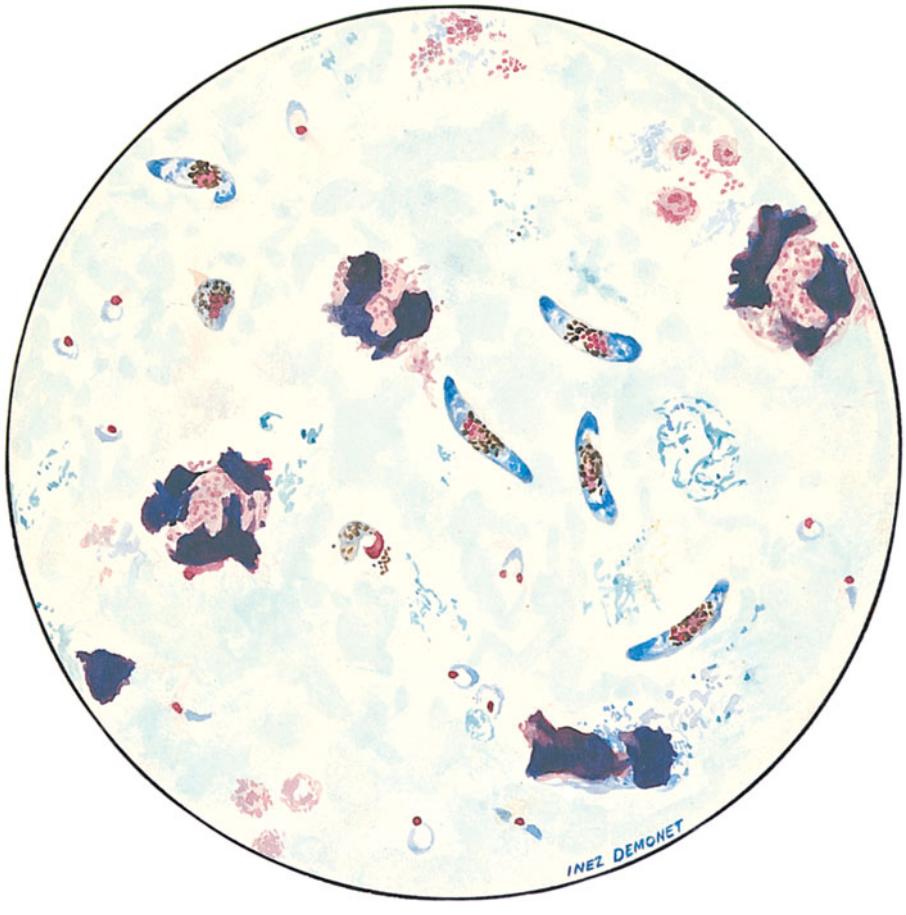


FIGURE 7 *P. falciparum:* morphology in Giemsa-stained thick blood smears. There are multiple small trophozoites (rings) and banana (crescent)-shaped gametocytes. Two gametocytes with variant shapes are present at 10 o'clock. Outside areas of endemicity, parasites on a thick film are normally fewer than in this figure. (Reproduced from reference 85.)

TABLE 3 Alternative methods for diagnosis of malaria

Principle	Method	Comments	Advantages	Disadvantages	Reference(s)
Giemsa stain	Thick film	Gold standard for detection	Screens large volume of blood	Species identification sometimes difficult	16
	Thin film	Traditional method for species identification	Preserves morphology of parasite in erythrocyte	Less sensitive than thick film examination	25
Fluorescent DNA/RNA stains	Acridine orange-stained films	Requires fluorescent microscope	Good sensitivity	May be difficult to read	27, 47
	Centrifugation/AO (QBC)[a]	Requires fluorescent microscope	Rapid	Species identification difficult	46, 63, 72
	Flow cytometry		Automated	Poor sensitivity	80
Hemozoin detection	Dark-field microscopy	Abundant pigment in macrophages suggests poor prognosis		Poor sensitivity	38
	Automated blood analyzers	Detection in unsuspected cases	Automated	Poor sensitivity	31
Nucleic acid detection	DNA/RNA hybridization	First nucleic acid-based method		Poor sensitivity, technically difficult	5, 23
	PCR	Care required to prevent contamination/ false-positive results	Sensitivity comparable to or better than thick film; species identification; best approach to detect mixed infections	Expensive, lengthy procedure	20, 70, 76, 83
Antigen detection	Detection of HRP-2 (Parasight-F, ICT Malaria Pf, PATH Falciparum Malaria IC Test)	Immunochromatographic dipstick, *P. falciparum* only	Convenient, rapid, minimal training required	Expensive, cannot be used to monitor therapy, detects only *P. falciparum*	6, 44, 77, 78, 86
	Detection of HRP-2 and aldolase (ICT Pf/Pv, Binax Now ICT Malaria Test)	Immunochromatographic dipstick, *P. falciparum* and non-*P. falciparum*	Convenient, rapid, minimal training required	Expensive, cannot be used to monitor therapy	19, 78, 87
	Detection of parasite LDH (OptiMAL)	Immunochromatographic dipstick, *P. falciparum* and non-*P. falciparum*	Convenient, rapid, minimal training required	Expensive	24, 37, 56, 61

[a] QBC, Quantitative Buffy Coat techinique.

Special Considerations in Areas of Intense Malaria Transmission

For returning travelers or residents of areas of low to moderate malaria transmission, it is generally safe to conclude that a positive malaria smear confirms the diagnosis of clinical malaria. In areas of intense transmission, however, such as much of sub-Saharan Africa or some areas in Southeast Asia, diagnosis of clinical malaria on the basis of a positive peripheral smear is difficult. In such areas both nonmalarial fevers and asymptomatic parasitemia are common; indeed, the large majority of individuals may harbor *P. falciparum.* Therefore, fever due to nonmalarial causes may frequently coincide with parasitemia. At a population level it is possible to estimate the fraction of fevers attributable to malaria (29), but this does not help in individual diagnosis. Since high parasitemias are more likely to be associated with fever than low parasitemias, it is tempting to create definitions of clinical malaria based on fever and parasitemia above a specified cutoff. Unfortunately, in many areas of endemicity no cutoff is satisfactory. For example, in Northern Ghana the pretest probability that a child with fever has clinical malaria is 60%. Finding a positive malaria smear raises that probability only to 70%. To obtain a posttest probability of 80% that the fever is due to malaria requires a parasitemia threshold of 20,000 parasites/μl and leads to a sensitivity in the clinical definition of only 55% (64a). In such situations it may be entirely reasonable not to perform a malaria smear and to treat all fevers as malaria empirically. Whether or not it is

cost-effective to attempt to make a laboratory diagnosis of malaria in a given area of endemicity depends on a complex interaction between the local attributable fraction of fevers due to malaria, the cost and side effect profile of recommended first-line antimalarial agents, and the cost and accuracy of available diagnostic methods.

Treatment and Prevention

Prophylaxis and therapy of malaria have been recently reviewed (17, 18, 48). *P. falciparum* resistant to chloroquine is present in all areas of endemicity with the exception of Central America and the Caribbean. In addition, resistance to other drugs including sulfadoxine-pyramethamine (Fansidar) and mefloquine is present in many areas and is expanding rapidly. Distribution of drug resistance in *P. falciparum* as of June 2005 is summarized in Table 4. Current information on the distribution of drug-resistant *P. falciparum* can be obtained from the Centers for Disease Control and Prevention, Malaria Hotline, in Atlanta, Ga. (phone: 770-488-7788). In vitro susceptibility testing of *P. falciparum* has been described (64) but is not available for clinical use. The combinations of quinine or quinidine and doxycycline or of atovaquone and proguanil remain effective against most strains of *P. falciparum.* In Africa, the combination of an artemisinin derivative with a variety of antimalarials including lumefantrine, sulfadoxine-pyramethamine, or amodiaquine has shown promising results and is replacing chloroquine as the first-line therapy for outpatient malaria recommended by many African Ministries of Health. None of the artemisinin derivatives, however, is licensed in the United States. Therapy of chloroquine-resistant *P. falciparum* is a complex and rapidly evolving field, and consultation with an infectious diseases specialist is essential. Chloroquine-resistant *P. vivax* emerged in a few areas in the 1990s (3, 54, 60, 67) and has continued to spread (2). More-detailed information on specific antimalarial agents and on specialized in vitro susceptibility test methods can be found in chapters 150 and 152, respectively.

Drug and Vaccine Development

In the face of rapidly developing drug resistance, development of new antimalarial drugs and, ultimately, an effective malaria vaccine is a high priority. The sequencing of the *P. falciparum* genome (26) and subsequent characterization of the transcriptome and proteome (30) have helped identify a number of potential new drug targets. Even before completion of the *P. falciparum* genome sequence, data mining of the unassembled sequence data led to identification of inhibitors of an isoprenoid biosynthesis pathway with in vitro and in vivo activity against *Plasmodium* (39). Since completion of the genome sequence, analysis of metabolic pathways has led to identification of additional drug targets in several pathways including serine proteases involved in hemoglobin degradation in the food vacuole; protein, DNA, and fatty acid synthesis in the apicoplast; and electron transport in the mitochondrion. These and other advances in antimalarial drug development have been recently reviewed (65, 90).

There remain many difficult obstacles to overcome in malaria vaccine development. *P. falciparum* is a complex, multistage pathogen that uses a variety of strategies to evade the human immune response, including stage-specific antigen expression, antigenic variation, and widespread allelic diversity of immune targets. Nonetheless, two lines of evidence suggest that a malaria vaccine may be attainable. First, residents of areas of endemicity who do not succumb to malaria in early childhood develop a limited, clinical immunity that greatly reduces their risk of severe disease and death when they become infected. This immunity is largely mediated by antibodies directed against antigens expressed on the merozoite stage (66). Second, human volunteers immunized with radiation-attenuated sporozoites develop solid sterile immunity to sporozoite challenge (13). Immunity induced with irradiated sporozoites is apparently mediated by T-cell responses directed against infected hepatocytes (15). A number of candidate malaria vaccines have been tested in humans including synthetic peptide vaccines (55, 57, 79), recombinant viral particle vaccines (73), DNA vaccines (82), and heterologous prime-boost combinations of DNA and recombinant viral vaccines (52). Although these vaccines have induced immune responses and, in some cases, short-term, partial protection (1, 73), much work remains before an effective vaccine can be deployed.

BABESIA

Introduction

The genus *Babesia* includes approximately 100 species that are transmitted by ticks of the genus *Ixodes* and infect a variety of wild and domestic animals (35). The *Babesia* species are grouped loosely into small *Babesia* (trophozoites measuring 1.0 to 2.5 μm), including *B. microti* and *B. gibsoni,* and the large *Babesia* (trophozoites measuring 2.5 to 5.0 μm), including *B. bovis* and *B. canis.* This morphological division is generally supported by molecular phylogenetic analysis, except that *B. divergens,* which has small trophozoites, groups with the large babesias (35). In the United States, *B. microti* and several recently described, unnamed species, WA1 (62), CA1 (58), and MO1 (32), infect humans. In Europe, the bovine parasites *B. bovis* and *B. divergens* have been isolated from human

TABLE 4 Distribution of drug-resistant *P. falciparum*[a]

Region	Chloroquine resistance	Resistance to second line antimalarials
Caribbean, Central America	No	No
South America	Yes	Sulfadoxine-pyramethamine (Amazon Basin), mefloquine (Amazon Basin)
West Africa	Yes	Sulfadoxine-pyramethamine, mefloquine
East Africa	Yes	Sulfadoxine-pyramethamine
Central Asia	Yes	Sulfadoxine-pyramethamine
Southeast Asia	Yes	Sulfadoxine-pyramethamine, mefloquine, halofantrine, quinine

[a] Based on information from U.S. Centers for Disease Control and Prevention (http://www.cdc.gov/ncidod/emergplan/box23.htm) as of 11 August 2001 and updated with information from the World Health Organization Roll Back Malaria Department (http://www.who.int/malaria/resistance.html) as of 24 June 2005.

patients. These geographic ranges are not strict; recently, infection by *B. microti* has been reported from both Europe (33, 50) and Japan (84), and infection with a *B. divergens*-like organism was reported in the northwest United States (34).

Life Cycle

While the life cycle of *Babesia* is roughly similar to that of *Plasmodium,* there are a number of important differences (Fig. 8). First, no exoerythrocytic stage has been described for *Babesia;* sporozoites injected by the bite of an infected tick invade erythrocytes directly. Within the erythrocyte, the trophozoites reproduce by binary fission rather than schizogony. While gametocytes presumably exist, they have not been morphologically distinguished from trophozoites. As in *Plasmodium,* fertilization of gametes occurs in the insect intestine within the blood meal and the zygotes pass through the intestinal epithelium to the hemolymph. In *Plasmodium,* sporogony takes place in oocysts attached to the transluminal aspect of the intestine and sporozoites released from oocysts migrate to the salivary glands; in *Babesia,* fertilized gametes migrate through the intestinal wall, through the hemolymph, and on to the salivary glands, where they develop into sporoblasts. The final production of sporozoites from sporoblasts occurs only when the tick begins to take a blood meal; the resulting sporozoites are injected into the host during the final hours of attachment and feeding.

Epidemiology

Babesiosis is transmitted by the bite of infected *Ixodes scapularis* ticks, and outbreaks of human infection have been described in the Northeast, Midwest, and West Coast of the United States; in Europe; and, recently, in Japan (84). Relatively few cases of babesiosis have been reported in the United States: 10 to 12 cases per year in New York and Nantucket over the past 30 years (35). However, because babesiosis is a mild and self-limiting infection in most persons,

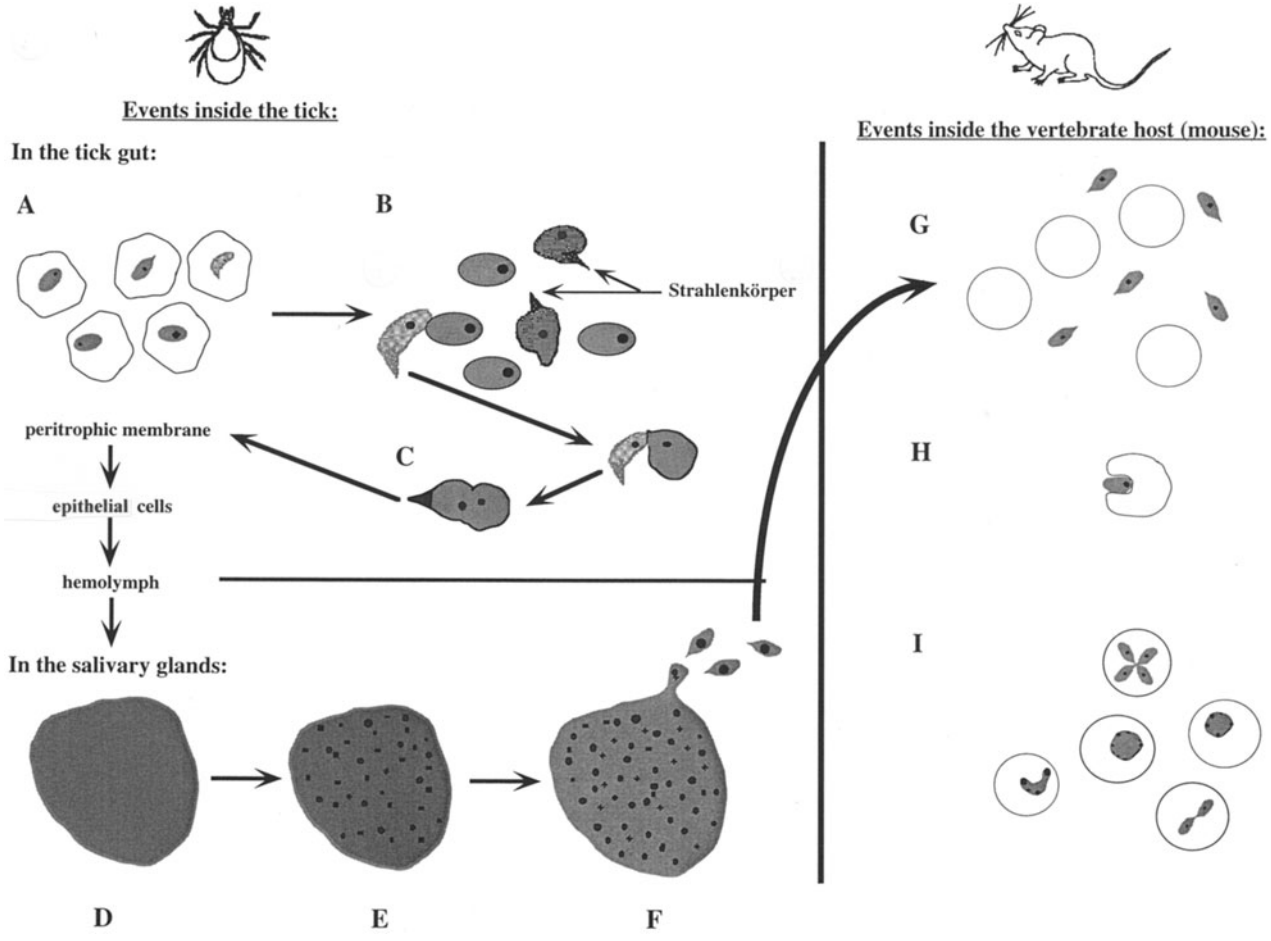


FIGURE 8 Life cycle of *Babesia.* Events in the tick begin with the parasites still visible in consumed erythrocytes. Some are beginning to develop Strahlenkörper forms (specialized apical organelles) (A). The released gametes begin to fuse (B). The zygote then goes on to infect and move through other tissues within the tick (C) to the salivary glands. Once a parasite has infected the salivary acini, a multinucleate but undifferentiated sporoblast is formed (D). After the tick begins to feed, the specialized organelles of the future sporozoites form (E). Finally, mature sporozoites bud off the sporoblast (F). As the tick feeds on its vertebrate host, these sporozoites are inoculated into the host (G). Sporozoites (or merozoites) contact a host erythrocyte and begin the process of infection by invagination (H). The parasites become trophozoites and can divide by binary fission within the host erythrocyte, creating the various ring forms and crosses seen on stained blood smears (I). Illustrations are not to scale. (Reproduced with permission from reference 35).

the number of unreported infections may be much higher. Indeed, serological surveys of blood donors have shown a seroprevalence of 3 to 8% for antibodies to *B. microti* (35). With the exception of a few cases attributed to newly described *Babesia* spp. (32, 34, 58, 62), babesiosis in the United States results from infection with *B. microti.* In Europe, only about 30 cases of babesiosis have been reported, principally infections of splenectomized individuals with *B. divergens;* however, these infections are clinically serious, with a mortality rate of 42% (28). A handful of cases of transfusion-associated babesiosis, usually involving transmission of *B. microti* from an asymptomatic donor, have been reported from the United States.

The host ranges of *B. microti* and *B. divergens* are wide, and virtually any mammal that serves as a host for *I. scapularis* ticks can be a reservoir of infection. *Babesia* spp. share both vectors and animal reservoirs, i.e., *Ixodes* ticks and white-footed mice, with several other tick-borne pathogens, including the agents of Lyme disease and human granulocytic ehrlichosis. Not surprisingly, there have been reports of coinfection with *B. microti* and *Borrelia burgdorferi,* and approximately 10% of Lyme disease patients in areas where babesias are endemic may be infected with *B. microti.* Lyme disease patients coinfected with *B. microti* experienced more-severe symptoms than those infected with either agent alone (41).

Clinical Disease

There is a wide range of clinical presentations of babesiosis, from mild or asymptomatic infection through a fulminant illness clinically similar to malaria and characterized by high fever, myalgias, malaise, fatigue, hepatosplenomegaly, and anemia. In general, infection with *B. microti* in the United States tends to occur in nonsplenectomized individuals and to be relatively mild. Infection with the more recently described babesias from the United States and with *B. divergens* in Europe tends to occur in splenectomized or immunocompromised individuals and to be clinically more severe. Overall, mortality among clinically apparent cases of *B. microti* infection in the United States is 5%, while that in *B. divergens* infection in Europe is 40%. In both the United States and Europe, risk factors for severe disease include increasing age, splenectomy, and immunocompromise.

Diagnosis

The diagnosis of babesiosis should be considered for a patient with the appropriate clinical symptoms and a history of travel to areas of endemicity, exposure to ticks, or recent blood transfusion. Examination of Giemsa-stained thin blood smears is the most direct approach to diagnosis. The appearance of *Babesia* in thin films is shown in Fig. 9. Although the trophozoites of *Babesia* can be confused with

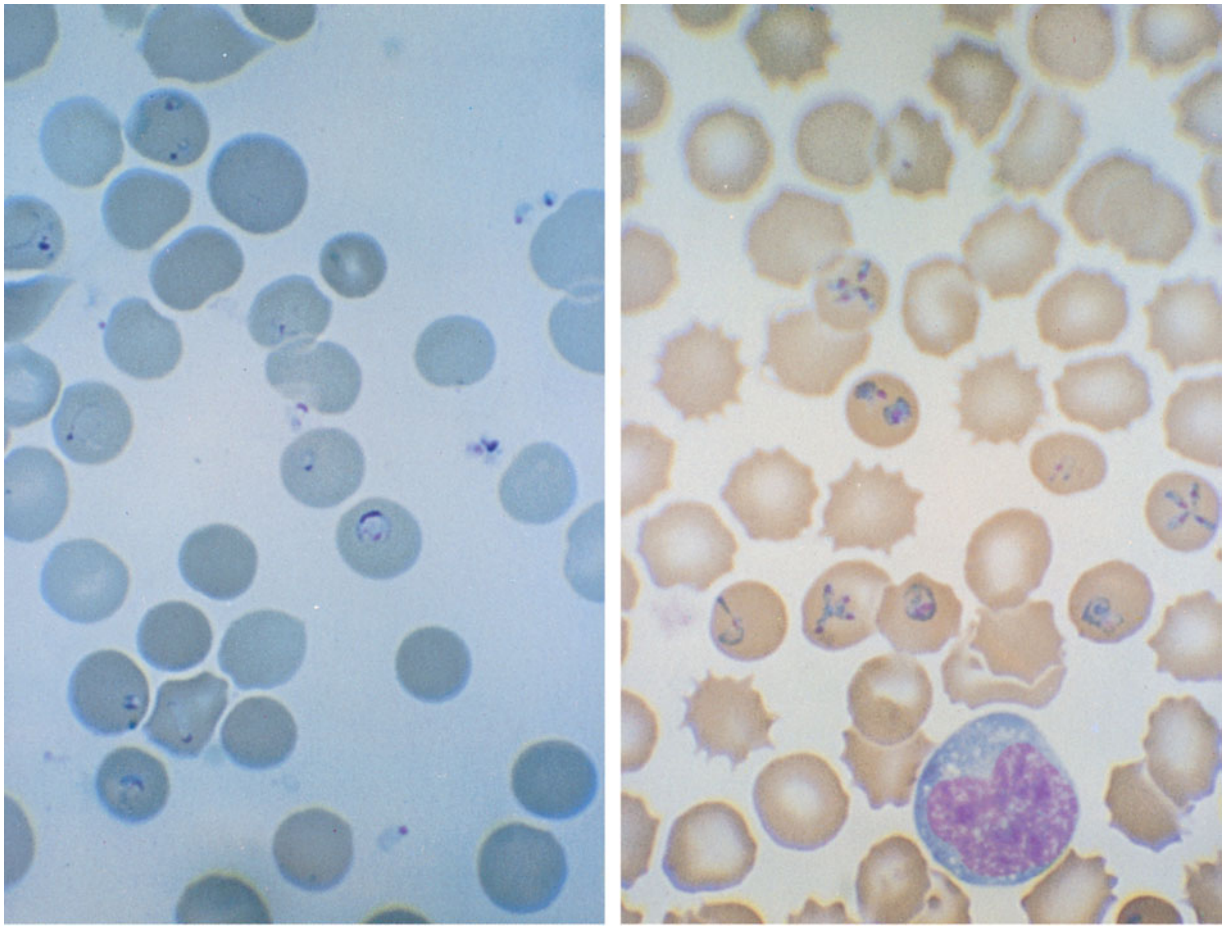


FIGURE 9 *Babesia* spp. in thin blood films. (Left) *B. microti* in human blood: parasites in erythrocytes and extracellular parasites; (right) *Babesia* sp. in classic Maltese cross (tetrad) configuration, a form diagnostic for babesiosis but rare in slides from humans infected with *B. microti.*

Plasmodium rings, particularly the small ring trophozoites of *P. falciparum, Babesia* can be distinguished from *P. falciparum* by several criteria. *Babesia* trophozoites are quite variable in size (1 to 5 μm), and the smallest are smaller than *P. falciparum* rings. Extracellular trophozoites and multiply infected erythrocytes are more common in *Babesia*. The cytoplasm of the larger *Babesia* trophozoites frequently contains a clear vacuole, which is rare or absent in *P. falciparum*. Finally, diagnostic tetrads, the Maltese cross, though rare in clinical specimens, may be present in *Babesia*. Parasitemias may be very low, particularly in chronic infections in nonsplenectomized patients; in such cases diagnosis may require serologic testing (12), hamster inoculation (8), or PCR amplification (59).

Treatment and Prevention

Mild cases of infection with *B. microti* usually resolve spontaneously. In more serious cases treatment with clindamycin and quinine or with atovaquone and azithromycin is standard. In very severe cases of *B. microti* infection and in *B. divergens* infection in splenectomized or immunosuppressed patients, antimicrobial therapy may be supplemented with exchange transfusion. Appropriate personal protective measures including use of long pants, long-sleeved shirts, and insect repellent may reduce the risk of infection when outdoors in areas of endemicity.

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Leishmania and *Trypanosoma*

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Leishmania spp. and *Trypanosoma* spp. are protozoa belonging to the family *Trypanosomatidae.* Leishmaniasis is principally a zoonosis, and the organisms are obligate intracellular parasites transmitted to humans by bites from an infected female sand fly. For *Leishmania* in the Old World, there is only one subgenus, *Leishmania;* however, in the New World, the genus has been split into subgenera *(Leishmania* and *Viannia)* according to the development of the organism in the digestive tract (peripylarian or suprapylarian) of the sand fly (30). Depending on the geographic area, many different species can infect humans, producing a variety of diseases (cutaneous, diffuse cutaneous, mucocutaneous, and visceral diseases) (Table 1). The spectrum of clinical presentation is dependent on the host's cell-mediated immune response (68).

Trypanosoma spp. are hemoflagellate protozoa that live in the blood and tissue of the human host. American trypanosomiasis (Chagas' disease) is produced by *Trypanosoma cruzi,* which belongs to the family *Schizotrypanum* and is confined to the American continent. *Trypanosoma rangeli* belongs to the family *Tejaria,* produces an asymptomatic infection, and is also present only on the American continent. African trypanosomiasis (sleeping sickness) is caused by *T. brucei gambiense* and *T. brucei rhodesiense* species belonging to the family *Trypanozoon* and is confined to the central belt of Africa. African trypanosomes and *T. rangeli* are transmitted directly into the bite wound by salivary secretions from the insect vector, whereas *T. cruzi* is transmitted through contamination of the bite wound with the feces from the reduviid bug (Table 2). The first documented case of human trypanosomiasis caused by *T. evansi* was detected in India (78).

LEISHMANIA SPP.

Recent estimates suggest that there are approximately 350 million people at risk of acquiring leishmaniasis, with 112 million currently infected. More than 400,000 new cases are reported annually (33). New species of *Leishmania,* particularly in the New World, are being detected frequently. The taxonomy of leishmaniasis is controversial and in a state of dynamic flux. Species differentiation is currently based on molecular techniques rather than geographical distribution and clinical presentation (18, 24, 51).

Life Cycle and Morphology

The parasite has two distinct phases in its life cycle (amastigote and promastigote) (Fig. 1). The amastigote stage (Leishman-Donovan body) is found in reticuloendothelial cells of the mammalian host. The amastigote form is small, is round or oval, measures 3 to 5 μm, and contains a large nucleus and small kinetoplast (Fig. 1 and 2). This stage undergoes multiplication within the reticuloendothelial cells of the host.

Upon ingestion during a blood meal by the insect vector (sand fly), the amastigote transforms into the promastigote stage (Fig. 3). Promastigotes multiply in the gut of the insect, transform to metacyclic promastigotes, and migrate to the hypostome of the sand fly, where they are released when the next blood meal is taken. The complete life cycle in the sand fly is from 4 to 18 days. Upon inoculation into the bite site, the promastigote changes to the amastigote form after being engulfed by tissue macrophages. This form change helps to defeat the host's immune response. Changes in the parasite's surface molecules play an important role in macrophage attachment and evading the host's immune response, including manipulating the macrophage's signaling pathways (58, 68, 77).

The life cycles of *Leishmania* organisms are similar for cutaneous, mucocutaneous, and visceral leishmaniasis, except that infected reticuloendothelial cells can be found throughout the body in visceral leishmaniasis.

Epidemiology

All adult female sand flies transmitting leishmaniasis belong to the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World. There are >30 species of sand flies that can transmit leishmaniasis. The disease is considered primarily a zoonosis, with natural reservoirs including rodents, opossums, anteaters, sloths, and dogs. In certain areas of the world where the disease is endemic, the infection can be transmitted by a human-vector-human cycle. The infection may also be transmitted by direct contact with an infected lesion or mechanically through bites by stable or dog flies. Greater than 90% of cutaneous leishmaniasis cases occur in Afghanistan, Algeria, Brazil, Iran, Iraq, Peru, Saudi Arabia, and Syria. There has been an increase in the number of cases among military personnel deployed in Afghanistan, Iraq, and

TABLE 1 Features of human leishmanial infections

Species	Disease type[a]	Recommended specimen	Geographic distribution
L. (L.) donovani	VL MCL, CL, DL	Bone marrow, spleen Skin or mucosal macrophages	Africa and Asia
L. (L.) infantum [*L. (L.) chagasi* (31)]	VL	Bone marrow, spleen	Africa, Europe, Mediterranean area, Southwest Asia, Central and South America
L. tropica	CL	Skin macrophages	Afghanistan, India, Turkey, former USSR
L. major	CL	Skin macrophages	Middle East, Afghanistan, Africa, former USSR
L. aethiopica	CL, CDL, MCL	Skin or mucosal macrophages	Ethiopia, Kenya, Yemen, former USSR
L. (L.) mexicana	CL, DCL	Skin macrophages	Texas, Belize, Guatemala, Mexico
L. (V.) brasiliensis	CL, MCL	Skin or mucosal macrophages	Central and South America
L. (V.) peruviana	CL	Skin macrophages	Panama, Colombia, Costa Rica
L. (L.) garnhami	CL	Skin macrophages	Venezuela
L. (V.) colombiensis	CL	Skin macrophages	Colombia, Panama
L. (L.) venezuelensis	CL	Skin macrophages	Venezuela
L. (V.) lainsoni, L. (V.) shawii	CL	Skin macrophages	Brazil
L. (L.) amazonensis	CL, DCL	Skin macrophages	Brazil, Venezuela
L. (V.) naiffi	CL	Skin macrophages	Brazil, Caribbean islands
L. (L.) pifanoi	CL, DCL		Brazil, Venezuela

[a] CL, cutaneous leishmaniasis; DCL, diffuse cutaneous leishmaniasis; DL, dermal leishmanoid; MCL, mucocutaneous leishmaniasis; VL, visceral leishmaniasis.

Kuwait (19). Most of the diagnosed cases of mucocutaneous leishmaniasis are from Bolivia, Brazil, and Peru.

Visceral leishmaniasis may exist as an endemic, epidemic, and sporadic disease. The disease is a zoonosis except in India, where kala azar is an anthroponosis. The nature reservoirs are wild *Canidae* and various rodents for *L. donovani;* dogs, other *Canidae,* and rats for *L. infantum;* and *Canidae* and cats for *L. infantum (L. chagasi)* in the Americas. Individuals with post-kala azar dermal leishmaniasis may be very important reservoirs for maintaining the infection during interendemic cycles. More than 90% of the cases of visceral leishmaniasis are found in Bangladesh, Brazil, India, Nepal, and Sudan.

Clinical Disease

Depending on the species involved, infection with *Leishmania* spp. can result in cutaneous, diffuse cutaneous, mucocutaneous, or visceral disease (Table 1) (48). A large number of disease variations have been described, which makes classical disease categories confusing (18). In areas of endemicity, leishmania coinfection with human immunodeficiency virus (HIV)-positive patients is common. If coinfected patients remain severely immunocompromised, approximately one-quarter will die within the first month of being diagnosed with leishmaniasis. The leishmanial infection will manifest itself like an opportunistic infection, and parasites will be detected in atypical sites (42).

The first sign of cutaneous disease is the appearance of a firm, painless papule at or near the insect bite site. The incubation period may be as short as 2 weeks *(L. major)* or as long as several months to 3 years *(L. tropica* and *L. aethiopica)*. Papules may be intensely pruritic and will grow to 2 cm or more in diameter. Lesions may progress from a simple papule or erythematous macule to a nodule and ulcerate within days to weeks. In simple cutaneous leishmaniasis, the infection remains localized at the insect bite site where a definite self-limiting granulomatous response develops. Lesions have been mistaken for basal cell carcinoma, tropical pyoderma, sporotrichosis, or cutaneous mycobacterial infections.

Mucocutaneous leishmaniasis is produced most often by the *L. braziliensis* complex. The primary lesions are similar to those found in other infections of cutaneous leishmaniasis. Untreated primary lesions may develop into the mucocutaneous form in up to 80% of the cases. Metastatic spread to the nasal or oral mucosa may occur in the presence of the active

TABLE 2 Characteristics of trypanosomiasis

Characteristic	*T. brucei rhodesiense*	*T. brucei gambiense*	*T. cruzi*	*T. rangeli*
Vector	Tsetse fly (*Glossina*)	Tsetse fly *(Glossina)*	Reduviid bug *(Panstrongylus, Rhodnius, Triatoma)*	Reduviid bug *(Rhodnius)*
Primary reservoir	Animal	Humans	Animals	Animals
Illness	Acute, <9 mo	Chronic, months to years	Acute, chronic	Asymptomatic
Epidemiology	Anthropozoonosis[a]	Anthroponosis[b]	Anthropozoonosis	Anthropozoonosis
Diagnostic stage	Trypomastigote	Trypomastigote	Trypomastigote, amastigote	Trypomastigote
Recommended specimens	Blood, CSF, chancre, lymph node aspirate	Blood, CSF, chancre, lymph node aspirate	Blood, lymph node, chagoma aspirate	Blood

[a] Anthropozoonosis: transmission involving a human-animal-human cycle.
[b] Anthroponosis: transmission involving a human-human cycle.

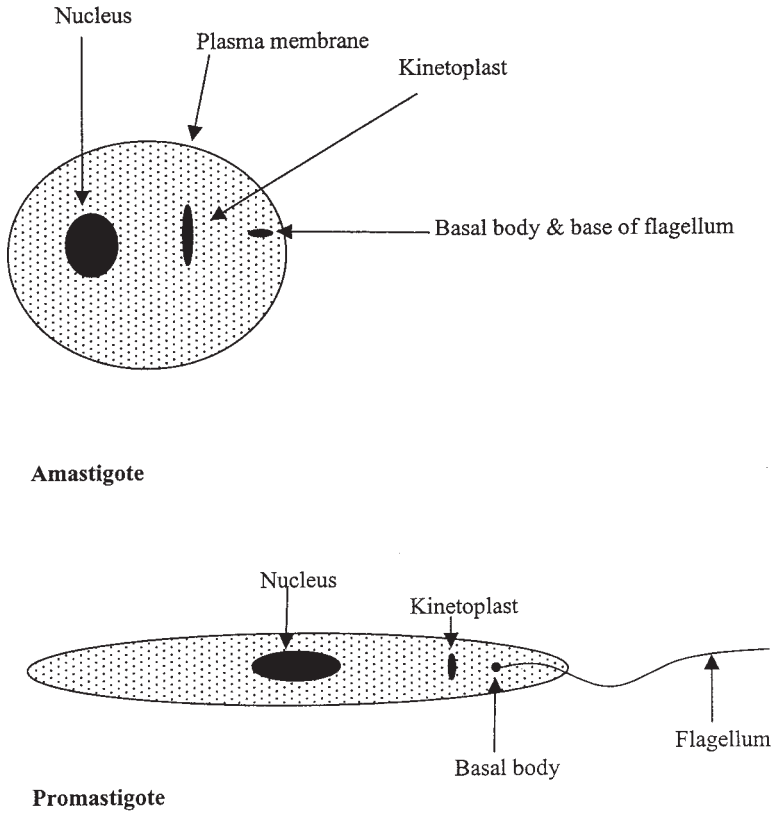


FIGURE 1 Life cycle stages of *Leishmania* spp.

primary lesion or many years later after the primary lesion has healed. Mucosal lesions do not heal spontaneously, and secondary bacterial infections are frequent and may be fatal. A small number of mucocutaneous leishmaniasis cases have been reported with *L. donovani* and *L. aethiopica*. Differential diagnosis has included lymphoma, midline granuloma, Wegener's granulomatosis, paracoccidioidomycosis, histoplasmosis, cutaneous mycobacterial infection, and leprosy.

Clinical features of the visceral disease vary from asymptomatic, self-resolving infections to frank visceral leishmaniasis. The incubation period may be as short as 10 days and as long as 2 years; usually it is within 2 to 4 months. Common symptoms include fever, anorexia, malaise, weight loss, and, frequently, diarrhea. Individuals coinfected with HIV may not elicit common symptoms seen in immunocompetent individuals, whereas the leukopenia will be unusually severe (42). Common clinical signs include nontender hepatomegaly and splenomegaly, lymphadenopathy, and occasional acute abdominal pain; darkening of facial, hand, foot, and abdominal skin (kala azar) is often seen in light-skinned persons in India. Anemia, cachexia, and marked enlargement of liver and spleen are noted as the disease progresses. Death may ensue after a few weeks, or after 2 to 3 years in chronic cases. The majority of infected individuals will be asymptomatic or have very few or minor symptoms that will resolve without therapy. Differential diagnosis in the acute stage will include amebic liver abscess, Chagas' disease, malaria, typhoid, typhus, and schistosomiasis. Subacute or chronic disease has been confused with bacteremia, brucellosis, histoplasmosis, leukemia, lymphoma, malaria, mononucleosis, and schistosomiasis.

Postdermal leishmaniasis or post-kala azar dermal leishmaniasis is a condition seen in India and the Sudan in some patients unsuccessfully treated for visceral leishmaniasis. This syndrome is rarely seen in Latin America but has been reported to occur in patients coinfected with HIV. The macular or hypopigmented dermal lesions are associated with few parasites, whereas erythematous and nodular lesions are associated with abundant parasites. This condition must be differentiated from leprosy, syphilis, and yaws.

Diagnosis

In areas where the disease is endemic, the diagnosis may be made on clinical grounds. Prolonged fever, progressive weight loss, anemia, leukopenia, hypergammaglobulinemia, and pronounced hepatomegaly and splenomegaly are highly suggestive of visceral leishmaniasis. The development of one or more chronic skin lesions with a history of exposure in an area of endemicity is suggestive of cutaneous leishmaniasis. Definitive diagnosis depends on detecting either the amastigotes in clinical specimens or the promastigotes in culture. All cutaneous lesions should be thoroughly cleaned with 70% alcohol, and extraneous debris, the eschar and exudates, should be removed. After debridement, with precautions being taken to prevent bleeding, the base of the ulcer can be scraped with a scapel blade to obtain an exudate for slide preparation, culture, or PCR. Specimens can be collected from the margin of the lesion by aspiration, scraping, or punch biopsy or by making a slit with a scalpel blade. Material scraped from the wall of the slit should be smeared onto a number of slides. Laboratorians may want to contact

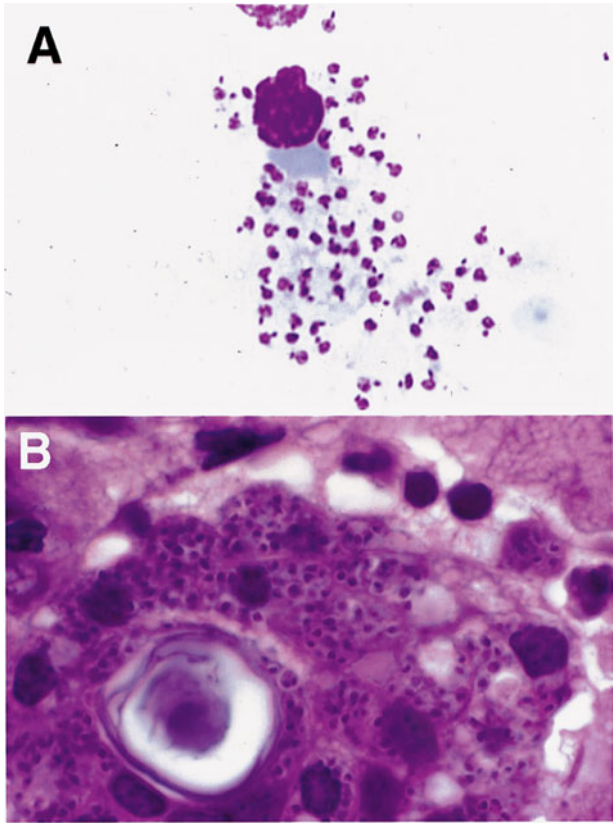


FIGURE 2 (A) *Leishmania donovani* amastigotes from splenic press preparation (Giemsa stain); (B) *Leishmania donovani* amastigotes in liver (hematoxylin and eosin stain).

their state public health laboratory or the Centers for Disease Control and Prevention (CDC) for diagnostic information and help in specimen selection and available tests.

The core of tissue from a punch biopsy can be used to make imprints or touch preparations on a slide. This core can also be submitted for histological examination. Recognition of amastigotes in tissues is more difficult than in smears or imprints because the organisms tend to be crowded within the cells, appear smaller, and are cut at various angles. Fine-needle aspiration can also be performed by using a sterile syringe containing sterile preservative-free buffered saline (0.1 ml) and a 26-gauge needle. The needle is inserted under the outer border of the lesion, the needle is rotated several times, and tissue fluid is aspirated into the needle. Tissue obtained by splenic puncture yields the highest rate of positive specimens; however, this procedure carries significant risk to the patient, particularly those with coagulation disorders. Other specimens for the detection of visceral leishmaniasis include lymph node aspirates, liver biopsy, sternal aspirates, iliac crest bone marrow, and buffy coat preparations of venous blood. Amastigotes with reticuloendothelial cells have been detected in bronchoalveolar lavage fluid, pleural effusions, and biopsy specimens collected from the gastrointestinal tract and oropharynx of HIV-positive patients (42). Individuals with post-kala azar dermal leishmaniasis have large numbers of parasites in the skin, particularly those with erythematous and nodular lesions (43).

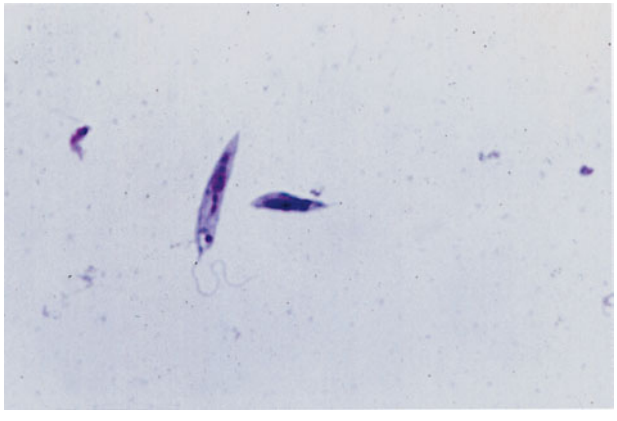


FIGURE 3 *Leishmania donovani* promastigotes (Giemsa stain).

Amastigote stages will be found within macrophages or close to disrupted cells (Fig. 2). This stage can be recognized by its shape, size, staining characteristics, and especially the presence of an intracytoplasmic kinetoplast. The cytoplasm will stain light blue, and the nucleus and kinetoplast will stain red or purple with Giemsa stain. Molecular techniques (none are FDA approved) for the detection of leishmanial DNA or RNA have been used for diagnosis, prognosis, and species identification. These methods are considered to be more sensitive than slide examination or culture, particularly for the detection of mucocutaneous leishmaniasis (3, 30, 33, 35, 37, 49, 57, 59, 62, 71, 75, 79). Generally, organisms in mucocutaneous lesions are scant and difficult to detect microscopically. Because infections caused by *Leishmania* subgenus *Viannia* are considered to be more aggressive and are more likely to result in treatment failure, molecular techniques to identify the organism to the species level can be very important (41, 44, 76).

If material is to be cultured, it must be collected aseptically. Tissues should be minced prior to culture. Culture media successfully employed to recover organisms include Novy, MacNeal, and Nicolle's medium (NNN) and Schneider's Drosophila medium supplemented with 30% fetal bovine serum. Cultures, incubated at 25°C, should be examined twice weekly for the first 2 weeks and once a week thereafter for up to 4 weeks before the culture is declared negative. Promastigote stages can be detected microscopically in wet mounts and then stained with Giemsa to observe their morphology.

Animals such as the golden hamster can be inoculated with patient material. Animals are inoculated intranasally for cutaneous and mucocutaneous leishmaniasis and intraperitoneally for visceral leishmaniasis. It may take 2 to 3 months before an animal becomes positive. A combination of tissue smears, culture, and animal inoculation may be needed to optimize the laboratory diagnosis of the infection. PCR testing, if available, can be used as a supplemental diagnostic procedure (62, 65).

The leishmanin (Montenegro) test (not available in Canada or the United States), a delayed-hypersensitivity reaction, is useful for epidemiological surveys of a population to identify groups at risk of infection. Positive reactions are usually seen in cutaneous and mucocutaneous leishmaniasis; however, patients with active visceral and diffuse cutaneous leishmaniasis exhibit negative reactions. Post-kala azar patients may also exhibit a negative reaction. This test is of no value for the diagnosis of visceral leishmaniasis.

Serologies (none are FDA approved) are available for research or epidemiologic purposes; however, they are not very useful for the diagnosis of mucocutaneous and visceral

leishmaniasis. In kala azar, there is a large increase in gamma globulins, both immunoglobulin G (IgG) and IgM. This is the basis for the aldehyde or formol-gel test, which has been used as a screening test in areas of endemicity (55). The addition of 1 drop of formalin to 1 ml of serum promotes the precipitation of immunoglobulins. A number of serologies including indirect fluorescent antibody (IFA), enzyme-linked immunoassay (ELISA), and immunoblot tests have been developed for diagnostic purposes; however, they are not widely available except in areas of endemicity (10, 13, 64). An ELISA and dipstick test using *L. infantum/L chagasi* recombinant k39 antigen has good sensitivity and specificity in diagnosing visceral leishmaniasis in immunocompetent people (12, 74). Visceral leishmaniasis patients coinfected with HIV may have no detectable antileishmania antibodies (11, 42). Serologic testing is available at some referral laboratories and the CDC. The detection of urinary antigens has been used for the diagnosis of visceral leishmaniasis (5).

Treatment and Prevention

Lesions in simple cutaneous leishmaniasis generally heal spontaneously. Treatment options have included cryotherapy, heat, surgical excision of lesions, and chemotherapy. Although the optimal treatment for cutaneous leishmaniasis is unknown, standard therapy consists of injections of antimonial compounds. Response to therapy varies depending on the species of *Leishmania* and the type of disease (70); therefore, it is important to identify the species of *Leishmania* causing the infection (79). Antimonial therapy has been given intralesionally and parenterally. The two commonly used pentavalent antimonials are sodium stibogluconate (Pentostam) and a meglumine antimoniate (Glucantime). The drug of choice for treatment of kala azar is sodium stibogluconate (Pentostam). Pentamidine isethionate is an alternative therapy for treatment of kala azar. Amphotericin B and liposomal amphotericin B can be used when the disease does not respond to antimonial or pentamidine therapy. Allopurinol, either alone or in combination with antimonials, has been effective in treating cutaneous leishmaniasis. Hexadecylphosphocholine (miltefosine) is an oral alkylphosphocholine analogue that has proven to be effective in treating cutaneous and visceral leishmaniasis even with patients coinfected with HIV (67). The risk of relapse is quite high within the first 6 to 12 months posttherapy. It is possible that macrophages may harbor quiescent parasites for life (54). Frequent relapses have been noted to occur in immunocompromised and HIV-positive patients and in patients unable to complete therapy due to drug toxicity (46). Patients clinically cured of *L. (V.) braziliensis,* which is noted for its chronicity, latency, and metastasis with mucosal membrane involvement, have been found to be PCR positive up to 11 years posttherapy (52). To ensure that treatment has been effective, follow-up smears and cultures should be done 1 to 2 weeks posttherapy. PCR testing has been used to monitor the progress of therapy (52, 61).

In areas where leishmaniasis is endemic, vaccination is still a major goal for eliminating leishmaniasis. This has been practiced by inoculating the serous exudate from naturally acquired lesions of cutaneous leishmaniasis into an inconspicuous area of the body of a nonimmune person. Vaccines against other forms of leishmaniasis have not been effective. Other possible prevention methods include spraying dwellings with residual insecticides and applying insect repellents to the skin. Fine-mesh bed netting, including insecticide-treated nets, may also be used and is an alternative to household spraying. Reservoir control has been unsuccessful in most areas, although in areas where canines may be a reservoir host, pyrethroid-impregnated collars are being used to prevent infections. Individuals with lesions should be warned to protect the lesion from insect bites with some type of covering. Patients should also be educated about the possibility of autoinoculation or infection.

AMERICAN TRYPANOSOMIASIS

■ *Trypanosoma cruzi*

American trypanosomiasis (Chagas' disease) is a zoonosis caused by *Trypanosoma cruzi.* There are 100 million persons at risk of infection in 18 Latin American countries, and 16 to 18 million persons are actually infected (42). Patients can present with either acute or chronic disease. Chagas' disease was considered a disease of rural areas; however, it is now ubiquitous due to social pattern changes of rural to urban migration. A very serious problem is disease acquisition through blood transfusion (50). A large number of patients with positive serology can remain asymptomatic.

Life Cycle and Morphology

Trypomastigotes (Fig. 4 and 5) are ingested by the reduviid bug (triatomids, kissing bugs, or conenose bugs) as it obtains a blood meal. The trypomastigotes transform into epimastigotes (Fig. 4 and 6) that multiply in the posterior portion of the midgut. After 8 to 10 days, metacyclic trypomastigotes develop from the epimastigotes. These metacyclic trypomastigotes are passed in the feces.

Humans contract Chagas' disease when the reduviid bug defecates while taking a blood meal and metacyclic trypomastigotes in the feces are rubbed or scratched into the bite wound or onto mucosal surfaces. In humans, *T. cruzi* can be found in two forms, as amastigotes and trypomastigotes (Fig. 4, 6, and 7). The trypomastigote form is present in the blood and infects the host cells. The amastigote form multiplies within the cell, eventually destroying the cell, and both amastigotes and trypomastigotes are released into the blood.

The trypomastigote is spindle shaped and approximately 20 μm long and characteristically assumes a C or U shape in stained blood films (Fig. 6). Trypomastigotes occur in the blood in two forms, a long, slender form and a short, stubby one. The nucleus is situated in the center of the body, with a large, oval kinetoplast located at the posterior end. A flagellum arises from the basal body and extends along the outer edge of an undulating membrane until it reaches the anterior end of the body, where it projects as a free flagellum. When the trypomastigotes are stained with Giemsa stain, the cytoplasm stains blue and the nucleus, kinetoplast, and flagellum stain red or violet.

The amastigote (2 to 6 μm in diameter) is indistinguishable from those found in leishmanial infections. It contains a large nucleus and a rod-shaped kinetoplast that stains red or violet with Giemsa stain, and the cytoplasm stains blue (Fig. 2 and 7).

Epidemiology

Chagas' disease is a zoonosis occurring throughout the American continent, including Central and South America, Texas, and California (56). It involves reduviid bugs living in close association with reservoirs (dogs, cats, armadillos, opossums, raccoons, and rodents). Human infections occur mainly in rural areas where poor sanitary and socioeconomic conditions and poor housing provide excellent breeding places for reduviid bugs. Chagas' disease is found in 18 countries in

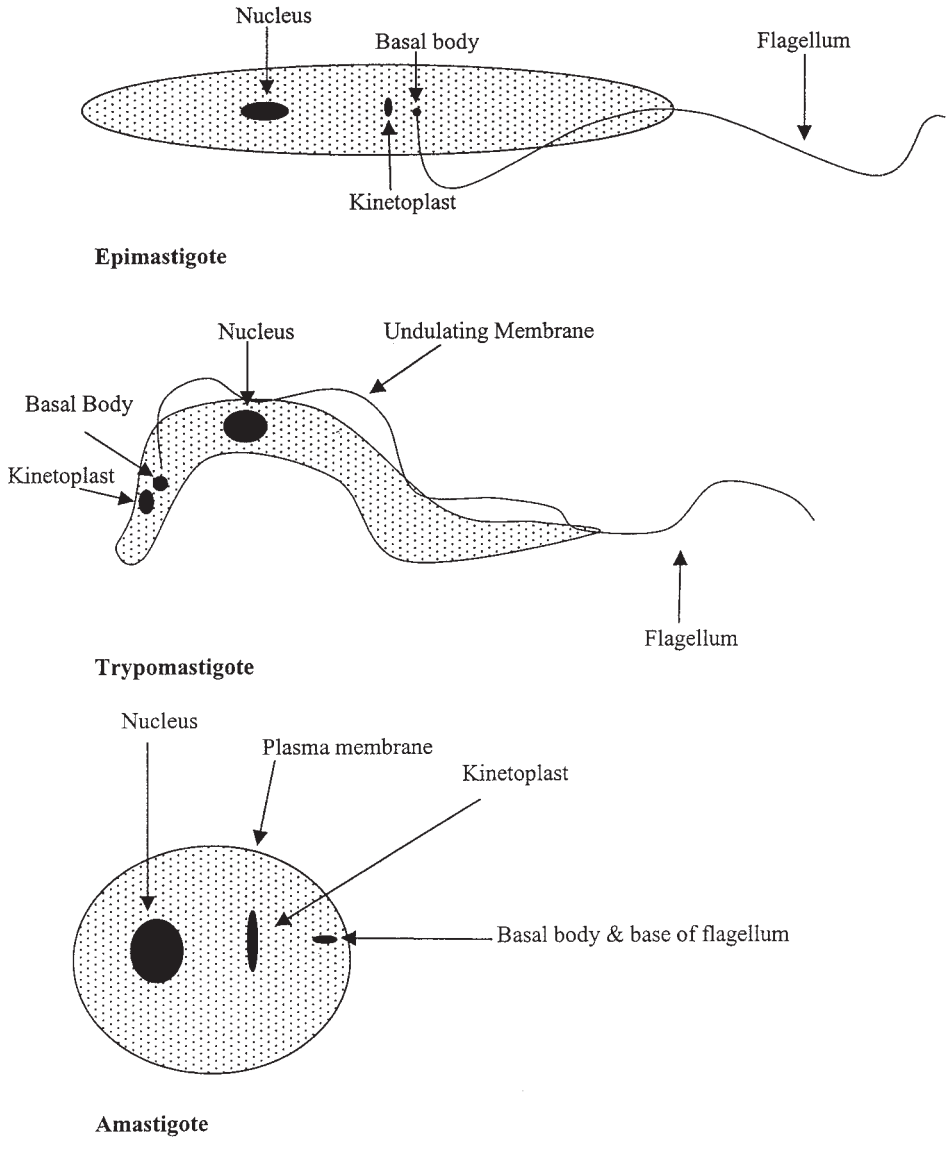


FIGURE 4 Life cycle stages of trypanosomes.

Central and South America. The disease distribution has been broken into two ecological zones: the Southern Cone, where the reduviid vector lives inside the human home, and the Northern South America, Central America, and Mexico zone, where the reduviid lives inside and outside the home. Strains of *T. cruzi* have large differences in infectivity of potential vectors, antigenicity, histotropism, pathogenicity, and response to therapy (4, 6, 7, 16). Most human infections may be due to *T. cruzi* subgroup II (34). Transmission by blood transfusion is a serious concern in areas of endemicity, and serologic screening of blood donors can be used, whereas in areas where the disease is not endemic questionnaires may be used to defer prospective donors from areas of endemicity (63, 73).

Clinical Disease

In addition to contracting *T. cruzi* infections through the insect's bite wound or exposed mucous membranes, one can be infected by blood transfusion, placental transfer, organ transplant, and accidental ingestion of parasitized reduviid bugs (73). A localized inflammatory reaction may ensue at the infection site with development of a chagoma (erythematous subcutaneous nodule) or Romaña's sign (edema of the eyelids and conjunctivitis).

Acute systemic signs occur around the second to third week of infection and are characterized by high fevers; hepatosplenomegaly; myalgia; erythematous rash; acute myocarditis; lymphadenopathy; and subcutaneous edema of face, legs, and feet. The acute phase of Chagas' disease in immunosuppressive patients is manifested as acute myocarditis or acute encephalitis with a high mortality rate. Most acute cases resolve over a period of 2 to 3 months into an asymptomatic chronic stage. Approximately 30% of these individuals develop myocarditis or symptoms associated with denervation of the digestive tract (31).

Chronic Chagas' disease is diagnosed more commonly than the acute phase. Symptoms of the chronic phase are related to the damage sustained during the acute phase of the disease.

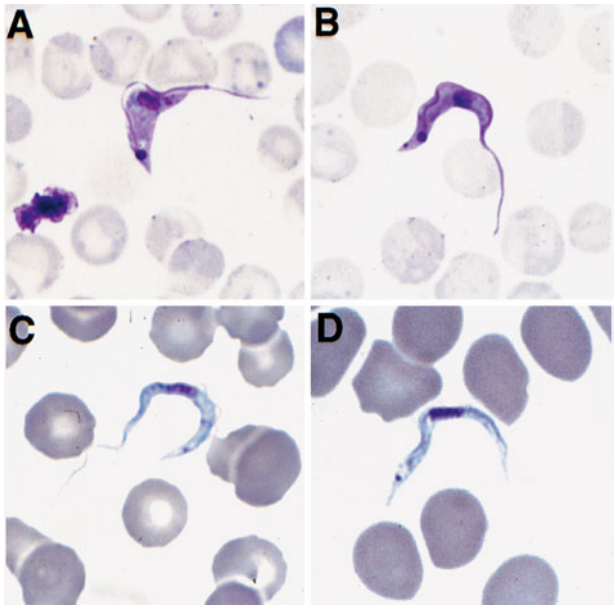


FIGURE 5 (A and B) *Trypanosoma cruzi* trypomastigotes; (C and D) *Trypanosoma brucei gambiense* trypomastigotes.

Chronic Chagas' disease may develop years or decades after undetected infection or after the diagnosis of acute disease. The most frequent clinical sign of chronic Chagas' disease is cardiomyopathy manifested by cardiomegaly and conduction changes. Some patients are more likely to have megaesophagus or megacolon. The "mega" condition has been associated with the destruction of ganglion cells, resulting in dysmotility causing dysphagia, aspiration, and regurgitation in patients with megaesophagus and severe constipation in megacolon. In chronic Chagas' disease, autoimmunity may also be responsible for tissue destruction in addition to the tissue destruction caused by the parasite. Reactivation of Chagas' disease in HIV-positive patients usually leads to very high parasitemia (25). Central nervous system (CNS) involvement is seldom observed, but in HIV-coinfected individuals, CNS involvement is frequently noted, and acute fatal meningoencephalitis and granulomatous encephalitis have been described for these patients (42). Congenital transmission can occur in both acute and chronic phases of the disease. Infants of seropositive mothers should be monitored for up to a year after birth to rule out infection.

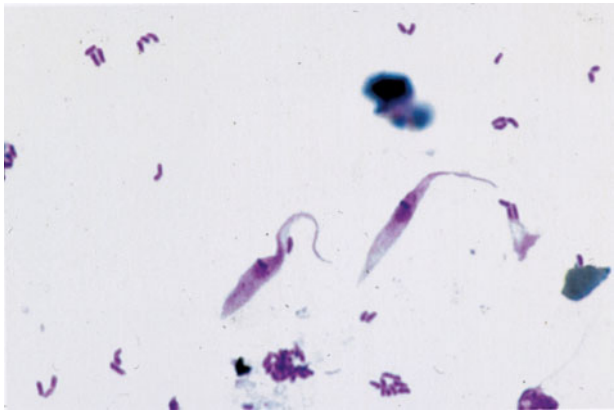


FIGURE 6 *Trypanosoma cruzi* epimastigotes (Giemsa stain).

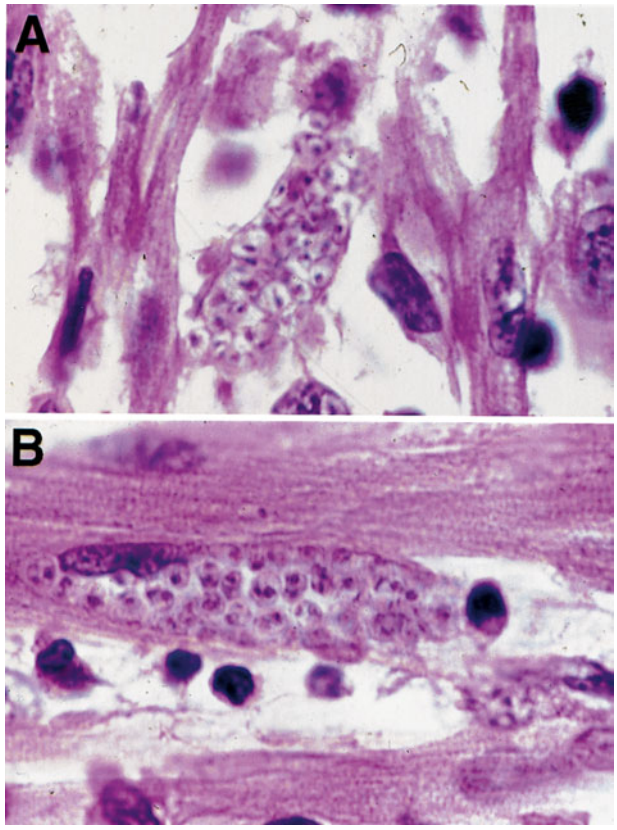


FIGURE 7 (A and B) *Trypanosoma cruzi* amastigotes in heart muscle (hematoxylin and eosin stain).

Diagnosis

Health care personnel working with specimens from patients suspected of having Chagas' disease should follow the blood-borne pathogen guidelines. Trypomastigotes are highly infectious.

The definitive diagnosis depends on demonstration of trypomastigotes in the blood, amastigote stages in tissues, or positive PCR and serologic tests. Aspirates from chagomas and enlarged lymph nodes can be examined for amastigotes and trypomastigotes. Histological examination of biopsy specimens may also be done. Trypomastigotes may be easily detected in the blood in acute disease; however, in chronic disease, this stage is rare or absent, except during febrile episodes. Trypomastigotes appear in the blood about 10 days after infection and persist through the acute phase. Laboratorians may want to contact their state public health laboratory or the CDC for diagnostic information and help in specimen selection and available tests.

Trypomastigotes may be detected in blood by using thin and thick blood films or the buffy coat concentration technique (72). The stain of choice is Giemsa for both amastigote and trypomastigote stages. Although not routinely available except in specialized centers, PCR (not FDA approved) has been used to detect as few as one trypomastigote in 20 ml of blood and has been useful in treatment follow-up (14, 21, 50). In areas where kala azar occurs, amastigote stages look

similar and infections of *L. donovani* and *T. cruzi* must be differentiated by either PCR, immunoassay, culture (epimastigote in *T. cruzi* versus promastigote in *L. donovani*), serologic tests, animal inoculation, or xenodiagnosis techniques (21). Patient history, including geographic and/or travel history, and confirmation of organisms in striated muscle rather than reticuloendothelial tissues are very strong evidence for *T. cruzi* rather than *L. donovani* as the causative agent.

Aspirates, blood, and tissues can also be cultured. The medium of choice is NNN. Cultures, incubated at 25°C, should be examined for epimastigote stages twice weekly during the first 2 weeks and once per week thereafter for up to 4 weeks before they are considered negative. If available, laboratory animals (rats or mice) can be inoculated and the blood can be observed for trypomastigotes.

Although xenodiagnosis is used less frequently for clinical diagnosis in areas of endemicity, trypanosome-free reduviid bugs are allowed to feed on individuals suspected of having Chagas' disease. The feces, hemolymph, hindgut, and salivary glands can be examined microscopically for flagellated forms over a period of 3 months, or PCR methods can be used to detect infected bugs. Xenodiagnosis is positive in less than 50% of the seropositive patients. Some patients may develop a severe anaphylactic reaction to the reduviid bug's salivary secretion.

Serological tests (none are FDA approved) using blood and saliva for the diagnosis of Chagas' disease include complement fixation (Guerreiro-Machado test), chemiluminescence, IFA, indirect hemagglutination, and ELISA. Most of these tests use an epimastigote antigen, and cross-reactions have been noted for patients infected with *Trypanosoma rangeli, Leishmania* spp., *Toxoplasma gondii*, and hepatitis (23, 33). Most lack sensitivity and specificity, and the WHO recommends testing with two conventional assays before accepting a positive result (40). The use of synthetic peptides and recombinant proteins has improved the sensitivity and specificity of the serodiagnostic techniques (1, 15, 28, 29, 38, 63). Due to the lack of sensitivity and specificity of serologic tests, it is recommended that at least two different serologic test methods be used to screen blood donor populations (60). Follow-up blood specimens should be reexamined 1 to 2 months after therapy by the techniques described above. Serologic testing is available at referral laboratories and the CDC.

Treatment and Prevention

Nifurtimox (Lampit) and benznidazole (Radamil) reduce the severity of acute Chagas' disease, but they have little impact on the progression of chronic Chagas' disease. Other drugs, allopurinol, fluconazole, itraconazole, and ketoconazole, have been used to treat a limited number of patients. Surgery has been successfully used to treat cases of chagasic heart disease, megaesophagus, and megacolon.

Until recently, control of Chagas' disease has been mainly through the use of insecticides to eliminate the reduviid vector. Construction of reduviid-proof dwellings and health education are essential for effective control programs. Bed nets are also effective in preventing infections. Serologic screening of blood products for transfusion from areas in which the disease is endemic is highly recommended. An alternative approach to serologic testing for blood donors is the use of questionnaires to defer prospective donors from areas of endemicity.

■ *Trypanosoma rangeli*

T. rangeli infects humans and other vertebrates in both Central and South America and is often found in areas where *T. cruzi* is also present (26, 39). Human infections are asymptomatic, and trypomastigotes have been noted to persist in the blood for longer than a year. *T. rangeli* and *T. cruzi* can use the same triatomid vector to transmit infections. *T. rangeli* infections can be transmitted by inoculation of triatomid saliva during feeding or by the vector's feces (27). In some areas, *T. rangeli* infections are five to six times more frequent than infections with *T. cruzi*. Trypomastigotes can be detected from the blood of infected patients by using thin and thick blood smears and buffy coat concentration techniques. The parasites can be stained with Giemsa or Wright stains. Microscopically, the trypomastigote cannot be differentiated from African trypanosomes, which do not occur in the Americas. PCR methods have been used to detect infections in humans and vectors (22, 27). Infections can also be detected by xenodiagnosis. In addition, blood can be cultured (Tobies medium or NNN) or injected into laboratory animals (mice) and examined for epimastigotes and trypomastigotes, respectively. Although there are no serologic tests to detect *T. rangeli* infections, serologic cross-reactions have been noted to occur with tests for *T. cruzi* (2). There are no treatment recommendations for *T. rangeli* infections.

AFRICAN TRYPANOSOMIASIS

African trypanosomiasis is limited to the tsetse fly belt of Central Africa, where there are over 60 million people at risk for African trypanosomiasis (20). The West African (Gambian) form of sleeping sickness, noted for its chronicity and responsible for 99% of the sleeping sickness cases, is caused by *T. brucei gambiense,* whereas the East African (Rhodesian) form, noted for its acute morbidity and mortality, is caused by *T. brucei rhodesiense* (66). *T. brucei gambiense* infections can last for months to years with slow CNS involvement. In some areas of endemicity, civil strife has disrupted both the health care infrastructure and vector control, which has led to a resurgence of this disease.

Life Cycle and Morphology

T. brucei rhodesiense and *T. brucei gambiense* are closely related and morphologically indistinguishable. In the past, differentiation was based on clinical signs and geographic area; however, differentiation can now be accomplished using isoenzyme characteristics and DNA and RNA methods.

The trypomastigote forms (Fig. 4 and 6) in the blood range from long, slender-bodied organisms with a long flagellum to short, fat, stumpy forms without a free flagellum (14 to 33 μm long and 1.5 to 3.5 μm wide). The short, stumpy forms are the infective stage for the tsetse fly.

Using Giemsa or Wright stains, the granular cytoplasm stains pale blue and contains dark blue granules and possibly vacuoles. The centrally located nucleus stains reddish. The kinetoplast is located at the organism's posterior end and stains reddish; the remaining intracytoplasmic flagellum (axoneme) may not be visible. The flagellum arises from the kinetoplast, as does the undulating membrane. The flagellum runs along the edge of the undulating membrane until the undulating membrane merges with the trypanosome body at the organism's anterior end. At this point the flagellum becomes free to extend beyond the body. Trypanosomal forms are ingested by the tsetse fly when a blood meal is taken and transform to epimastigotes (Fig. 4). The organisms multiply in the gut of the fly, and after approximately 2 weeks, the organisms migrate back to the salivary glands. Humans are infected when metacyclic forms from the salivary glands are introduced into the bite site when the blood meal is taken. The trypomastigote has the ability to change the surface coat of the

outer membrane, helping the organism evade the host's humoral immune response (32).

Epidemiology

The development cycle in the tsetse fly varies from 12 to 30 days and averages 20 days. Fewer than 10% of the tsetse flies become infective after obtaining blood from infected patients. Both female and male tsetse flies can transmit the infection.

Although there is no evidence of animal-to-human transmission of *T. brucei gambiense,* trypanosomal strains isolated from hartebeest, kob, chickens, dogs, cows, and domestic pigs in West Africa are identical to those isolated from humans in the same area. Evidence suggests that transmission may be entirely interhuman. The tsetse fly vectors of Rhodesian trypanosomiasis are game feeders that may transmit the disease from human to human or from animal to human.

Clinical Disease

After a bite by an infected tsetse fly, a local inflammatory reaction that resolves spontaneously within 1 to 2 weeks can be detected at the bite site. The trypomastigotes gain entrance to the bloodstream, causing a symptom-free low-grade parasitemia that may continue for months. The infection may selfcure during this period without development of symptoms or lymph node invasion. Chancres may be confused with insect bites and bacterial skin infections.

The clinical course and disease progression are more acute with *T. brucei rhodesiense* than with *T. brucei gambiense* infections. Diagnostic symptoms include irregular fever, lymph node enlargement (particularly those of the posterior triangle of the neck, known as Winterbottom's sign, which is prominent in *T. brucei gambiense* infections), delayed sensation to pain (Kerandel's sign), and erythematous skin rashes. In addition to lymph node involvement, the spleen and liver become enlarged. With Gambian trypanosomiasis, the blood-lymphatic stage (stage I) may last for years before the sleeping sickness syndrome occurs (CNS involvement, stage II).

Laboratory findings include anemia, granulocytopenia, increased sedimentation rate, and marked increases in serum IgM. The sustained high IgM levels are a result of the parasite producing variable antigen types to evade the patient's defense system (32). In an immunocompetent host, the lack of elevated serum IgM rules out African trypanosomiasis. Diagnostic differential may include brucellosis, malaria, relapsing fever, and typhoid.

Upon trypomastigote invasion of the CNS, the sleeping-sickness stage of the infection is initiated (stage II). Gambian trypanosomiasis is characterized by steady progressive meningoencephalitis, behavioral changes, apathy, confusion, coordination loss, and somnolence. *T. brucei rhodesiense* produces a more rapid, fulminating disease, and death may occur before there is extensive CNS involvement. In the terminal phase of the disease, the patient becomes emaciated, leading to profound coma and death, usually from secondary infections. Cerebrospinal fluid (CSF) findings include increased protein and IgM levels, lymphocytosis, and morular cells of Mott. Morular (mulberry) cells are altered plasma cells whose cytoplasm is filled with proteinaceous droplets. Morular cells are not seen in all patients; however, they are characteristic of African trypanosomiasis. The diagnostic differential may include cryptococcosis, HIV, meningitis, Parkinson's disease, psychiatric disorders, and space-occupying lesions.

Diagnosis

Definitive diagnosis depends upon demonstration of trypomastigotes in blood, lymph node aspirate, sternum bone marrow, and CSF. Trypomastigotes can be more readily detected in body fluids in infections due to *T. brucei rhodesiense* than in those due to *T. brucei gambiense* because of higher parasitemias. Due to periodicity, parasite numbers in the blood vary, and a number of techniques must be used to detect the trypomastigotes. Laboratorians may want to contact their state public health laboratory or the CDC for diagnostic information and help with specimen selection and available tests.

Trypomastigotes are highly infectious, and health care personnel must be cautious and adhere to bloodborne-pathogen precautions when handling blood, CSF, or aspirates. Blood can be collected from either finger stick or venipuncture. Venous blood should be collected in a tube containing EDTA. Multiple blood exams should be performed before trypanosomiasis is ruled out. Parasites are found in high numbers in the blood during the febrile period and in low numbers in the afebrile periods. If CSF is examined, a volume greater than 1 ml, preferably 5 ml or more, should be collected. In addition to thin and thick blood films, a buffy coat concentration method is recommended to detect the parasites. Parasites can be detected on thick blood smears when numbers are greater than 2,000/ml, with hematocrit capillary tube concentration when numbers are greater than 100/ml, and on an anion-exchange column when numbers are greater than 4/ml. Unfortunately, anion-exchange is not easily adapted to clinical laboratories or field studies (20). In suspected and confirmed cases of trypanosomiasis, a lumbar puncture is mandatory to rule out CNS involvement (stage II). CSF examination must be conducted by using centrifuged sediments (17). The CSF should be examined immediately because the trypomastigotes begin to autolyse within 10 min. Detection of trypomastogotes in the CSF allows immediate classification of stage II illness (CNS involvement). In cases in which trypomastigotes are in undetectable numbers in the blood, they may be seen in aspirates of inflamed lymph nodes; however, attempts to demonstrate them in tissue are not practical. Blood and CSF specimens should be examined during therapy to evaluate the clinical response and 1 to 2 months after therapy. ELISA has been used to detect antigen in serum and CSF. This method could also be used for clinical staging of the disease to determine whether there was CNS infection and as a follow-up to therapy. Referral laboratories have used molecular methods (PCR, not FDA approved) to detect infections and differentiate species, but these methods are not routinely used in the field (9, 20, 46).

Serologic techniques (not FDA approved) that have been used for epidemiologic screening include IFA, ELISA, indirect hemagglutination assay, the card agglutination trypanosomiasis test (CATT), and LATEX/*T. b. gambiense*. CATT is effective in screening the population for suspected cases of *T. brucei gambiense* but not *T. brucei rhodesiense*. Major serodiagnostic problems with CATT include false-positive results due to malarial infections and the fact that many in the population have elevated antibody levels due to exposure to animal trypanosomes that are noninfectious to humans. CATT does not differentiate between current and past infections (20, 36, 69). Both CATT and LATEX/*T. b. gambiense* have good negative predictive values (20). Markedly elevated serum and CSF IgM concentrations are of diagnostic value. CSF antibody titers should be interpreted with caution because of the lack of reference values and the possibility of CSF containing serum due to a traumatic tap. Intrathecal production of immunoglobulins can be found in a number of neuroinflammatory diseases. LATEX/IgM has been developed for field use to measure CSF concentrations of IgM (20).

Antibodies to galactocerebrosides and neurofilaments as well as elevated concentrations of interleukin-10 may be

markers for CNS involvement, all of which have been used to monitor CNS infections (20, 47).

Small laboratory animals (rat and guinea pig) have been used to detect infections. *T. brucei rhodesiense* is more adaptable to cultivation and animal infection than *T. brucei gambiense;* however, cultivation is not practical for most diagnostic laboratories.

Treatment and Prevention

Suramin (Bayer 205, Naphuride, Antrypol) is the drug of choice for treating the early blood or lymphatic stage of *T. brucei rhodesiense* infections, whereas pentamidine isethionate (Lomidine) is the drug of choice for treating the early stages of *T. brucei gambiense* infections (8, 45, 66). Both drugs require parenteral administration, and side effects can be quite severe. Suramin and pentamidine isethionate do not cross the blood-brain barrier; therefore, melarsoprol (mel B, Arsobal) is the drug of choice when CNS involvement is suspected. A major complication of melarsoprol is reactive encephalitis, occurring in 5 to 15% of the patients, for whom there is a 50% mortality rate. A new 10-day course with reduced dosage has been found to be effective in treating *T. brucei gambiense* infections (45, 66).

Difluoromethyornithine (DFMO, Eflornithine, Ornidyl) is a cytostatic drug effective against the acute and late stages of *T. brucei gambiense* infections. It is less effective in late-stage disease in patients coinfected with HIV, and patients were more likely to relapse after treatment (42). Therapy failure rates have reached as high as 50% (53). The effectiveness of therapy can be judged microscopically by the absence of trypomastigotes in the blood, lymph fluid, or CSF and by a decrease in CSF white blood cells. CSF antibodies (IgM) decrease, as do levels of interleukin-10, after successful therapy (47). Any individual treated for African trypanosomiasis should be monitored for 2 to 3 years after completion of therapy. About 2% of the patients treated for CNS disease experience relapses.

Population-screening programs have been used to control *T. brucei gambiense* infections. The use of vector control measures has met with limited success. The release of sterile male flies to mate with females has had some success in eliminating vectors (7). The most effective control measures include an integrated approach to reduce the human reservoir of infection and the use of insecticide and fly traps; however, economic resources are limited and effective programs cannot be sustained. Persons visiting areas in which the infection is endemic should wear protective clothing (long-sleeved shirts and long trousers). Other measures include reduction in vegetation around human settlements, insect repellents, bed netting, and screens. Chemoprophylaxis is not recommended due to drug toxicity. Vaccines are not available.

OTHER TRYPANOSOMES INFECTING HUMANS

Trypanosoma evansi

The first human case of *T. evansi* was diagnosed in India (78). This organism is normally considered to be a parasite of animals (buffalo, camels, cattle, horses, and rats) and has a very wide geographic distribution (Africa, Asia, and Central and South America). The infection is transmitted mechanically by blood-sucking insects such as stable or horse flies. In animals, the incubation period is 5 to 60 days and the severity of the disease varies from no symptoms to weakness, weight loss, anemia, abortions, and death. In the above-mentioned human case, the patient complained of transient fevers and sensory disorders. Fever peaks were noted every 7 to 10 days, and large numbers of parasites were detected in the blood at the time of fevers. No parasites were observed in the CSF. The patient was treated with suramin. Laboratory diagnosis is usually done by examination of blood and lymph node aspirates or biopsy specimens. *T. evansi* cannot be differentiated from *T. brucei gambiense, T. brucei rhodesiense,* or *T. rangeli* microscopically.

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Toxoplasma

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TAXONOMY

Phylum Apicomplexa, Class Conoidasida, Subclass Coccidiasina, Order Eucoccidiorida, Suborder Eimeriorina, Family Sarcocystidae, (*Sarcocystis*), Subfamily Toxoplasmatinae, Genus *Toxoplasma*

LIFE CYCLE

Toxoplasma gondii is a protozoan parasite that infects most species of warm-blooded animals, including humans. Members of the cat family Felidae are the only known definitive hosts for the sexual stages of *T. gondii* and thus are the main reservoirs of infection. The three stages of this obligate intracellular parasite are (i) tachyzoites (trophozoites), which rapidly proliferate and destroy infected cells during acute infection; (ii) bradyzoites, which slowly multiply in tissue cysts; and (iii) sporozoites in oocysts (Fig. 1). Tachyzoites and bradyzoites occur in body tissues; oocysts are excreted in cat feces. Cats become infected with *T. gondii* by carnivorism or by ingestion of oocysts. Cats that are allowed to roam outside are much more likely to become infected than domestic cats that are confined indoors. After tissue cysts or oocysts are ingested by the cat, viable organisms are released and invade epithelial cells of the small intestine, where they undergo an asexual cycle followed by a sexual cycle and then form oocysts, which are then excreted. The unsporulated (i.e., uninfective) oocyst takes 1 to 5 days after excretion to become sporulated (infective). Although cats shed oocysts for only 1 to 2 weeks, large numbers may be shed, often exceeding 100,000 per g of feces. Oocysts can survive in the environment for several months to more than a year and are remarkably resistant to disinfectants, freezing, and drying but are killed by heating to 70°C for 10 min.

EPIDEMIOLOGY AND TRANSMISSION

Serologic prevalence data indicate that toxoplasmosis is one of the most common infections of humans throughout the world. Because *T. gondii* organisms are rarely detected in humans with toxoplasmosis, serologic examination is used to indicate the presence of the infection by detecting *Toxoplasma*-specific antibodies. The prevalence of positive serologic titers increases with age. Infection is more common in warm climates and at lower altitudes than in cold climates and mountainous regions. This distribution is probably related to conditions favoring the sporulation and survival of oocysts. Variations in the prevalence of infection between geographic areas and between population groups within the same locale are also probably due to differences in exposure. A high prevalence of infection in France (65 to 85%) has been related to a preference for eating raw or undercooked meat. However, a high prevalence in Central America has been related to the frequency of stray cats in a climate favoring the survival of oocysts. Among U.S. military recruits in 1962, seroprevalence rates of up to 30% were found in people living along the sea coast, with rates of less than 1% in the Rocky Mountains and the desert Southwest. More recent data comparing antibody prevalence in U.S. military recruits in 1962 and 1989 indicated a one-third decrease in seropositivity (80). The overall seroprevalence in the United States as determined with specimens collected by the Third National Health and Nutritional Assessment Survey (NHANES III) between 1988 and 1994 among persons 12 or more years of age was found to be 22.5%, with seroprevalence among women of childbearing age (15 to 44 years) of 15% (43). No statistically significant differences were found in *T. gondii* antibody prevalence between NHANES III and NHANES 1999–2000 samples (42).

Human infection may be acquired in several ways: (i) ingestion of undercooked infected meat containing *T. gondii* cysts; (ii) ingestion of the oocyst from fecally contaminated hands, food, or water; (iii) organ transplantation or blood transfusion; (iv) transplacental transmission; and (v) accidental inoculation of tachyzoites. The two major routes of transmission of *Toxoplasma* to humans are oral and congenital. In humans, ingesting either the tissue cyst or the oocyst results in the rupture of the cyst wall, which releases organisms that invade the intestinal epithelium, disseminate throughout the body, and multiply intracellularly. The host cell dies and releases tachyzoites, which invade adjacent cells and continue the process. The tachyzoites are pressured by the host's immune response to transform into bradyzoites and form tissue cysts, most commonly in skeletal muscle, myocardium, and brain; these cysts may remain throughout the life of the host. Recrudescence of clinical disease may occur if the host becomes immunosuppressed and the cysts rupture, releasing the parasites.

Prevention

Risk factors for *T. gondii* infection identified in epidemiologic studies include eating raw or undercooked pork, mutton, lamb, beef, mincemeat products, or wild game meat; cleaning

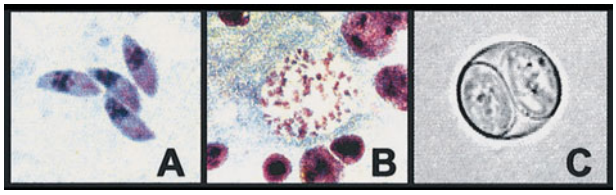


FIGURE 1 Three life stages of *T. gondii*. (A) Tachyzoites, Giemsa stain. (B) Cyst with bradyzoites in brain tissue, Giemsa stain. (C) Sporulated oocysts, unstained. (Photographs courtesy of J. P. Dubey, U.S. Department of Agriculture, Beltsville, Md.)

the cat litter box; contact with soil (gardening and yard work); and eating raw or unwashed vegetables or fruits (44). Recommendations for prevention of toxoplasmosis in pregnant women were discussed at a conference at the Centers for Disease Control and Prevention and published (5). These recommendations included the following: (i) food should be cooked to safe temperatures (beef, lamb, and veal roasts and steaks to at least 145°F; pork, ground meat, and wild game to 160°F; poultry to 180°F); (ii) fruits and vegetables should be peeled or washed thoroughly before eating; (iii) cutting boards, dishes, counters, utensils, and hands should always be washed with hot, soapy water after they have contacted raw meat, poultry, seafood, or unwashed fruits or vegetables; (iv) pregnant women should wear gloves when gardening and during any contact with soil or sand because cat waste might be in soil or sand, and they should wash their hands afterwards; (v) pregnant women should avoid changing cat litter if possible. If no one else is available to change the cat litter, pregnant women should use gloves and then wash their hands thoroughly. The litter box should be changed daily because *T. gondii* oocysts require more than one day to become infectious. Pregnant women should be encouraged to keep their cats inside and not adopt or handle stray cats. Cats should be fed only canned or dried commercial food or well-cooked table food, not raw or undercooked meats.

CLINICAL SIGNIFICANCE

Toxoplasmosis can be categorized into four groups: (i) acquired in the immunocompetent patient; (ii) acquired or reactivated in the immunodeficient patient; (iii) congenital; and (iv) ocular. Methods of diagnosis and their interpretations may differ for each clinical category.

Acquired infection with *Toxoplasma* in immunocompetent individuals is generally an asymptomatic infection. However, 10 to 20% of patients with acute infection may develop cervical lymphadenopathy and/or a flu-like illness. The clinical course is benign and self-limited; symptoms usually resolve within weeks to months.

Immunodeficient patients often have central nervous system (CNS) disease but may have myocarditis or pneumonitis. In patients with AIDS, toxoplasmic encephalitis is the most common cause of intracerebral mass lesions and is thought to be due to reactivation of chronic infection. Toxoplasmosis in patients being treated with immunosuppressive drugs may be due to either newly acquired or reactivated latent infection.

Congenital toxoplasmosis results from an acute primary infection acquired by the mother during pregnancy. The incidence and severity of congenital toxoplasmosis vary with the trimester during which infection was acquired. Because treatment of the mother may reduce the severity of symptoms in the infant due to congenital infection, prompt and accurate diagnosis is extremely important. Many infants with subclinical infection at birth will subsequently develop signs or symptoms of congenital toxoplasmosis; however, treatment may help prevent subsequent symptoms.

Ocular toxoplasmosis, an important cause of chorioretinitis in the United States, may be the result of congenital or acquired infection. Acquired infection is now thought to be more common than congenital infection. Congenitally infected patients are often asymptomatic until the second or third decade of life, when lesions develop in the eye presumably due to cyst rupture and subsequent release of tachyzoites and bradyzoites. Chorioretinitis is characteristically bilateral in patients with congenital infection but is often unilateral in individuals with acute acquired *T. gondii* infection.

In general, physicians treat *T. gondii* infection in four circumstances: (i) pregnant women with acute infection, to prevent fetal infection (11, 24, 28); (ii) congenitally infected infants (31, 51); (iii) immunosuppressed persons, usually with reactivated disease (6, 7, 81); and (iv) acute and recurrent ocular disease (38). Drugs are also prescribed for preventive or suppressive treatment in human immunodeficiency virus (HIV)-infected persons (4). The currently recommended drugs work primarily against the actively dividing tachyzoite form of *T. gondii* and do not eradicate encysted organisms (bradyzoites).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Serum, plasma, cerebrospinal fluid (CSF), ocular fluid, and amniotic fluid may be tested for antibodies and/or parasite DNA.

Collection for Determination of Parasite DNA

To determine parasite DNA, blood samples should be collected with an anticoagulant; CSF, ocular fluid, and amniotic fluids do not need an anticoagulant. All samples should be shipped and stored at 4°C prior to testing.

Collection for Antibody Determination

Blood specimens to be tested for the presence of antibodies should be allowed to clot and centrifuged, and the serum should be removed and shipped to a reference laboratory. Hemolysis does not seem to interfere with the antibody reaction in most tests. Serum and CSF specimens may be stored for several days at 4°C or frozen for longer storage. Specimens may be shipped at ambient temperature unless they are to be in transit for more than 1 week or be subjected to temperatures above 30°C. To avoid evaporation of small volumes, ocular fluids should be stored and shipped frozen. CSF and ocular fluid should be tested in parallel with a serum sample drawn on the same date. Long-term storage should take place at –20°C or below.

If the determination of immune status is the reason for testing, a single serum specimen is satisfactory; acute- and convalescent-phase specimens are not necessary. In situations in which determining the time of infection is important, specimens drawn at least 3 weeks apart may or may not be useful. In most cases, detection of an increasing immunoglobulin G (IgG) or IgM titer is not possible because the titers have already reached a plateau by the time the initial sample is drawn. If two specimens are to be compared, they should be tested together. Results from tests done at different times, in different laboratories, or with different procedures should not be compared quantitatively, only qualitatively, as positive or negative.

For tests other than serology, contact a reference laboratory for instructions *before* collecting specimens to ensure proper collection and handling.

DIRECT EXAMINATION

Microscopy

Only very rarely can the diagnosis of toxoplasmosis be documented by the observation of parasites in patient specimens by microscopy. Secretions, excretions, body fluids, and tissues are potential specimens for direct observation of parasites but are generally unrewarding. Fluid specimens such as heparinized blood or CSF should be centrifuged, and the sediment should be smeared on a microscope slide. The slides should be air dried, fixed in methanol, and stained with Giemsa for microscopic examination. Tachyzoites may be observed as free organisms or within host cells such as leukocytes. Well-preserved tachyzoites are crescent shaped and stain well, but degenerating organisms may be oval and stain poorly. Tissue imprints stained with Giemsa may reveal *T. gondii* cysts.

Antigen Detection

Immunologic techniques have been used to identify parasites in tissue sections or tissue cultures; fluorescein isothiocyanate- or peroxidase-labeled antisera may be useful in detecting tachyzoites in tissue sections. Enzyme immunoassay (EIA) antigen detection techniques lack sensitivity for human samples and are not recommended.

Nucleic Acid Detection Techniques

PCR technology for *Toxoplasma* has been used to detect congenital infections, toxoplasmic encephalitis in AIDS patients, and ocular disease with various degrees of success. The most important use of PCR appears to be in the prenatal diagnosis of congenital toxoplasmosis. When maternal serological results indicate potential infection during pregnancy, PCR of amniotic fluid has been shown to be more sensitive for the confirmation of fetal infection than the conventional methods of inoculation of mice and tissue culture cells, and fetal blood testing for IgM (23, 36). PCR technology for *Toxoplasma* is offered at the Toxoplasma Serology Laboratory, Palo Alto, Calif., and by a few commercial laboratories. Commercial kits are not yet available. The requestor should be aware that the reliability of PCR tests may vary widely (32, 67).

ISOLATION PROCEDURES

Parasites can be isolated with limited success by inoculating patient tissue or body fluids into either mice or tissue culture cells. Fresh tissue samples are ground in saline with a mortar and pestle and inoculated into the peritoneum of mice or directly into tissue culture flasks. The mice should be monitored for 4 to 6 weeks; if the organism is virulent for mice, the parasites can often be demonstrated in the peritoneal fluid after 5 to 10 days. However, if the organism is relatively avirulent for mice, as is usually the case, the mice may not be killed by the infection. If they survive for 6 weeks, serum samples should be obtained for testing. If antibodies are present, the mouse brain should be examined for the presence of *T. gondii* cysts. If cysts are not observed, the murine host may not have been the ideal host. Inoculate additional mice with brain homogenate from the initially inoculated mice and observe and recheck after 6 weeks. *T. gondii* grows in a variety of tissue culture cells. A cytopathic effect may be detected on direct examination after 24 to 96 h in culture. Giemsa staining may reveal parasite structure, but parasitized cells may be difficult to detect. Immunofluorescence allows more sensitive detection of the organisms. The following procedure has been used with some success for parasite isolation from amniotic fluids (16). Centrifuge a 10-ml sample of amniotic fluid at 1,000 × *g*. Resuspend the sediment in 8 ml of minimum essential medium. Inoculate 1 ml into coverslip cultures of human embryonic fibroblast cell line MRC5 in 24-well plates. Incubate the cultures for 96 h with one change of medium at 24 h; fix the cultures with cold acetone. Examine the coverslips by indirect immunofluorescence for the presence of *T. gondii.* The use of tissue culture cells for isolation permits a more rapid diagnosis than mouse inoculation; both methods can be useful for diagnosing congenital toxoplasmosis.

SEROLOGIC TESTS

Many tests for the detection of antibodies to *Toxoplasma* have been used since Sabin and Feldman developed the methylene blue dye test (DT) (66). Commercial kits for agglutination tests, indirect fluorescent antibody (IFA) tests, and EIA are available worldwide. Because of difficulties in obtaining specimens from patients with clinically documented toxoplasmosis, commercial kit sensitivity and specificity may not be based on documented case specimens but rather on a comparison of results obtained with another kit. Consequently, the true sensitivity and specificity of a kit are generally not known or determined. The rates stated by the manufacturer or published in articles may vary depending on the samples chosen for testing. Sensitivity and specificity rates determined in prospective studies, when random samples are tested as received for *Toxoplasma* testing, will usually differ from those determined in retrospective studies, when the samples have been chosen as potential problem samples to increase the probability of detecting false-positive or false-negative reactions.

When laboratory personnel decide to initiate *Toxoplasma*-specific antibody testing or when they decide to switch to a different antibody detection kit, the user must carefully review the manufacturer's package insert and published literature for information on the sensitivity and specificity rates. The user should perform an in-laboratory comparison of kits by using positive and negative samples confirmed by a toxoplasmosis reference laboratory. Tables 1 and 2 list commercial kits currently available in the United States and references to published evaluations. However, the test kit industry is in a great deal of flux; company and kit names may change.

In the United States, initial testing for the presence of IgG antibodies in most laboratories is usually performed with an EIA or IFA commercial kit. Results may be stated in international units (based on the WHO international standard reference serum for *Toxoplasma* (68) distributed by the PHLS Reference Unit, Public Health Laboratory, Singleton Hospital, Swansea SA2 8QA, Wales, United Kingdom), as an index (specific to each kit), as an optical density value (specific to each kit), or as a geometric mean titer. Numerical results are not comparable from kit to kit; comparison may be made only qualitatively as negative (nonreactive or not infected) or positive (reactive or infected). Although elevated *Toxoplasma*-specific IgG levels have been suggested as an indicator of recent infection, high levels may last for many years after primary infection and should not be relied upon for this purpose.

To more definitely distinguish acute and chronic infections, detection of *Toxoplasma*-specific IgM antibodies has been used. The most important use of IgM test results is that a negative reaction essentially excludes recent infection. A guide to the general interpretation of *Toxoplasma* IgG and

TABLE 1 Toxoplasma IgG kits available commercially in the United States

Type of test and company[a]	Kit	Reference(s)
IFA		
GenBio	ImmunoFA Toxoplasma IgG	
Hemagen	VIRGO Toxo IgG	
Inverness	Toxoplasma IgG	
Meridian	Toxoplasma IgG	
EIA		
Abbott Labs	IMx Toxo IgG	15, 25, 33, 35, 46, 65
	AxSYM Toxo IgG	18, 29, 62, 77
Bayer Diagnostics	Immuno1	65
	ADVIA Centaur	60
Beckman Coulter	ACCESS Toxo G	15
bioMérieux Vitek	VIDAS Toxo IgG	25, 29, 35, 62, 77, 83
Bio-Rad	Platelia Toxo G	35, 62
Biotecx	OptiCoat Toxo IgG ELISA	
Biotest Diagnostics	Toxo IgG	
Diagnostic Products Corp.	IMMUNLITE Toxoplasma IgG	18, 21, 83
Diamedix	Toxoplasma IgG	
DiaSorin	Toxoplasma IgG	
	LIAISON	62
GenBio	ImmunoDOT TORCH	
Hemagen	Toxoplasma IgG	
Trinity Biotech	CAPTIA Toxoplasma gondii IgG	
Inverness	Toxo IgG II	
Latex		
Biokit	Toxogen	

[a] Abbott Labs, Diagnostics Division, North Chicago, Ill.; Bayer Diagnostics, Tarrytown, N.Y.; Beckman Coulter, Fullerton, Calif.; Biokit USA, Lexington, Mass.; bioMérieux, Hazlewood, Mo.; BioRad, Hercules, Calif.; Biotecx Laboratories, Houston, Tex.; Biotest Diagnostics Corp., Denville, N.J.; Diagnostic Products Corp., Los Angeles, Calif.; Diamedix Corp., Miami, Fla.; DiaSorin, Stillwater, Minn.; GenBio, San Diego, Calif.; Hemagen Diagnostics, Waltham, Mass.; Inverness Medical Professional Diagnostics, Princeton, N.J.; Meridian Bioscience, Cincinnati, Ohio.

IgM serology results is presented in Table 3. IFA IgM titers generally increase within 1 week of the onset of symptoms and revert to negative within 6 to 9 months of infection. False-positive reactions caused by rheumatoid factor and false-negative reactions caused by blockage by *Toxoplasma*-specific IgG may occur in IFA IgM and indirect EIA for IgM when whole-serum samples are tested. To decrease the effects of these interfering factors, specimens should be treated to obtain only the IgM fraction for testing.

The IgM capture EIA eliminates potential interference by IgG and other isotypes by binding only IgM antibodies; unbound antibodies are removed by washing, thus eliminating the need for serum fractionation. The most important advantage of the IgM capture EIA compared to IFA is the increased detection of congenital infections: the IgM enzyme-linked immunosorbent assay (ELISA) was positive for 73% of serum samples from newborn infants with proven congenital toxoplasmosis, whereas only 25% of the same serum samples were found positive by an IFA IgM test (59). Although the capture EIA system is more efficient at detecting acute infections, some persons may have undetectable or low-level IgM antibodies, and some persons may have detectable IgM antibodies beyond 2 years postinfection (30). Therefore, determining the relative time of infection is not possible with this system alone. Many commercial companies market an EIA kit for IgM: some use the indirect EIA format with a serum pretreatment step, while others use the IgM capture format. False-positive IgM reactions due to unknown factors may be a problem with commercially available kits (85).

The Toxoplasma IgG avidity test is an additional tool to help rule out that a patient's infection occurred within the prior 4 to 5 months (67). Results are based on the measurement of the avidity of *Toxoplasma*-specific IgG antibodies. Depending on the method used, a high avidity result indicates infection acquired more than 3 to 5 months before. However, a low avidity result does not indicate a recently acquired infection because low avidity antibodies may be detectable for a year postinfection. Commercial kits are not available in the United States but are available in other countries from Ani Labsystems (2), bioMérieux (56), and DiaSorin (62).

Other tests may be of assistance in determining current infection. Assays for *Toxoplasma*-specific IgA antibodies should always be performed in addition to IgM assays for newborns suspected of congenital infection (13, 84). The presence of *Toxoplasma*-specific IgE antibodies may also contribute to the determination of acute infections (22). Immunoblot assays may be useful in determining congenital infections (63) and ocular infections (69). These and other assays (45, 53) are available in the United States at the Toxoplasma Serology Laboratory, Palo Alto Medical Foundation, Palo Alto, Calif. (phone: 650-853-4828) and at many of the Toxoplasma Reference Laboratories in Europe.

A guideline for the clinical use and interpretation of serologic tests for *T. gondii* was published (9) by the Clinical and

TABLE 2 Toxoplasma IgM kits available commercially in the United States

Type of test and company[a]	Kit	Reference(s)
IFA		
GenBio	ImmunoFA Toxoplasma IgM	
Hemagen	VIRGO Toxo IgM	
Inverness	Toxoplasma gondii IgM	
Meridian	Toxoplasma IgM	
EIA		
Abbott Labs	IMx Toxo IgM	15, 35, 46, 50, 85
	AxSYM Toxo IgM	14, 29, 62
Bayer Diagnostics	Immuno1	
	ADVIA Centaur	60
Beckman Coulter	ACCESS Toxo M	14, 15, 20, 48
bioMérieux Vitek	VIDAS Toxo IgM	3, 14, 29, 35, 62, 85
Bio-Rad	Platelia Toxo IgM	15, 35, 47, 62, 85
Biotecx	OptiCoat Toxo IgM	
Biotest Diagnostics	Toxo IgM	
Diagnostic Products Corp.	IMMULITE	21, 83
Diamedix	Toxoplasma IgM	79
DiaSorin	Toxoplasma IgM	
	LIAISON	62
Hemagan	Toxoplasma IgM	
Trinity Biotech	CAPTIA Toxoplasma gondii IgM	
Inverness	Toxo IgM	

[a]For information on companies, see Table 1, footnote *a*.

Laboratory Standards Institute (formerly called the National Committee for Clinical Laboratory Standards or NCCLS) and is available for purchase on their website at http://www.clsi.org.

CLINICAL USE OF IMMUNODIAGNOSTIC TESTS

Determination of Immune Status

An algorithm for serological testing for immune status and acute acquired infection is shown in Fig. 2. Three situations in which baseline information about an individual's immune status would be useful include the following: (i) before conception; (ii) before receiving immunosuppressive therapy; and (iii) after the initial determination of positive HIV-1 status. Screening one serum specimen with a sensitive test for IgG antibodies, such as DT, IFA, or EIA, is sufficient. A negative test result indicates that the patient has not been infected. A positive result of any degree indicates infection with *T. gondii* at some undetermined time.

Diagnosis of Acute Acquired Infections

If an acute acquired infection is suspected, the patient's serum specimen should be tested for the presence of *Toxoplasma*-specific antibodies (Fig. 2). A negative result in DT, IgG IFA test, or IgG EIA essentially excludes the diagnosis of acute *Toxoplasma* infection in an immunocompetent person. Demonstration of seroconversion from a negative titer to a positive titer or of more than a fourfold increase in titer confirms the diagnosis when specimens drawn several weeks apart are tested at the same time with the same test. However, such situations are rare because specimens are usually drawn after titers have peaked, too late to observe titer changes after initial infection. Results of an EIA for IgM and an IgG avidity assay can provide additional evidence for or against acute infection when IgG antibodies are present (72). A negative IgM test essentially rules out infection in the previous 6 months. A positive IgM titer combined with a positive IgG titer may be suggestive of acute infection, due to persistent IgM antibodies, or may be a false-positive reaction. If the patient is pregnant, an IgG avidity test should then be performed. A high avidity result in the first 12 weeks of pregnancy essentially rules out an infection acquired during gestation. A low IgG avidity result cannot be used as an indicator of recent infection, because some individuals have persistent low IgG avidity for many months after infection. Immunodiagnosis of acute infection in a pregnant woman should be confirmed by a toxoplasmosis reference laboratory prior to intervention (45).

The presence of typical lymphadenopathy suggestive of acute toxoplasmosis, the presence of a high DT or IgG IFA titer (≥300 IU/ml or ≥1:1,000), and the presence of specific IgM are indicative of acute infection (54, 57). If the patient has clinical illness compatible with toxoplasmosis but the IgG titer is low, a follow-up test 3 weeks later should show an increase in the antibody titer if the illness is due to acute toxoplasmosis and the host is not severely immunosuppressed.

Diagnosis of Congenital Infection

Diagnosis of congenital toxoplasmosis involves diagnosing acute infection in a pregnant woman, demonstrating infection in the fetus, or documenting infection in the newborn infant (66). Congenital toxoplasmosis occurs when a woman passes the infection to her fetus after acquiring a primary infection during pregnancy or, more rarely, when a pregnant woman is immunocompromised and a previously acquired infection is reactivated. The rate of transmission of infection

TABLE 3 Guide to general interpretation of *Toxoplasma* serology results obtained with IgG and IgM commercial assays

IgG result	IgM result	Report/interpretation for humans except infants
Negative	Negative	No serological evidence of infection with *Toxoplasma*.
Negative	Equivocal	Possible early acute infection or false-positive IgM reaction. Obtain a new specimen for IgG and IgM testing. If results for the second specimen remain the same, the patient is probably not infected with *Toxoplasma*.
Negative	Positive	Possible acute infection or false-positive IgM result. Obtain a new specimen for IgG and IgM testing. If results for the second specimen remain the same, the IgM reaction is probably a false positive.
Equivocal	Negative	Indeterminate: obtain a new specimen for testing or retest this specimen for IgG in a different assay.
Equivocal	Equivocal	Indeterminate: obtain a new specimen for both IgG and IgM testing.
Equivocal	Positive	Possible acute infection with *Toxoplasma*. Obtain a new specimen for IgG and IgM testing. If results for the second specimen remain the same or if the IgG becomes positive, both specimens should be sent to a reference laboratory with experience in the diagnosis of toxoplasmosis for further testing.
Positive	Negative	Infected with *Toxoplasma* for more than 1 year.
Positive	Equivocal	Infected with *Toxoplasma* for probably more than 1 year or false-positive IgM reaction. Obtain a new specimen for IgM testing. If results with the second specimen remain the same, both specimens should be sent to a reference laboratory with experience in the diagnosis of toxoplasmosis for further testing.
Positive	Positive	Possible recent infection within the last 12 months, or false-positive IgM reaction. Send the specimen to a reference laboratory with experience in the diagnosis of toxoplasmosis for further testing.

to the fetus ranges from 11% in the first trimester to 90% in the late third trimester, with an overall transmission rate of approximately 40 to 50%.

In France and Austria, the prevention, diagnosis, and treatment of congenital toxoplasmosis begin with mandatory serologic testing of all women before or soon after conception. Although the cost-effectiveness of adopting this approach for all pregnant women in the United States has not been established, this approach does serve as a model for managing individual pregnant patients (11, 34, 36, 64).

Immunocompetent women who have IgG antibody before conception are considered immune and so at very little risk for transmission of infection to the fetus. Women who are seronegative are considered at risk for infection and in France are tested monthly during pregnancy for IgG antibody. If a woman is first tested after conception and has *Toxoplasma*-specific IgG antibodies, IgM and IgG avidity testing should be done to determine if acute infection has occurred during pregnancy (72) (Fig. 2). When the diagnosis of acute toxoplasmosis has been made for a pregnant woman, she can be treated and the fetus can be tested for evidence of infection. The strategy used by Daffos et al. (11) involved initiating treatment with spiramycin once acute maternal infection was indicated and then obtaining amniotic fluid and fetal blood samples between 20 and 26 weeks of gestation for testing (Fig. 3). Amniotic fluid PCR is the recommended test of choice to establish the intrauterine diagnosis of congenital toxoplasmosis (73, 74, 82). In addition, fetal ultrasound examinations were performed every 2 to 4 weeks until delivery to search for several nonspecific signs of infection: cerebral or hepatic calcifications, hydrocephalus, hepatomegaly, or ascites.

If collected, fetal blood should be tested for *Toxoplasma*-specific IgG, IgM, and IgA antibodies. Clotted blood should be inoculated into mice or tissue culture cells to demonstrate parasitemia. Nonspecific markers of infection should be tested for; these include leukocytes, eosinophils, platelets, total IgM, gamma-glutamyltransferase, and lactate dehydrogenase. Most infected fetuses have one or more abnormal nonspecific tests, most commonly an elevated total IgM level or an elevated gamma-glutamyltransferase level (11, 34, 64). Demonstrating *Toxoplasma*-specific IgM or IgA antibodies in fetal serum or isolating the parasite from fetal leukocytes is a definitive diagnosis of fetal infection.

Diagnosis of Infection in the Newborn

Diagnosis of *Toxoplasma* infection in the newborn is made through a combination of serologic testing, parasite isolation, and nonspecific findings (51, 58, 63, 64, 71). An attempt should be made to isolate *T. gondii* from the placenta, amniotic fluid, and cord blood if the diagnosis has not already been established (Fig. 4). *T. gondii* has been isolated from 95% of the placentas of congenitally infected newborns when the mother has not been treated and from approximately 81% when the mother has been treated. However, *T. gondii* can be isolated from the placentas of uninfected newborns as well. The child's serum should be tested for total IgG and IgM antibody levels and *Toxoplasma*-specific IgG, IgM, and IgA antibodies. CSF should be analyzed for cells, glucose, protein, total IgG antibody, and *Toxoplasma*-specific IgG and IgM antibodies and should be directly examined for *T. gondii* tachyzoites. A child suspected of having congenital toxoplasmosis should have a thorough general, neurologic, and ophthalmologic examination and a computed tomographic scan of the head (magnetic resonance imaging does not demonstrate calcifications). Because the diagnosis can take several months to confirm, clinicians may have to treat patients based upon early signs, symptoms, and serology while awaiting definitive confirmation. Although the complexity of diagnosing congenital infection necessitates the use of multiple costly laboratory tests, the benefit of early diagnosis and treatment and the cost of unnecessary treatment justify establishing the correct diagnosis.

Persistent or increasing IgG antibody levels in the infant compared with the mother as measured by the DT or IFA test, and/or positive result for *Toxoplasma*-specific IgM or IgA are diagnostic of congenital infection. Demonstration by immunoblotting that the newborn has serum antibodies that are directed against unique *Toxoplasma* epitopes not found in the mother's serum is also evidence of congenital infection (67, 70).

Placental leak can occasionally lead to false-positive IgM measurements in the newborn. However, because the half-life of IgM is 3 to 5 days, repeat testing at 1 week should

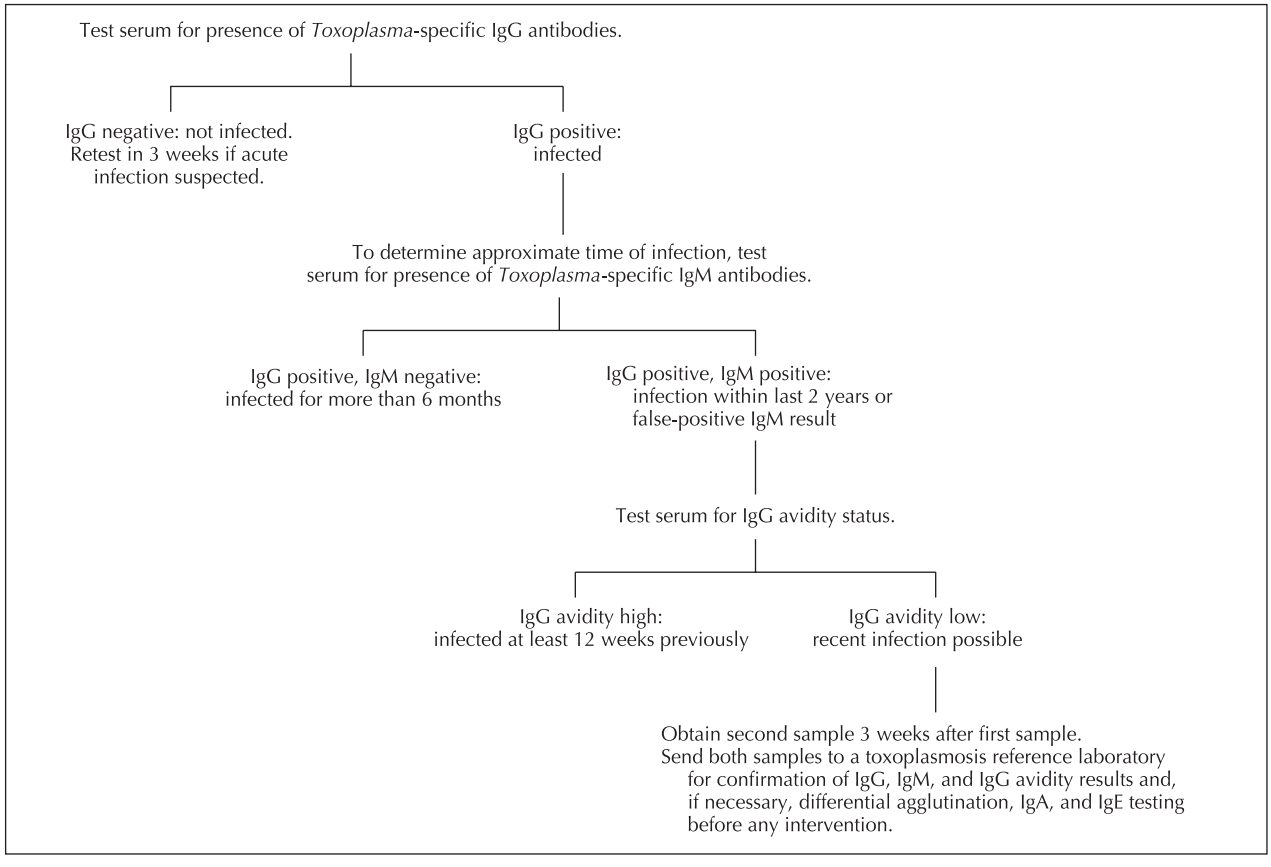


FIGURE 2 Algorithm for the serodiagnosis of toxoplasmosis in people greater than 1 year of age.

show a significant reduction in antibody titer if the child is not infected. Passively transferred maternal IgG has a half-life of approximately 1 month. Maternal antibodies can be detected for several months and have been reported up to 1 year of age. The untreated congenitally infected newborn will begin to produce *Toxoplasma*-specific IgG antibody within approximately 3 months. Treatment of the infected child may delay antibody production until 9 months of age and on rare occasion may prevent production altogether. Persistence of a positive IgG result for a child 12 months old confirms infection. Demonstration of a decrease in antibody load (*Toxoplasma*-specific IgG antibody divided by total IgG) can be helpful in differentiating maternal antibody from fetal antibody.

Although rarely performed, demonstration of IgM antibody or local *Toxoplasma*-specific IgG antibody production in CSF not contaminated with peripheral blood can help to confirm the diagnosis of congenital toxoplasmosis. The calculation is

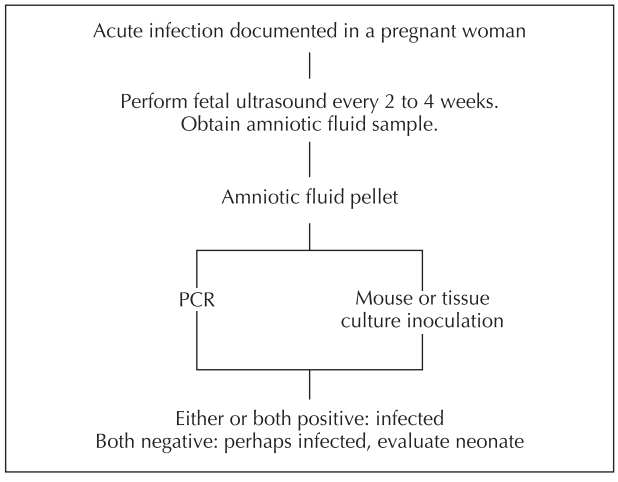


FIGURE 3 Algorithm for the diagnosis of antenatal congenital toxoplasmosis.

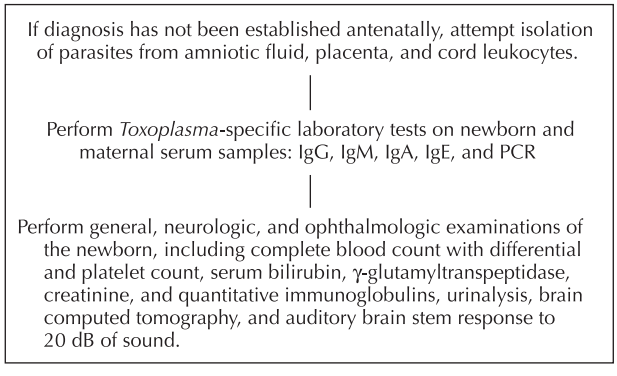


FIGURE 4 Algorithm for the diagnosis of neonatal toxoplasmosis.

made by dividing the *Toxoplasma*-specific antibody titer in the body fluid by the *Toxoplasma*-specific antibody titer in the serum and multiplying the result by the concentration of gamma globulin in serum divided by the concentration of gamma globulin in the body fluid. A result of four or greater corresponds to significant antibody production.

A long-term prospective study is currently under way in the United States to define optimal therapeutic regimens for the treatment of congenital toxoplasmosis (1, 51). Clinicians should contact Rima McLeod, University of Chicago Hospitals, Chicago, Ill. (phone: 773-834-4152), regarding the treatment of infected children.

Diagnosis of Ocular Infection

Toxoplasma chorioretinitis results from both acute infection and congenital infection (37, 38). In addition to demonstrating IgG antibody to *Toxoplasma* in the serum of a person with compatible eye lesions, demonstration of the local production of antibody and detection of parasite DNA in aqueous humor have been used to document active ocular toxoplasmosis (26). When the antibody load formula described in "Diagnosis of Infection in the Newborn" above is used to calculate results obtained in eye fluids and with results obtained in serum, a value of 8 or greater suggests acute ocular toxoplasmosis. If the serum DT titer is greater than 1:1,000, it is usually not possible to calculate local antibody production.

Diagnosis of Infection in the Immunocompromised Host

A wide variety of immunosuppressed hosts, including patients with lymphoma, leukemia, multiple myeloma, carcinoma, neuroblastoma, thymoma, systemic lupus erythematosus, scleroderma, and autoimmune hemolytic anemia and patients with kidney, liver, and heart transplants, have been described as having severe, often fatal, toxoplasmosis. The disease is most often related to reactivation of latent infection and commonly involves the CNS, although a wide spectrum of clinical manifestations have been reported. Diagnosis can be very difficult for these patients, as IgM antibody is usually not detectable and the presence of IgG antibody only confirms chronic infection. In the absence of serologic evidence of acute infection, diagnosis can be confirmed by demonstration of the organism histologically or cytologically as replicating within tissue or by isolation or identification of its nucleic acids in a site such as amniotic fluid, CSF, bronchoalveolar fluid, or placenta, in which the encysted organism would not be present as part of a latent infection.

Persons undergoing organ or bone marrow transplantation can benefit from pretransplant testing for *Toxoplasma*-specific IgG antibodies to determine immune status because they are at risk for either acute acquired infection if they are seronegative before transplantation, or at risk for reactivation if they are seropositive before transplantation (78). Those with acute acquired infection usually develop detectable *Toxoplasma*-specific IgG and IgM antibodies, while those with reactivation do not have a detectable *Toxoplasma*-specific IgM response. Seronegative transplant recipients of hearts from seropositive donors can develop toxoplasmic myocarditis that mimics organ rejection.

Toxoplasmic encephalitis is the most frequent CNS opportunistic infection of AIDS patients and is uniformly fatal if untreated. Among AIDS patients who died from 1992 through 1997 in the United States, 7.2% developed toxoplasmic encephalitis during the course of the syndrome (41). It is recommended that all HIV-infected persons be tested for *Toxoplasma*-specific IgG antibodies soon after the diagnosis of HIV infection to detect latent infection (4). If they are *Toxoplasma* seropositive, adult or adolescent patients who have a $CD4^+$ T-lymphocyte count of $<100/\mu l$ should be administered prophylaxis against toxoplasmic encephalitis with trimethoprim-sulfamethoxazole (TMP-SMZ). Most AIDS patients with toxoplasmic encephalitis have demonstrable IgG antibodies to *T. gondii*. However, approximately 3% of AIDS patients with toxoplasmic encephalitis do not have *Toxoplasma*-specific antibody in their serum. Local production of *Toxoplasma*-specific IgG antibody in CSF has been demonstrated for persons with AIDS and with toxoplasmic encephalitis (10). When the formula described above for toxoplasmosis in the newborn is used, a result of >1 corresponds to significant antibody production.

TREATMENT

In general, physicians treat *T. gondii* infection in four circumstances: (i) pregnant women with acute infection to prevent fetal infection (11, 17, 36); (ii) congenitally infected infants (31, 51); (iii) immunosuppressed persons, usually with reactivated disease (6, 7, 40, 49, 52); and (iv) acute and recurrent ocular disease (19, 37, 75, 76). Drugs are also prescribed for preventive or suppressive treatment in HIV-infected persons (4). The currently recommended drugs work primarily against the actively dividing tachyzoite form of *T. gondii* and do not eradicate encysted organisms (bradyzoites).

The most common drug combination used to treat congenital toxoplasmosis consists of pyrimethamine and a sulfonamide (sulfadiazine is recommended in the United States), plus folinic acid in the form of leucovorin calcium to protect the bone marrow from the toxic effects of pyrimethamine. Pyrimethamine inhibits dihydrofolate reductase, which is important in the synthesis of folic acid and produces a reversible depression of the bone marrow. Sulfonamines inhibit synthesis of dihydrofolic acid, also important in the synthesis of folic acid. The two drugs work synergistically against *T. gondii*. Due to toxicity, pyrimethamine and sulfadiazine therapy is generally recommended for use in pregnant women after the 17th week of gestation (55, 66). After the 18th week, pyrimethamine and sulfadiazine may be given if fetal infection is confirmed by amniocentesis or cordocentesis. Spiramycin (available through the FDA; phone: 301-796-1600) is recommended for pregnant women with acute toxoplasmosis when fetal infection has not been confirmed in an attempt to prevent transmission of *T. gondii* from the mother to the fetus (55, 66). Randomized prospective studies of treatment during acute infection in pregnant women have not been performed. Some researchers have questioned, or been unable to demonstrate, the effectiveness of treatment during pregnancy in preventing congenital infection (28, 84) or sequelae in infants (27). One hypothesis for the lack of effectiveness is that *T. gondii* tachyzoites transform into bradyzoites within days of infection, probably coinciding with the serological and cell-mediated responses (27). Therefore, by the time an infection is detected in a pregnant woman tachyzoites may have already been transmitted to the fetus, inflicted damage, and been converted to encysted bradyzoites that do not respond to therapy. Nevertheless, a multicenter observational study found that the treatment of acute *T. gondii* infection in pregnancy was associated with a reduction of sequelae in infants, but not a reduction in maternal-fetal transmission (24).

Pyrimethamine and sulfadiazine (plus leucovorin) are the drugs generally used to treat infants with congenital toxoplasmosis and have led to improved outcomes compared with historic controls (51, 66).

In immunosuppressed persons with toxoplasmosis, a regimen of pyrimethamine and sulfadiazine plus leucovorin is the preferred treatment (6, 7, 52, 55). Clindamycin is a second alternative for use in combination with pyrimethamine and leucovorin for those who cannot tolerate sulfonamides (6, 7, 12, 49, 55). Leucovorin prevents the hematologic toxicities associated with pyrimethamine therapy. Atovaquone in combination with either pyrimethamine or sulfadiazine has sufficient activity to be considered for treatment in some less severely affected adult patients (8, 55). The role of other drugs in the treatment of systemic toxoplasmosis has not been defined by controlled trials. In general, alternative drugs such as azithromycin, clarithromycin, and dapsone should be used in combination with another drug, preferably pyrimethamine (52).

Because relapse often occurs after toxoplasmosis in HIV-infected patients, maintenance therapy (secondary prophylaxis) with pyrimethamine plus sulfadiazine (first choice) or pyrimethamine plus clindamycin (alternative) is recommended for the patient (4). Secondary prophylaxis for toxoplasmosis may be discontinued in patients with a sustained increase in $CD4^+$ counts (e.g., 6 months) to >200 cells/μl in response to highly active antiretroviral therapy if the patient has completed the initial therapy and has no symptoms or signs attributable to toxoplasmosis. For prophylaxis to prevent an initial episode of *T. gondii* in *Toxoplasma*-seropositive persons with a $CD4^+$ T-lymphocyte counts less than 100 cells/μl, trimethoprim-sulfamethoxazole is recommended as the first choice with alternatives consisting of dapsone plus pyrimethamine, or atovaquone with or without pyrimethamine. Leucovorin is given with all regimens including pyrimethamine (4).

Pyrimethamine and sulfadiazine are often used for persons with ocular disease (38). Clindamycin, in combination with other antiparasitic medications, is also frequently prescribed for ocular disease (19). A variety of newer agents have been tried in the treatment of ocular toxoplasmosis including atovaquone (61), rifabutin, trovafloxacin, azithromycin, and clarithromycin (39). In addition to antiparasitic drugs, physicians may add corticosteroids to reduce ocular inflammation. However, the optimal treatment for ocular toxoplasmosis remains to be defined by controlled trials.

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Pathogenic and Opportunistic Free-Living Amebae

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BACKGROUND

Small, free-living amebae belonging to the genera *Naegleria, Acanthamoeba,* and *Balamuthia* have been identified as agents of central nervous system (CNS) infections of humans and other animals (6, 8, 10, 13, 17, 19, 23, 24, 26–31, 36, 43, 52, 53, 55). Only one species of *Naegleria (Naegleria fowleri)*, several species of *Acanthamoeba* (e.g., *Acanthamoeba castellanii, A. culbertsoni, A. hatchetti, A. healyi, A. polyphaga, A. rhysodes, A. astronyxis,* and *A. divionensis*), and the only known species of *Balamuthia, Balamuthia mandrillaris,* are known to cause disease. *Acanthamoeba* spp. also cause infection of the human cornea, *Acanthamoeba* keratitis (4, 11, 21, 35, 47, 48, 54, 56). Additionally, both *Acanthamoeba* spp. and *B. mandrillaris* have been identified as agents of cutaneous infections in humans (5, 32, 45, 46). According to a recent report, *Sappinia diploidea,* a free-living ameba normally found in soil contaminated with the feces of elk and buffalo, was identified in an excised brain lesion from a 38-year-old immunocompetent man who developed a bifrontal headache, blurry vision, and loss of consciousness following a sinus infection. This finding clearly indicates that there are probably other small amebae that may also cause encephalitis in humans (15).

N. fowleri and *Acanthamoeba* spp. are commonly found in soil, freshwater, sewage and sludge, and even dust in air. Several species of *Acanthamoeba* have also been isolated from brackish water and seawater and from ear discharges, pulmonary secretions, nasopharyngeal mucosa samples, maxillary sinus samples, mandibular autografts, and stool samples (9, 17, 24, 27, 28, 38, 42, 44, 54). These amebae normally feed on bacteria and multiply in their environmental niche as free-living organisms. *Acanthamoeba* spp. have also been known to harbor *Legionella* spp., *Mycobacterium avium,* and other bacterial pathogens such as *Listeria monocytogenes, Burkholderia pseudomallei, Vibrio cholerae,* and *Escherichia coli* serotype O157, which signifies a potential expansion of public health importance of these organisms (14, 17, 24, 54). Additionally, pure cultures of *A. polyphaga* have been used to isolate *Legionella pneumophila* (37), *Legionella anisa* (22), and recently *Mycobacterium massiliense* (1) from human clinical specimens such as sputa, liver and lung abscess specimens, and even human feces. Obligate intracellular pathogens such as *Chlamydia, Chlamydophila,* and *Chlamydia*-like bacteria have been found in ~5% of *Acanthamoeba* isolates, and *Chlamydophila pneumophila,* a respiratory pathogen, can survive and grow within *Acanthamoeba* (14). Whether endosymbiont-bearing *Acanthamoeba* strains serve as reservoirs for these bacteria, some of which are potential pathogens for humans, is unknown.

Although *B. mandrillaris* has been recently isolated from soil (12, 39), not much is known about the environmental niche of *B. mandrillaris* and its feeding habits. It is, however, believed that its habitat is similar to those of *Acanthamoeba* and *Naegleria.* Excellent reviews (17, 24, 28, 42, 54) and books (27, 44) have been published on the biology, disease potential, pathology, pathogenicity, and epidemiology of these amebae.

The concept that these small, free-living amebae may occur as human pathogens was proposed by Culbertson and colleagues, who isolated *Acanthamoeba* sp. strain A-1 (now designated *A. culbertsoni*) from tissue culture medium thought to contain an unknown virus (7). They also demonstrated the presence of amebae in brain lesions of mice and monkeys that died within a week after intracerebral inoculation with *A. culbertsoni.* Culbertson hypothesized that similar infections might exist in nature in humans. In 1965, Fowler and Carter were the first to describe a fatal infection due to free-living amebae in the brain of an Australian patient (6). The infection is now believed to have been due to *N. fowleri* (6).

NAEGLERIA MENINGOENCEPHALITIS

In 1966, Butt et al. (6, 27) described the first case of CNS infection caused by *N. fowleri* in the United States and coined the term "primary amebic meningoencephalitis" (PAM). PAM is an acute fulminating disease with an abrupt onset that occurs generally in previously healthy children and young adults who have had contact with freshwater about 7 to 10 days before the onset of symptoms. It is characterized by a severe headache, spiking fever, a stiff neck, photophobia, and a coma leading to death within 3 to 10 days after the onset of symptoms (6, 27, 28, 43). The portal of entry of the *N. fowleri* amebae is the nasal passages. When people swim in lakes and other bodies of freshwater that harbor these amebae, the amebae may enter the nostrils of the swimmers, make their way into the olfactory lobes via the cribriform plate, and cause acute hemorrhagic necrosis leading to destruction of the olfactory bulbs and the cerebral cortex. Only a few patients have survived. Upon autopsy, large numbers of amebic trophozoites, many with ingested erythrocytes and brain tissue, are usually seen interspersed with brain tissue (Fig. 1). It is believed that *N. fowleri* directly ingests brain tissue by

producing food cups, or amebostomes, as well as exerting contact-dependent cytolysis, possibly mediated by a multicomponent system consisting of a heat-stable hemolytic protein, a heat-labile cytolysin, and/or phospholipase enzymes (17, 25). Cysts of *N. fowleri* are not usually seen in brain tissue. It was believed until recently that *N. fowleri* infects only humans. In March 1997, however, a report of the first case of PAM in a South American tapir was published, indicating that PAM can occur in animals other than humans (23). A recent report of PAM in Holstein cattle, associated with drinking of surface waters, indicates that this disease is probably more common than is currently appreciated (51).

ACANTHAMOEBA ENCEPHALITIS

Several species of *Acanthamoeba (A. culbertsoni, A. castellanii, A. polyphaga, A. astronyxis, A. healyi,* and *A. divionensis)* have been known to cause a chronic granulomatous amebic encephalitis (GAE), primarily in immunosuppressed (either because of iatrogenic suppression or because of human immunodeficiency virus infection or AIDS), chronically ill, or otherwise debilitated persons with no previous history of exposure to recreational freshwater (27, 28, 31, 54). GAE has an insidious onset and is usually chronic, lasting for more than a week and sometimes even for months (10, 26–28, 30, 55). It is characterized by a headache, confusion, dizziness, drowsiness, seizures, and sometimes hemiparesis. Cerebral hemispheres are usually the most heavily affected CNS tissue. They are often edematous, with extensive hemorrhagic necrosis involving the temporal, parietal, and occipital lobes. Amebic trophozoites and cysts are usually scattered throughout the tissue. Many blood vessels are thrombotic with fibrinoid necrosis and cuffed by polymorphonuclear leukocytes, amebic trophozoites, and cysts (Fig. 2). Multinucleated giant cells forming granulomas may be seen in immunocompetent patients. Some patients, especially those with human immunodeficiency virus infection or AIDS, develop chronic ulcerative skin lesions, abscesses, or erythematous nodules (26–28, 32, 45, 46, 55). It is believed that the route of invasion of and penetration into the CNS is hematogenous, probably from a primary focus in the lower respiratory tract or the skin (26–28). *Acanthamoeba* spp. also cause infections of the CNSs of animals besides humans. Such infections have been recorded in gorillas, monkeys, dogs, ovines, bovines, horses, and kangaroos (28, 36).

Because of the confusion that existed in the earlier literature with regard to the nomenclature of *Acanthamoeba* and *Hartmannella* spp., some workers in the field referred to these amebae as members of the *Hartmannella-Acanthamoeba* group or simply as "*H-A* amebae." Since no true *Hartmannella* species has been found to be pathogenic to humans, all references in the literature to *Hartmannella* in human tissues should be corrected to read *Acanthamoeba* or *Balamuthia.*

BALAMUTHIA (LEPTOMYXID) ENCEPHALITIS

It was believed that all cases of GAE were caused by *Acanthamoeba* spp. Although a number of these cases were confirmed by serologic techniques as being caused by *Acanthamoeba* spp., the causative organisms in a few cases could not be definitively identified. Since cysts were found in the brain tissues of the patients, it was believed that the infections were caused by some other species of *Acanthamoeba* that did not cross-react with the anti-*Acanthamoeba* sera used in the serologic test (28, 54). *B. mandrillaris* (leptomyxid ameba) was definitively identified by an indirect immunofluorescence test as the causal agent in these cases (5, 8, 13, 19, 28, 36, 52, 53). The rabbit anti-*B. mandrillaris* serum used in the indirect immunofluorescence test was made by using culture-derived *Balamuthia* organisms that were isolated from the CNS tissue of a baboon (52). It is now known that a number of primates, including gorillas, gibbons, baboons, orangutans, and monkeys, as well as dogs, sheep, and horses, have died of CNS infections caused by *B. mandrillaris* (13, 28, 29, 36, 52). According to a recent report, Hispanic Americans have a high incidence of *B. mandrillaris* infection. It is not clearly known whether this is due to environmental factors, genetic predisposition, limited access to medical care, or other socioeconomic factors and pressures (40).

The pathology and pathogenesis of *B. mandrillaris*-induced GAE are similar to those of *Acanthamoeba*-induced GAE. Both trophozoites and cysts are found in CNS tissue (Fig. 3), and their sizes overlap those of *Acanthamoeba* trophozoites and cysts (13, 28, 29, 36, 52, 53). Hence, it is difficult to differentiate *Balamuthia* from *Acanthamoeba* spp. in tissue sections under the light microscope. In some cases, *Balamuthia* trophozoites in tissue sections appear to have more than one nucleolus in the nucleus (Fig. 3). In such cases, it may be possible to distinguish *Balamuthia* amebae from *Acanthamoeba* organisms on the basis of nuclear morphology, since *Acanthamoeba* trophozoites have only one nucleolus. In most cases, electron microscopy, immunohistochemical techniques, or both are necessary to identify *Balamuthia* organisms. Ultrastructurally, the cysts are characterized by three layers in the cyst wall: an outer wrinkled ectocyst, a middle structureless mesocyst, and an inner thin endocyst (52, 53). *Balamuthia* amebae are antigenically distinct from *Acanthamoeba* organisms; they can easily be distinguished by immunofluorescence or other immunochemical assays (Fig. 4) (8, 13, 19, 28, 29, 36, 52, 53).

ACANTHAMOEBA KERATITIS

Acanthamoeba spp. also cause a painful vision-threatening disease of the human cornea, *Acanthamoeba* keratitis. If the infection is not treated promptly, it may lead to ulceration of the cornea, loss of visual acuity, and eventually blindness and enucleation (4, 11, 18, 23, 35, 47, 48, 56). The first case of *Acanthamoeba* keratitis in the United States was reported in 1973 in a south Texas rancher with a history of trauma to his right eye (18). Both trophozoite and cyst stages of *A. polyphaga* were demonstrated to be present in corneal sections and were repeatedly cultured from corneal scrapings and biopsy specimens. Between 1973 and July 1986, 208 cases were diagnosed and reported to the Centers for Disease Control (47, 48, 54). The numbers of cases increased gradually between 1973 and 1984, and a dramatic increase began in 1985. An in-depth epidemiologic and case control study (47, 48) revealed that a major risk factor was the use of contact lenses, predominantly daily-wear or extended-wear soft lenses, and that patients with *Acanthamoeba* keratitis were significantly more likely than controls to use homemade saline solution instead of commercially prepared saline (78 and 30%, respectively), to disinfect their lenses less frequently than recommended by the lens manufacturers (72 and 32%), and to wear their lenses while swimming (63 and 30%). *Acanthamoeba* keratitis is characterized by severe ocular pain, a 360° or partial paracentral stromal ring infiltrate, recurrent corneal epithelial breakdown, and a corneal lesion refractory to the commonly used ophthalmic antibacterial medications. *Acanthamoeba* keratitis in the early stages is frequently misdiagnosed as herpes simplex virus keratitis because of the irregular epithelial lesions, stromal infiltrative keratitis, and edema

that are commonly seen in herpes simplex virus keratitis (18, 56). A nonhealing corneal ulcer is often the first clue that *Acanthamoeba* keratitis may be the problem. It is estimated that as of August 2005, more than 4,000 cases of *Acanthamoeba* keratitis have occurred in the United States. Since the diseases caused by *Acanthamoeba* are not reportable in the United States, the actual number may be much higher.

MORPHOLOGY

N. fowleri in its trophic form is a small, slug-like ameba measuring 10 to 35 μm long that exhibits an eruptive locomotion by producing smooth hemispherical bulges. The posterior end, termed the uroid, appears to be sticky and often has several trailing filaments. During its life cycle, this ameba produces a transient pear-shaped biflagellate stage, resulting from altered environmental conditions, and smooth-walled cysts (Fig. 5 to 8). The flagellates do not have cytostomes (mouths). Cysts are usually spherical and measure 7 to 15 μm, and the cyst wall may have one or more pores plugged with a mucoid material (34).

An *Acanthamoeba* organism is a slightly larger ameba (15 to 45 μm) that produces from the surface of its body fine, tapering, hyaline projections called acanthopodia (Fig. 9). It has no flagellate stage but produces a double-walled cyst (10 to 25 μm) (Fig. 10) with a wrinkled outer wall (the ectocyst) and a stellate, polygonal, or even round inner wall (the endocyst) (34).

Balamuthia trophozoites are in general irregular in shape; a few, however, may be slug-like. Actively feeding amebae may be 12 to 60 μm long, with a mean length of 30 μm (Fig. 11). The trophic forms, while feeding on tissue culture cells, produce broad pseudopodia without any clearly discernible movement. However, when tissue culture cells are destroyed, the trophozoites resort to a spider-like walking movement by producing finger-like determinate pseudopodia (53). Like *Acanthamoeba, Balamuthia* does not have a flagellate stage. Cysts are generally spherical and measure 6 to 30 μm in diameter (Fig. 12). Under the light microscope, each cyst appears to have an irregular and slightly wavy outer wall and a round inner wall. A layer of refractile granules immediately below the inner cyst wall is often seen in mature cysts. Under the electron microscope, the cyst wall can be seen to consist of three walls: a thin, irregular outer ectocyst; a thick, electron-dense inner endocyst; and a middle amorphous layer, the mesocyst (52, 53).

Acanthamoeba, Balamuthia, and *Naegleria* are predominantly uninucleate, although binucleate forms are occasionally seen. The nucleus is characterized by a large, dense, centrally located nucleolus. *Naegleria* amebae exhibit a promitotic pattern of cell division wherein the nucleolus and the nuclear membrane persist during nuclear division. *Acanthamoeba* spp., however, divide by conventional mitosis, in which the nucleolus and the nuclear membrane disappear during cell division. The pattern of nuclear division in *Balamuthia* is termed metamitosis: the nuclear membrane breaks down, and the nucleolus eventually disappears (53).

CLINICAL AND LABORATORY DIAGNOSIS

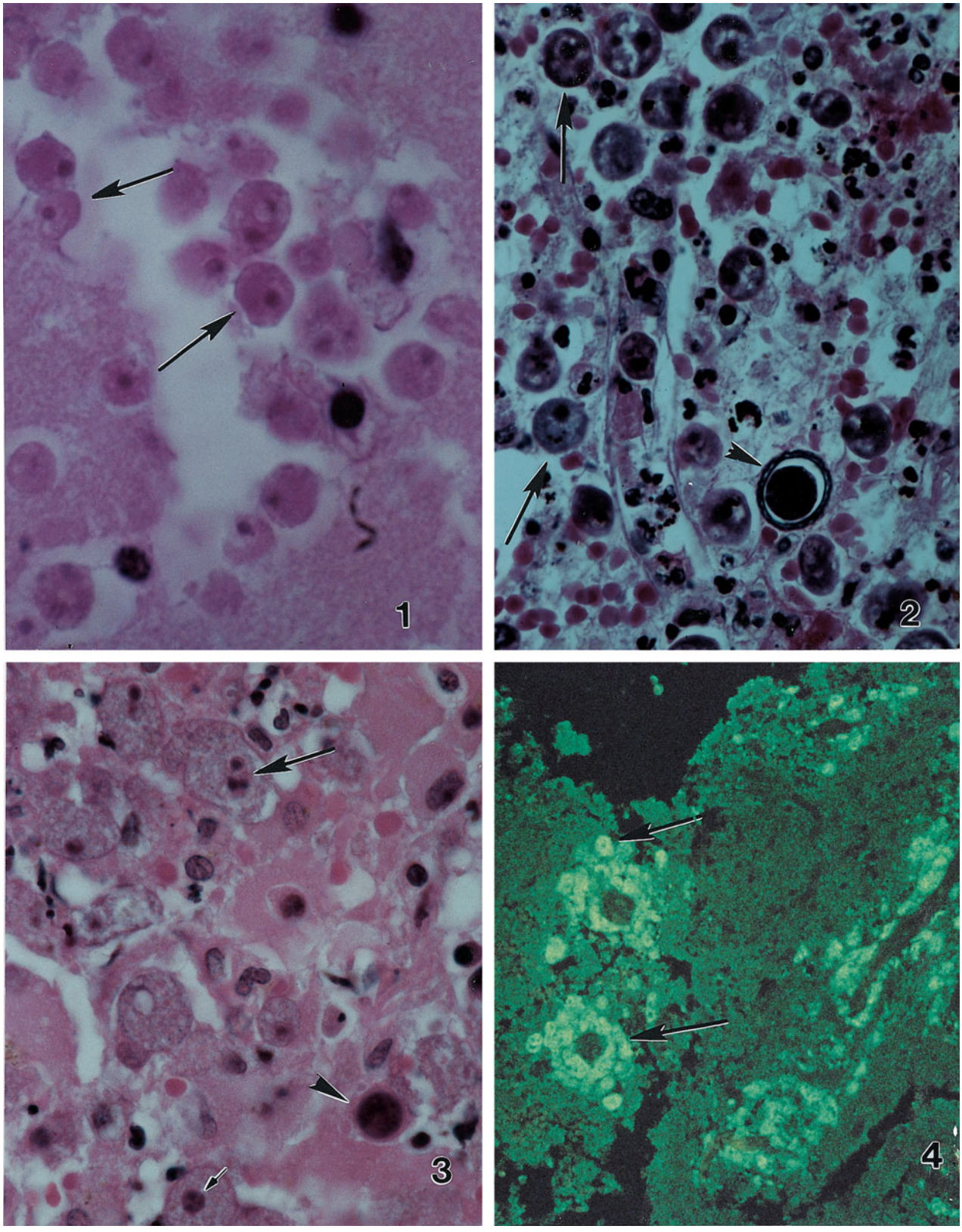
No distinctive clinical features differentiate PAM from pyogenic or bacterial meningitis. In PAM the cerebrospinal fluid (CSF) is usually pleocytotic, with a preponderance of polymorphonuclear leukocytes and no bacteria. The CSF pressure may be elevated. The CSF glucose level may be normal or slightly reduced, but the CSF protein level is increased, ranging from 1 to 10 mg/ml. Microscopic detection of amebic organisms in the CSF is the only means of diagnosing PAM. CSF should be examined in situ microscopically for the presence of *N. fowleri* amebae with directional movement. Giemsa or trichrome staining should be performed on CSF smears to visualize the nuclear morphology of the amebae. Gram staining is not useful in the detection of amebae. Further, a false-positive Gram stain may lead to inaccurate diagnosis and hence to inappropriate therapy, which will result in death. A recent report describing the transplantation of kidneys and a liver from a donor infected with *N. fowleri* (20) underscores the importance of a correct and timely diagnosis. Sequencing of the 5.8S rRNA gene and the internal transcribed spacers 1 and 2 (ITS1 and ITS2) of *N. fowleri* can not only differentiate *N. fowleri* from other *Naegleria* spp. but can also be used in the genotypic analysis of *N. fowleri* strains (58). However, this methodology has not been used to identify *N. fowleri* infections in clinical cases. *Acanthamoeba* trophozoites can be identified, although rarely, in the CSF, but *Balamuthia* organisms have never been seen in the CSF. They have, however, been identified in stained brain and/or skin biopsy specimens and also have been isolated in culture (5, 10, 19, 28, 36, 52, 53). *Acanthamoeba* organisms have also been recovered and identified from corneal scrapings as well as biopsy samples. Recently, direct examination of a patient's cornea with the confocal microscope has been used to diagnose *Acanthamoeba* keratitis (35). A large number of studies have sequenced the 18S rRNA gene to understand the phylogeny of *Acanthamoeba*. However, only a few studies have used this technique to identify *Acanthamoeba* keratitis or *Acanthamoeba* GAE by using patient specimens (3, 49). Similarly, molecular techniques have rarely been used to diagnose *B. mandrillaris* infections, although PCR technology is available to specifically identify *Balamuthia* DNA from cultured organisms as well as from tissue specimens (2, 13, 57). Efforts are, however, under way to develop real-time PCR protocols to simultaneously identify *Acanthamoeba, Balamuthia,* and *N. fowleri* in CSF specimens (35a; Y. Qvarnstrom, G. S. Visvesvara, R. Sriram, B. Barnes, and A. J. da Silva, *Proc. XIth Int. Meet. Biol. Pathog. Free-Living Amoebae*, České Budějovice, Czeck Republic, 2005).

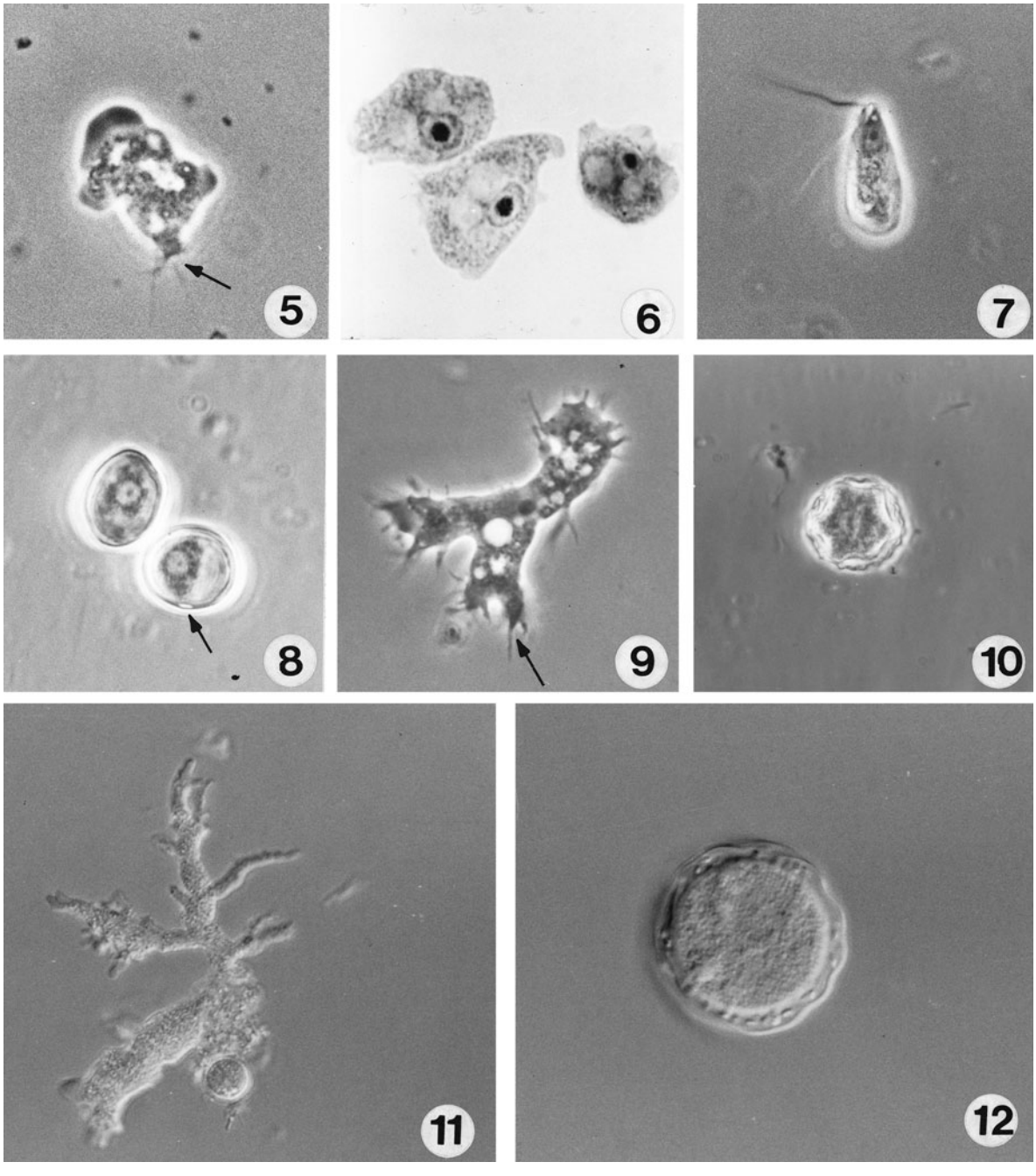
FIGURE 1 Large numbers of *N. fowleri* trophozoites (arrows) in a section of CNS tissue, showing extensive necrosis and destruction of brain tissue. Magnification, ×742.

FIGURE 2 *A. culbertsoni* trophozoites (arrows) and a cyst (arrowhead) around a blood vessel in a section of CNS tissue from a GAE patient. Magnification, ×643.

FIGURE 3 *B. mandrillaris* trophozoites and a cyst (arrowhead) in a brain section from a GAE patient. Note the double (small arrow) and triple (large arrow) nucleolar elements within the nuclei of the trophozoites. Magnification, ×544.

FIGURE 4 Immunofluorescence localization of *B. mandrillaris* in a brain section from a GAE patient. Note the fluorescent amebae (arrows) around blood vessels. Magnification, ×247.


1
2
3
4



FIGURES 5 through 8 *N. fowleri.* (Figure 5) Trophozoite, phase contrast (note the uroid and filaments at arrow); (Figure 6) trophozoite, trichrome stain; (Figure 7) biflagellate, phase contrast; (Figure 8) smooth-walled cyst, phase contrast (note the pore at the arrow). All magnifications, ×1,100.

FIGURES 9 and 10 *A. castellanii.* (Figure 9) Trophozoite, phase contrast (note the acanthopodia at the arrow); (Figure 10) double-walled cyst, phase contrast. Both magnifications, ×1,100.

FIGURES 11 and 12 *B. mandrillaris.* (Figure 11) Trophozoite, phase contrast; (Figure 12) cyst, phase contrast. Both magnifications, ×1,500.

COLLECTION, HANDLING, AND STORAGE OF SPECIMENS

For isolation of the etiologic agent, CSF, small pieces of tissue (brain, lung, skin, or corneal biopsy material), or corneal scrapings from the affected area must be obtained aseptically. The specimens should be kept at room temperature (24 to 28°C) and should never be frozen. The specimens may be kept at 4°C for short periods but never for more than 24 h. Personnel handling the specimens must take appropriate precautions, such as wearing surgical masks and gloves and

working in a biological safety cabinet. Remaining tissues must be preserved in 10% neutral buffered Formalin so that they can be examined histologically for amebae (50).

METHODS OF EXAMINATION

Direct Examination

Direct examination of the sample as a wet mount preparation is of paramount importance in the diagnosis of PAM and other diseases caused by these amebae. Since the amebae tend to attach to the surface of the container, the container should be shaken gently; then, a small drop of fluid is placed on a clean microscope slide and covered with a no. 1 coverslip. The CSF may have to be centrifuged at 500 × *g* for 5 min to concentrate the amebae. After the specimen has been centrifuged, most of the supernatant is carefully aspirated and the sediment is gently suspended in the remaining fluid. A drop of this suspension is prepared as described above for microscopic observation.

The slide preparation should be examined under a compound microscope with 10× and 40× lens objectives. Phase-contrast optics are preferable. If regular bright-field illumination is used, the slide should be examined under diminished light. The slide may be warmed to 35°C (to promote amebic movement), and amebae, especially *N. fowleri,* if present, can easily be detected by their active directional movements. Rarely, flagellates with two flagella may be seen. Currently, this is the most sensitive method available for the diagnosis of PAM.

Permanently Stained Preparations

A small drop of the sedimented CSF or other sample is placed in the middle of a slide, which is allowed to stand in a moist chamber for 5 to 10 min at 37°C. This will allow any amebae to attach to the surface of the slide. Several drops of warm (37°C) Schaudinn's fixative are dropped directly onto the sample, which is allowed to stand for 1 min. The slide is then transferred for 1 h to a Coplin jar containing the fixative. It may be stained with Wheatley's trichrome or Heidenhain's iron hematoxylin stain. Corneal scrapings smeared onto microscope slides may be fixed with methanol and stained with Hemacolor stain (Harleco, a division of EM Industries, Inc.) (28, 54).

ISOLATION

The recommended procedure for isolating free-living pathogenic amebae from biological specimens is as follows.

Materials

1. Page's ameba saline (17, 34). Physiological saline or phosphate-buffered saline solutions that are normally available in the clinical laboratories are not suitable, as the sodium chloride concentrations in these solutions will prevent the growth of amebae, especially *N. fowleri.*

2. Petri dishes containing 1.5% Difco agar made with Page's ameba saline (nonnutrient agar plate) (50). These plates can be stored at 4°C for up to 3 months. Chocolate agar with blood, Trypticase soy agar, and Lowenstein-Jensen agar have been used sometimes. These are not suitable because bacteria that coat the plates or bacteria from the clinical sample may overgrow and either prevent the growth of amebae, especially when they are in small numbers, or obscure their presence.

3. 18- to 24-h-old culture of *E. coli* or *Enterobacter aerogenes*
4. Sterile distilled water
5. Bacteriologic loop
6. Fine spatula made of nichrome wire
7. Sterile Pasteur pipettes with rubber bulbs
8. Sterile 1-ml serologic pipettes
9. Sterile screw-cap test tubes, 100 by 13 or 125 by 16 mm
10. Moist chamber
11. Vaspar (1:1 petrolatum-paraffin), melted
12. Microscope slides, 3 by 1 or 3 by 2 in. (7.6 by 2.5 or 7.6 by 5 cm)
13. Coverslips, 22 mm square, no. 1 thickness

Preparation of Agar Plates

1. Remove plates from the refrigerator, and place them in a 37°C incubator for 30 min.

2. Add 0.5 ml of ameba saline to a slant culture of *E. coli* or *Enterobacter aerogenes.* Gently scrape the surface of the slant with a sterile bacteriologic loop (do not break the agar surface). Using a sterile Pasteur pipette, gently and uniformly suspend the bacteria. Add 2 or 3 drops of this suspension to the middle of a warmed (37°C) agar plate, and spread the bacteria over the surface of the agar with a bacteriologic loop. The plate is then ready for inoculation.

Inoculation of Plates with Specimens

1. For CSF samples, centrifuge the CSF at 500 × *g* for 5 to 8 min. With a sterile serologic pipette, carefully transfer all but 0.5 ml of the supernatant to a sterile tube, and store the tube at 4°C for possible future use. Mix the sediment with the remaining fluid. With a sterile Pasteur pipette, place 2 or 3 drops in the center of the agar plate precoated with bacteria, and incubate in room air at 37°C.

2. For tissue samples, triturate a small piece of the tissue in a small amount of ameba saline. With a sterile Pasteur pipette, place 2 or 3 drops of the mixture in the center of the agar plate. Incubate the plate in room air at 37°C for CNS and lung tissues and at 30°C for tissues from other sites (skin, cornea).

3. Handle water and soil samples in the same manner as CSF and tissue specimens, respectively.

4. Control cultures are recommended for comparative purposes, although care should be exercised to prevent cross-contamination of patient cultures.

Examination of Plates

1. Using the low-power (10×) lens objective of a microscope, observe the plates daily for 7 days for amebae.

2. If you see amebae anywhere, circle that area with a wax pencil. With the fine spatula, cut a small piece of agar from the circled area, and place it face down on the surface of a fresh agar plate precoated with bacteria; incubate as described above. Both *N. fowleri* and *Acanthamoeba* spp. can easily be cultivated in this way and, with periodic transfers, maintained indefinitely. When the plate is examined under a microscope, the amebae will look like small blotches, and if they are observed carefully, their movement can be discerned. After 2 to 3 days of incubation, the amebae will start to encyst. If a plate is examined after 4 to 5 days of incubation, trophozoites as well as cysts will be visible. *B. mandrillaris,* however, will not grow on agar plates seeded with bacteria. While *B. mandrillaris* can be grown on monkey kidney or lung fibroblast cell lines and human brain microvascular endothelial cells and axenically in a complex medium (12, 16, 39, 42, 52, 53), such techniques are not routinely available.

Identification and Culture

Identification of living organisms to the genus level is based on characteristic patterns of locomotion, morphologic features of

the trophic and cyst forms, and results of enflagellation experiments. Immunofluorescence or immunoperoxidase tests using monoclonal or polyclonal antibodies (available at the Centers for Disease Control and Prevention) will be helpful in differentiating *Acanthamoeba* spp. from *B. mandrillaris* in fixed tissue and in identifying the species, especially *Acanthamoeba* spp., in fixed tissue (8, 13, 19, 28, 36, 52, 53).

Enflagellation Experiment

1. Mix 1 drop of the sedimented CSF containing amebae with about 1 ml of sterile distilled water in a sterile tube, or with a bacteriologic loop, scrape the surface of a plate that is positive for amebae, transferring a loopful of scraping to a sterile tube that contains approximately 1 ml of distilled water.

2. Gently shake the tube, and transfer a drop of this suspension to the center of a coverslip whose edges have been coated thinly with petroleum jelly. Place a microscope slide over the coverslip, and invert the slide. Seal the edges of the coverslip with Vaspar. Place the slide in a moist chamber, and incubate as before for 2 to 3 h. In addition, incubate the tube as described above.

3. Periodically examine the tube and the slide preparation microscopically for free-swimming flagellates. *N. fowleri* has a flagellate stage; *Acanthamoeba* spp. and *B. mandrillaris* do not. If the sample contains *N. fowleri,* about 30 to 50% of the amebae will have undergone transformation into pear-shaped biflagellate organisms (Fig. 7).

Other Culture Methods

Axenic Culture

Acanthamoeba spp. can easily be cultivated axenically, without the addition of serum or host tissue, in many different types of nutrient media, e.g., Proteose Peptone-yeast extract-glucose medium, Trypticase soy broth medium, and chemically defined medium (50). *N. fowleri,* however, requires media containing fetal calf serum or brain extract, e.g., Nelson's medium. A chemically defined medium has only recently been developed for *N. fowleri* (33). *B. mandrillaris* cannot be cultivated on agar plates with bacteria. It can, however, be cultivated on mammalian cell lines (12, 16, 39, 52, 53) or a complex axenic medium (41).

Axenic cultures of *Acanthamoeba* spp. and *N. fowleri* can be established as follows. An actively growing 24- to 36-h-old ameba culture is scraped from the surface of the plate, suspended in 50 ml of ameba saline, and centrifuged at 500 × *g* for 5 min. The supernatant is aspirated, and the sediment is inoculated into Proteose Peptone-yeast extract-glucose medium or Nelson's medium, depending on the ameba isolate, and incubated at 37°C. Gentamicin, to a final concentration of 50 μg/ml, is added aseptically to the medium before the amebae are inoculated. Three subcultures into the antibiotic-containing medium at weekly intervals are usually sufficient to eliminate the associated bacteria (*E. coli* or *Enterobacter aerogenes*).

Cell Culture

Acanthamoeba spp., *B. mandrillaris,* and *N. fowleri* can also be inoculated onto many types of mammalian cell cultures. Shell vial cultures normally used in the isolation of viruses are suitable for the isolation of the amebae provided that antifungal agents (amphotericin B) are not included in the antibiotic mix. The amebae grow vigorously in these cell cultures and produce cytopathic effects somewhat similar to those caused by viruses (17, 42, 52, 53). Because of such cytopathic effects, *Acanthamoeba* organisms were mistaken for transformed cell types presumed to contain viruses and were erroneously termed lipovirus and Ryan virus (27, 54).

Animal Inoculation

Two-week-old Swiss Webster mice weighing 12 to 15 g each can be infected with these amebae. The mice are anesthetized with ether, and a drop of ameba suspension is instilled into their nostrils. Mice infected with *N. fowleri* die within 5 to 7 days after developing characteristic signs such as ruffled fur, aimless wandering, partial paralysis, and finally coma and death. Mice infected with *Acanthamoeba* spp. and *B. mandrillaris* may die of acute disease within 5 to 7 days or may die of chronic disease after several weeks. In all cases, the presence of amebae in the mouse brain can be demonstrated either by culture or by histologic examination.

Antigenic and Molecular Characteristics

Pathogenic *N. fowleri* is morphologically indistinguishable from nonpathogenic *Naegleria* at the trophic stage. Differences between these amebae, however, have been demonstrated antigenically by the gel diffusion, immunoelectrophoretic, and indirect immunofluorescence techniques (17, 24, 28, 42, 54). Antigenic differences have also been shown among various species of *Acanthamoeba* (17, 24, 28, 54). Some recent studies indicate that *N. fowleri* can be distinguished from other species of *Naegleria* on the basis of isoenzyme patterns as well as the sequencing of the 5.8S rRNA gene and ITS1 and ITS2 (17, 24, 28, 42, 54, 58). Analyses of isoenzyme patterns, mitochondrial DNA, and small-subunit RNA have also been carried out for various *Acanthamoeba* species and isolates in an attempt to understand inter- and intraspecies diversity and phylogenetic relationships (3, 24, 42, 49). Similarly, a PCR assay for the detection of *B. mandrillaris* has been described previously (2). Recently, a PCR assay to detect *B. mandrillaris* in Formalin-fixed archival tissue specimens has also been described (13).

Serology

The serologic techniques discussed here have been developed as research tools and are not routinely available to clinical laboratories. Complement fixation antibody to *Acanthamoeba* spp. has been shown to be present in the sera of patients with upper respiratory tract distress and those with optic neuritis and macular disease (17, 24, 28, 42, 54). Kenney (42, 54) demonstrated increasing titers of complement fixation antibody to *Acanthamoeba* in three successive samples of serum collected over a 2-month period from each of two patients. Precipitin antibody has also been shown to be present in the serum of a patient suffering from *A. polyphaga* keratitis (18, 54). Recently, an increase in ameba immobilization antibody titers over a 16-month period was demonstrated in the serum of a Nigerian patient, who made a partial recovery from an *A. rhysodes*-induced CNS disease (28, 54).

An antibody response to *N. fowleri,* however, has not yet been defined. Most of the patients with *Naegleria* PAM died very shortly after infection (5 to 10 days), before they had time to produce detectable levels of antibody. In one case, however, in which the patient survived PAM caused by *N. fowleri,* an indirect immunofluorescence titer of 4,096 against *N. fowleri* was detected in the serum after 42 days of hospitalization (28, 42, 43, 54).

Since the recognition of *Balamuthia* GAE is relatively new, not much information is available on the serologic response of patients infected with *Balamuthia.* According to a recent report, immunofluorescent-antibody titers were determined for four patients with confirmed *Balamuthia* GAE infection, 61

patients with encephalitis of unknown causes, and 12 healthy controls. Titers ranging from 64 to 2,048 were recorded for the sera of patients with *Balamuthia* GAE, whereas six sera from patients with encephalitis of unknown causes (10% of the sera) had titers of 64 and above and none of the control sera had titers of 64 or above. It is therefore possible that patients with *Balamuthia* GAE may be diagnosed premortem by using this serologic test (42, 54).

TREATMENT

Pathogenic *N. fowleri* is exquisitely susceptible to amphotericin B in vitro (6, 17, 27, 28, 42, 43). At least several patients with PAM were reported to have recovered after receiving intrathecal and intravenous injections of amphotericin B alone or in combination with miconazole or treatment with amphotericin B in a hyperbaric chamber (17, 28, 42, 43). Although a few patients with *Acanthamoeba* sp. GAE have survived, most have died in spite of treatment with several drug combinations. According to a recent report, a Spanish AIDS patient with *Acanthamoeba* group II GAE completely recovered from this disease after surgery and treatment with sulfadiazine and fluconazole (30). Culbertson found sulfadiazine to be active against experimental *Acanthamoeba* infections in mice (17, 28, 42, 54). Jones et al. (18) found that paromomycin, clotrimazole, and hydroxystilbamidine isethionate were active against *A. polyphaga* in vitro. Several patients with *Acanthamoeba* keratitis have been successfully treated with different drug combinations administered over a long period. For example, in one study, treatment with 0.1% propamidine isethionate (Brolene) eye drops and 0.15% dibromopropamidine ointment together with topical neomycin sulfate was successful in the management of *Acanthamoeba* keratitis (56). In another study, 1% clotrimazole used in combination with propamidine isethionate and a compound of bacitracin, neomycin, and polymyxin B (Neosporin) for four patients gave excellent results (11). In a recent study, azithromycin and several phenothiazine compounds protected rat glioma cells from destruction by *Acanthamoeba* (41). Medical cure has also been achieved with topical administration of polyhexamethylene biguanide (21) or topical application of hexamidine isethionate, an analogue of pentamidine (4). A recent study carried out with 111 patients with *Acanthamoba* keratitis indicates that treatment with a combination of 0.02% polyhexamethylene biguanide and 0.1% propamidine isethionate hourly for 3 days and subsequently up to six times a day for 6 to 8 weeks is associated with a good prognosis (21), and this may prove to be the treatment of choice. The prognoses of patients without CNS infection but with disseminated cutaneous ulcers due to *Acanthamoeba* spp. are good. For example, a patient with Down's syndrome and an immunoglobulin A deficiency who also had undergone cadaveric renal transplantation developed a biopsy-confirmed *Acanthamoeba* skin ulcer. The patient was successfully cured of the infection after prolonged (more than 8 months) therapy with a regimen that included topical as well as systemic administration of a combination of drugs. His skin ulcers were cleansed twice daily with chlorhexidine gluconate solution followed by topical application of 2% ketoconazole cream. He also received pentamidine isethionate intravenously for 1 month and thereafter was given oral itraconazole therapy for 8 months; this regimen resulted in complete healing of the cutaneous ulcers (46).

Recent in vitro studies indicate that *B. mandrillaris* is susceptible to pentamidine isethiocyanate and that patients with *B. mandrillaris* infection may benefit from treatment with this drug (41). Although most patients with *B. mandrillaris* GAE have died of this disease, two patients from California and a patient from New York have survived after treatment initially with pentamidine isethionate and subsequently with a combination of sulfadiazine, clarithromycin, fluconazole, and 5-fluorocytosine (8, 19).

Use of trade names is for identification only and does not imply endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

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Intestinal and Urogenital Amebae, Flagellates, and Ciliates

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Giardia lamblia and *Entamoeba histolytica* infections are two of the most common protozoal infections seen worldwide and are of serious concern on a global scale due to their prevalence and the pathogenicity of the causative agents. Most of the other protozoa described in this chapter are nonpathogenic organisms. Nevertheless, they need to be detected and differentiated from true pathogens in clinical specimens because they are indicators of exposure to fecal contamination and examination of additional specimens may reveal pathogenic protozoa. The pathogenic and nonpathogenic organisms are categorized as indicated in Table 1; however, reports of disease in patients infected with nonpathogenic species are found in the literature.

Microscopic examination of stool specimens continues to be the main tool used in the laboratory diagnosis of intestinal amebic, flagellate, and ciliate infections. The goal of microscopy is to identify pathogenic protozoa, differentiate between these and nonpathogenic species, and properly discriminate among various artifacts that may be present. Other methods for detection include antigen detection assays such as enzyme immunoassays (EIAs), immunochromatographic assays, and direct fluorescent-antibody (DFA) assays for the detection of pathogens such as the *E. histolytica*/*Entamoeba dispar* group, *E. histolytica,* and *G. lamblia.* Culture may be useful under certain limited situations. Nucleic acid-based techniques have been developed but are not yet widely available. All of these methods may not be available or necessary in every instance. See the sections below and refer to chapters 16, 18, and 135 for additional information.

AMEBAE

Taxonomy

The amebae that parasitize the intestinal tracts of humans belong to four genera: *Entamoeba, Endolimax, Iodamoeba,* and *Blastocystis*. They all belong to the phylum Sarcomastigophora, subphylum Sarcodina, which contains organisms that move by means of cytoplasmic protrusions called pseudopodia. It is noteworthy that *Dientamoeba fragilis,* once classified as an ameba, is now grouped with the flagellates. Even though this reclassification has occurred, *D. fragilis* is still identified on the basis of morphologic comparison to amebae.

Description of the Agents

Of the seven species of intestinal amebae, *E. dispar, Entamoeba hartmanni, Entamoeba coli, Endolimax nana,* and *Iodamoeba bütschlii* are nonpathogenic for humans. *E. histolytica* is pathogenic for humans, causing invasive intestinal and extraintestinal amebiasis. The pathogenicity of *Blastocystis hominis* is still controversial.

Epidemiology, Transmission, and Prevention

All amebae have a common and relatively simple life cycle. The cyst is the infectious form and is acquired by ingestion of contaminated material such as water and food or by direct fecal-oral transmission. Once the cyst arrives in the intestinal tract, excystation occurs, releasing trophozoites. Encystment occurs in the colon, presumably when conditions become unfavorable for the trophozoites. Cysts are passed in the feces and remain viable in the environment for days to weeks in water and soil if protected from desiccation. Improvements in sanitary conditions are necessary to prevent infections in areas where the organisms are endemic. Research to develop a vaccine against *E. histolytica* is ongoing, but none is currently available.

Collection, Transport, and Storage of Specimens

For detection of the amebae, laboratories receive predominantly stool specimens for examination. Both fresh and preserved specimens are useful for the diagnosis of infection, depending on the methodology employed and the circumstances of the laboratory. If fresh specimens are received for the detection of organism motility, they must be examined quickly; wet mounts for the detection of motility cannot be performed on preserved specimens. Other sample types such as aspirates and tissue samples may be received and are appropriate for testing depending on the organism suspected. For a more detailed description of collection, refer to chapter 133 of this Manual.

Direct Examination

Microscopy

All diagnostic stages of the amebae (trophozoite and cyst) can be detected in fecal specimens, the most common specimen submitted to the laboratory. The key morphologic features of

TABLE 1 Intestinal and urogenital amebae, flagellates, and ciliates of humans

Parasite	Pathogenic	Nonpathogenic
Amebae	*Entamoeba histolytica*[a] *Blastocystis hominis*	*Entamoeba dispar*[b] *Entamoeba hartmanni* *Entamoeba coli* *Entamoeba polecki* *Entamoeba gingivalis*[c] *Endolimax nana* *Iodamoeba bütschlii*
Flagellates	*Giardia lamblia* *Trichomonas vaginalis* *Dientamoeba fragilis*	*Chilomastix mesnili* *Pentatrichomonas (Trichomonas) hominis* *Trichomonas tenax*[c] *Enteromonas hominis* *Retortamonas intestinalis*
Ciliates	*Balantidium coli*	

[a] A distinction between *E. histolytica* and *E. dispar* cannot be made on the basis of morphology unless ingested RBCs are seen in the cytoplasm of the trophozoite.

[b] In rare instances, *Entamoeba moshkovskii* may be found in human stool specimens. A free-living ameba, it is nonpathogenic and morphologically identical to *E. histolytica*/*E. dispar* (119).

[c] *E. gingivalis* and *T. tenax* are found in the oral cavity and related specimens.

amebae must be used to differentiate among the various species and to distinguish between somatic cells and other material. Trophozoites must be distinguished from epithelial cells and macrophages. Cysts must be distinguished from polymorphonuclear cells. Also, yeast, pollen, molds, food particles, and other debris present in feces may cause confusion (Table 2; Fig. 1 and 2).

Morphologic examination of fecal specimens can be accomplished with fresh wet mount preparations, wet mounts of concentrated material, and permanent-stained smears. Each of these three types of preparations may be useful for visualizing certain key characteristics. Stained and unstained wet mounts of concentrated material can also be useful for identification, particularly for certain cysts such as those of *Entamoeba coli* and *I. bütschlii.* Iodine will provide color and contrast, both of which may aid in the identification of organisms in wet preparations. However, morphologic examination with permanent-stained smears by oil immersion microscopy (magnification, ×1,000) is the most useful procedure (20).

Trophozoite motility is visible only in saline wet mounts of fresh feces and is often difficult to detect. The arrangement, size, and pattern of nuclear chromatin help differentiate species within the genus *Entamoeba* from other intestinal amebae. The size and position of the nuclear karyosome are also important morphologic features. A ring of nuclear chromatin surrounding the karyosome, resembling a bull's eye, is characteristic of *Entamoeba. Endolimax, Iodamoeba,* and the flagellate *Dientamoeba* lack peripheral chromatin. The cytoplasm of the trophozoites may contain granules and ingested material such as erythrocytes (RBCs), bacteria, yeasts, and molds.

The characteristics of cysts are less variable than those of trophozoites. To aid in differentiation among the genera, the cytoplasm should be examined for the presence of chromatoidal bodies and vacuoles, particularly the large glycogen vacuole seen in *I. bütschlii.*

Evaluation, Interpretation, and Reporting of Results

It is important to remember that identification may not be possible on the basis of one morphologic feature or the characteristics of a single organism in the preparation. Nuclear and cytoplasmic features can vary within species and may overlap between species, making identification challenging. Mixed infections are not uncommon and can be missed in a cursory examination. A complete, overall assessment of the slide is necessary for correct identification. It is important to use an accurate micrometer to measure life cycle stages. Size is reliable only for the differentiation of *E. histolytica/E. dispar* from *E. hartmanni.* Also, on permanent-stained smears, shrinkage may occur, affecting the apparent size of the organism. Results of microscopy should clearly indicate the full taxonomic name of the organisms detected along with the forms of the organisms seen (cysts versus trophozoites). Except for *Blastocystis,* quantitation of the amebae on the final report is not appropriate.

E. histolytica

Epidemiology, Transmission, and Prevention

E. histolytica can be found worldwide but is more prevalent in tropical and subtropical regions. In areas where the organism is endemic, up to 50% of people may be infected. In temperate climates with poor sanitation, infection rates can approach those seen in tropical regions. Humans are the primary reservoir; infection occurs by ingestion of cysts from fecally contaminated material such as water and food. Sexual transmission also occurs.

Asymptomatic *E. histolytica* infection is equally distributed between the genders, while invasive amebiasis affects men predominantly. In the United States, groups with a higher incidence of amebiasis include immigrants from South and Central America and Southeast Asia. Also, residents of the southern United States and institutionalized individuals are more likely than others to be infected. In one study, short-term travelers to areas where *E. histolytica* and *E. dispar* are endemic were found to be at higher risk of infection with the pathogenic species, *E. histolytica,* than were residents, who were more likely to harbor the nonpathogenic species, *E. dispar* (126). In homosexual males, the infection is often transmitted by sexual behavior, with up to 30% found to be infected in certain studies. These infections are most often asymptomatic. Among human immunodeficiency virus (HIV)-infected patients in the United States, the incidence of diagnosed *E. histolytica* disease is low (13.5 cases per 10,000 person-years) (74). HIV-infected individuals in non-Western countries, such as Taiwan and Korea, do have a higher risk of invasive amebiasis, in contrast to findings from the United States (50, 87). These differing rates of invasive disease may be attributed to the higher endemicity of *E. histolytica* in the Asia-Pacific regions (51).

Clinical Significance

Among the estimated 500 million people infected each year with *E. histolytica,* there are approximately 50 million cases of colitis and liver abscess and 100,000 deaths (131). The discrepancy between the number of people infected with *E. histolytica* and the morbidity and mortality rates is explained by the existence of two morphologically similar yet distinct species: one capable of producing disease and the other not.

TABLE 2 Key features of trophozoites and cysts of common intestinal amebae[a]

Organism	Trophozoites	Cysts
E. histolytica/*E. dispar*	Size[b]: 12–60 μm; invasive forms, >20 μm Motility: Progressive, directional, rapid Nucleus[c]: 1; peripheral chromatin evenly distributed; karyosome small, compact, centrally located; may resemble *E. coli* Cytoplasm: Finely granular, like "ground glass"; may contain bacteria Note: RBCs in cytoplasm diagnostic for *E. histolytica* infection	Size: 10–20 μm; spherical Nucleus: Mature cyst, 4; immature cyst, 1 or 2; peripheral chromatin fine, uniform granules, evenly distributed; karyosome small, compact, centrally located Cytoplasm: Chromatoidal bodies may be present; elongate with blunt rounded edges; may be round or oval
E. hartmanni	Size: 5–12 μm Motility: Nonprogressive Nucleus: 1; peripheral chromatin like *E. histolytica*/*E. dispar,* may appear as solid ring; karyosome small, compact, centrally located or eccentric Cytoplasm: Finely granular, bacteria, no RBCs Note: Accurate measurement essential for differentiation from *E. histolytica*/*E. dispar*	Size: 5–10 μm; spherical Nucleus: Mature cyst, 4; immature cyst, 1 or 2 (very common); peripheral chromatin fine, evenly distributed, may be difficult to see; karyosome small, compact, centrally located Cytoplasm: Chromatoidal bodies usually present, like in *E. histolytica*/*E. dispar*
E. coli	Size: 15–50 μm Motility: Sluggish, nondirectional Nucleus: 1; peripheral chromatin clumped and uneven, may be solid ring; karyosome large, not compact, diffuse, eccentric Cytoplasm: Granular, usually vacuolated; contains bacteria, yeast, no RBCs Note: Can resemble *E. histolytica*/*E. dispar;* coinfection seen; stained smear essential	Size: 10–35 μm; spherical, rarely oval or triangular Nucleus: Mature cyst, 8; occasionally ≥16; immature cyst, ≥2; peripheral chromatin coarsely granular, unevenly arranged; may resemble *E. histolytica*/*E. dispar;* karyosome small, usually eccentric but may be central Cytoplasm: Chromatoidal bodies less frequent than in *E. histolytica*/*E. dispar;* splintered, with rough, pointed ends Note: May be distorted on permanent-stained smear due to poor penetration of fixative
E. nana	Size: 6–12 μm Motility: Sluggish, nonprogressive Nucleus: 1; no peripheral chromatin; karyosome large, "blot like" Cytoplasm: Granular, vacuolated; may contain bacteria Note: May be tremendous nuclear variation; can mimic *E. hartmanni* and *D. fragilis*	Size: 5–10 μm; oval, may be round Nucleus: Mature cyst, 4; immature cyst, 2; no peripheral chromatin; karyosome smaller than those in trophozoites but larger than those in *Entamoeba* spp. Cytoplasm: Chromatoidal bodies rare; small granules occasionally seen
I. bütschlii	Size: 8–20 μm Motility: Sluggish, nonprogressive Nucleus: 1; no peripheral chromatin; karyosome large, may have "basket nucleus" Cytoplasm: Coarsely granular, may be highly vacuolated; bacteria, yeast, and debris may be seen Note: Stained smear essential; nucleus may appear to have a halo with chromatin granules fanning around karyosome	Size: 5–20 μm; oval to round Nucleus: Mature cyst, 1; no peripheral chromatin; karyosome large, usually eccentric Cytoplasm: No chromatoidal bodies; small granules occasionally present Note: Glycogen present, large, compact, well-defined mass; cysts may collapse owing to large glycogen vacuole space
B. hominis[d]	Very difficult to identify; rarely seen	Size: 6–40 μm; generally round Description: Usually characterized by a large, central body (looks like a large vacuole) surrounded by small, multiple nuclei; central body area can stain various colors (trichrome) or remain clear

[a] Adapted from reference 30.
[b] Size ranges are based on wet preparations (with permanent stains, organisms usually measure 1 to 2 μm less).
[c] Nuclear and cytoplasmic descriptions are based on permanent-stained smears.
[d] Description of central body form.

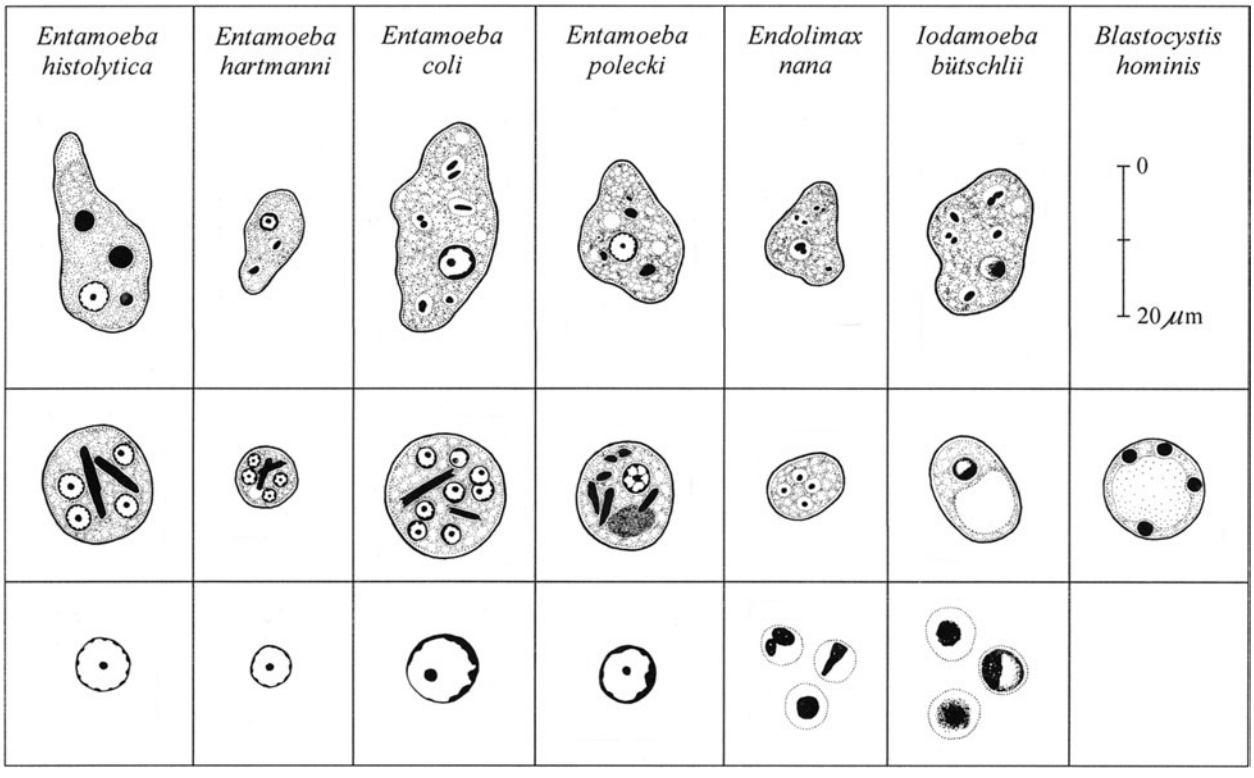


FIGURE 1 Intestinal amebae of humans. (Top row) Trophozoites. *E. histolytica* is shown with ingested RBCs. This is the only microscopic finding that allows differentiation of the pathogenic species *E. histolytica* from the nonpathogenic species *E. dispar.* An ameboid form of *B. hominis* is rarely seen and is difficult to identify. (Middle row) Cysts. For *B. hominis* the central body form is depicted. (Bottom row) Trophozoite nuclei, shown in relative proportion.

The development of axenic culture methods was a key step in confirming the existence of two species. Using organisms obtained by such cultures, Sargeaunt and Williams (104) performed isoenzyme analysis of several glycolytic enzymes and identified electrophoretic banding patterns, or zymodemes. Two groups were identified on the basis of these patterns: pathogenic zymodemes (invasive isolates) and nonpathogenic zymodemes (noninvasive isolates). The zymodeme patterns represent stable genetic differences and do not interconvert (90). Additional genetic, biochemical, and immunologic evidence has supported the existence of two distinct species. Diamond and Clark (25) redescribed the two species as *E. histolytica* Schaudinn 1903, which is the invasive human pathogen, and *E. dispar* Brumpt 1925, which is noninvasive and which does not cause disease.

Infection with *E. histolytica/E. dispar* can result in different clinical presentations: asymptomatic infection, symptomatic infection without tissue invasion, and symptomatic infection with tissue invasion. The majority of infections with *E. histolytica/E. dispar* are asymptomatic. Individuals with such infections will have a negative or weak serologic response and will primarily pass cysts in their stools. Zymodeme analysis shows that most asymptomatic individuals are infected with the noninvasive species *E. dispar* (130, 131). However, it appears that infection with both *E. histolytica* and *E. dispar* can be asymptomatic, with cyst stages being passed in the stool (11). Asymptomatic *E. histolytica* infection may be due to the existence of genetically distinct invasive and noninvasive strains of *E. histolytica* (135).

Intestinal disease results from the penetration of the amebic trophozoites into the intestinal tissues. Approximately 10% of infected individuals will have clinical symptoms, presenting as dysentery, colitis, or, rarely, ameboma. The incubation period varies from a few days to several months. Various molecules such as adhesins, amebapores, and proteases have been associated with lysis of the colonic mucosa in intestinal amebiasis (27). The 260-kDa galactose- or *N*-acetylgalactosamine-specific lectin of *E. histolytica* is an important virulence factor, mediating the attachment of the ameba to the intestinal epithelium and contact-dependent cytolysis (96). Symptoms of amebic dysentery include diarrhea with cramping, lower abdominal pain, low-grade fever, and the presence of blood and mucus in stool. The ulcers produced by intestinal invasion by trophozoites start as superficial localized lesions that deepen into the classic flask-shaped ulcers of amebic colitis. The ulcers are separated by segments of normal tissue but can coalesce. Amebae can be found at the advancing edges of the ulcer but usually not in the necrotic areas. Abdominal perforation and peritonitis are rare but serious complications. A more chronic presentation occurs with amebic colitis. It is characterized by intermittent diarrhea over a long period and can be misdiagnosed as ulcerative colitis or irritable bowel syndrome. Ameboma, a localized tumor-like lesion, results from chronic ulceration and may be mistaken for malignancy. Histologically, it consists of granulomatous tissue.

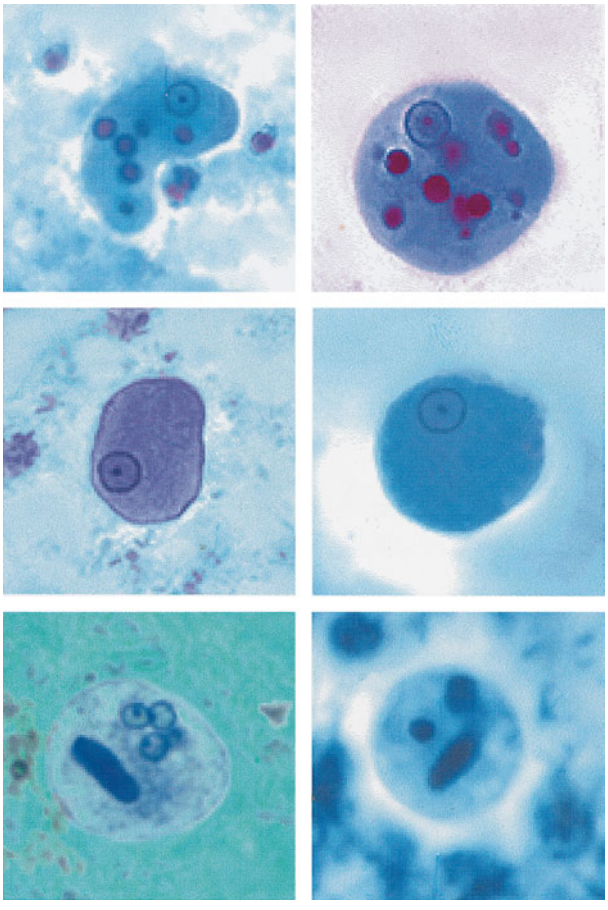


FIGURE 2 (Top row) *Entamoeba histolytica* trophozoites. Note the ingested RBCs in the cytoplasm. (Middle row) *E. histolytica/E. dispar* trophozoites. Note the absence of RBCs in the cytoplasm. (Bottom row) *E. histolytica/E. dispar* cysts. These cysts cannot be identified to the species level on the basis of morphology. Organisms are stained with Wheatley's trichrome stain. (Courtesy of L. Garcia.)

Extraintestinal disease occurs with the hematogenous spread of the organism. It can occur with or without previous symptomatic intestinal infection. The liver is the most common site of extraintestinal disease, followed by the lungs, pericardium, brain, and other organs. Symptoms can be acute or gradual and may include low-grade fever, right-upper-quadrant pain, and weight loss. Up to 5% of individuals with intestinal symptoms develop liver abscess. However, up to 50% of individuals with liver abscess have no history of gastrointestinal disease.

Direct Examination

The laboratory diagnosis of amebiasis can be made by the examination of feces, material obtained from sigmoidoscopy, tissue biopsy specimens, and abscess aspirates. Serologic testing is also useful for the diagnosis of extraintestinal amebiasis. A summary of the laboratory techniques and their performance characteristics is presented in Table 3.

Microscopy

As discussed above, the most important part of the standard microscopic examination of stool and other specimens is the permanent-stained smear. Direct wet preparations and concentration procedures may also be useful (Fig. 1; Table 2; see also chapter 135). Detection of trophozoites and cysts does not, however, allow differentiation of the pathogenic species, *E. histolytica,* from the nonpathogenic species, *E. dispar* (Fig. 1 and 2). The presence of ingested RBCs in the cytoplasm of the trophozoites is commonly regarded as diagnostic of *E. histolytica* infection. However, Haque et al. (43) found that 16% of *E. dispar* isolates had ingested RBCs; thus, this distinction between the two species is not absolute (119, 120). In tissue specimens, only the trophozoite is found, and its presence is considered diagnostic of invasive *E. histolytica* disease.

Antigen Detection

For a more definitive differentiation of *E. histolytica* and *E. dispar,* methods other than microscopy are necessary. Zymodeme analysis can accomplish this differentiation but requires culture of the organisms from the specimen and is too expensive and complex for routine laboratory use. More recently, antigen detection methods for the detection and differentiation of *E. histolytica* and *E. dispar* have become commercially available in the United States (TechLab, Blacksburg, Va.; Alexon-Trend, Ramsey, Minn.; and Biosite Inc., San Diego, Calif.). The assays are designed for use with fresh, fresh-frozen, or unfixed human fecal specimens. Depending on the kit used, the group *E. histolytica/E. dispar* or the individual species can be detected in the feces. Only Tech Labs offers an *E. histolytica*-specific kit, *E. Histolytica II*, which detects the Gal- or GalNAc-binding lectin specific to the pathogenic species (43, 95). All the antigen detection methods are relatively simple and are more sensitive and specific than microscopy (Table 3) (33, 43, 89). They cannot be used with preserved stool, as the fixative appears to denature the antigens. Although some reports of the use of preserved stool have appeared (133), more work is needed to identify additional antigens that withstand fixation before it would be practical to offer antigen tests in laboratories that receive only preserved stool specimens. Antigen assays have been used to test a number of other sample types such as serum, pus, and saliva (1, 42); the detection of antigen in serum may prove to be a sensitive means of diagnosing amebic liver abscess and intestinal disease (42, 119). Because fewer than 10% of patients with amebic liver abscesses have concurrent intestinal disease with amebae detectable in the stool, methods such as routine ova and parasite examination are not useful. Microscopic examination or culture of pus from liver abscesses likewise lacks sensitivity. In a study by Haque et al. (42), serum antigen detection by using the Tech Labs *E. Histolytica II* kit was a sensitive method for diagnosis with samples collected prior to treatment with metronidazole. Serum antigenemia appears to clear after treatment, suggesting possible utility to monitor therapy; however, this use is still experimental (42, 119).

Nucleic Acid Detection Techniques

Nucleic acid amplification techniques, such as PCR, have been developed for the detection and differentiation of *E. histolytica* and *E. dispar.* Traditional PCR has been applied to specimens such as feces and liver or brain aspirates and can detect both trophozoite and cyst DNA (28, 101, 136). More recently, real-time PCR has been used for the detection of *E. histolytica* and *E. dispar* (7, 103) and for the simultaneous detection of *E. histolytica*, *G. lamblia,* and *Cryptosporidium parvum* (124) in stool. Different extraction techniques, some of which are relatively simple, and the use of fresh and preserved material have been reported (101, 121, 125). Stool can present challenges

TABLE 3 Sensitivity and specificity of diagnostic tests for amebiasis[a]

Test and specimen type	Colitis		Liver abscess
	Sensitivity (%)	Specificity (%)	Sensitivity (%)
Microscopy			
Stool	<60	10–50	<10
Abscess fluid	NA[b]	NA	<25
Culture with isoenzyme analysis	Lower than antigen or PCR tests	Gold standard	<25
Stool antigen detection (ELISA)[c]	>95	>95	Usually negative
Serum	65 (early)	>90	~75 (late); ~100 (first 3 days)
Abscess fluid	NA	NA	~100 (before treatment)
Saliva	Not done	Not done	70
PCR			
Stool	>70	>90	Not done
Serum antibody detection (ELISA)	>90	>85	70–80 (acute stage); >90 (convalescent stage)

[a] Reprinted from reference 119 with permission.
[b] NA, not available.
[c] ELISA, enzyme-linked immunosorbent assay.

due to the presence of PCR inhibitors (84), so the inclusion of an amplification control is useful (124).

PCR has proven to be more sensitive and specific than microscopy and at least as sensitive as antigen detection, depending on the study (Table 3) (40, 119). For routine use in clinical laboratories, a PCR method would ideally involve a relatively simple sample preparation procedure and allow the use of preserved material. Because PCR and antigen detection may be approximately equal in sensitivities, an antigen detection test may be preferable due to technical ease of use and shorter time required.

Serologic Tests

Serologic testing is a useful aid, in conjunction with antigen detection or PCR, for the diagnosis of symptomatic, invasive disease. Multiple methods have been used, including indirect hemagglutination, complement fixation, latex agglutination, and EIA (119). Of patients with biopsy-proven intestinal amebiasis, 85% have serum antibodies. For patients with extraintestinal disease, serologic tests have a sensitivity approaching 99%. For asymptomatic intestinal diseases, serology is generally not useful unless the patient has invasive infection. Persons infected with *E. dispar* do not produce detectable antibodies. After cure of invasive amebiasis, serum antibodies may persist for up to 10 years; this can complicate diagnosis in areas where infection is endemic (57).

EIAs for the detection of immunoglobulin M (IgM) and IgG are widely used, and most are based on the detection of antilectin antibodies, which appear over 1 week after symptoms of *E. histolytica* infection (119). Indirect hemagglutination is useful and highly specific but may lack sensitivity compared to EIAs (119). Several investigators have reported the utility of IgA antibody testing and a link to partial immunity in individuals with detectable antilectin IgA (41, 42, 116).

Evaluation, Interpretation, and Reporting of Results

Until all laboratories have the capability to discriminate between *E. histolytica* and *E. dispar,* the method of laboratory reporting must change. In January 1997, a World Health Organization panel of experts made recommendations concerning the reporting and treatment of amebiasis (131). If microscopic diagnosis is made on the basis of the detection of trophozoites and/or cysts and no method is used to differentiate the two species, the report should indicate "*E. histolytica*/*E. dispar* detected." Laboratory reports must indicate whether trophozoites and/or cysts are present, due to differences in therapy.

Treatment

On the basis of the 1997 World Health Organization conference, treatment is not recommended for *E. dispar* infections; if *E. histolytica*/*E. dispar* is detected (no differentiation of species) in symptomatic patients, the physician must evaluate the total clinical presentation to decide whether treatment is indicated. The detection of *E. histolytica* requires treatment of the patient regardless of the symptoms.

The drugs used for the treatment of amebiasis are of two classes: luminal amebicides for cysts (paromomycin, iodoquinol, and diloxanide furoate) and tissue amebicides for trophozoites (metronidazole, tinidazole, and dehydroemetine). Invasive disease should be treated with a tissue amebicide followed by a luminal amebicide. Tissue amebicides are not appropriate for treatment of asymptomatic infections (cysts). No high-level resistance to amebicides has been detected to date. Follow-up stool examination is always necessary because of potential treatment failures. Chemoprophylaxis is never appropriate because it may lead to drug resistance and limit the utility of drugs such as metronidazole (123, 131).

B. hominis

Epidemiology, Transmission, and Prevention

B. hominis is a common intestinal parasite of humans, with a worldwide distribution. Depending on the geographic location, it may be detected in 1 to 40% of fecal specimens.

Since its first description in 1912, the taxonomic classification has changed and is still somewhat unclear. Once classified as a yeast, it is now considered a protozoan grouped with the amebae. Silberman et al. (112) reported that the analysis of rRNA indicates that *B. hominis* should be classified with the stramenopiles (examples are brown algae such as kelp, diatoms, slime nets, and water molds). Genetic, biochemical, and immunologic analyses have revealed that great diversity exists within the species (19, 75, 86). Further studies are needed to determine whether these differences constitute separate species or strains and whether there is any epidemiologic significance associated with this diversity.

The life cycle includes amebic forms, cyst stages, and the central body form, which is the classic form seen in human stool specimens. *B. hominis* produces pseudopods and reproduces by binary fission or sporulation. It is strictly anaerobic and is capable of ingesting bacteria and other debris. The membrane-bound central body occupies up to 90% of the cell and may function in reproduction. Both thin- and thick-walled cysts have been observed. The thick-walled cysts are thought to be responsible for external transmission via the fecal-oral route; the thin-walled cysts are thought to cause autoinfection (82). Human-to-human, animal-to-human, and waterborne modes of transmission have also been proposed (71, 86, 134).

Clinical Significance

The role of *B. hominis* in human disease is still controversial, and studies supporting and refuting its pathogenicity have been published (77, 110, 137). Regardless of the number of publications on each side of this debate, clinicians may decide to treat patients with *B. hominis* infection. When *B. hominis* is present in large numbers in the absence of other pathogens, it may be the cause of gastrointestinal disease. The most common symptoms cited include recurrent diarrhea without fever, vomiting, and abdominal pain. The symptoms may be more pronounced and prolonged in patients with underlying conditions such as HIV infection, neoplasia, and abnormal intestinal tract function (49, 122). Other studies suggest that symptomatic patients receiving treatment for *B. hominis* infection may improve due to the elimination of another, undetected pathogen (77). In a study of HIV-infected individuals, Albrecht et al. (5) concluded that even in patients with severe underlying immunodeficiencies, *B. hominis* is not pathogenic and its detection does not justify treatment.

Direct Examination

Microscopy

Diagnosis of infection is made by detection of the organism, typically the central body form, by routine microscopic stool examination (Table 2; Fig. 1 and 3). The size of the central body form can vary tremendously, from 6 to 40 μm. Examination of permanent-stained smears is the procedure of choice. Exposure to water before fixation (for the concentration method) will lyse the trophozoites and central body forms, yielding false-negative results. Some type of quantitation (few, moderate, or many) should be included in the laboratory report.

Other Diagnostic Methods

Culture of the organism from stool is possible and has been reported to be more sensitive than microscopy but is not routinely available (72). A serologic response to *B. hominis* has been detected using techniques such as EIA and fluorescent-antibody testing (29, 138). It is suggested that this antibody response supports the role of *B. hominis* as a human pathogen but is not useful for diagnosis. In asymptomatic individuals, a serologic response may require exposure of up to 2 years before it is detectable (58).

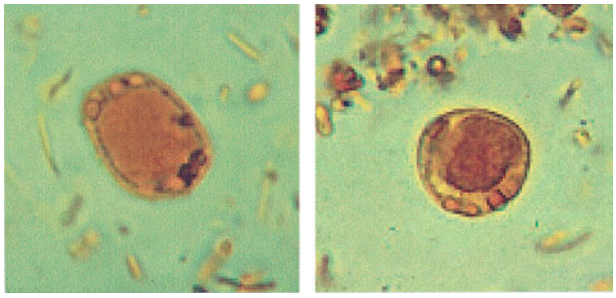


FIGURE 3 *Blastocystis hominis* on an iodine wet mount preparation. Note the peripheral nuclei and the central body form. (Courtesy of L. Garcia.)

Treatment

Until the role of *B. hominis* as an intestinal pathogen is clearly established, treatment decisions must be based on the overall clinical presentation. Current recommendations for treatment include the use of metronidazole, iodoquinol, or trimethoprim-sulfamethoxazole (2). Metronidazole appears to be the most appropriate choice at present. In vitro data on the susceptibility of *B. hominis* to various drugs are limited (137), but resistance to metronidazole has been reported (45). A failure to clear organisms from the stool has been demonstrated in patients treated with metronidazole and trimethoprim-sulfamethoxazole.

NONPATHOGENIC AMEBAE

The other species of intestinal amebae are considered nonpathogenic; except for *Entamoeba polecki,* they have a worldwide distribution and are more prevalent in warmer climates. They must, however, be differentiated from the pathogenic species, *E. histolytica.* A permanently stained smear is often essential to accomplish this goal (Table 2; Fig. 1).

E. hartmanni is a separate species that is morphologically similar to *E. histolytica/E. dispar* (Fig. 4). Size is the key differentiating characteristic. *Entamoeba coli* trophozoites may be difficult to differentiate from *E. histolytica/E. dispar* trophozoites on wet preparations. The mature cyst of *Entamoeba coli* may be refractory to fixation, making it less visible in permanent-stained smears but still detectable by the wet mount method (Fig. 5). It has been reported as the most common ameba

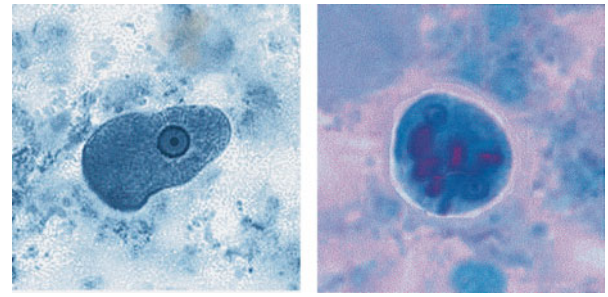


FIGURE 4 *Entamoeba hartmanni* trophozoite (left) and cyst (right). Note the "bull's eye" nucleus in the trophozoite and the numerous chromatoidal bars within the cyst. Organisms are stained with Wheatley's trichrome stain. (Courtesy of L. Garcia.)

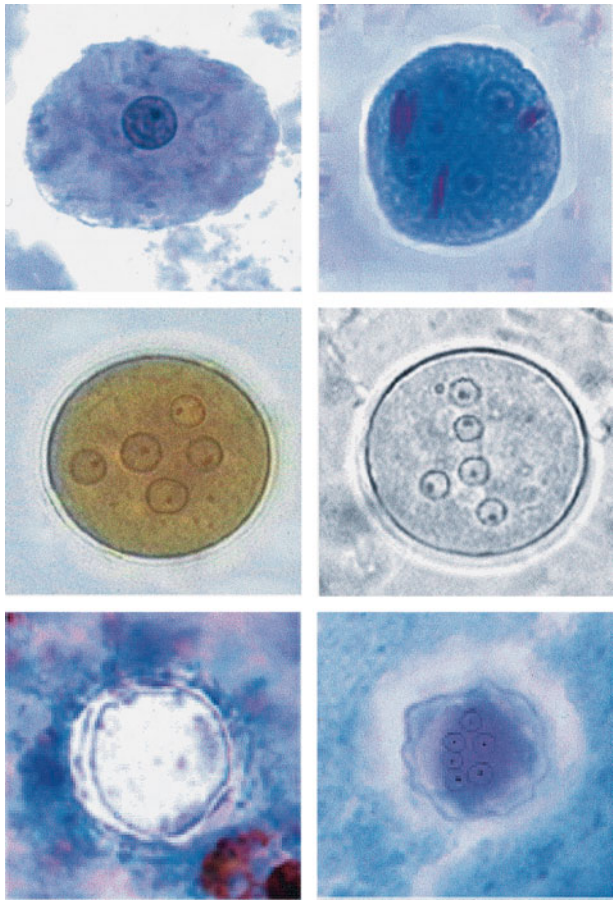


FIGURE 5 (Top row) *Entamoeba coli* trophozoite (left) and cyst (right). Note the large eccentric karyosome in the trophozoite and the chromatoidal bars with sharp pointed ends in the cyst. (Middle row) *Entamoeba coli* cyst in iodine (left) and in saline (right). The nuclei are visible; the presence of five or more nuclei is confirmatory for *Entamoeba coli.* (Bottom row) *Entamoeba coli* cyst (left; trichrome) and cyst (right; trichrome). Both cysts are very distorted and shrunken, although the nuclei can be seen in the cyst on the right. This type of shrinkage is typical of *Entamoeba coli* cysts on permanent-stained smears. (Courtesy of L. Garcia.)

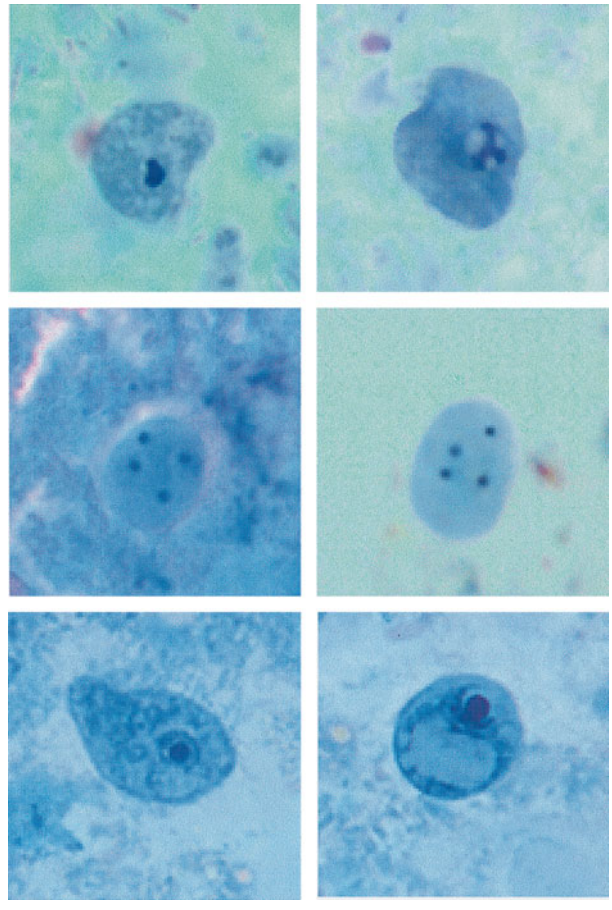


FIGURE 6 (Top row) *Endolimax nana* trophozoites. Note the typical nucleus on the left and the nuclear variation on the right. (Middle row) *E. nana* cysts. Note the typical four nuclei, none of which have peripheral chromatin. (Bottom row) *I. bütschlii* trophozoite (left) and cyst (right). Note the "basket nucleus" in the cyst, along with the large glycogen vacuole. Organisms are stained with Wheatley's trichrome stain. (Courtesy of L. Garcia.)

isolated from human stool specimens. *E. polecki* is associated with pigs, and in certain areas of the world, such as Papua New Guinea, it is the most common human intestinal parasite. The trophozoite shares characteristics with both *E. histolytica*/*E. dispar* and *Entamoeba coli;* the cyst normally has one nucleus. *Entamoeba gingivalis* was the first parasitic ameba of humans to be described. It is found in the soft tartar between teeth and can be recovered from sputum. In the trophozoite, the cytoplasm often contains ingested leukocytes. A cyst form has not been observed.

E. nana, like *E. hartmanni,* is one of the smaller intestinal amebae. It is seen in most populations as frequently as *Entamoeba coli.* There is a great deal of nuclear variation, and it can mimic *D. fragilis* and *E. hartmanni* (Fig. 6). The cysts of *E. nana* are usually oval, and both the trophozoites and the cysts are commonly present in fecal material. *I. bütschlii* has the same distribution as other nonpathogenic amebae but is less common than *Entamoeba coli* and *E. nana.* The trophozoite of *I. bütschlii* may be similar to that of *E. nana,* and differentiation between them is difficult. The cyst is very characteristic; it is round to oval and may contain a large glycogen vacuole (Fig. 6).

Treatment is not recommended for infections with any of the nonpathogenic amebae. Methods of prevention of infection with all these amebae include improved personal hygiene and improved sanitary conditions.

FLAGELLATES

Taxonomy

The flagellates that parasitize the intestinal tracts of humans belong to six genera in the phylum Sarcomastigophora, subphylum Mastigophora: *Giardia, Dientamoeba, Trichomonas, Chilomastix, Enteromonas,* and *Retortamonas* (Fig. 7).

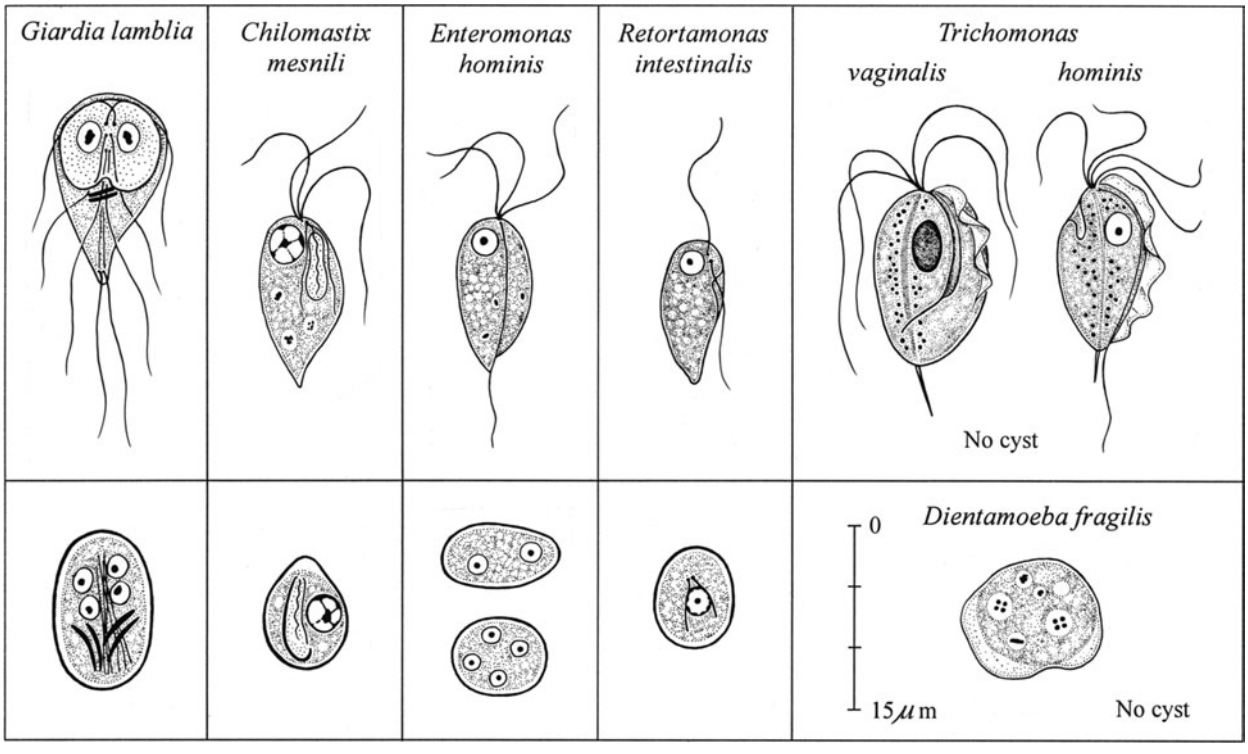


FIGURE 7 Intestinal and urogenital flagellates of humans. (Top row) Trophozoites. *T. vaginalis* is found in urogenital sites; all other flagellates are intestinal. (Bottom row) Cysts. *D. fragilis* trophozoite is shown; no cyst stage.

Description of the Agents

Some flagellates are commensals that reside in the intestinal tract and are harmless to the individual. The flagellates that are pathogenic to humans include *G. lamblia, D. fragilis,* and *Trichomonas vaginalis.* The nonpathogenic organisms are *Chilomastix mesnili, Enteromonas hominis, Retortamonas intestinalis,* and *Pentatrichomonas (Trichomonas) hominis.* As mentioned above, *D. fragilis* has been reclassified as a flagellate and appears to be closely related to the trichomonads.

Epidemiology, Transmission, and Prevention

Transmission of flagellates, with the exception of *T. vaginalis,* is initiated via the ingestion of contaminated food or water. Of the members of the six genera, all except *D. fragilis* and *Trichomonas* spp. are transmitted in a cyst form. To date, only a trophozoite form has been observed for *D. fragilis* and *Trichomonas* spp. Once in the intestine, the organism excysts, releasing trophozoites that attach to the intestinal epithelium. Completion of the life cycle in humans culminates in the release of viable cysts into the environment via the feces. Infection with any of the flagellates indicates exposure to feces regardless of the pathogenicity of the organism; preventing this exposure is the key. Measures to improve sanitary conditions are necessary to prevent the spread of infection. As yet there are no human flagellate vaccines available.

Collection, Transport, and Storage of Specimens

For the detection of flagellates and ciliates, laboratories receive predominantly stool specimens for microscopic examination. String test samples can be submitted for *Giardia* as well. Fresh or preserved specimens can be submitted, but preserved stool is the specimen of choice for most routine clinical laboratories. If fresh specimens are submitted and observed for motility, they must be examined within a specific time period depending on the consistency of the stool. Stool specimens for immunoassay or DFA assay can be fixed in formalin, which can also be used for an ova and parasite exam if immunoassays are negative for *G. lamblia*. Additional information on specimen collection and transport can be found in chapter 133 of this Manual.

Direct Examination

Microscopy

In the clinical laboratory, wet preparations and permanent-stained smears of fecal material are still the predominant specimens used to diagnose infections with flagellates. The flagellates have greater morphologic diversity relative to one another than do the amebae (described above), making determination of the genus easier (Table 4; Fig. 7). To aid in the identification of the trophozoite, key features to be noted are the shape, size, number, and position of flagella; the number of nuclei; and the presence of a spiral groove, cytostome, and characteristic features such as a sucking disk or undulating membrane. Typically, the size, shape, and number of nuclei are diagnostic characteristics used to identify cysts. Examination of permanent-stained smears is always recommended for diagnosis of infection because the wet mount may not be reflective of all organisms and stages present in the specimen.

Other Diagnostic Methods

Antigen-based tests for organisms such as *G. lamblia* are increasing in popularity due to the ease of use and increased

TABLE 4 Key features of trophozoites and cysts of common intestinal and urogenital flagellates[a]

Organism	Trophozoites	Cysts
D. fragilis	Size[b]: 5–15 μm; shaped like amebae Motility: Nonprogressive; pseudopodia are angular Flagella: None Nucleus[c]: 1 (40%) or 2 (60%); no peripheral chromatin; karyosome clusters of 4–8 granules Note: Trophozoites not visible in unstained preparation; variation in size between trophozoites can exist on the same smear; cytoplasm is finely granular, and vacuoles may be present	No cyst stage
G. lamblia	Size: 10–20 μm long, 5–15 μm wide; pear shaped Motility: Falling leaf Flagella: 4 lateral, 2 ventral, 2 caudal Nucleus: 2; not visible in unstained preparation; small central karyosomes present Note: Sucking disk is prominent on ventral side of trophozoite; organism is spoon shaped from side view	Size: 8–19 μm long, 7–10 μm wide; oval, ellipsoidal, or round Nucleus: 4 nuclei usually located on one end; not distinct in unstained preparation; no peripheral chromatin; karyosomes smaller than those in trophozoite Cytoplasm: Staining can cause shrinkage where cytoplasm pulls away from the cyst wall; 4 median bodies present Note: Poorly defined longitudinal fibers may be present
C. mesnili	Size: 6–24 μm long, 4–8 μm wide; pear shaped Motility: Stiff, rotary Flagella: 3 anterior, 1 in cytostome Nucleus: 1; not visible in unstained preparation Cytoplasm: Prominent cytostome extends over one-third to one-half of body; spiral groove across ventral surface can be hard to see; vacuoles present	Size: 6–10 μm long, 4–6 μm wide; lemon shaped with anterior hyaline knob Nucleus: Same as trophozoite; difficult to see in unstained preparation; indistinct central karyosome present Cytoplasm: Curved fibril alongside of cytostome known as "shepherd's crook"
P. hominis	Size: 5–15 μm long, 7–10 μm wide; pear shaped Motility: Jerky, rapid Flagella: 3–5 anterior, 1 posterior (extends beyond end of body) Nucleus: 1; not visible in unstained preparation Cytoplasm: Central longitudinal axostyle; undulating membrane runs the entire length of body Note: May have tremendous nuclear variation; can mimic *E. hartmanni* and *D. fragilis*	No cyst stage
T. vaginalis	Size: 7–23 μm long, 5–15 μm wide Motility: Jerky, rapid Flagella: 3–5 anterior, 1 posterior Nucleus: 1; not visible in unstained preparation Note: Undulating membrane extends one-half the length of body; no free posterior flagella	No cyst stage
E. hominis	Size: 4–10 μm long, 5–6 μm wide; oval Motility: Jerky Flagella: 3 anterior, 1 posterior Nucleus: 1; not visible in unstained preparation Note: One side of the body is flat; posterior flagellum extends free posteriorly or laterally	Size: 4–10 μm long, 4–6 μm wide; elongate or oval in shape Nucleus: 1–4; not visible in stained preparation Note: Resemble *E. nana* cysts; fibrils or flagella usually not seen
R. intestinalis	Size: 4–9 μm long, 3–4 μm wide; pear shaped or oval Motility: Jerky Flagella: 1 anterior, 1 posterior Nucleus: 1; not visible in unstained preparation Note: Very difficult to identify; rarely seen; prominent cytostome which extends half the length of body	Size: 4–9 μm long, 5 μm wide; pear or lemon shaped Nucleus: 1; not visible in unstained preparation Note: Resemble *Chilomastix* cysts; bird beak fibril arrangement; shadow outline of cytostome with supporting fibrils extends above nucleus

[a] Adapted from reference 30.
[b] Size ranges are based on wet preparations (with permanent stains, organisms usually measure 1 to 2 μm less).
[c] Nuclear and cytoplasmic descriptions are based on permanent-stained smears.

throughput compared to microscopy. In addition, nucleic acid amplification methods have been developed to detect *T. vaginalis* in clinical samples and *G. lamblia* in water and clinical samples but are not yet routinely available in the clinical laboratory. Both antigen and amplification tests for the flagellates will be discussed in more detail below. Serologic assays are available for the diagnosis of *G. lamblia* infection but are not routinely used in the clinical setting.

Evaluation, Interpretation, and Reporting of Results

It is important that a representative portion of the slide, either a wet mount or a permanent smear, be scanned before a final opinion on a specimen is given. It is also important to use an accurate micrometer to measure life cycle stages. Reports of microscopy results should clearly indicate the full taxonomic names of the organisms detected along with the forms of the organisms seen (cysts versus trophozoites). Quantitation of the number of organisms seen is not appropriate for the flagellates.

G. lamblia

Epidemiology, Transmission, and Prevention

G. lamblia is an intestinal flagellate that infects both humans and animals and is the most common cause of intestinal parasitosis in humans worldwide. Because the literature refers to this organism as *G. lamblia, Giardia intestinalis,* and *Giardia duodenalis,* it is evident that there is still debate about the classification and nomenclature of this flagellate. Previous work, based primarily on structural variations of the parasite, resulted in the proposal of three species, *Giardia muris* (mice), *Giardia agilis* (amphibians), and the intestinalis group *(Giardia duodenalis)* (3, 30). Further studies support the existence of additional species, *Giardia ardeae* (herons), *Giardia psittaci* (parakeets), and *Giardia microti* (voles and muskrats). Also, it appears that *G. lamblia (intestinalis)* is a species complex that should contain the species *G. microti* as well (83). Further studies are needed to elucidate species designations and groupings. Although these species have been proposed, *G. lamblia* is the name predominantly used in the United States and will continue to be used throughout this chapter.

It is now accepted that there is considerable genetic diversity within *G. lamblia.* Based on molecular biology, the genus *Giardia* is subdivided into major genotypes containing subgenotypes. The major genotypes of *G. lamblia* that are associated with human infections are assemblages A and B. Assemblage A is associated with a mixture of both human and animal isolates, whereas assemblage B is typically associated with human isolates only. Most zoonotic infections from animal to human occur with assemblage A (38).

Infection with *G. lamblia* occurs through fecal-oral transmission or the ingestion of cysts in contaminated food or water, and an inoculum of only 10 to 100 cysts is sufficient for human infection (99). Individuals more commonly infected in developed countries are children in day care centers, hikers, and immunocompromised individuals. Among immunocompromised individuals, infections have been documented in people with AIDS and hypogammaglobulinemia and those affected by malnutrition. Prevalence rates for this pathogen range from 1 to 7% in industrialized countries and from 5 to 50% in developing countries. Of the intestinal flagellates, *G. lamblia* is the flagellate most frequently isolated in the United States. In high-risk domestic settings, such as day care centers, prevalence rates can reach 90% (93). A *Giardia* vaccine is available for dogs and cats; this may affect the prevalence of infections in humans (88).

Infection occurs when viable cysts are ingested, excyst, and transform into trophozoites. After excystation, the trophozoite, which appears to have a propensity for the duodenum, attaches to the mucosal epithelium. Trophozoites attach to the epithelium via a sucking disk located on the ventral side of the parasite. During the course of infection, the parasites remain attached to the epithelium and do not invade the mucosa. In the intestine, trophozoites divide by binary fission to produce two identical daughter trophozoites. As they move down toward the large intestine, the trophozoites encyst, and infective cysts are excreted into the environment (30).

Clinical Significance

The majority of individuals infected with *G. lamblia* are asymptomatic. Asymptomatic versus symptomatic infection may be due to the existence of two different strains of *Giardia* with different levels of virulence. Preliminary studies have grouped *G. lamblia* into two groups, group A and group B. Group A appears to be more pathogenic and is associated with symptomatic infection. Isoenzyme and molecular studies show that group A and group B differ from one another and appear to be no more related than *E. histolytica* and *E. dispar.* The genes homologous between group A and group B *Giardia* isolates show an identity of approximately 81 to 89% (83, 91, 92). In symptomatic individuals, acute *G. lamblia* infections can mimic infections with other protozoal, viral, and bacterial pathogens. After an incubation period of approximately 12 to 20 days, patients can experience nausea, chills, low-grade fever, epigastric pain, and a sudden onset of watery diarrhea. Diarrhea is often explosive and presents as foul smelling without the presence of blood, cellular exudate, or mucus. Individuals can develop subacute or chronic infections with symptoms such as recurrent diarrhea, abdominal discomfort and distention, belching, and heartburn. In patients with chronic cases of giardiasis, diarrhea can lead to dehydration, malabsorption, and impairment of pancreatic function (14).

Direct Examination

Microscopy

Diagnosis of *G. lamblia* infection is typically established by the microscopic examination of stool for the presence of cysts and/or trophozoites. Stained smears are more helpful in identifying the trophozoite stage of the infection, although this stage can be identified in wet mount preparations (Table 4; Fig. 7 and 8). Examination of stool specimens may not be diagnostic because cyst forms can be trapped in mucus, making them difficult to detect on smears. Also, the excretion pattern of cysts can be cyclical. In these instances, other methods, such as the string test (Entero-Test; HDC Corp., San Jose, Calif.), should be used to obtain clinical samples. The string test procedure consists of a patient swallowing a weighted capsule containing gelatin and a tightly wound string. After approximately 5 h, the string is removed from the patient and the adherent material is examined as a wet mount or permanent smear (30). Endoscopy can also be used to collect clinical specimens. Because this procedure is invasive, it is used only for diagnosis of disease in patients with perplexing clinical presentations (93).

Antigen Detection

Other assays that have gained wide acceptance for the detection of *G. lamblia* in stool specimens are EIAs, DFA assays, and immunochromatographic assays. These methods

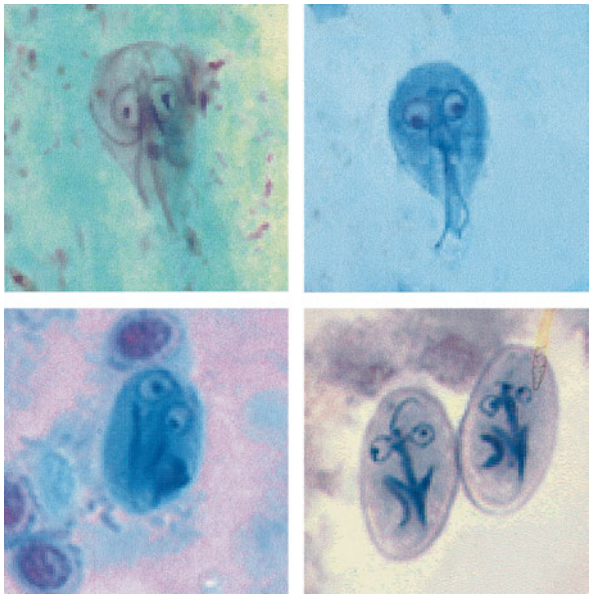


FIGURE 8 (Top row) *Giardia lamblia* trophozoites. Note the typical nuclei, axonemes, and curved median bodies. (Bottom row) *G. lamblia* cysts. The cysts on the right are stained with iron-hematoxylin. Wheatley's trichrome stain. (Courtesy of L. Garcia.)

have very high specificities (90 to 100%) and sensitivities that range from 65 to 100% (32, 56, 59, 76). Although these methods have proven to be fairly sensitive and specific compared to the routine staining of fecal smears (trichrome staining), other important pathogens may be missed if a wet mount and permanent-stained smear are not examined. The DFA assay widely used is the Merifluor reagent assay (Meridian Bioscience, Inc., Cincinnati, Ohio), but it does require the availability of a fluorescent microscope. DFA assays have a sensitivity and specificity of >95%, and there are reagents to detect *C. parvum* as well. Immunoassays are available and are useful for testing large numbers of patient specimens, and some studies have shown the performance of the EIA to be excellent compared to that of the ova and parasite exam (31, 56, 76). Two available immunoassays are the ProSpecT *Giardia* EZ (Alexon-Trend) and the ColorPAC *Giardia* assay (Becton Dickinson and Company, Sparks, Md.) (59). If the patient is immunocompromised, has traveled, or is suspected of coming into contact with another pathogen, the ova and parasite examination should be performed. Some studies suggest that the use of an immunoassay does not eliminate the need for more than one stool specimen. Positive specimens from asymptomatic patients may be missed if only one stool specimen is tested using an immunoassay (39). Some immunoassays are affected by specimens contaminated with blood. It was documented in one study that blood can interfere with the performance of the ProSpecT assay and potentially cause false-positive results (59). Immunochromatographic detection methods have also been developed for the clinical laboratory (31, 33, 34, 97). The ImmunoCard STAT (Meridian Bioscience, Inc.) has been developed to detect *G. lamblia* and *C. parvum* antigens in stool specimens by using rapid solid-phase qualitative immunochromatography. Patients negative by the ImmunoCard STAT that remain symptomatic should be tested using a routine ova and parasite exam (34). The Triage Micro parasite panel (Biosite Inc.) is an immunoassay that can detect not only *G. lamblia* antigens but also those of *E. histolytica/E. dispar* and *C. parvum* in stool specimens. Antigens in stool specimens are immobilized on a membrane containing specific antibodies to the respective intestinal parasites. The formation of color bars occurs on a specific area of the membrane depending on the parasite present in the stool. All of these assays described can be run using formalin-fixed stool. This is important in the event that the laboratory would need to run an ova and parasite exam on the same specimen. Although there are several methods now available for the detection of *G. lamblia,* parameters such as workload, skill levels of technologists, and the availability of necessary equipment should be considered in choosing a specific method.

Nucleic Acid Detection Techniques

Some investigators are now using molecular tools to diagnose *G. lamblia* infection with clinical samples (6, 38, 124). One group has developed an oligonucleotide microarray with excellent sensitivity and specificity for the detection of *G. lamblia* and other intestinal parasites in stool specimens. The assay was also able to discriminate between *Giardia* assemblages A and B (127). A multiplex PCR assay to detect specific genotypes of *G. lamblia* has also been developed which may potentially aid in epidemiologic investigations of outbreaks (85). It is important to note that molecular detection of *G. lamblia* has not been widely implemented and there are no commercial assays approved by the Food and Drug Administration for clinical use to date.

Because of the potential for contamination of municipal water supplies, routine monitoring of water for parasitic protozoa is recommended as a public health control measure. *G. lamblia* cysts can remain viable for 2 to 3 months in cold water and are fairly resistant to killing by routine chlorine treatments. DFA assays are commonly used to detect *G. lamblia* cysts in water samples, but PCR is also being used for this purpose (102). The advantage of PCR is the increased sensitivity compared to those of fluorescence assays or assays that use special stains. Research continues to enhance the ability of investigators to detect viable organisms, which is a better indication of poor water quality (129).

Treatment

For individuals diagnosed with giardiasis, the treatment of choice is metronidazole or nitazoxanide (2). Alternatives include tinidazole, paromomycin, furazolidone, and quinacrine (2) or other nitroimidazoles such as ornidazole and secnidazole (35). In most immunocompetent hosts, infection is self-limiting; however, treatment will lessen the duration of symptoms and prevent transmission. Other drugs that have been used to treat giardiasis are albendazole, mebendazole, and bacitracin (78), and there are other therapeutics on the horizon. Albendazole, mebendazole, and paromomycin have lower activity against *Giardia* than do the nitroimidazoles (35). Resistance to metronidazole and other agents has been observed clinically and in vitro (123). In vitro resistance-testing assays are available but lack the standardization required for the clinical laboratory (115).

D. fragilis

Epidemiology, Transmission, and Prevention

D. fragilis is found worldwide and is known to cause a noninvasive diarrheal illness in humans. Despite the lack of external

flagella, this parasite is currently classified as a flagellate but has historically been grouped with the amebae. Electron microscopy and antigenic analysis have aided in the classification of this organism and have demonstrated that it is closely related to *Trichomonas* and *Histomonas* species. Phylogenetic analysis of small-subunit rRNA sequences has also confirmed the relationship between *D. fragilis* and *Histomonas* (36, 111). Currently *D. fragilis* is grouped in the phylum Parabasala, class *Trichomonadea,* family *Trichomonadidae,* and possibly the subfamily *Tritrichomonadinae* (36). Colonization in humans is similar to that seen with other intestinal parasites; typically, the cecum and the proximal part of the colon are affected. Because *D. fragilis* does not have a cyst stage as part of its life cycle, the mode of transmission is less well understood. One hypothesis concerning the spread of *D. fragilis* is that transmission occurs within the eggs of *Enterobius vermicularis* or *Ascaris lumbricoides* (12). Several studies provide circumstantial evidence to support *E. vermicularis* as a vector, documenting a greater-than-expected coinfection rate with the two organisms (54).

Though *D. fragilis* has a worldwide distribution, prevalence rates for this organism vary quite substantially from 0% in Prague to 42% in Germany (54). There appears to be a higher prevalence in certain groups of individuals such as missionaries, Native Americans living in Arizona, and institutionalized individuals (132). In the United States, the prevalence is reported to be quite low. This may be due to underreporting attributable to difficulties associated with identifying the organism in clinical samples. In addition, Johnson and Clark (55) identified the existence of two genetically distinct types of *D. fragilis* with a resulting sequence divergence of approximately 2% (55, 94). The degree of divergence appears to be similar to that seen with *E. histolytica* and *E. dispar.* Since asymptomatic infections occur, this finding may lend support to the possibility that two distinct species exist, though there is no consensus on this matter (54, 55).

Clinical Significance

The frequency of symptomatic disease ranges from 15 to 25% in adults, and symptomatic disease is more common in children, in whom up to 90% of those infected have clinical signs (13, 93). Symptoms include fatigue, insufficient weight gain, diarrhea (often intermittent), abdominal pain, anorexia, and nausea. Studies have linked *D. fragilis* infection to biliary infection (118), irritable bowel syndrome (10), allergic colitis (23), and diarrhea in HIV-infected patients (69). One case report describes a patient who presented in the emergency room with acute appendicitis which was attributed to *D. fragilis* infection (105). Some individuals, mainly children, also experience unexplained peripheral blood eosinophilia (30). Diarrhea is seen predominantly during the first 1 to 2 weeks after the onset of disease. The number of *D. fragilis* organisms can vary greatly from day to day, which is similar to those of other intestinal protozoa. Abdominal pain can persist for 1 to 2 months (13). Although *D. fragilis* has been implicated in the above-mentioned clinical situations, the organism is isolated from patients with no apparent clinical symptoms. In addition, the lack of an animal model affects researchers' ability to detect specific pathological manifestations (54).

Direct Detection

Microscopy

Diagnosis of *D. fragilis* infection is similar to that of infections with other intestinal protozoa, and detection of the trophozoite in fresh or preserved stool is warranted to establish infection. Laboratories using direct microscopy may be at a disadvantage since the trophozoite can degenerate if not placed in fixative. Nonfixed trophozoites can appear rounded and refractile and are more difficult to identify microscopically. Trophozoites have been recovered from soft and formed stools, indicating the need to evaluate all types of samples (13). The recovery of *D. fragilis* is greatly enhanced by the collection of at least three stool specimens (20, 93). Morphologically, the trophozoite of *D. fragilis* contains one or two nuclei (binucleate), with two nuclei being more common. Well-trained laboratory personnel can identify *D. fragilis* trophozoites in stool specimens, but because no cyst stage exists, diagnosis from the wet mount can be difficult. Use of a permanent-stained smear is the recommended procedure for detection (Table 4; Fig. 7 and 9).

Other Diagnostic Methods

Culture has been shown to be more sensitive than microscopy; however, it is not recommended for the routine clinical laboratory (54). Antigen and antibody techniques have been used as diagnostic tools for the detection of *D. fragilis* but are not commercially available (17, 18). *D. fragilis* DNA has been detected using PCR with fresh, unpreserved stool. This technique has the promise of being more sensitive than the microscopy (117).

Treatment

The treatment of choice for symptomatic individuals is iodoquinol (diiodohydroxyquin) (2). Metronidazole, iodoquinol, and tetracycline have been used to treat children (30). Additional therapeutic options include diphetarsone, carbarsone, erythromycin, hydroxychinoline, paromomycin, and secnidazole (37, 54). If *E. vermicularis* is detected concomitantly, the treatment regimen should also include mebendazole (13).

T. vaginalis

Epidemiology, Transmission, and Prevention

T. vaginalis is a pathogenic flagellate that infects the urogenital tracts of males and females. The infection is primarily a sexually transmitted disease (STD) and is thought to be the most common curable STD among sexually active young women. There are an estimated 7.4 million new cases each year in the United States; worldwide, there are 180 million cases of trichomoniasis each year (128). The incidence of trichomoniasis differs depending on the population examined, varying from 5 to 60% in various studies. Factors such as lower socioeconomic

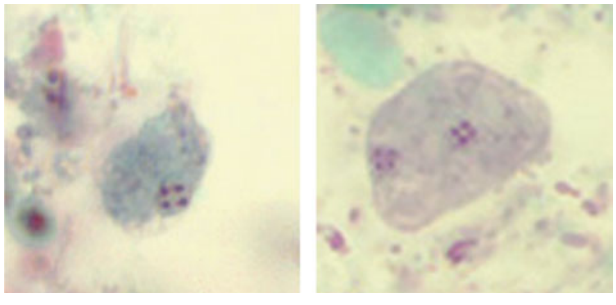


FIGURE 9 *Dientamoeba fragilis* trophozoites. The trophozoites can have one nucleus (left) or two nuclei (right). No cyst stage is known for this organism. Trophozoites are stained with Wheatley's trichrome stain. (Courtesy of L. Garcia.)

status, multiple sex partners, and poor personal hygiene are linked to a higher incidence of infection.

The estimated infection rates cited in the literature may indeed be too low because (i) trichomoniasis is not a reportable disease in the United States and other countries; (ii) the infection, particularly in men, can be asymptomatic; and (iii) laboratory tests used for diagnosis vary in their sensitivities. Despite these rates of infection and their serious medical consequences, trichomoniasis has not received adequate attention from public health and STD prevention programs (106).

The life cycle of *T. vaginalis* includes only the trophozoite stage; there is no cyst stage. The organism is similar in morphology to the other trichomonads and is characterized by a pear shape with a prominent axostyle and an undulating membrane that stops halfway down the side of the trophozoite (Table 4; Fig. 7 and 10). It is a facultative anaerobe that divides by binary fission, and it cannot survive long outside the host.

Clinical Significance

Infection in females can result in vaginitis, cervicitis, and urethritis (106). The classic vaginal discharge is described as copious, liquid, greenish, frothy, and foul smelling. The onset of symptoms, such as intense vaginal and vulvar pruritus and discharge, is often sudden and occurs during or after menstruation. The vaginal pH is usually elevated above the normal pH of 4.5, and dysuria occurs in 20% of women with *T. vaginalis* infection. Infection has also been associated with premature rupture of membranes, premature birth, and posthysterectomy cuff infections (21, 81, 114). In men, the most common symptomatic presentation is urethritis. Up to 50% of infected women are asymptomatic carriers. In men, the majority of infections are asymptomatic. Asymptomatic carriers serve as a reservoir for transmission and also remain at risk for developing disease. Trichomoniasis has been implicated as a cofactor in the transmission of HIV (53, 68). In one study, men with symptomatic *Trichomonas* urethritis were found to have increased HIV concentrations in seminal plasma compared to HIV-infected men without urethritis (47).

Neonates can acquire the organism during passage through the infected birth canal. It is estimated that 2 to 17% of female babies acquire trichomoniasis by direct vulvovaginal contamination (68). Reports have also documented *T. vaginalis* as a cause of neonatal pneumonia (79).

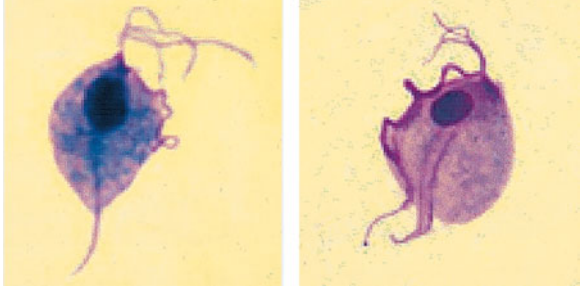


FIGURE 10 *Trichomonas vaginalis* trophozoites. Note the undulating membrane, which comes about halfway down the body. Although the membrane appears to come down farther on one of the trophozoites (right), this appearance occurs because of the photographic angle. Also, note the axostyle that penetrates the bottom of the organism. No cyst stage is known for this organism. Trophozoites are stained with Giemsa stain. (Courtesy of L. Garcia.)

Direct Detection

Microscopy

The diagnosis of *T. vaginalis* infection is commonly based on the examination of wet preparations of vaginal and urethral discharges, prostatic secretions, and urine sediments. Permanent stains such as Papanicolaou and Giemsa stains can be used, but the organisms may be difficult to recognize (65). For wet preparations, vaginal specimens are routinely collected during a speculum examination, but studies suggest that self-collected or tampon-collected specimens may be used successfully (46, 108). Specimens should be mixed with a drop of physiologic saline and examined microscopically within 1 h under low power (magnification, ×100) with reduced illumination. Specimens should never be refrigerated. The presence of actively motile organisms with jerky motility is diagnostic. The movement of the undulating membrane may be seen as the motility of the trophozoite diminishes. Polymorphonuclear cells are often present. The sensitivity of the wet preparation test is between 50 and 70%, depending on the skill of the microscopist and other factors. Perhaps the most important factor affecting sensitivity is the time between collection and examination of the specimen. Viability of the organism is essential for the detection of motility on the wet mount and drops off precipitously with time. Amies gel agar transport medium can maintain the viability for culture of *T. vaginalis* on swabs held at room temperature for 24 ± 6 h prior to inoculation of the specimen into a culture pouch (48). Because the morphology of *P. hominis*, a nonpathogenic intestinal flagellate, is very similar to that of *T. vaginalis*, care must be taken to ensure that specimens are not contaminated with fecal material.

Culture

Culture has greater sensitivity (>80%) than the wet mount method and is considered the "gold standard" method for the detection of *T. vaginalis*. Specimens must be collected properly and inoculated immediately into the appropriate medium, such as modified Diamond's, Trichosel, or Hollanders medium. Due to cost and convenience, this approach is not routinely used. Culture systems (InPouchTV [BioMed Diagnostics, San Jose, Calif.] and the system of Empyrean Diagnostics, Inc., Mountain View, Calif.) that allow direct inoculation, transport, culture, and microscopic examination are commercially available (9, 26). In situations where immediate transport of specimens is not feasible, the use of these transport/culture devices should be encouraged. Studies have also shown that a delayed inoculation protocol is as sensitive as immediate inoculation, allowing the results of microscopy to be used to determine whether further culture is necessary (109). Serologic testing is not useful for the diagnosis of trichomoniasis.

Antigen Detection

Several antigen detection methods have been developed for *T. vaginalis* and offer the advantage of being rapid and easy to perform. A latex agglutination test (TV Latex; Kalon Biological, Surrey, United Kingdom) has been shown to have excellent sensitivity (4) but is not available in the United States. An immunofluorescence assay (Light Diagnostics *T. vaginalis* DFA; Chemicon International, Temecula, Calif.) is available in the United States for testing directly from patient samples. Two immunochromatographic capillary flow assays are available commercially for the qualitative detection of *T. vaginalis* antigens from vaginal swabs. The OSOM Trichomonas Rapid Test (Genzyme Diagnostics, Cambridge, Mass.) is a dipstick assay providing results in 10 min and has

been shown to have good sensitivity and specificity (83.3 and 98.8%, respectively) compared to a combined standard of wet mount and culture (52). The Xenostrip Tv Trichomonas vaginalis test (Xenotope Diagnostics, Inc., San Antonio, Tex.) is a rapid point-of-care test that has demonstrated sensitivities of 67 to 80% and specificities of >99% in published studies (66, 98).

Nucleic Acid Detection Techniques

The Affirm VPIII (Becton Dickinson and Company) is a direct DNA probe test for the detection of organisms from vaginal swabs associated with vaginosis/vaginitis. It tests for the three most common syndromes associated with increased vaginal discharge: bacterial vaginosis *(Gardenerella vaginalis)*, candidiasis *(Candida albicans)*, and trichomoniasis. The assay has good sensitivity and specificity compared with wet mount and culture for *T. vaginalis* (90 and 98%, respectively [package insert, Affirm VPIII, version no. 670160JAAG, Becton Dickinson and Company, Sparks, Md.]). Nucleic acid-based amplification methods, such as PCR, for the detection of *T. vaginalis* have been reported in the literature, but none are yet commercially available. These amplification tests have demonstrated varying sensitivities depending on the genomic target, specimen type, and sex of the patient (44, 73, 100). PCR has a sensitivity of 85 to 100% with vaginal swabs; its sensitivity with urine is lower, ranging from 60 to 80% (61, 70, 106). For men, PCR with urine and urethral swabs has been reported and appears to be more sensitive than conventional methods (62, 107).

While the wet mount provides a rapid result at a low cost, tests with the increased sensitivities of nucleic acid probes or PCR may be indicated because of the impact of *T. vaginalis* infections on pregnancy and the link with HIV transmission (48, 106). An algorithm to reflex specimens with negative wet mounts to culture or a more sensitive methodology may be a useful diagnostic approach.

Treatment

The treatment of choice for *T. vaginalis* infections is metronidazole, with oral therapy recommended over topical treatment (16). More recently, tinidazole has received U.S. Food and Drug Administration approval for the treatment of trichomoniasis in adults (2) and may be used as a first-line agent or for refractory cases previously treated with metronidazole (22). Metronidazole is the recommended therapy for treatment for symptomatic infection during pregnancy. There are no data to support treatment for asymptomatic infections as studies have failed to demonstrate that metronidazole therapy prevents preterm birth (16, 63). All sexual partners of infected individuals should also receive treatment. Treatment failure with metronidazole is most often due to noncompliance or reinfection. True resistance to metronidazole has been documented and appears to be increasing (22, 113). While not routinely available, methods have been published for the in vitro determination of susceptibility. These methods have not been standardized, and the results can vary based on assay conditions (8, 80).

NONPATHOGENIC FLAGELLATES

C. mesnili is found worldwide and is generally considered nonpathogenic. Unlike *D. fragilis, C. mesnili* has both a trophozoite and a cyst stage. The organism is acquired through the ingestion of contaminated food or water and resides in the cecum and/or colon of the infected human or animal. The trophozoite is 6 to 24 μm long and contains a characteristic spiral groove that runs longitudinally along the body (Table 4; Fig. 7 and 11). Motility of the organism can sometimes be seen in fresh preparations, and the spiral groove may be exposed as the organism turns. Flagella are difficult to see in stained preparations. The trophozoite contains one nucleus, with a cytostome or oral groove in close proximity. The pear-shaped cyst retains the cytoplasmic organelles of the trophozoite, with a single nucleus and curved cytostomal fibril. Observing the organism in permanent-stained preparations makes identification more definitive.

P. hominis, formerly referred to as *Trichomonas hominis,* is a nonpathogenic flagellate that is similar to *D. fragilis* in that only the trophozoite stage has been observed. Although the organism is cosmopolitan in nature and is recovered from individuals with diarrhea, it is still considered nonpathogenic. The trophozoites typically inhabit the cecum. They are pyriform and contain an undulating membrane that runs the length of the parasite. The use of permanent smears is recommended for observation of these organisms in clinical specimens. The trophozoites may stain weakly, making them difficult to detect on stained smears (30).

Two additional nonpathogenic intestinal flagellates are *E. hominis* and *R. intestinalis.* Both *E. hominis* and *R. intestinalis* are found in warm or temperate climates, and infection is acquired through the ingestion of cysts. When clinical specimens are examined, it is important to note that cysts of *E. hominis* can resemble those of *E. nana,* although *E. nana* cysts containing two nuclei are rare. Because of the small sizes of *E. hominis* and *R. intestinalis,* it is difficult to detect these organisms even when permanent-stained smears are examined. This may lead to the underreporting of both organisms. *R. intestinalis* has been recovered from the pancreatic juice of a patient with small lesions of the pancreatic duct (60).

In general, treatment is not recommended for infections with the nonpathogenic flagellates. Improved personal hygiene and sanitary conditions are key methods for the prevention of infection.

CILIATES

Balantidium coli

Taxonomy

B. coli belongs to the phylum Ciliophora, the members of which are protozoa possessing cilia in at least one stage of their life cycles. The ciliophora also have two different types of nuclei, one macronucleus and one or more micronuclei.

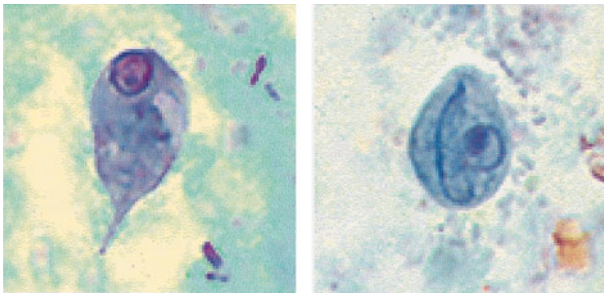


FIGURE 11 *Chilomastix mesnili* trophozoite (left) and cyst (right). Note the oral groove (feeding groove) at the right side of the trophozoite (clear area) and the curved fibril (shepherd's crook) in the cyst. Also note the typical pear or lemon shape of the cyst. Organisms are stained with Wheatley's trichrome stain. (Courtesy of L. Garcia.)

Description of the Agent

This organism has both the trophozoite and cyst forms as part of its life cycle (Table 5; Fig. 12). The cyst form is the infective stage. After ingestion of the cysts and excystation, trophozoites secrete hyaluronidase, which aids in the invasion of the tissue. The trophozoite, which is oval and covered with cilia, is easily seen in wet mount preparations under low-power magnification. The cytoplasm contains both a macronucleus and a micronucleus, in addition to two contractile vacuoles. Motile trophozoites can be observed in fresh wet preparations, but the specimen must be observed soon after collection. The trophozoite is somewhat pear shaped and also contains vacuoles that may harbor debris such as cell fragments and ingested bacteria. Cyst formation takes place as the trophozoite moves down the large intestine.

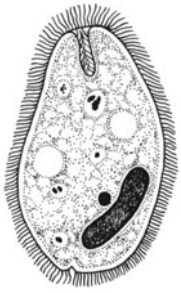
Epidemiology, Transmission, and Prevention

B. coli exists in animal reservoirs such as pigs and chimpanzees. The organism is the only pathogenic ciliate and the largest pathogenic protozoan known to infect humans. Transmission occurs by the fecal-oral route following ingestion of the cysts in contaminated food or water. Infection is more common in warmer climates and in areas where humans are in close contact with pigs. As with other intestinal protozoa, poor sanitary conditions lead to a higher incidence of infection.

Clinical Significance

Infection with *B. coli* is most often asymptomatic; however, symptomatic infection can occur, resulting in bouts of dysentery similar to amebiasis (15, 30). In addition, colitis caused by *B. coli* is often indistinguishable from that caused by *E. histolytica*. Symptoms typically include diarrhea, nausea, vomiting, headache, and anorexia. Fluid loss can be dramatic, as seen in some patients with cryptosporidiosis. The organism can invade the submucosa of the large bowel, and ulcerative abscesses and hemorrhagic lesions can occur. The shallow ulcers and submucosal lesions that result from invasion are prone to secondary infection by bacteria and can be problematic for the patient (24, 64). Death due to invasive *B. coli* infection has been reported (24). Even though the organism can invade the underlying mucosa, it does not typically spread to other organs. Infections associated with extraintestinal sites have been described on rare occasions (24, 64, 67).

TABLE 5 Key features of the ciliate *B. coli*

Stage	Characteristics
Trophozoite 	Shape and size: Ovoid with tapering anterior end; 50–100 μm long, 40–70 μm wide Motility: Rotary, boring; may be rapid Nuclei: 1 large kidney-bean-shaped macronucleus may be visible in unstained preparation; 1 small round micronucleus adjacent to macronucleus, difficult to see Cytoplasm: May be vacuolated; may contain ingested bacteria and debris; anterior cytostome Cilia: Body surface covered with longitudinal rows of cilia; longer near cytostome Note: May be confused with helminth eggs or debris on a permanent-stained smear; concentration or sedimentation examination recommended
Cyst 	Shape and size: Spherical or oval; 50–70 μm in diam Nuclei: 1 large macronucleus, 1 micronucleus, difficult to see Cytoplasm: Vacuoles are visible in young cysts; in older cysts, internal structure appears granular Cilia: Difficult to see within the thick cyst wall

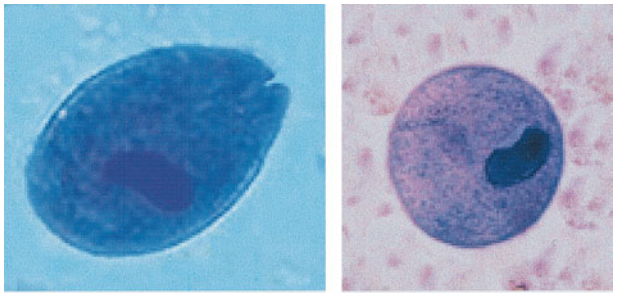


FIGURE 12 *Balantidium coli* trophozoite (left) and cyst (right). Note the single macronucleus visible in both the trophozoite and the cyst. The much smaller micronucleus is not visible. Organisms are stained with Wheatley's trichrome stain. (Courtesy of L. Garcia.)

Direct Examination

Microscopy

Either ova and parasite examination of feces or histologic examination of intestinal biopsy specimens establishes the diagnosis of *B. coli* infections. The diagnosis can be established only by demonstrating the presence of trophozoites in stool or tissue samples (67). It is very easy to identify these organisms in wet preparations and concentrated stool samples. Conversely, it can be challenging to identify *B. coli* from trichrome-stained permanent smears because the organisms are so large and have a tendency to overstain. This makes the organism less discernible and increases the chance of misidentification.

Treatment

The treatment of choice for *B. coli* infection is tetracycline, although it is considered an investigational drug when used in this context (2). Metronidazole and iodoquinol are therapeutic alternatives used in some cases (30).

SUMMARY

Clinical laboratories are now given more choices for testing in diagnostic parasitology, with assays ranging from microscopy, culture, antigen detection, and nucleic acid amplification techniques. Molecular biology has the promise to deliver more sensitive and specific methods, but to date these methods have not been fully adapted to the clinical diagnostic laboratory. Some researchers have implemented their own laboratory-developed amplification assays for better detection of intestinal protozoa such as *Giardia,*

Cryptosporidium, and *Trichomonas*. In addition to these amplification tests, rapid point-of-care tests are available for organisms such as *T. vaginalis*. Results can now be available in real time for the clinician to manage patients directly in the exam setting. Even though the field of diagnostic parasitology is changing, clinical laboratories are still faced with using microscopy for routine work-up for stool specimens due to the slow implementation of some of the new methods by commercial diagnostic companies. Not all laboratories are equipped to develop home brew molecular tests and rely on the diagnostic industry to bring products to the market. Some laboratories have switched to antigen-based methods, but many still rely on microscopy because antigen-based methods cannot detect all potential pathogens in a given stool specimen. Microscopy, as we know, cannot differentiate between pathogenic and nonpathogenic amebae and the different genotypes of *Giardia*. On the horizon are newer methodologies, such as the microarray, which would allow for the detection of multiple pathogens from a clinical specimen. Some manufacturers are developing assays for the detection of stool parasites using chemiluminescent and bead-based technologies. These exciting new areas will help increase the options that the clinical parasitology laboratory has for the diagnosis of intestinal parasitic infections.

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Isospora, Cyclospora, and *Sarcocystis**

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Isospora (Cystoisospora), Cyclospora, and *Sarcocystis* are intestinal coccidia infecting humans (Fig. 1 and 2). They have varied life cycles, epidemiology, treatment requirements, and diagnostic methods. Oocysts of these coccidia are found in the feces of humans (Table 1), and diagnosis is based ultimately on demonstrating oocysts in human stool samples.

TAXONOMY

Isospora, *Cyclospora,* and *Sarcocystis* are in the Phylum Apicomplexa, Subclass Class Sporozoasida, Subclass Coccidiasina, Order Eucoccidiorida, Suborder Eimeriorina. *Cyclospora cayetanensis* is in the Family Eimeriidae, while *Sarcocystis hominis* and *S. suihominis* are in the Family Sarcocystidae. The placement of *Isospora belli* is currently under debate as to whether it should be in the Family Eimeriidae or moved to the Family Sarcocystidae.

DESCRIPTION OF ORGANISM, EPIDEMIOLOGY, TRANSMISSION, AND PREVENTION

Life Cycles

Isospora belli

The life cycle is direct (monoxenous), but evidence exists that it can be facultatively heteroxenous (use two hosts). *Isospora belli* oocysts are passed in the feces unsporulated or partially sporulated (Fig. 1C and D and 2A and B). Oocysts generally complete sporulation within 72 h, although 1 to >5 days have been reported depending on temperature (59). The prepatent period, i.e., the time it takes for unsporulated oocysts to appear in the feces after sporulated oocysts are ingested, is 9 to 17 days (28, 54). The patent period, i.e., the time from when oocysts first are excreted in the feces until they can no longer be observed in the feces, is quite variable and depends on the immune status of the infected individual. Oocysts can usually be found for 30 to 50 days in immunocompetent patients, while immunosuppressed patients may continue to shed oocysts for 6 months or more (60). This prolonged oocyst shedding in immunosuppressed patients is presumably due to recycling of one or more merogonous stages or activation of dormant extraintestinal unizoites (Fig. 3).

Developmental stages of *I. belli* have been reported from intestinal biopsy specimens of the duodenum, jejunum, and occasionally ileum, and oocysts can be aspirated directly from the duodenal contents. Intestinal development occurs predominantly in epithelial cells, although meronts (schizonts) are occasionally reported from the lamina propria or submucosa (6). At least two generations of meronts, as well as macrogametocytes (female sexual stage), microgametocytes (male sexual stage), and unsporulated oocysts, have been documented in these reports.

I. belli sporozoites/merozoites are capable of traveling extraintestinally and becoming dormant as unizoite tissue cysts (Fig. 3) in a variety of tissues, including intestine, mesenteric lymph nodes, liver, and spleen (52, 57). These cysts are commonly termed "unizoites" or "hypnozoites." Unizoic cysts in histological sections are thick walled and measure 12 to 22 by 8 to 10 μm, each containing a single dormant sporozoite/merozoite measuring about 8 to 10 by 5 μm (52, 57). Presumably, the cysts are capable of reactivating patent infections once immunity wanes.

The existence of these unizoic cysts has led to speculation that a paratenic (transport) host may be involved in the life cycle of *I. belli* (52). Paratenic hosts are known to occur in the *Isospora* species that infect cats and dogs.

Isospora natalensis

The life cycle of *I. natalensis* is presumably monoxenous (direct). The oocysts are smaller and more spherical that those of *I. belli* (Table 1). Oocysts are passed in the feces unsporulated. At ambient temperature, some oocysts completed sporulation within 24 h (26). The prepatent and patent periods are unknown. One individual passed unsporulated oocysts for at least 4 days (18). Oocysts of *I. natalensis* have been reported only from patients from South Africa. The validity of this species is questionable.

Cyclospora cayetanensis

The life cycle of *C. cayetanensis* is monoxenous and involves only humans as hosts. Oocysts are passed in the feces unsporulated (Fig. 1A and 2C). At room temperature (23 to 25°C), low numbers of oocysts may sporulate within 10 to 12 days (37, 74) (Fig. 1B). However, many oocysts require 3 to 4 weeks for sporozoites to fully develop (S. J. Upton,

*This chapter contains information presented in chapter 134 by Ynes R. Ortega and Michael Arrowood in the eighth edition of this Manual.

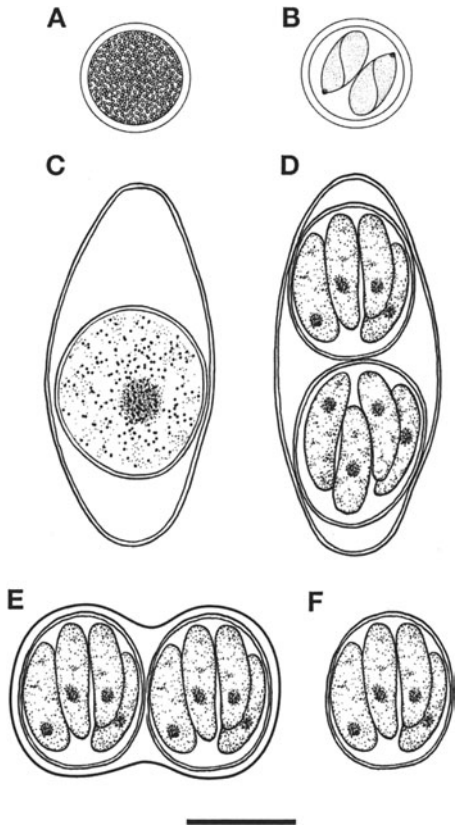


FIGURE 1 Line drawings of unsporulated and sporulated oocysts of C. *cayetanensis* (A and B) and *I. belli* (C and D) and sporulated oocyst (E) and sporocyst (F) of *Sarcocystis* species from humans.

unpublished data). The precise prepatent period is not yet known. However, the onset of clinical signs following infection generally averages 7 to 8 days postinfection and lasts 2 to 3 weeks but may range from 1 to >100 days. The length of time oocysts are shed in the feces is highly variable. Oocysts may be shed in the feces anywhere from 7 days to several months. Indigenous infections are primarily confined to tropical, subtropical, or warm temperate regions of the world. Outbreaks occur in other areas of the world due to contaminated foodstuffs.

Developmental stages of *C. cayetanensis* generally occur within epithelial cells of the lower duodenum and jejunum (4, 64, 75). There are two asexual generations followed by sexual stages and oocysts. Stages develop in supranuclear locations within enterocytes (75).

Sarcocystis hominis

The life cycle of *S. hominis* is heteroxenous. Humans are definitive hosts for *S. hominis*, and bovids are the intermediate hosts. Infection occurs when infected meat which is not cooked or is not thoroughly cooked is ingested. Known intermediate hosts include cattle *(Bos taurus)*, American bison *(Bison bison)*, water buffalo *(Bubalus bubalis)*, and European bison or Wisent *(Bison bonasus)*. These intermediate hosts harbor sarcocysts (muscle cysts) that are infective when ingested by humans. Infective bradyzoites (dormant merozoite-like stages) are present in the sarcocysts. The bradyzoites penetrate the human intestinal epithelium and develop as sexual stages (macrogametocytes and microgametocytes) in cells in the lamina propria of the intestine. Fertilization occurs and the oocysts sporulate in the lamina propria. The oocysts are "*Isospora*"-like and contain two sporocysts each with four sporozoites. The oocyst wall often ruptures as the oocyst makes its way to the intestinal lumen. This results in the shedding of individual sporocysts in the feces. Both oocysts with two sporocysts and individual sporocysts can be seen in the feces of humans with intestinal *Sarcocystis* infection (Fig. 1E and F).

Oocysts and sporocysts are fully sporulated when passed in the feces. In human volunteers, the prepatent period has been reported as 8 to 39 days and patent infections can last as long as 18 months. *Sarcocystis hominis* occurs on all continents, anywhere cattle or buffaloes have access to human feces and humans ingest raw or undercooked beef.

Sarcocystis suihominis

The life cycle of *S. suihominis* is similar to that described above for *S. hominis* except that pigs are the intermediate hosts. The prepatent period is 9 to 10 days, and patency is in

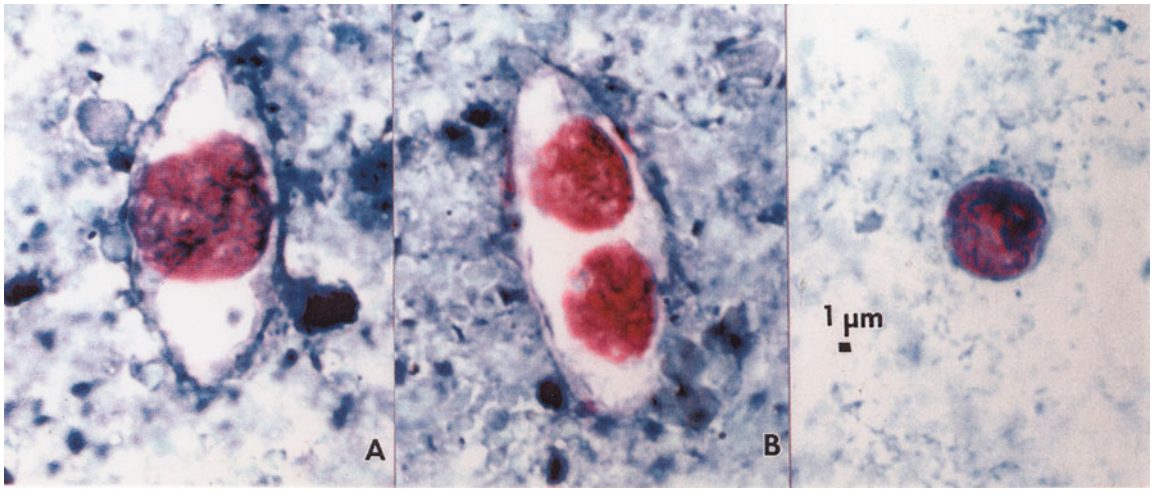


FIGURE 2 Modified Kinyoun's acid-fast-stained smears demonstrating an *I. belli* oocyst with a sporont (A), an *I. belli* oocyst with two sporoblasts (B), and a *C. cayetanensis* oocyst with a sporont.

TABLE 1 Structural data for *Isospora (Cystoisospora)*, *Cyclospora,* and *Sarcocystis* coccidial oocysts and sporocysts found in the feces of humans

Organism	Mean size (range)[a]	
	Oocysts	Sporocysts
I. belli	32 by 14 (20–36 by 10–19)	14 by 10 (12–17 by 7–11)
I. natalensis	Not given (25–30 by 21–24)	17 by 12 (not given)
C. cayetanensis	9 by 9 (8–8 by 10–10)	6 by 4 (6–7 by 3–4)
S. hominis	Not given (19 by 15)	15 by 9 (13–17 by 8–11)
S. suihominis	19 by 13 (19–20 by 12–15)	14 by 11 (12–14 by 10–11)
Sarcocystis lindemanni	Not applicable	Not applicable

[a] Data have been rounded to the nearest micrometer.

excess of 36 days (39). *S. suihominis* presumably occurs on all continents, anywhere swine have access to human feces and humans ingest raw or undercooked pork.

Human Muscular *Sarcocystis* Infection

Sarcocysts have been reported as incidental findings from both skeletal and cardiac muscle of nearly 60 humans worldwide. There are at least seven distinct types of sarcocysts present in human muscles (2). Many of these sarcocysts appeared to be similar to sarcocysts found in nonhuman primates. Humans seem to be an occasional incidental intermediate host for some *Sarcocystis* spp. in wildlife.

Epidemiology, Transmission, and Prevention

I. belli

I. belli is primarily found in tropical, subtropical, and warm temperate regions, but reports of indigenous infections from temperate areas have also been published. Most cases of infection in temperate areas involve foreign travel or homosexual contact. Unlike *Cyclospora,* large outbreaks of isosporiasis have not been reported. Transmission is via ingestion of sporulated oocysts and possibly the ingestion of raw or undercooked tissues from unknown paratenic hosts. Improving sanitation in areas of endemicity will decrease transmission of *I. belli.*

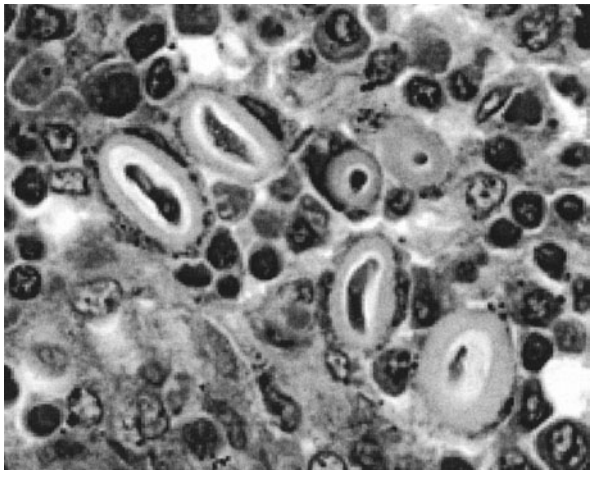


FIGURE 3 Toluidine blue-stained 1-μm section demonstrating several unizoic cysts of *I. belli*. Note the thick wall that surrounds each single zoite.

C. cayetanensis

Cyclospora is endemic in Central and South America, the Caribbean, Mexico, Indonesia, Asia, Nepal, Africa, India, southern Europe, and the Middle East. Infections in most temperate areas are correlated with the consumption of imported, contaminated fruits and vegetables such as basil (42), raspberries (38, 39, 41, 45), lettuce (10, 19), mesclun (37), or snow peas (13). One outbreak of diarrheal disease in Chicago was originally correlated with stagnant tap water (43), but no oocysts were ever recovered from a water storage tank reputed to harbor the parasite and infected individuals had attended a catered party 8 days previously (37). *C. cayetanensis* DNA has been found in water used for drinking by humans in Guatemala (21).

Better washing of produce may help remove *Cyclospora* oocysts, but many of the fruits are delicate and cannot withstand much manipulation. Most of the produce items implicated as transmitting *Cyclospora* are consumed raw, and this does not lend itself to prevention by thermal means. Nonthermal treatments such as high hydrostatic pressure (51) have been shown to inactivate *Toxoplasma gondii* oocysts, and these methods may be effective in inactivating *Cyclospora* on produce.

Sarcocystis Species

Human intestinal *Sarcocystis* species is potentially present in any region in the world where cattle, buffaloes, and swine have access to human feces and the life cycle can be maintained. The cycle has not been detected in the United States. Cultural habits that include ingestion of raw meat or undercooked meat products help maintain this life cycle in areas of endemicity. Cooking meat until an internal temperature of >67°C is achieved kills *T. gondii* tissue cysts (22), and this temperature should also kill tissue cysts of human infective *Sarcocystis* species in meat products. Preventing cattle, buffaloes, and swine from consuming human feces will also break the cycle in areas of endemicity.

Most cases of human muscular *Sarcocystis* infection have been reported from the Far East (1). It is presumed that humans become infected by ingesting sporocysts in contaminated water (1) or food.

CLINICAL DISEASE

I. belli

I. belli can cause serious and sometimes fatal disease in immunocompetent humans. Symptoms of *I. belli* infection include diarrhea, steatorrhea, headache, fever, malaise,

abdominal pain, vomiting, dehydration, and weight loss (6, 9, 79). Blood is not usually present in the feces. Eosinophilia is observed in many patients (11). The disease is often chronic, with parasites present in the feces or biopsy specimens for several months to years. Recurrences are common. Disease is more severe in infants and young children (58).

Clinical disease in *I. belli* infections is usually more severe in immunocompromised patients than in immunocompetent patients. Diarrhea produced by *I. belli* infection in AIDS patients is often very fluid and secretory-like and leads to dehydration requiring hospitalization (11). Fever and weight loss are also common findings. Other opportunistic pathogens are also common copathogens in these patients. *I. belli*-induced intestinal lesions and responses to chemotherapy are usually similar to those observed in immunocompetent patients. *I. belli* has been observed in patients with concurrent Hodgkin's disease (6), non-Hodgkin's lymphoproliferative disease (36, 70), human T-cell leukemia virus type I-associated adult T-cell leukemia (35), and acute lymphoblastic leukemia (88). These patients respond to specific anti-*I. belli* treatment. It was suggested in one report that treatment with prednisolone led to transient immunosuppression and severe *I. belli* infection in one patient (59). The patient recovered without specific treatment.

Extraintestinal cyst-like stages have been documented in AIDS patients and may play a role in relapse of infection (32, 52). These usually contain a single merozoite-like stage (Fig. 3), and many thousands of these stages can be present (52).

Infections with *I. belli* in the gallbladder epithelium (3, 31, 33) and endometrial epithelium (15) have been reported. Clinical signs in patients with parasites in these locations are not specific for coccidiosis, and parasites are located after tissue biopsy as part of a diagnostic workup. The parasites probably reach these sites as merozoites from the gut or as zoites from extraintestinal locations, and the epithelial cells of these tissues are permissive to parasite entrance and multiplication.

C. cayetanensis

Nonbloody diarrhea is the main clinical symptom of *C. cayetanensis* infection. Some individuals can be infected and show no clinical signs. In most immunocompetent patients, typical symptoms of cyclosporiasis include cycles of diarrhea with anorexia, malaise, nausea, and cramping and periods of apparent remission (37).

C. cayetanensis infection can be associated with biliary disease in both immunosuppressed patients and immunologically normal patients (72). Developmental stages of *C. cayetanensis* have been seen in the gallbladder epithelium of AIDS patients with acalculous cholecystitis by light (89) and transmission electron microscopy (14). Oocysts can be observed in the bile of patients with active biliary disease.

Intestinal *Sarcocystis* Infections

Clinical *Sarcocystis* infections in humans can manifest primarily as intestinal disease if infected meat is ingested or as muscular disease if sporocysts are ingested (27). Intestinal disease occurs soon after consumption of infected meat (3 to 6 h) and is characterized by nausea, abdominal pain, and diarrhea. Intestinal disease can be more severe in individuals who have additional enteropathogens present in the gut. Intestinal *Sarcocystis* infection combined with invasion by gram-positive bacteria has been associated with over 20 cases of segmental enterocolitis in Thailand (7, 8). Experimental studies with human volunteers have produced more severe disease in those who have ingested pork containing *S. suihominis* than in those who have ingested beef containing *S. hominis* (27). Some individuals can be infected and show no clinical signs.

Muscular *Sarcocystis* Infections

Muscular *Sarcocystis* infections in humans are usually considered subclinical or only associated with mild clinical signs (56). Many of the over 50 reported cases have been incidental findings (2) or associated with eosinophilic myostitis (80). Clinical signs associated with an outbreak of human muscular *Sarcocystis* infection occurred in 7 of 15 members of a U.S. combat unit in Malaysia (1). The signs developed about 3 weeks after the troops returned from the jungle and were fever, myalgias, bronchospasm, fleeting puritic rashes, transient lymphadenopathy, and subcutaneous nodules. Eosinophilia, elevated erythrocyte sedimentation rate, and elevated levels of muscle creatinine kinase were present in these troops (1).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

The results obtained in the diagnostic laboratory are only as good as the material they are presented with for testing. Choosing the appropriate sample and sample fixative is extremely important (33). Universal precautions should be followed when handling fresh stool samples. If samples are to be sent to another laboratory for diagnosis, then they should be fixed in an appropriate fixative. A 5 to 10% formalin solution is an appropriate fixative for stools suspected of containing intestinal coccidia. Formalin fixation does not interfere with some of the immunodetection methods currently employed to detect *Cryptosporidium* and *Giardia,* which is a drawback of polyvinyl alcohol fixative. Oocyst structure can last several months when stools are stored at 4°C in formalin fixatives.

A single negative stool specimen is not conclusive in the examination of stools for coccidial parasites; a total of three or more stool specimens collected on subsequent days need to be examined before coccidial infection can be ruled out. Liquid stool samples can be concentrated by centrifugation, and the pellet can be used for examination using wet mounts and concentration techniques or in stained smears. Large numbers of oocysts may make diagnosis less challenging but do not always translate directly to severity of clinical signs. Some individuals may excrete oocysts and be asymptomatic.

Microscopy

Oocysts of *Isospora* and *Cyclospora* and sporocysts of *Sarcocystis* species are readily identified in fresh unstained wet mounts based on their characteristic sizes and morphologies (Table 1 and Fig. 1). This is especially true if oocysts and sporocysts are present in high numbers. Autofluorescence of oocysts of *Isospora* and *Cyclospora* and sporocysts of *Sarcocystis* is an especially useful tool (5, 23, 49, 81) and has replaced many of the staining techniques previously used for these parasites in laboratories equipped with appropriate fluorescent microscopes.

Concentration techniques like formalin-ethyl acetate (rarely formalin-ether) sedimentation or sucrose centrifugal flotation are helpful when few oocysts are present. Sucrose centrifugal flotation has been found to be superior to formalin-ether sedimentation for demonstrating oocysts of *Cyclospora* (47). The same should be true for *Isospora* and *Sarcocystis*. Direct wet smears can be very useful, and their utility approaches that of sucrose centrifugal flotation when coupled with autofluorescence examination (47). Staining

procedures may adversely affect the autofluorescence of oocysts and sporocysts.

Stained fecal smears have been widely used to demonstrate *Cyclospora* oocysts and to a lesser extent *Isospora* oocysts (23, 25, 67). *Isospora* and *Cyclospora* oocysts stain red with the modified Kinyoun's acid-fast stain, and this method is widely used (Fig. 2). The main drawback is that staining can be variable and some oocysts do not stain (85). Oocysts usually do not stain with trichrome, chromotrope, or Gram-chromotrope (85). Some *Cyclospora* oocysts stain light blue with Giemsa (85). Variations on the safranin staining technique stain *Cyclospora* oocysts orange or pinkish orange, and heating and other treatments have been used to increase the staining frequency of oocysts.

Culture

In vitro culture of coccidial parasites is most often used as a tool to study developmental biology or identify active chemotherapeutic agents. It presently has limited use in diagnosis of active human infection.

There are no reports on development of *Cyclospora,* and only one report of development of *I. belli* in culture has been published (73). Bradyzoites of *Sarcocystis suihominis* undergo sexual development and produce oocysts in cell culture (55). Schizont stages of human *Sarcocystis* species have not been reported.

Antigen Detection

The inability to produce stages in cell cultures and provide a source of diagnostic antigens has limited the usefulness of antigen detection for these coccidial parasites of humans.

Nucleic Acid Detection Techniques

No nucleic acid detection techniques have been developed for use in diagnosis of human *Sarcocystis* infections. More efforts have been placed on nucleic acid-based tests for *Isospora* and *Cyclospora.*

Detection of *I. belli* by PCR using primers based on small-subunit ribosomal RNA sequences and Southern blot hybridization has been reported (61). This method was effective for detecting *I. belli* DNA in duodenal biopsies and in stool samples. A slight cross-reactivity to *T. gondii* was observed in a nested-PCR version of this test (61).

Much attention has been placed on molecular methods to detect *Cyclospora* oocysts in stools (82, 84), in water samples (21, 77), and on produce (42, 63, 76) because of the numerous outbreaks of *Cyclospora* infections. Because *Cyclospora* organisms are closely related to *Eimeria* species (68, 69) from vertebrates, it is important that tests designed to detect *Cyclospora* in the water or on produce be examined for cross-reactivity to *Eimeria* (44). *Cyclospora* species infecting mammals other than humans may also be present in water samples or on produce, and proofs of specificity are needed for these tests designed to look at environmental sources of *Cyclospora* and to detect *Cyclospora* on produce.

Quantitative PCR assays have been developed for *Cyclospora* oocysts in stool samples (82). This method detected the DNA from as few as one oocyst of *C. cayetanensis* per 5 ml of reaction volume.

Serologic Tests

The inability to obtain usable quantities of antigens from *Isospora, Cyclospora,* and *Sarcocystis* has greatly limited the use of serological diagnostic tests for these parasites. It is very difficult to obtain enough oocysts from feces to conduct serological tests. Wang et al. (86) reported the development of an immunoglobulin G (IgG) and IgM enzyme-linked immunosorbent assay based on using *C. cayetanensis* oocysts as antigen. This study is questionable because the investigators obtained the oocysts from experimentally infected guinea pigs. Well-controlled studies indicate that humans are the only suitable host for *C. cayetanensis* (24). Attempts were made to develop an immunofluorescent-antibody test to detect IgG, IgA, and IgM specific for *Cyclospora* using sectioned oocysts (12). None of four stool-positive patients reacted in the immunofluorescent-antibody test.

TREATMENT

I. belli

The drug of choice for the treatment of *Isospora* is trimethoprim-sulfamethoxazole. Trimethoprim-sulfamethoxazole administered two to four times a day for 10 to 14 days results in a clearance of parasites, decrease in diarrhea, and decrease in abdominal pain within a mean of 2.5 days after treatment (66). Patients with human immunodeficiency virus type 1 (HIV-1) infection and $CD4^+$ cell counts less than 200 should receive secondary prophylaxis with trimethoprim-sulfamethoxazole once daily or three times a week as relapse is common. It is likely that secondary prophylaxis is not needed once the $CD4^+$ count exceeds 200, as has been demonstrated for many opportunistic pathogens in patients with HIV-1 infection.

For patients unable to tolerate sulfonamides due to allergy or intolerance, there is no standard treatment. Pyrimethamine is an effective alternative for treatment or prophylaxis in patients with sulfonamide allergies (87). Pyrimethamine should be given with folinic acid to minimize bone marrow suppression. Another alternative agent is ciprofloxacin, a fluoroquinolone that inhibits topoisomerase. In a randomized study of 22 patients with isosporiasis and HIV infection, all of the 10 patients who received trimethoprim-sulfamethoxazole had a cessation of diarrhea within 2 days and 10/12 of those who received ciprofloxacin had a cessation of diarrhea within 4.5 days (83). All three patients (two with diarrhea and one without) who had persistent *Isospora* in their stools responded to trimethoprim-sulfamethoxazole (83). In patients who responded to ciprofloxacin, continued prophylaxis with ciprofloxacin prevented recurrence of disease (83). Nitazoxanide has also been used effectively to treat *I. belli* infections in three patients (20, 71).

Other therapies have had variable efficacy in treating *I. belli* enteritis. Roxithromycin given orally decreased the diarrhea in an AIDS patient suffering from chronic *I. belli*-induced diarrhea that was not responsive to trimethoprim-sulfamethoxazole or pyrimethamine treatments. *I. belli* was not detected in stool samples while the patient was on roxithromycin (62). Spiramycin initially provided clinical improvement in an AIDS patient, but after a month on therapy the disease recurred (34).

In a study of eight AIDS patients with *I. belli* enteritis, treatment with diclazuril resulted in a resolution of diarrhea in four of eight patients (46). Diclazuril was reported to successfully treat *I. belli* in an AIDS patient who was hypersensitive to trimethoprim-sulfamethoxazole and pyrimethamine; however, the diarrhea eventually recurred (48). For two patients with *I. belli* infection, the combination of albendazole and ornidazole was effective in clearing this infection (17). Treatment with other antiprotozoal agents such as metronidazole, tinidazole, quinacrine, and furazolidone appears to be of little value in this infection (9, 78, 79, 87).

C. cayetanensis

The drug of choice for the treatment of *Cyclospora* infection is trimethoprim-sulfamethoxazole (53, 65). Clearance of parasites, decrease in diarrhea, and decrease in abdominal pain occurred within a mean of 2.5 days after treatment. Patients with HIV-1 infection and $CD4^+$ cell counts less than 200 should receive secondary prophylaxis with trimethoprim-sulfamethoxazole, as relapse occurs in up to 50% of patients with AIDS without prophylaxis. It is likely that secondary prophylaxis is not needed once the $CD4^+$ count exceeds 200, as has been demonstrated for many opportunistic pathogens in patients with HIV-1 infection.

For patients unable to tolerate sulfonamides due to allergy or intolerance there is no standard treatment. Nitazoxanide has been evaluated for activity against a broad range of pathogenic protozoa and helminths (16, 29), and in these studies its efficacy for the treatment of cyclosporiasis has been about 70%. It should be appreciated, however, that only a few patients with cyclosporiasis were treated in any of these studies (16, 29). Another alternative agent is ciprofloxacin, a fluoroquinolone that inhibits topoisomerase. In a randomized study of 20 patients with cyclosporiasis, all of the 9 patients who received trimethoprim-sulfamethoxazole had a cessation of diarrhea within 3 days and 10/11 of those who received ciprofloxacin had a cessation of diarrhea within 4 days. Only 7/11 patients treated with ciprofloxacin cleared the organism from their stool (83). Anecdotal data suggest that the following drugs are ineffective: albendazole, azithromycin, pyrimethamine, nalidixic acid, norfloxacin, tinidazole, metronidazole, quinacrine, tetracycline, doxycycline, and diloxanime furoate (L. M. Weiss, unpublished data).

S. hominis and *S. suihominis*

There is no known treatment or prophylaxis for intestinal infection, myositis, vasculitis, or related lesions in humans due to sarcocystosis. For six patients in Thailand with segmental necrotizing enteritis, treatment consisted of surgical resection of the affected areas of the intestine with antibiotics for the associated bacterial infection (7, 8). Given the efficacy of trimethoprim-sulfamethoxazole against other coccidian infections and its activity in vitro against *Sarcocystis neurona* (50), it is reasonable to assume that this drug may have some efficacy in *Sarcocystis* infection. There is a case report of albendazole having efficacy in an outbreak of eosinophilic myositis due to *Sarcocystis* spp. (1). It is likely that steroids have a role in decreasing the inflammatory response in cases of myositis and vasculitis due to *Sarcocystis* infection, but this has never been evaluated in a controlled trial. Ponazuril has had efficacy against asexual stages of *S. neurona* in a mouse model of encephalitis but has not been used in human infections (30; D. S. Lindsay and S. M. Mitchell, unpublished data).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Both *I. belli* and *C. cayetanensis* are usually identified by stool examination and are rarely misidentified when isolated from human feces. Quantitation of the number of organisms found per high-power field is not required. Stool examinations that are reported as negative should indicate that at least three stool examinations are needed to detect 95% of infected individuals and that using a single specimen may miss as many as 30% of infected patients. If acid-fast or similar stains are positive, then this should be indicated in the report. Computer report notes can indicate that trimethoprim-sulfamethoxazole is the drug of choice for treatment of these parasites. PCR can be used for the identification of these organisms, but it is not commercially available and should be indicated as an experimental test. Serology is not currently used for diagnosis of these diseases in humans. Of these coccidia, only *C. cayetanensis* is a reportable disease to the State Health Department and Centers for Disease Control and Prevention as it has been associated with outbreaks and the contamination of human food sources.

S. hominis and *S. suihominis* can be identified by their characteristic morphology in stool specimens or in tissue biopsy specimens. Due to the rarity of these infections, confirmation of the observed organisms should be obtained from experts in parasitology. Computer reports should indicate if confirmation of the identification of these organisms has been obtained. There is no requirement for reporting *Sarcocystis* spp. to the State Department of Health or Centers for Disease Control and Prevention.

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*Cryptosporidium**

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Cryptosporidium spp. inhabit the brush borders of the gastrointestinal and respiratory epithelium of various vertebrates (27), causing enterocolitis, diarrhea, and cholangiopathy in humans (19). Immunocompetent children and adults with cryptosporidiosis usually have a short-term illness accompanied by watery diarrhea, malabsorption, and weight loss. In immunosuppressed persons, however, the infection can be protracted and life-threatening (48). *Cryptosporidium* spp. are well-recognized water- and food-borne pathogens, having caused many outbreaks of human diarrheal disease in the United States and other countries (25).

TAXONOMY

Cryptosporidium spp. belong to the family Cryptosporidiidae, which is a member of the phylum Apicomplexa. The exact placement of Cryptosporidiidae in Apicomplexa is uncertain. It was long considered a member of the class Coccidea, in the order of Eimeriida or Eucoccidiorida. Recent phylogenetic studies, however, indicate that *Cryptosporidium* spp. are more related to gregarines than to coccidia (16). Extracellular gregarine-like reproductive stages in *C. andersoni* and *C. parvum* have been described (90). Thus, *Cryptosporidium* spp. are no longer considered coccidian parasites.

Recently, the taxonomy of *Cryptosporidium* has gone through revisions as the result of extensive molecular genetic studies and biologic characterizations of parasites from various animals (109). The validity of several early-described species has been established, such as *C. parvum* in ruminants and humans; *C. muris* in rodents; *C. wrairi* in guinea pigs; *C. felis* in cats; *C. meleagridis*, *C. baileyi*, and *C. galli* in birds; and *C. saurophilum* and *C. serpentis* in reptiles. Several new *Cryptosporidium* species have been named from parasites of mammals, such as *C. hominis* (previously known as *C. parvum* genotype 1 or the human genotype) almost exclusively in humans, *C. andersoni* (previously known as *C. muris*-like or *C. muris* bovine genotype) and *C. bovis* (previously known as *Cryptosporidium* bovine genotype B) in weanling calves and adult cattle, *C. canis* (previously known as *C. parvum* dog genotype) in dogs, and *C. suis* (previously known as *Cryptosporidium* pig genotype I or *C. parvum* pig genotype) in pigs. Two new species have also been found in fish: *C. molnari* and *C. scophthalmi*. Thus, there are at least 16 established *Cryptosporidium* species in fish, reptiles, birds, and mammals. There are also many host-adapted *Cryptosporidium* genotypes that do not yet have designated species names, such as *Cryptosporidium* horse, rabbit, mouse, ferret, deer mouse, skunk, squirrel, bear, deer, deer-like, cervine, fox, mongoose, wildebeest, duck, woodcock, snake, tortoise, goose I and II, muskrat I and II, opossum I and II, marsupial I and II, and pig II genotypes (109).

DESCRIPTION OF AGENT

Cryptosporidium spp. are intracellular parasites that primarily infect epithelial cells of the stomach, intestine, and biliary ducts. In birds and in severely immunosuppressed persons, the respiratory tract is sometimes also involved. The infection site varies according to species, but almost the entire development of *Cryptosporidium* spp. occurs between the two lipoprotein layers of the apical membrane of the epithelial cells (27), with the exception of *C. molnari* and *C. scophthalmi*, for which oogonial and sporogonial stages are located deeply within the epithelial cells (1).

Cryptosporidium infections in humans or other susceptible hosts start with the ingestion of viable oocysts. Upon contact with gastric and duodenal fluid, four sporozoites are liberated from each excysted oocyst, invade the epithelial cells, and develop to trophozoites surrounded by a parasitophorous vacuole. Within the epithelial cells, trophozoites undergo several generations of asexual amplification called merogony, leading to the formation of different types of meronts containing 4 to 8 merozoites. The latter differentiate into sexually distinct stages called macrogamonts and microgamonts (containing microgametes) in a process called gametogony. New oocysts are formed in the epithelial cells from the fusion of macrogamonts and microgametes, sporulate in situ in a process called sporogony, and contain four sporozoites. It is believed by some that about 20% are "thin walled" and may excyst within the digestive tract of the host, leading to the infection of new cells (autoinfection). The remaining 80% of oocysts are excreted into the environment; are resistant to low temperature, high salinity, and most disinfectants; and can initiate infection in a new host without further development upon ingestion. The time from ingestion of infective oocysts to the completion of endogenous development and excretion of new oocysts varies with species, hosts, and infection doses; it is usually between 4 and 14 days (27).

*This chapter contains information presented in chapter 134 by Ynes R. Ortega and Michael Arrowood in the eighth edition of this Manual.

The only extracellular stages in *Cryptosporidium* life cycle are oocysts, which are the environmental stage of the parasite, and released sporozoites, merozoites, and microgametes, which are briefly in the lumen of the digestive tract (27). However, recently, a gregarine-like extracellular stage has been described for *C. andersoni* and *C. parvum,* which undergoes multiplication through syzygy, a sexual reproduction process involving the end-to-end fusion of two or more parasites (90).

To date, eight *Cryptosporidium* spp. have been reported to exist in humans: *C. hominis, C. parvum, C. meleagridis, C. felis, C. canis, C. muris, C. suis,* and *Cryptosporidium* cervine genotype. Humans are most frequently infected with *C. hominis* and *C. parvum*. The former almost exclusively infects humans and thus is considered an anthroponotic parasite, whereas the latter infects mostly humans and domestic and wild ruminants and thus is considered a zoonotic pathogen. Other species, such as *C. meleagridis, C. felis,* and *C. canis,* are less common. In contrast, *C. muris, C. suis,* and *Cryptosporidium* cervine genotypes have been found only in a few human cases (107, 109, 110). Despite an earlier suggestion that unusual zoonotic species usually infect immunocompromised persons, results of a recent study in Peru suggest that there is no significant difference in the distribution of *Cryptosporidium* species between AIDS patients and children living in the same geographic area (13).

EPIDEMIOLOGY, TRANSMISSION, AND PREVENTION

Cryptosporidium spp. have a worldwide distribution, and their oocysts are ubiquitously present in the environment. There were 2,769 to 3,787 annual cases of reported human cryptosporidiosis in the United States during 1999–2002 (42). Humans can acquire cryptosporidiosis through several transmission routes, such as direct contact with infected persons or animals and consumption of contaminated water (drinking or recreational) or food. However, the relative role of each in the transmission of *Cryptosporidium* infection in humans is unclear.

Anthroponotic versus Zoonotic Transmission

Studies in the United States and Europe have shown that cryptosporidiosis is more common in homosexual men than in persons in other human immunodeficiency virus (HIV) transmission categories (40), indicating that direct person-to-person or anthroponotic transmission of cryptosporidiosis is common. Contact with persons with diarrhea has been identified as a major risk factor for sporadic cryptosporidiosis in the United States, the United Kingdom, and Australia (47, 88, 92). This is exemplified by the high prevalence of cryptosporidiosis in day care facilities and nursing homes in developed countries.

Only a few case control studies assessed the role of zoonotic transmission in the acquisition of cryptosporidiosis in humans. In the United States, the United Kingdom, and Australia, contact with farm animals is a major risk factor in sporadic cases of human cryptosporidiosis (37, 47, 88, 92). Contact with pigs, dogs, or cats is also a risk factor for cryptosporidiosis in children in Guinea-Bissau and Indonesia (55, 72), although it is a protective factor in Australia (88). A weak association was observed between the occurrence of cryptosporidiosis in HIV-positive persons and contact with dogs, but not other animals (36). In other studies, no increased risk in the acquisition of cryptosporidiosis was associated with contact with animals (75, 82).

The distribution of *C. parvum* and *C. hominis* in humans is probably a good indicator of the transmission routes. Thus far, studies conducted in tropical countries such as Peru, Thailand, Malawi, Uganda, Kenya, and South Africa showed a predominance of *C. hominis* in children or HIV-positive adults. This is also true for the United States, Canada, Australia, and Japan. In Europe, however, several studies have shown slightly higher prevalence of *C. parvum* than *C. hominis* in both immunocompetent and immunocompromised persons (110). In contrast, Kuwaiti children are almost exclusively infected with *C. parvum* (99). The differences in the distribution of *Cryptosporidium* genotypes in humans are considered an indication of differences in infection sources (58); the occurrence of *C. hominis* in humans is most likely due to anthroponotic transmission, whereas the predominance of *C. parvum* in a population has been considered the result of zoonotic transmission. Thus, in most tropical countries, it is possible that anthroponotic transmission of *Cryptosporidium* plays a major role in human cryptosporidiosis, whereas in Europe, both anthroponotic and zoonotic transmissions are important. Indeed, in areas with a high percentage of infections due to *C. parvum*, massive slaughtering of farm animals and restriction of visits to farms during foot-and-mouth disease outbreaks can result in a reduction in the proportion of human infections due to *C. parvum* (45).

Nevertheless, recent subtyping studies based on sequence analyses of the 60-kDa glycoprotein (GP60) gene have shown that not all *C. parvum* infections in humans are results of zoonotic transmission (2, 63, 107). Among several *C. parvum* subtype families identified, IIa and IIc (previously known as Ic) are the two most common families. The former has been identified in both humans and ruminants, thus acting as a zoonotic pathogen, whereas the latter has been seen only in humans (2, 107) and thus is an anthroponotic pathogen. In Lima, Peru, all *C. parvum* infections in children and HIV-positive persons are due to the subtype family IIc, indicating that anthroponotic transmission of *C. parvum* is common in certain areas (109). Even in the United Kingdom, where zoonotic transmission is known to play a significant role in the transmission of human cryptosporidiosis, anthroponotic transmission of *C. parvum* is also common (63).

Waterborne Transmission

Epidemiologic studies have frequently identified water as a major route of *Cryptosporidium* transmission in areas where the disease is endemic (29, 79, 106). In most tropical countries, *Cryptosporidium* infections in children usually peak during the rainy season, and waterborne transmission is considered a major route of cryptosporidiosis in these areas (9, 10, 77, 80, 100).

Seasonal variations in the incidence of human *Cryptosporidium* infection in industrialized nations have also been attributed to waterborne transmission (12, 22, 68, 92). In the United States, there are two annual peaks in the number of cryptosporidiosis cases among HIV-positive persons: one in spring and one in late summer (50, 94). In the general population, there is also an annual late summer peak in sporadic cases of cryptosporidiosis (92). It is generally accepted that the late summer peak of cryptosporidiosis cases is due to recreational activities such as swimming and water sports, suggesting that waterborne transmission may be important in cryptosporidiosis epidemiology.

The role of drinking water in sporadic *Cryptosporidium* infection is not clear. For Mexican children living near the United States border, cryptosporidiosis is associated with consumption of municipal water instead of bottled water (57). In England, there is an association between the number of glasses of tap water drunk at home each day and the occurrence of sporadic cryptosporidiosis (47). In the United States,

drinking untreated surface water was identified as a risk factor for the acquisition of *Cryptosporidium* in a small case control study (29), and residents living in cities with surface-derived drinking water generally have higher levels of antibodies against *Cryptosporidium* antigens in their blood than those living in cities with ground water as drinking water, indicating that drinking water plays a role in the transmission of human cryptosporidiosis (28). Nevertheless, case control studies conducted in both immunocompetent persons and AIDS patients in the United States have failed to show a direct linkage of *Cryptosporidium* infection to drinking water (56, 95).

Numerous waterborne outbreaks of cryptosporidiosis have occurred in the United States, Canada, the United Kingdom, France, Australia, Japan, and other industrialized nations (25, 61, 91). These include outbreaks associated with both drinking water and recreational water (swimming pools and water parks). After the massive cryptosporidiosis outbreak in Milwaukee in 1993, the water industry has adopted more stringent treatments of source water. Currently, the number of drinking water-associated outbreaks is in decline in the United States and the United Kingdom. Even though five *Cryptosporidium* species are commonly found in humans, thus far only *C. parvum* and *C. hominis* are associated with cryptosporidiosis outbreaks, with *C. hominis* responsible for more outbreaks than *C. parvum* (68, 81, 107). This is even the case for the United Kingdom, where *C. parvum* is more common than *C. hominis* in the general population.

Foodborne Transmission

The role of food in the transmission of cryptosporidiosis is much less clear. *Cryptosporidium* oocysts have been isolated from several foodstuffs, and these have been associated mainly with fruits, vegetables, and shellfish (26, 89). Direct contamination of food by fecal materials from animals or food handlers has been implicated in several foodborne outbreaks of cryptosporidiosis in industrialized nations. In most instances, human infections were usually due to consumption of contaminated fresh produce and nonpasteurized apple cider or milk (71, 85).

Very few case control studies have examined the role of potentially contaminated food as a risk factor in the acquisition of *Cryptosporidium* infection in areas where the disease is endemic. A pediatric study in Brazil failed to show any association between *Cryptosporidium* infection and diet or type of food hygiene (82). Case control studies conducted in the United States, the United Kingdom, and Australia have actually shown a lower prevalence of *Cryptosporidium* infection in immunocompetent persons with frequent consumption of raw vegetables (47, 88, 92). Nevertheless, it is estimated that about 10% of *Cryptosporidium* infections in the United States are foodborne (70).

Prevention

As for any pathogens that are transmitted by the fecal-oral route, good hygiene is the key in preventing the acquisition of *Cryptosporidium* infection (53). Immunosuppressed persons especially should take necessary precautions in preventing the occurrence of cryptosporidiosis (65). These include washing hands after going to bathrooms, changing diapers, and contacting pets or soil (including gardening); avoiding drinking water from lakes and rivers, swallowing water in recreational activities, and drinking nonpasteurized milk, milk products, and juices; and following safe-sex practices (avoiding oral-anal contact). During cryptosporidiosis outbreaks or when a community advisory to boil water is issued, individuals should boil water for 1 min to kill the parasite or use a tap water filter capable of removing particles less than 1 μm in diameter. Immunosuppressed persons also should avoid eating raw shellfish and should not eat uncooked vegetable salads and unpeeled fruits when traveling to areas where cryptosporidiosis is endemic (such as tropical countries) (65).

CLINICAL SIGNIFICANCE

In developing countries, human *Cryptosporidium* infection occurs mostly in children younger than 5 years old, with peak occurrence of infections and diarrhea in children less than 2 years of age (9, 10, 77). Frequent symptoms include diarrhea, abdominal cramps, vomiting, headache, fatigue, and low-grade fever (79). The diarrhea can be voluminous and watery but usually resolves within 1 to 2 weeks without treatment. Not all infected children have diarrhea or other gastrointestinal symptoms, and the occurrence of diarrhea in children with cryptosporidiosis can be as low as 30% in community-based studies (10). Even subclinical cryptosporidiosis exerts a significant adverse effect on child growth, as infected children with no clinical symptoms experience growth faltering, both in weight and in height. Children can have multiple episodes of cryptosporidiosis, implying that the anti-*Cryptosporidium* immunity in children is short-lived or incomplete (9, 10, 77). Cryptosporidiosis has been associated with increased mortality in developing countries in hospitalized children (100).

In developed countries, pediatric cryptosporidiosis occurs in older children than in developing countries, probably due to later exposures to a contaminated environment as a result of better hygiene (99). Cryptosporidiosis is also common in elderly people attending nursing homes where person-to-person transmission probably also plays a major role in the spread of *Cryptosporidium* infections (76). In the general population, a substantial number of adults are probably susceptible to *Cryptosporidium* infection, as sporadic infections occur in all age groups in the United States and the United Kingdom and traveling to developing countries and consumption of contaminated food or water can frequently lead to infection (22, 37, 92). Hemodialysis patients with chronic renal failure more commonly develop cryptosporidiosis (101).

Unlike in developing countries, immunocompetent persons with sporadic cryptosporidiosis in industrialized nations usually have diarrhea (37, 88). The median number of stools per day during the worst period of the infection is 7 to 9.5 (88). Other common symptoms include abdominal pain, vomiting, and low-grade fever (37, 88). Patients infected with *C. hominis* are more likely to have joint pain, eye pains, recurrent headache, dizziness, and fatigue than those infected with *C. parvum* (46). The duration of illness has a mean or median of 9 to 21 days, with a median loss of 5 work or study days and hospitalization of 7 to 22% of patients (22, 37, 88).

Cryptosporidiosis is common in immunocompromised persons, including AIDS patients, persons with primary immunodeficiency, and cancer and transplant patients undergoing immunosuppressive therapy (48, 69). It is frequently associated with chronic, life-threatening diarrhea (48). In HIV-positive persons, the occurrence of cryptosporidiosis increases as the $CD4^+$ lymphocyte cell counts fall, especially below 200 cells/μl (48, 67). Manabe et al. described four clinical syndromes of cryptosporidiosis in the United States: chronic diarrhea (36% of patients), cholera-like disease (33%), transient diarrhea (15%), and relapsing illness (15%) (64). Sclerosing cholangitis and other biliary involvements, however, are also very common in AIDS patients with cryptosporidiosis (19). Symptoms of cryptosporidiosis in AIDS patients vary in severity, duration, and responses to drug treatment (64, 67). Much of this variation can be explained by the degree of

immunosuppression (67). In addition, variation in the infection site (gastric infection, proximal small intestine infection, or ileo-colonic infection, versus pan-enteric infection) has been seen in AIDS patients with cryptosporidiosis, and this anatomic localization may also contribute to differences in disease severity and survival (60). Cryptosporidiosis in AIDS patients is associated with increased mortality and shortened survival (64).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

At the moment, almost all active *Cryptosporidium* infections are diagnosed by analysis of stool specimens. Stool specimens are usually collected fresh or in fixative solutions such as 10% buffered formalin (31). Stool specimens fixed in formalin are preferred for routine diagnosis of cryptosporidiosis. For outbreak investigations, *Cryptosporidium* spp. present are sometimes genotyped by PCR methods, which require the use of fresh stool specimens or stools preserved in 2.5% potassium dichromate. It is recommended that whenever possible multiple specimens (three specimens passed at intervals of 2 to 3 days) from each patient should be examined if *Cryptosporidium* infection is suspected and the examination of the initial stool specimen is negative. This is because carriers with low oocyst shedding are common (87), and examination of single specimens can lead to the detection of only 53% of infections (39).

Examination of intestinal or biliary biopsy specimens is sometimes used in the diagnosis of cryptosporidiosis in AIDS patients (39). However, the sensitivity of the diagnosis depends on the location of tissues examined; the duodenum is usually infected with *Cryptosporidium* only in severe infection, and the terminal ileum has significantly higher detection rates than the duodenum (39). Thus, upper endoscopic biopsies are much less sensitive than lower endoscopic biopsies in diagnosing cryptosporidiosis. However, lower endoscopy is generally considered too invasive and risky for many AIDS patients.

DIRECT EXAMINATION

A variety of methods are used in the detection of *Cryptosporidium* in stool specimens, including microscopy, culture, antigen detection, and nucleic acid detection techniques (6).

Microscopy

Stool specimens can be examined directly for *Cryptosporidium* oocysts by microscopy of direct wet-mount or stained fecal materials if the number of oocysts in specimens is high. *Cryptosporidium* oocysts in humans generally measure 4 to 6 μm. Occasionally, *C. muris* oocysts are also found, which are more elongated and measure 6 to 9 μm. More often, oocysts present are concentrated using either traditional ethyl acetate or Weber-modified ethyl-acetate concentration methods (104). Concentrated stool specimens can be examined by microscopy in several ways. *Cryptosporidium* oocysts can be detected by bright-field microscopy in direct wet mounts. This allows the observation of oocyst morphology and more accurate measurement of oocysts, which is frequently needed in biologic studies. Differential interference contrast can be used in microscopy, which produces better images and visualization of internal structures of oocysts. Morphology and morphometrics measurements, however, are generally not enough for *Cryptosporidium* species differentiation (109), as many *Cryptosporidium* species look similar by microscopy and have similar morphometrics measurements.

More often, *Cryptosporidium* oocysts in concentrated stool specimens are detected by microscopy after staining of the fecal smears. Many special stains have been used in the detection of *Cryptosporidium* oocysts, but modified acid-fast stains are the most commonly used (6). Modified acid-fast staining is commonly used in developing countries because of its low cost, ease of use, lack of need for special microscopes, and ability to simultaneously detect several other pathogens such as *Isospora* and *Cyclospora* (Fig. 1). Two stains widely used in detecting *Cryptosporidium* oocysts are the modified Ziehl-Neelsen acid-fast staining and modified Kinyoun's acid-fast stains (6). Oocysts are stained bright red to purple against a blue or green background in modified acid-fast staining (Fig. 1).

Recently, immunofluorescence assays (IFA) have been used increasingly in *Cryptosporidium* oocyst detection by microscopy, especially in industrialized nations. Compared to acid-fast staining, IFA has higher sensitivity and specificity (52). Many commercial IFA kits are marketed for the detection of *Cryptosporidium;* some of these kits include reagents allowing simultaneous detection of *Giardia* cysts (Table 1). Oocysts appear apple green against a dark background in immunofluorescence microscopy (Fig. 2). Because of its high sensitivity and specificity, IFA has been used by some as the "gold standard" or reference test (33, 52). It has been shown that most antibodies used in immunofluorescent detection of *Cryptosporidium* recognize carbohydrate epitopes on the oocyst wall (111). As the monoclonal antibodies used in commercial IFA kits react with oocysts of almost all *Cryptosporidium* species, IFA cannot identify organisms at the species level (38, 111).

The sensitivity of most microscopic methods is probably low. The detection limit for the combination of ethyl acetate concentration and IFA was shown to be 10,000 oocysts per gram of liquid stool and 50,000 oocysts per gram of formed stool (103, 105). The sensitivity of modified acid-fast staining was 10-fold lower (103), probably because acid-fast stains do not consistently stain all oocysts (30).

Culture

The in vitro cultivation of *Cryptosporidium* spp. remains inefficient despite recent advances (7). Thus, the low parasite yields and oocyst production have limited the usefulness of parasite culture in the diagnosis of *Cryptosporidium* infection. Nevertheless, in vitro cultivation of early *Cryptosporidium* developmental stages in several epithelial cell lines (HCT-8, MDBK, Caco-2, etc.) has been used widely in research studies to assess potential drugs and oocyst disinfection methods and to characterize parasite development, differentiation, and biochemistry (7). The recent development of procedures for long-term maintenance of *C. parvum* and *C. hominis* in cell and cell-free culture could promote the use of in vitro cultivation methods in routine diagnosis of *Cryptosporidium* infection (41).

Antigen Detection

Cryptosporidium infection can also be diagnosed by the detection of *Cryptosporidium* antigens in stool specimens by immunoassays. Antigen capture-based enzyme immunoassays (EIA) have been used in the diagnosis of cryptosporidiosis since 1990 (18, 33). In recent years, EIAs have gained popularity because they do not require experienced microscopists and can be used to screen a large number of samples. In clinical laboratories, several commercial EIA kits are commonly used (Table 1). High specificity (99 to 100%) has been generally reported for these EIA kits (21, 33, 52). Sensitivities, however, have been reported to range from 70% (52)

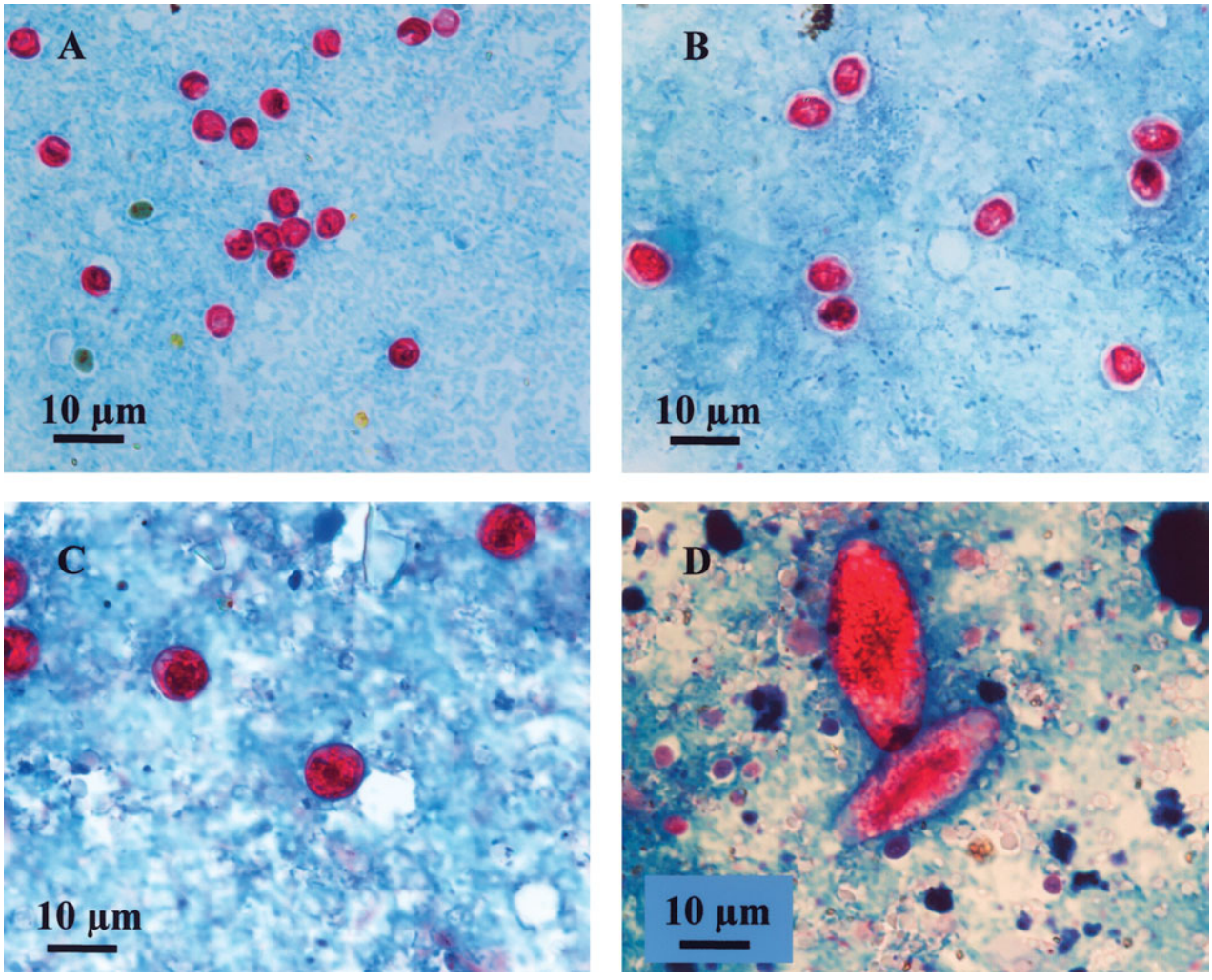


FIGURE 1 Oocysts of *Cryptosporidium hominis* (4 to 6 μm) (A), *Cryptosporidium muris* (6 to 8 μm) (B), *Cyclospora cayetanensis* (8 to 10 μm) (C), and *Isospora belli* (20 to 30 by 10 to 20 μm) (D) stained by the modified Ziehl-Neelsen acid-fast stain.

to 94 to 100% (11, 33). Nevertheless, occasional false positivity of EIA kits is known to occur in the detection of *Cryptosporidium* (18), and manufacturers' recalls of EIA kits have occurred because of high nonspecificity (3, 23). If a patient is in the carrier state or during self-cure, the number of oocysts may drop below the sensitivity levels of these kits (49, 52). Most of the EIA kits have been evaluated only with human stool specimens. Their usefulness in the detection of *Cryptosporidium* spp. in animals may be compromised by the high specificity of antibodies. For example, the ProSpecT *Cryptosporidium* EIA does not detect many *Cryptosporidium* species that are genetically distant from *C. parvum,* such as *C. muris, C. andersoni, C. serpentis,* and *C. baileyi* (38).

In the last few years, several lateral flow immunochromatographic assays have been marketed for rapid detection of *Cryptosporidium* in stool specimens (Table 1). In the few evaluation studies conducted with two of the assays, these assays have been shown to have high specificities (>98%) and sensitivity (98 to 100%) (32, 34, 52, 54). However, a recent study has shown a sensitivity of 68% for one of the assays (52). These rapid assays have also been affected by quality control problems and have been subjected to several manufacturers' recalls because of false-positive results (4, 5).

Nucleic Acid Detection Techniques

Molecular techniques, especially PCR and PCR-related methods, have been developed and used in the detection and differentiation of *Cryptosporidium* spp. for many years. Earlier PCR methods do not have the ability for species differentiation or genotyping and thus can be used only in the determination of the presence or absence of *Cryptosporidium* spp. The primer sequences of these techniques are mostly based on undefined genomic sequences from *C. parvum* bovine isolates, which tend to be more polymorphic than structural or housekeeping genes. Therefore, PCR methods targeting undefined *C. parvum* genomic sequences are unlikely to amplify efficiently DNA from *Cryptosporidium* spp. that are more distant from *C. parvum,* such as *C. muris, C. canis, C. felis,* and *C. suis* (51).

Several PCR-restriction fragment length polymorphism-based genotyping tools have been developed for the detection and differentiation of *Cryptosporidium* at the species level (20, 78, 98, 108). Most of these techniques are based

TABLE 1 Some commercial diagnostic assays for the detection of *Cryptosporidium* oocysts or antigens

Product	Manufacturer, country	CLIA certified	Intended use
Immunofluorescent assays			
Merifluor Cryptosporidium/Giardia	Meridian Biosciences, United States	Yes	Clinical and water
Crypto Cel	Cellabs, Australia	No	Clinical
Crypto/Giardia Cel	Cellabs, Australia	No	Clinical
Cryptosporidium/Giardia Direct Fluorescent Antigen Assay	IVD Research, Inc., United States	No	Clinical
Para-Tect Cryptosporidium/Giardia DFA Kit	Medical Chemical Corporation, United States	No	Clinical
Crypt-a-Glo	Waterborne Inc., United States	No	Clinical and water
Aqua-Glo G/C Kit	Waterborne Inc., United States	No	Water
Hydrofluor Combo	Strategic Diagnostics, United States	No	Water
Enzyme immunoassays			
Cryptosporidium Test	TechLab, United States	Yes	Clinical
ProSpecT Cryptosporidium Microplate	Remel, United States	Yes	Clinical
ProSpecT Giardia/Crypto Microplate	Remel, United States	Yes	Clinical
ProSpecT Cryptosporidium Rapid Assay	Remel, United States	Yes	Clinical
Triage Parasite Panel	Biosite, United States	Yes	Clinical
Cryptosporidium Stool Antigen ELISA	IVD Research, Inc., United States	No	Clinical
Crypto/Giardia CELISA	Cellabs, Australia	No	Clinical
Para-Tect Cryptosporidium Antigen Test Kit	Medical Chemical Corporation, United States	No	Clinical
Cryptosporidium Immunoassay Kit	SafePath Laboratories, United States	No	Veterinary
Cryptosporidium EIA Kit	Cypress Diagnostics, Belgium	No	Veterinary
Immunochromatographic assays			
ColorPAC Giardia/Cryptosporidium Rapid Assay Test Kit	Becton Dickinson, United States	Yes	Clinical
Contrast Giardia/Cryptosporidium Combo Rapid Assay	Genzyme, United States	Yes	Clinical
Xpect Cryptosporidium Kit	Remel, United States	Yes	Clinical
Xpect Giardia/Cryptosporidium Test Kit	Remel, United States	Yes	Clinical
ImmunoCard STAT! Cryptosporidium/ Giardia Test Kit	Meridian Bioscience, United States	No	Clinical
Crypto Strip Quick Test	Cypress Diagnostics, Belgium	No	Clinical
RIDA Quick Cryptosporidium	R-Biopharm, Germany	No	Clinical
RIDA Quick Cryptosporidium/Giardia Combi	R-Biopharm, Germany	No	Clinical
Cryptosporidium Dipstick	Cypress Diagnostics, Belgium	No	Veterinary

on the small subunit rRNA gene. Other genotyping techniques are designed mostly for the differentiation of *C. parvum* and *C. hominis* (73, 81, 96); as a result, they cannot detect and differentiate other *Cryptosporidium* spp. or genotypes. Their usefulness in the analysis of human stool specimens is compromised by their inability to detect *C. canis, C. felis, C. suis,* and *C. muris* (51).

Several subtyping tools have also been developed to characterize the diversity within *C. parvum* or *C. hominis* (110). One of the most commonly used techniques is microsatellite analysis (63). Although it is not a strict microsatellite locus by definition, results of a series of recent studies have shown high sequence polymorphism in the GP60 gene (also known as gp15/45/60, gp40/15) (2, 17, 99). Most of the genetic heterogeneity in this gene is present in the number of a trinucleotide repeat (TCA, TCG, or TCT), although extensive sequence differences are also present between groups (families) of subtypes. Other subtyping tools include sequence analysis of the 70-kDa heat shock protein gene, heteroduplex analysis and nucleotide sequencing of the double-stranded viral RNA, and single-strand conformation polymorphism-based analysis of the second internal transcribed spacer (110). A multilocus mini- and microsatellite subtyping tool for *C. parvum* and *C. hominis* has also been developed (63). The usefulness of subtyping tools has been demonstrated by the analysis of samples from foodborne and waterborne outbreaks of cryptosporidiosis (17, 35, 59, 107, 110).

SEROLOGIC TESTS

Serologic tests are sometimes used in studies of the prevalence of cryptosporidiosis in communities and epidemiologic investigations of cryptosporidiosis outbreaks. Electrophoretic and Western blot analysis showed that specific antibody response appeared between days 4 and 15 postinoculation. The two main target antigens had apparent molecular masses of 15 to 17 and 23 kDa (86). These two antigens, Cp17 (also called gp15) and Cp23 (also called the 27-kDa antigen), have been used by many researchers in the detection of *Cryptosporidium* antibodies by enzyme-linked immunosorbent assays (ELISA) or Western blotting (14, 28, 83, 84). Native Cp17 extracted by Triton from oocysts of *C. parvum* and recombinant *C. parvum* Cp23 expressed in *Escherichia coli* are usually used in these assays (83, 102). ELISA methods using these two antigens generally have higher sensitivity and specificity than earlier methods that use crude oocyst

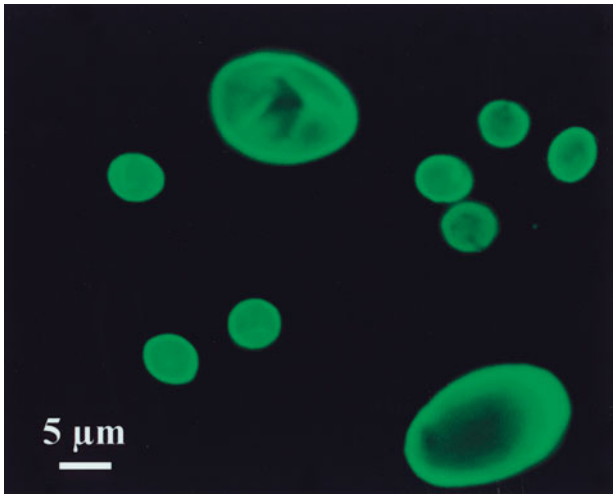


FIGURE 2 *Cryptosporidium hominis* oocysts (4 to 6 μm) and *Giardia duodenalis* cyst (11 to 14 μm by 7 to 10 μm) under immunofluorescence microscopy.

antigens (83). Most researchers use both antigens in serologic studies.

Western blotting or ELISA methods based on Cp17 and Cp23 have been used in research studies of *Cryptosporidium* transmission in immunocompromised persons (24), children (97), and the general community (28) and in investigations of cryptosporidiosis outbreaks (66). Recently, a multiplex bead assay based on these two antigens has been developed for the detection of *Cryptosporidium* antibodies in sera and oral fluids (74). These serologic assays are not intended for the diagnosis of active *Cryptosporidium* infection, as antibodies to both the 27- and 17-kDa antigens have a half-life of about 12 weeks (84). All these tests are not yet commercially available.

TREATMENT

Numerous pharmaceutical compounds have been screened for anti-*Cryptosporidium* activities in vitro or in laboratory animals (93). Some of those showing promising results have been used in the experimental treatment of cryptosporidiosis in humans, but few have been shown to be effective in controlled clinical trials (48). Oral or intravenous rehydration is used whenever severe diarrhea is associated with *Cryptosporidium* infection (43). Nitazoxanide (NTZ) is the only FDA-approved drug for the treatment of pediatric cryptosporidiosis. It has also been recently approved for the treatment of giardiasis in adult patients. Clinical trials have demonstrated that NTZ can shorten clinical disease and reduce parasite loads (8, 93). This drug, however, is not yet approved for the treatment of *Cryptosporidium* infections in immunodeficient people, even though it is likely to be partially effective in patients not severely immunosuppressed (8). For this population, paramomycin and spiramycin have been used in the treatment of some patients, but their efficacy remains unproven (48).

In industrialized nations, the most effective treatment and prophylaxis for cryptosporidiosis in AIDS patients is the use of highly active antiretroviral therapy (HAART) (15). It is believed that the eradication and prevention of the infection are directly related to the replenishment of $CD4^+$ cells in treated persons, rather than antiparasitic activities of these drugs (15), even though some of the protease inhibitors used in HAART, such as indinavir, nelfinavir, and ritonavir, have been shown to have anticryptosporidial activities in vitro and in laboratory animals (44). Relapse of cryptosporidiosis is common in AIDS patients who have stopped HAART (15, 62).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Cryptosporidiosis is a notifiable disease in most states in the United States. Thus, the detection of the parasite or its antigens in stools or tissues should be reported to the local health department in addition to the physicians. Because most routine diagnostic tests cannot differentiate *Cryptosporidium* species, the detection of *Cryptosporidium* oocysts or antigens in stools or other specimens should be reported as *Cryptosporidium* positive without referring to the nature of the species involved. From a public health point of view, the reporting of a significant number of cases above background levels in industrialized nations indicates the likely occurrence of outbreaks of cryptosporidiosis or false positivity of diagnostic kits (3–5, 23). In situations like this, it is crucial to have the test results verified with a confirmatory test such as IFA or PCR and to report them to the state or local public health department. The inclusion of both positive and negative controls in each test run will reduce the occurrence of test errors.

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Microsporidia*

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THE ORGANISMS

The nontaxonomic term "microsporidia" refers to a group of obligate intracellular, spore-forming protists belonging to the phylum Microspora. More than 140 microsporidial genera and 1,200 species that are parasitic in every major animal group have been identified (35). To date, seven genera *(Enterocytozoon, Encephalitozoon, Nosema, Pleistophora, Vittaforma, Trachipleistophora,* and *Brachiola)* and unclassified microsporidia, assigned to the collective group *Microsporidium,* have been implicated in human infections (Table 1).

Microsporidia develop intracellularly exclusively and have no metabolically active stages outside the host cell. A life cycle (Fig. 1) involving a proliferative merogonic sequence followed by a sporogonic sequence results in environmentally resistant spores of unique structure. Mature spores contain a tubular extrusion apparatus (polar tube) for injecting infective spore contents (sporoplasm) into the host cell.

Microsporidia are true eukaryotes because they have a membrane-bound nucleus, an intracytoplasmic membrane system, and chromosome separation on mitotic spindles, but they are unusual eukaryotes in that they have bacterium-like ribosomes, no recognizable mitochondria, no peroxisomes, and simple vesicular Golgi membranes. Compared with those of other eukaryotes, the genomes of microsporidia are reduced in size and complexity (29). The genome sizes of different microsporidia vary between 2.3 and 19.5 Mb, and the numbers of chromosomes range from 7 to 16 (57). The compactness of the microsporidial genomes results from the loss of genes and from the reduction of coding and noncoding elements. The small-subunit (SSU) rRNA genes of microsporidia, e.g., are significantly shorter than those of other eukaryotes and even those of some prokaryotes.

It has been postulated that, phylogenetically, microsporidia are ancient protists that diverged before the mitochondrial endosymbiosis (Fig. 2). However, genes related to some mitochondrial functions in *Encephalitozoon cuniculi* were sequenced, implying that microsporidia have retained a mitochondrion-derived organelle (29). Indeed, tiny organelles with double membranes were visualized by using antibodies against the mitochondrial heat shock protein 70 in the microsporidian *Trachipleistophora hominis* (58). Furthermore, phylogenetic analyses involving several genes found microsporidia to branch within the zygomycete group, indicating that their ancestors may have been a mitochondriate eukaryote and that microsporidia are a sister group of fungi (26, 30). Also, structural features of the organisms such as the presence of chitin in the spore wall, diplokaryotic nuclei, and electron-dense spindle plaques associated with the nuclear envelope suggest a possible relationship between fungi and microsporidia.

DESCRIPTION OF THE GENERA AND SPECIES

The species are illustrated in Fig. 1.

Nosema spp. develop in direct contact with host cell cytoplasm, nuclei are paired (diplokaryotic), divisions are by binary fission, and sporonts are disporoblastic. Only one species of human origin, *Nosema ocularum,* has been retained in this genus. It will probably require reclassification, but insufficient information is currently available for a new generic assignment. Spores measure 3 by 5 μm and have polar tubes with 9 to 12 coils.

Brachiola spp. have diplokaryotic nuclei and develop in direct contact with host cell cytoplasm. Additionally, the organisms produce electron-dense extracellular secretions and vesiculotubular appendages. Disporoblastic sporogony of *Brachiola vesicularum* produces 2.9- by 2-μm diplokaryotic spores containing 8 to 10 anisofilar coils of the polar filament arranged in one to three rows, usually two (5). On the basis of diplokaryotic nuclei, disporoblastic sporogony, and the formation of vesiculotubular secretions, *Nosema connori* and *Nosema algerae,* also discovered in human infections, have been reclassified as species of *Brachiola*. Spores of *Brachiola connori* measure 2.0 to 2.5 by 4.0 to 4.5 μm and contain polar tubes with approximately 11 coils (6). Spores of *Brachiola algerae* measure 3.7 by 2.0 μm and have 7 to 11 coils of the polar tube (33).

Vittaforma corneae, originally classified as *Nosema corneum,* was transferred to a new genus on the basis of ultrastructural features (42). Nuclei are diplokaryotic, sporogony is polysporoblastic, sporonts are ribbon shaped, and all stages including spores are individually enveloped by a cisterna of host endoplasmic reticulum studded with ribosomes. The spores contain polar tubes with five to seven coils and measure 1.2 by 3.8 μm.

* This chapter contains information presented in chapter 135 by Rainer Weber and Elizabeth U. Canning in the eighth edition of this Manual.

TABLE 1 Microsporidial species pathogenic in humans, and clinical manifestations

Microsporidial species	Clinical manifestations	
	Immunocompromised patients	Immunocompetent persons
Enterocytozoon bieneusi	Chronic diarrhea, wasting syndrome, "AIDS-cholangiopathy," cholangitis, acalculous cholecystitis, chronic sinusitis, chronic cough, pneumonitis	Self-limiting diarrhea in adults and children, traveler's diarrhea, asymptomatic carriers
Encephalitozoon hellem	Disseminated infection, keratoconjunctivitis, sinusitis, bronchitis, pneumonia, nephritis, ureteritis, cystitis, prostatitis, urethritis	Possibly diarrhea[a]
Encephalitozoon intestinalis (formerly *Septata intestinalis*)	Chronic diarrhea, cholangiopathy, sinusitis, bronchitis, pneumonitis, nephritis, bone infection, nodular cutaneous lesions	Self-limiting diarrhea, asymptomatic carriers
Encephalitozoon cuniculi	Disseminated infection, keratoconjunctivitis, sinusitis, bronchitis, pneumonia, nephritis, hepatitis, peritonitis, symptomatic and asymptomatic intestinal infection, encephalitis	Not described; two HIV-seronegative children with seizure disorder and presumed *E. cuniculi* infection presumably were immunocompromised
Pleistophora sp.	Myositis	Not described
Pleistophora ronneafiei	Myositis	Not described
Trachipleistophora hominis	Myositis, keratoconjunctivitis, sinusitis	Keratitis
Trachipleistophora anthropophthera	Disseminated infection	Not described
Brachiola connori (formerly *Nosema connori*)	Disseminated infection	Not described
Brachiola vesicularum	Myositis	Not described
Brachiola algerae (formerly *Nosema algerae*)	Myositis, nodular cutaneous lesions	Keratitis
Nosema ocularum	Not described	Keratitis
Vittaforma corneae (formerly *Nosema corneum*)	Disseminated infection	Keratitis
Microsporidium ceylonensis[b]	Not described	Corneal ulcer, keratitis
Microsporidium africanum[b]	Not described	Corneal ulcer, keratitis
Microsporidia (not classified)	Not described	Keratoconjunctivitis in a contact lens wearer

[a] *E. hellem* has been detected using PCR among persons with traveler's diarrhea and coinfection with *Enterocytozoon* sp. (41); thus, the pathogenic role of *E. hellem* remains unknown. Microscopic detection of *E. hellem* spores in stool has not been reported so far.

[b] *Microsporidium* is a collective generic name for microsporidia that cannot be classified because available information is not sufficient.

Pleistophora spp., including *Pleistophora ronneafiei,* which is pathogenic in humans, have unpaired nuclei, and all stages are multinucleate plasmodia, which divide into smaller multinucleate segments. Meronts have a thick, amorphous coat, which separates from the surface in sporogony to form a sporophorous vesicle. Sporogonic divisions give rise to a large and variable number of spores, packaged in the persistent sporophorous vesicle. The spores contain polar tubes with 9 to 12 coils and measure 2 to 2.8 by 3.3 to 4.0 μm (4).

T. hominis also forms spores in sporophorous vesicles, but these arise from repeated binary fissions and not from multinucleate plasmodia. The vesicles, which contain 2 to more than 32 spores, enlarge as the number of spores increases. The nuclei are unpaired in all stages of development. The pear-shaped spores measure 2.4 by 4.0 μm and have about 11 anisofilar coils (24, 27). *Trachipleistophora anthropophthera* is similar to *T. hominis* but appears to be dimorphic as two different forms of sporophorous vesicles and spores have been observed (47).

Enterocytozoon bieneusi develops in direct contact with host cell cytoplasm (Fig. 3). The proliferative and sporogonial forms are rounded multinucleate plasmodia with unpaired nuclei measuring up to 6 μm in diameter. The oval spores measure 0.7 to 1.0 by 1.1 to 1.6 μm. The polar tube, derived from electron-dense disks in sporonts, has five to seven isofilar coils that appear in two rows when seen in transverse section by transmission electron microscopy (16).

Encephalitozoon spp. develop intracellularly in parasitophorous vacuoles bounded by a membrane of presumed host cell origin. Nuclei of all stages are unpaired. Meronts divide repeatedly by binary fission and lie close to the vacuolar membrane. Sporonts appear free in the center of the vacuole and divide into two or four sporoblasts, which mature into spores. The spores measure 1.0 to 1.5 by 2.0 to 3.0 μm, and the polar tube has four to eight isofilar coils.

E. cuniculi was isolated from a range of animals before human infections were identified. Human isolates of three *Encephalitozoon* spp. and animal isolates of *E. cuniculi* are morphologically almost identical. In 1991, *Encephalitozoon hellem* was distinguished from *E. cuniculi* on the basis of different protein patterns found by sodium dodecyl sulfate-polyacrylamide gel electrophoresis separation and immunoblotting (18). In 1993, *Septata intestinalis* was described and named, based upon the morphological finding that the intracellular vacuoles containing the parasites showed a unique parasite-secreted fibrillar network surrounding the developing organisms, so that the

vacuoles appeared septate (3). Subsequently, on the basis of phylogenetic analyses, *S. intestinalis* was reclassified as *Encephalitozoon intestinalis,* and it was confirmed that the three *Encephalitozoon* spp. are indeed distinct organisms. Finally, with the use of immunological and molecular methods, three different strains of *E. cuniculi* have been identified which differ in animal host preferences and geographic distributions (20).

Microsporidium is a collective generic name for microsporidia that cannot be classified because available information is not sufficient. *Microsporidium ceylonensis* and *Microsporidium africanum* have been assigned to the group name (6).

NATURAL HABITATS

Human microsporidial infections have been documented globally. The sources of microsporidia infecting humans and their modes of transmission are uncertain. Ingestion of the environmentally highly resistant spores is probably the normal mode of transmission. Possible transmission by the aerosol route has also been considered because microsporidia have been found in respiratory specimens. Studies with mammals suggest that *Encephalitozoon* spp. can be transmitted transplacentally from mother to offspring, but no congenitally acquired human infections have been reported (6, 52).

Direct zoonotic transmission of microsporidia infecting humans has not been verified but appears likely because many microsporidial species can infect both humans and animals (15, 35) (Fig. 2A). *E. cuniculi* is considered to be a zoonotic parasite (34). Two of the three different strains of *E. cuniculi* which were detected in natural infections in rabbits and dogs have been isolated from human immunodeficiency virus (HIV)-infected patients (13, 20). In addition to documentation in humans, *E. hellem* has been detected in psittacines kept in aviaries and in a variety of wild birds, and *E. intestinalis* has been reported in single studies with domestic animals in Mexico (2) and gorillas in Uganda (35). *E. bieneusi* has been found mainly in humans, but the parasite has also been detected in domestic animals (pigs, cattle, cats, dogs, and chickens), in wildlife, and in rhesus monkeys experimentally infected with simian immunodeficiency virus (35). Analyses of the rRNA genes' internal transcribed spacers show considerable genetic variation among isolates of the species *E. bieneusi* of human and animal origin. Of 16 human-infecting *E. bieneusi* genotypes identified so far, four seem to have zoonotic potential as they have also been discovered in vertebrate hosts (Fig. 2B) (35). Also, an insect-pathogenic microsporidian, *B. algerae,* was isolated from three patients (11, 49, 50).

Epidemiologic studies suggest water contact as a risk factor for microsporidiosis, and *E. bieneusi, E. intestinalis,* and *V. corneae* homologous DNA has been detected in sewage effluent, groundwater, and surface water. Nevertheless, the importance of possible waterborne transmission requires further investigation.

CLINICAL SIGNIFICANCE

Although microsporidiosis appears to occur most frequently in persons infected with HIV, it is emerging as an infection in other immunocompromised hosts (such as organ transplant recipients and patients with idiopathic or other CD4 T lymphocytopenia) as well as in immunocompetent individuals (Table 1) (52). Microsporidiosis has been associated with abnormalities in structures and functions of infected organs, but the mechanisms of pathogenicity of the different microsporidial species are not sufficiently understood. Patients with severe cellular immunodeficiency appear to be at the highest risk for developing microsporidial disease. Unfortunately, little is known about immunity to this infection, although the importance of T cells has been demonstrated in experiments with athymic mice. It is not understood whether microsporidiosis in immunocompromised patients is primarily a reactivation of latent infection acquired prior to the state of suppressed immunity or whether microsporidial disease is caused by recently acquired infection.

E. bieneusi

E. bieneusi infects the enterocytes of the small intestine and epithelial cells of the biliary tree and respiratory tract. Clinical disease is probably caused by the continuous excess loss of epithelial cells. *Enterocytozoon* infection may be accompanied by alterations in small bowel physiology such as decreased brush border sucrase, lactase, and maltase activities, as well as malabsorption.

E. bieneusi is estimated to be one of the most important HIV-associated intestinal pathogens, present in 5 to 30% of patients with chronic diarrhea, weight loss, or cholangiopathy, particularly when CD4 lymphocyte counts are below 0.1×10^9/liter (55). Upper or lower respiratory tract infections have been detected in a few patients (54), but systemic infection due to *E. bieneusi* has not been documented. Case reports have documented that the parasite is also a cause of diarrhea in organ transplant recipients.

In immunocompetent adults and in children, *E. bieneusi* is associated with self-limited watery diarrhea (lasting up to 2 to 3 weeks), particularly among persons who reside or have traveled in tropical countries (41). Furthermore, the parasite has been identified among elderly persons with acute or chronic diarrhea.

Encephalitozoon spp.

Encephalitozoon spp. infect epithelial and endothelial cells, fibroblasts, macrophages, and possibly other cell types, as reported for humans and mammals. *Encephalitozoon* (identified as *E. cuniculi* because this was the only species known at that time) was initially identified in two children with a seizure disorder (53).

The spectrum of recognized *E. cuniculi*- and *E. hellem*-associated disease in patients with AIDS, organ transplant recipients, and otherwise immunocompromised patients includes keratoconjunctivitis, intraocular infection, sinusitis, bronchiolitis, pneumonitis, nephritis, ureteritis, cystitis, prostatitis, urethritis, hepatitis, sclerosing cholangitis, peritonitis, diarrhea, and encephalitis (13, 18, 52, 53). Clinical manifestations may vary substantially, ranging from an asymptomatic carrier state to organ failure.

E. intestinalis infects primarily enterocytes, but the parasite is also found in intestinal lamina propria, and dissemination to the kidneys, airways, and biliary tract appears to occur via infected macrophages (3). Chronic diarrhea and disseminated disease due to *E. intestinalis* have been diagnosed mainly in immunodeficient patients. A case of nodular cutaneous *E. intestinalis* infection was observed in a patient with AIDS (31). Furthermore, *E. intestinalis* has been detected in stool specimens from otherwise healthy children and adults, with or without diarrhea, living in Mexico (22) and among travelers with diarrhea returning from tropical countries.

Other Microsporidia

Different microsporidial species have been isolated from otherwise healthy persons who presented with keratoconjunctivitis,

severe keratitis, or corneal ulcers (Table 1) (9, 52). Also, keratoconjunctivitis due to microsporidia has been diagnosed in an immunocompetent contact lens wearer.

In immunocompromised patients, myositis has been linked to infections with different microsporidia, including *Pleistophora* sp. (32), *P. ronneafiei* sp. nov. (4), *T. hominis* (24), *B. vesicularum* (5), and *B. algerae* (11). *B. algerae* has also been isolated from erythematous skin nodules from a boy with acute lymphocytic leukemia (50).

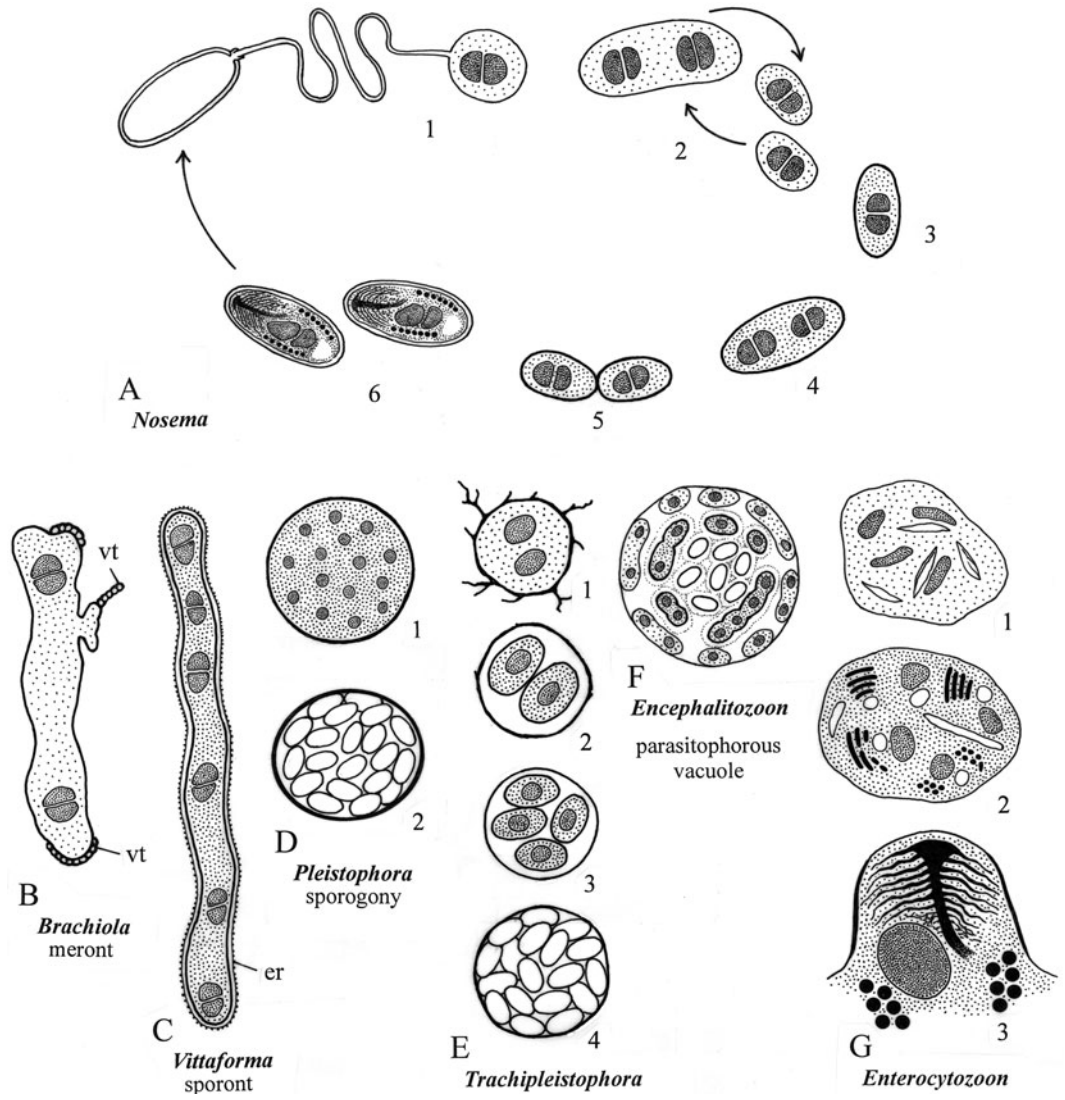
T. anthropophthera has been identified at autopsy in cerebral, cardiac, renal, pancreatic, thyroid, hepatic, splenic, lymphoid, and bone marrow tissue of patients with AIDS who initially presented with seizures (61). Disseminated infection due to *B. connori* was found at autopsy in a 4-month-old athymic male infant (Table 1).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Spores of enteropathogenic microsporidia can be detected in stool specimens or duodenal aspirates that have been fixed in 10% formalin or in sodium acetate-acetic acid-formalin, fresh stool samples, or biopsy specimens. The spores of microsporidia causing disseminated infection can usually be detected in fresh or fixed urine sediments, other body fluids (including sputum, bronchoalveolar lavage fluid, nasal secretions, cerebrospinal fluid, and conjunctival smears), corneal scrapings, or tissue. For histological examination, tissue specimens are fixed in formalin. For electron microscopy, fixation of tissue with glutaraldehyde is preferred. Collection of fresh material (without fixative) may be useful for cell culture and for molecular identification. Microsporidial spores are environmentally resistant and, if prevented from drying, can remain infectious for periods of up to several years.

DETECTION PROCEDURES

The most robust technique for the diagnosis of microsporidial infection is light microscopic detection of the parasites themselves. Spores, the stages at which microsporidia pathogenic in humans are usually identified, are small, ranging in size from 1 to 4 μm. Evaluation of patients with suspected intestinal microsporidiosis should begin with light microscopic examination of stool specimens, and microsporidia which cause systemic infection are best detected in urine sediments or other body fluids. Definitive species identification of



microsporidia is made by electron microscopy, antigenic analysis, or, preferably, molecular analysis (25, 56).

Examination of Stool Specimens

Preparation of Smears, Staining, and Microscopic Examination

Smears are prepared using 10 to 20 μl of unconcentrated stool that is very thinly spread onto slides. Most of the procedures that have been adapted for the concentration of ova and parasites fail to concentrate microsporidial spores present in stool specimens. The formalin-ethyl acetate concentration and different flotation methods remove significant amounts of fecal debris, and smears prepared from these concentrates appear easier to read by light microscopic examination than smears prepared from unconcentrated specimens. Such concentration techniques, however, lead to a substantial loss of microsporidial spores and may give false-negative results (51).

The most commonly used stains are chromotrope-based stains (51) and chemofluorescent optical brightening agents (45, 46), including calcofluor white M2R (American Cyanamid Corp., Princeton, N.J.), the Fungi-Fluor kit (Polysciences Inc., Warrington, Pa.), Fungiqual A (Medical Diagnostics, Kandern, Germany), Cellufluor (Polysciences Inc.), Uvitex 2B (Ciba Geigy, Basel, Switzerland; not commercially available), and other chemofluorescent stains. Regardless of which staining technique is used, the use of positive control material is essential. The detection of microsporidial spores requires adequate illumination and magnification, i.e., magnification of ×630 or ×1,000 (oil immersion). The differences in the sizes of *Enterocytozoon* spores (1 to 1.5 μm) and *Encephalitozoon* spores (2 to 3 μm) often permit a tentative identification of the genus from light microscopic examination of stool specimens.

Chromotrope-Based Staining Procedures

Microsporidial spores are ovoid and have a specific appearance when stained with chromotrope stains (Fig. 4 and 5, right panel) (51). The spore wall stains bright pinkish red, some spores appear transparent, and other spores show a distinct pinkish red-stained belt-like stripe that girds the spores diagonally or equatorially. Most background debris in stool specimens counterstains a faint green (or blue, depending on the staining technique). Some other fecal elements, such as yeasts and some bacteria, may also stain reddish, but they are distinguished from microsporidial spores by their sizes, shapes, and staining patterns.

Several modifications of the original chromotrope staining solution (51) have been proposed, including modifications of the counterstain and changes in the temperature of the standard chromotrope staining solution and in the

FIGURE 1 Generalized life cycle of microsporidia and identifying characteristics of the genera known to infect humans. Light stippling indicates merogonic stages; heavy stippling indicates sporogonic stages; no stippling indicates spores and sporoplasm. (A) Basic life cycle, illustrated by *Nosema.* Development may occur in direct contact with host cell cytoplasm *(Nosema*, *Brachiola*, and *Enterocytozoon)* or in isolation by host cell membranes *(Vittaforma* and *Encephalitozoon)* or a cyst-like sporophorous vesicle of parasite origin *(Pleistophora* and *Trachipleistophora)*.1, Sporoplasm: the infective stage emergent from the spore. It may have unpaired nuclei (monokaryotic) or two nuclei in close apposition (diplokaryotic), depending on the genus. 2, Merogony: proliferative stage. It may have a simple plasma membrane, but a surface coat is present in *Brachiola*, *Pleistophora,* and *Trachipleistophora*. Division can be by binary or multiple fission into two or more individuals. 3, Sporont: the first stage of sporogony. If not already present, a surface coat is added (this step is delayed in *Enterocytozoon*). 4, Sporogony: divisions culminating in spore production. Binary or multiple fissions give rise to sporoblasts. 5, Sporoblasts: end products of sporogony, which mature into spores. 6, Spores: resistant stages for transmission. Spores are characterized by an extrusion apparatus (polar tube), which serves to conduct the sporoplasm into a host cell. The polar tube may be of uniform diameter (isofilar) or show a sharp decrease in diameter in the most posterior coils (anisofilar). (B to G) Identifying characteristics of genera. (B) *Brachiola*. Members of this genus are diplokaryotic and disporoblastic; the life cycle is like that of *Nosema,* but meronts are of bizarre shapes and possess a surface coat with vesiculotubular structures (vt) embedded in it and extended from it. (C) *Vittaforma*. Members of this genus are diplokaryotic and polysporoblastic; all stages, including spores, are isolated in a close-fitting, ribosome-studded cisterna of host endoplasmic reticulum (er). (D) *Pleistophora*. Members of this genus are monokaryotic and polysporoblastic; meronts and sporonts are multinucleate stages called plasmodia; a thick surface coat is already present on meronts and sporonts (1); this coat separates from the sporogonial plasmodium to form a cyst-like vesicle, the sporophorous vesicle; the plasmodium divides within it to produce numerous spores (2). (E) *Trachipleistophora*. Members of this genus are monokaryotic and polysporoblastic. The meront surface coat has branched extensions (1); these are withdrawn when the coat separates to form the sporophorous vesicle around a uninucleate sporont; the sporont undergoes a series of binary fissions (2 and 3) and finally encloses numerous spores (4). (F) *Encephalitozoon*. Members of this genus are monokaryotic and di- or tetrasporoblastic; all stages of the life cycle, developing from the sporoplasm, occur concurrently within a host cell vacuole (parasitophorous vacuole); merogonic stages are appressed against the vacuole wall; sporogonic stages are free; the vacuole is finally packed with spores, so that it superficially resembles a sporophorous vesicle. (G) *Enterocytozoon*. Members of this genus are monokaryotic and polysporoblastic; meronts (1) have irregular nuclei and lucent clefts; sporonts (2) are multinucleate with rounded nuclei and have highly characteristic electron-dense disks, which are polar tube precursors; all spore organelles are formed prematurely, so that constriction around the sets of organelles and a nucleus (3) gives rise directly to almost mature spores. The surface coat is deposited only during constriction.

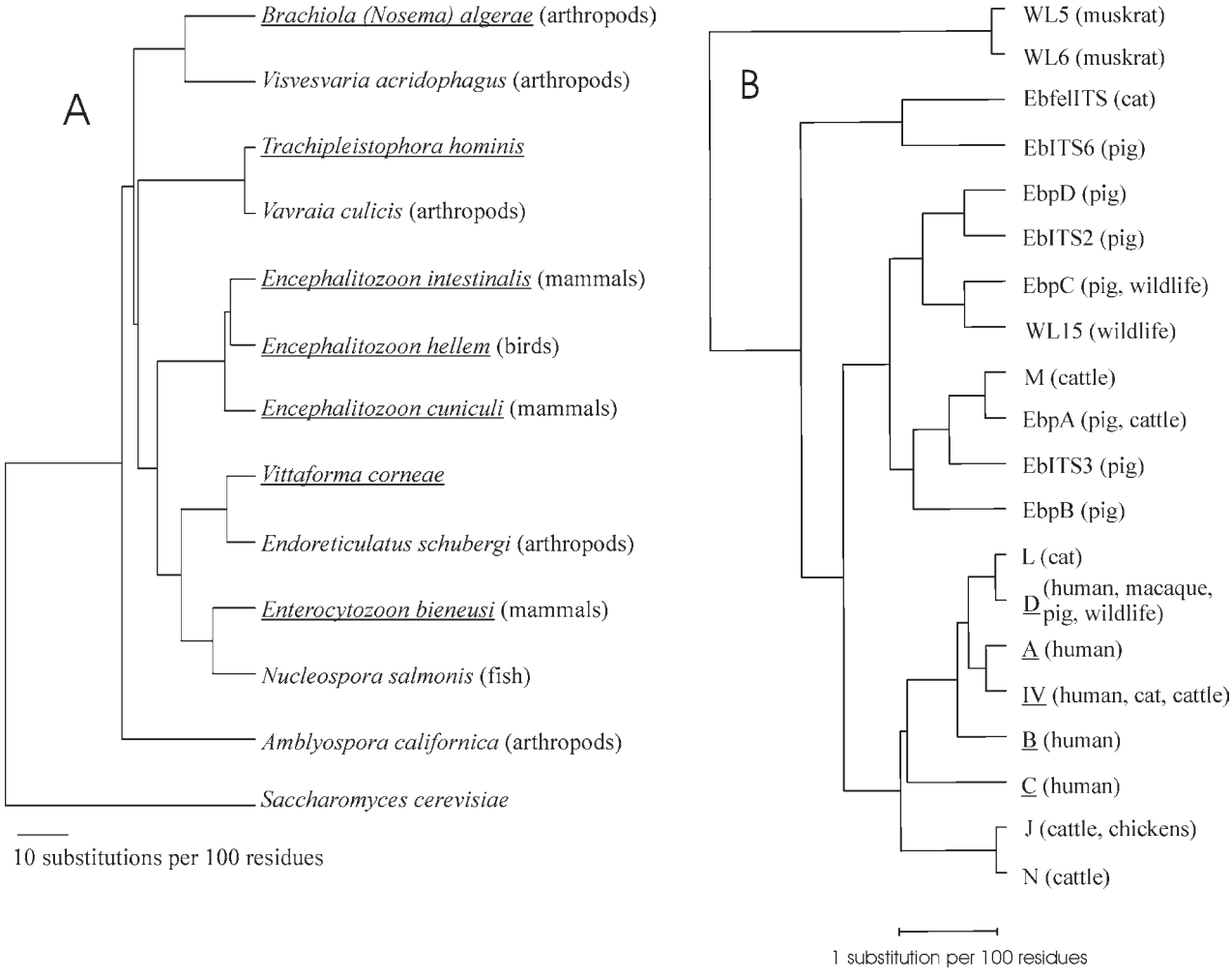


FIGURE 2 (A) Phylogeny of microsporidia generated from the SSU RNA gene sequences of microsporidian species identified in humans (underlined) and selected other species (Kimura's distance, unweighted pair group method of analysis). Known animal hosts are indicated in parentheses; the brewer's yeast *Saccharomyces cerevisiae* serves as an outgroup. No corresponding gene sequences are known for the human-infecting microsporidian species *P. ronneafiei*, *T. anthropophthera*, *B. connori* (formerly *N. connori*), *B. vesicularum*, *N. ocularum*, *M. ceylonensis*, and *M. africanum*. (B) Dendrogram of rRNA internal transcribed spacer sequences of human-infecting *E. bieneusi* genotypes (those confirmed in independent studies are underlined) and selected genotypes with animal hosts (host species are indicated in parentheses). Human-infecting genotypes cluster together, indicating a certain degree of host preference. The eight novel *E. bieneusi* genotypes recently identified in a single study of HIV-infected patients (43) cluster within the branch containing all other human-infecting genotypes (for a detailed dendrogram, see reference 43). All sequences are deposited in GenBank (Kimura's distance, unweighted pair group method of analysis). Adapted from reference 35.

staining time (19). An acid-fast trichrome stain (28) which permits visualization of acid-fast cryptosporidial oocysts as well as microsporidial spores on the same slide and a "quick-hot" Gram-chromotrope staining technique (40) have also been developed.

Chemofluorescent Agents

Chemofluorescent optical brightening agents are chitin stains, which require examination with a fluorescent microscope. With the correct wavelength illumination, the chitinous wall of the microsporidial spores fluoresces brightly, facilitating the detection of spores (45). However, staining is not specific, and fungi, of which small species may be present in fecal material, and other fecal elements may also be brightened. Some experience is necessary to distinguish the microsporidia.

Epidemiological comparisons of the chromotrope staining technique with methods that use chemofluorescent optical brighteners indicate that these tests are robust for routine use and that the sensitivities of both methods are similarly high (19). Some laboratories use both staining techniques because the chromotrope stains result in a highly specific visualization of spores whereas the chemofluorescent agents may be more sensitive but may produce false-positive results.

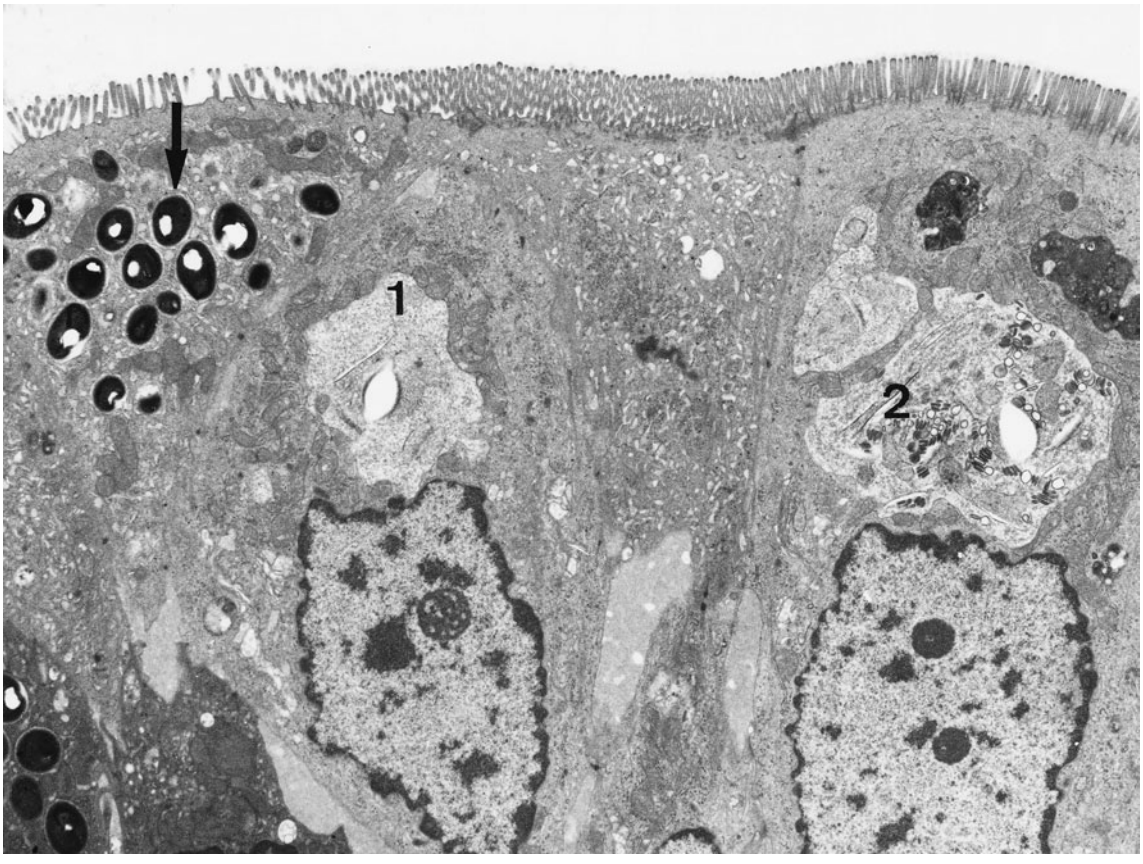


FIGURE 3 Transmission electron micrograph showing the duodenal epithelium of an HIV-infected patient infected with *E. bieneusi*. The different developmental stages between the enterocyte nuclei and the microvillus border include a proliferative plasmodium (1), late sporogonial plasmodia (2), and mature spores (arrow). Magnification, ×5,370. (Courtesy of M. A. Spycher, University Hospital, Zurich, Switzerland.)

Immunofluorescent-Antibody Tests

Immunofluorescence procedures for the detection of *Encephalitozoon* and *Enterocytozoon* microsporidial spores are promising but not commercially available. Diagnostic application of polyclonal antibodies in fecal samples has been hampered by high levels of background staining, cross-reactions with yeast and bacteria, and sensitivity lower than those of chromotrope or chemofluorescent staining procedures (19). Monoclonal antibodies against *Encephalitozoon* spp. (21) and *E. bieneusi* (1) have been generated, of which some have been evaluated for diagnostic purposes with stool specimens (commercially not available).

Cytological Diagnosis

Microsporidial spores have been detected in sediments obtained by centrifugation of body fluids for 10 min at 500 × *g*, including duodenal aspirates, bile, biliary aspirates, urine (Fig. 5, right panel), bronchoalveolar lavage fluid (Fig. 5, middle panel), and cerebrospinal fluid, and in smears of conjunctival swabs, sputum, and nasal discharge. Microscopic examination of stained smears of centrifuged duodenal aspirate obtained during endoscopy is a highly sensitive technique for the diagnosis of intestinal microsporidiosis. Because microsporidial infection often involves multiple organs, the detection of microsporidia in virtually any tissue or body fluid should prompt a thorough search of other sites. Particularly for patients with suspected disseminated microsporidiosis, urine specimens should be examined (52, 56).

Examination of Biopsy Specimens and Corneal Scrapings

Examination of duodenal and terminal ileal tissue has resulted in the detection of intestinal microsporidia, but the parasites are rarely found in colonic tissue sections. Microsporidial species causing disseminated infection have been found in almost every organ system (52, 61).

Only highly experienced pathologists have reliably and consistently identified microsporidia in tissue sections by using routine techniques such as hematoxylin and eosin staining. Ultrathin plastic sections stained with methylene blue-azure II-basic fuchsin or with toluidine blue may facilitate detection. In our experience, tissue Gram stains (Brown-Brenn or Brown-Hopps) have proved to be the most useful for the rapid and reliable identification of HIV-associated microsporidia in routine paraffin-embedded tissue sections (Fig. 5, left panel) and corneal scrapings (51, 56). The microsporidial spores are gram variable, and they are readily identified because of the contrasting dark blue or reddish staining against a faint brown-yellow background. Others prefer a silver stain (Warthin-Starry stain) (23), the chromotrope-based staining technique, or chemofluorescent agents.

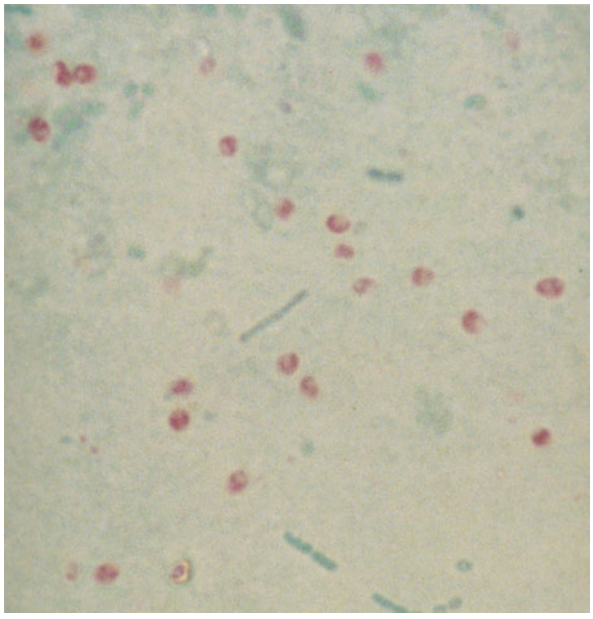


FIGURE 4 Smear of a stool specimen from a patient with AIDS and chronic diarrhea, showing pinkish red-stained spores of *E. bieneusi* that measure 0.7 to 1.0 by 1.1 to 1.6 μm. Chromotrope staining was used. Magnification, ×1,000 (oil immersion).

Molecular Techniques

Universal panmicrosporidian and genus- or species-specific primer pairs that target the rRNA genes and their application in the diagnosis of intestinal microsporidial infection have been described previously (57). The detection and identification of *E. bieneusi* and the different *Encephalitozoon* spp. have been successfully performed with fresh stool specimens, formalin-fixed stool specimens, intestinal tissue obtained by endoscopic biopsy, urine specimens, and other body fluids (12, 13). A real-time PCR method was developed for quantitation of *E. bieneusi* DNA (36) and *E. intestinalis* DNA (37) in stool specimens. Furthermore, in situ hybridization to visualize *E. bieneusi* in tissue sections has been developed (8).

ISOLATION OF MICROSPORIDIA

Microsporidia cannot be grown axenically. *Encephalitozoon* spp., *T. hominis, V. corneae,* and *B. algerae* have been isolated with different cell culture systems, including RK-13 (rabbit kidney), MDCK (Madin-Darby canine kidney), and MRC-5 (human embryonic lung fibroblast) cells and other cells (6, 14, 48). Only short-term in vitro propagation has been accomplished with *E. bieneusi* (48). The isolation of microsporidia has no relevance for diagnostic purposes but is an important research tool and is necessary for the development of immunoassay reagents.

IDENTIFICATION

The identification of microsporidia and their taxonomy have been based primarily upon ultrastructural characteristics. Microsporidial ultrastructure is unique and pathognomonic for the phylum, and ultrastructural features can distinguish all microsporidial genera (Fig. 1) (6). Nevertheless, morphologic features alone do not sufficiently characterize all microsporidial species pathogenic for humans. The characterization of the three *Encephalitozoon* spp., which share most of

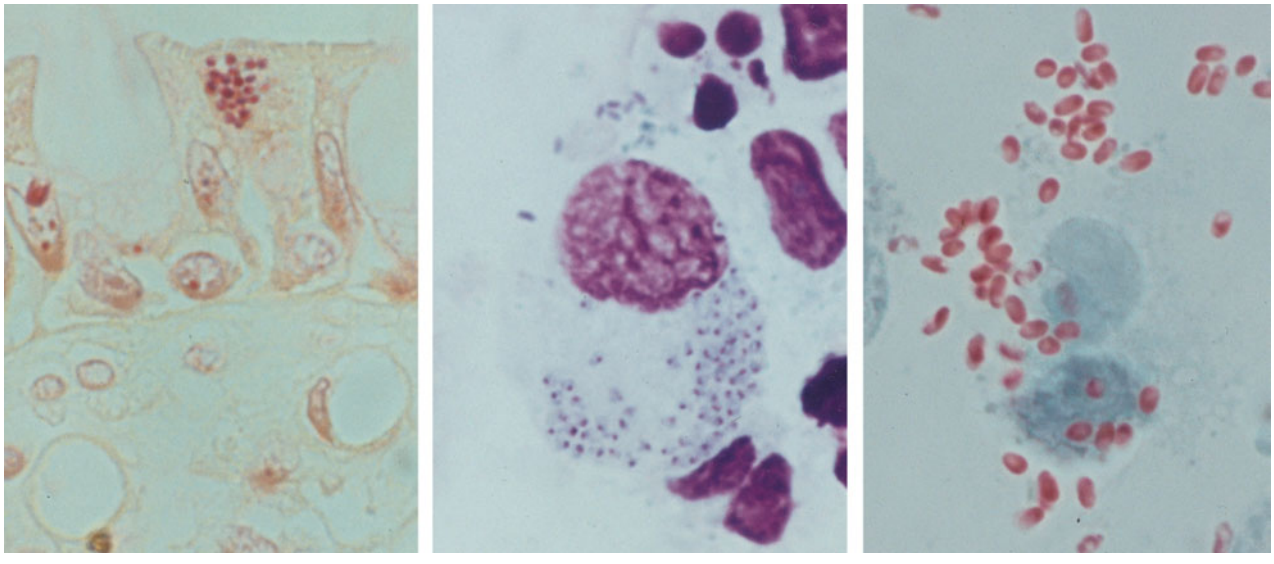


FIGURE 5 (Left) Terminal ileal tissue obtained by ileocolonoscopy from a patient with AIDS and chronic diarrhea due to *E. bieneusi* infection. Gram-labile microsporidial spores, measuring 0.7 to 1.0 by 1.1 to 1.6 μm, are found at a supranuclear location within small intestinal enterocytes. Brown-Brenn stain was used. Magnification, ×1,000. (Middle) Cytospin preparation of bronchoalveolar lavage fluid from a patient with AIDS and intestinal *E. bieneusi* infection, showing intracellular microsporidia. Giemsa stain was used. Magnification, ×1,000 (oil immersion). (Right) Urine sediment from a patient with AIDS and disseminated *E. cuniculi* infection, showing pinkish red-stained microsporidial spores measuring 1.0 to 1.5 by 2.0 to 3.0 μm. Chromotrope stain was used. Magnification, ×1,000 (oil immersion).

their morphologic features, requires antigenic or molecular analyses, which may also reveal subtype-specific variation (18, 20). Phylogenetic relationships of different microsporidia pathogenic in humans, including *E. bieneusi* (35), *Pleistophora*-like organisms (10), *E. cuniculi, E. hellem,* and *E. intestinalis* (59, 60), have been investigated by analyses of SSU rRNA sequences, protein-encoding genes, and gene sequences encoding the largest subunit of the RNA polymerase.

SEROLOGIC TESTS

Serologic assays (including the carbon immunoassay, indirect immunofluorescence test, and enzyme-linked immunoadsorbent assay and Western blot immunodetection) have been useful in detecting specific antibodies to *E. cuniculi* in several species of animals. However, the value of such tests for humans has controversially been discussed because of possible cross-reactivity of the spore wall antigens of the *Encephalitozoon* spp. Furthermore, the results of serologic studies with humans were not substantiated by the detection of parasites in individuals with antibody responses. Recently, by employing recombinant antigens of the polar tube of *E. cuniculi,* improved specificity was demonstrated, and the development of serodiagnostic tools seems feasible (44). *Enterocytozoon,* the most frequent microsporidian pathogen in humans, has not been continuously propagated to produce antigens.

ANTIMICROBIAL SUSCEPTIBILITY

Albendazole has been found to cause growth deformities of *Encephalitozoon* and *Vittaforma* and to reduce or eradicate the parasites propagated in cell cultures but does not destroy mature microsporidial spores, so these may sustain infection (17). Fumagillin, its analog TNP-470, and nikkomycin Z have been shown to inhibit completely or partially the replication or spore germination of *Encephalitozoon* and *Vittaforma* in vitro (17). Numerous antiprotozoal drugs and antibiotics have been tested in vitro, with negative findings. In vitro systems to investigate *E. bieneusi* are not available.

Treatment studies with humans are limited, and only two randomized controlled trials have been conducted. Albendazole can result in clinical cure of HIV-associated encephalitozoonosis in parallel with the cessation of spore excretion (38). In contrast, albendazole is not effective for *Enterocytozoon* infection and does not reduce the parasite load, although previous observations had suggested that clinical improvement may occur in some patients. Oral purified fumagillin appeared to eradicate *E. bieneusi* in many patients, but serious adverse events and parasitic relapse were observed (39).

E. bieneusi-associated diarrhea as well as systemic infection due to *Encephalitozoon* spp. is observed mainly in severely immunodeficient patients with CD4 lymphocyte counts below 0.05 to 0.1×10^9/liter. Observational studies showed that an improvement of immune functions by potent antiretroviral combination therapy results in complete clinical response and normalization of intestinal architecture which parallels the clearance of intestinal microsporidia (7).

INTERPRETATION AND REPORTING OF RESULTS

Microsporidia are predominantly opportunistic pathogens capable of causing disease in severely immunodeficient HIV-infected persons (with CD4 cell counts below 200/μl) and otherwise immunocompromised patients including organ transplant recipients. Therefore, stool examination or examination of other specimens is particularly indicated for these patient groups, and it is prudent to consider microsporidia as the etiologic agents when they are detected in clinical specimens from such patients. Furthermore, various microsporidial species may cause self-limited diarrhea or keratoconjunctivitis in immunocompetent and otherwise healthy persons.

Routine diagnosis of microsporidiosis is based upon microscopic detection of microsporidial spores. Although sensitive and specific, light microscopic examination does not allow the identification of the organisms to the genus and species level. This can be achieved in most cases by electron microscopy, which is relatively insensitive for the detection of microsporidia because only small samples are examined and sampling errors may occur, or by specific immunofluorescence staining of organisms and by PCR-based procedures (both are commercially not available).

Immunocompetent patients may excrete lower numbers of microsporidial spores in feces or urine, and therefore, the threshold of the current light microscopic detection procedures may not be sufficient for the reliable detection of microsporidia in this group.

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Nematodes

HARSHA SHEOREY, BEVERLEY-ANN BIGGS, AND PETER TRAYNOR

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A quarter of the world's population is infected with intestinal nematodes, and these parasites are a major cause of poor health. Most infections occur in resource-poor settings where sanitation is inadequate. Women and children have the greatest morbidity. Immigrants and refugees, travelers, and war veterans often unknowingly harbor helminths for years after leaving an area where the parasites are endemic. Clinical features of infection are usually directly proportional to the intensity of the worm infection. Effective school-based drug therapy programs are under way in many developing countries (14). Laboratory diagnosis is based primarily on microscopic examination of feces and differention of species based on the morphologies of eggs or larvae detected (Fig. 1–3). The sensitivity of this procedure depends on the stage of the infection and is affected by factors such as the variability of the number of eggs or larvae excreted.

TAXONOMY OF NEMATODES

There are various morphological features by which nematodes are identified. At a high taxonomic level, the nature of the anterior regions of the alimentary canal (e.g., the esophagus), the form of the head (presence, number of lips, teeth, etc.), and the form of the tail, especially in the male, are important features. At the species level, details of the male tail, such as the arrangement of caudal papillae and the length and shape of the spicules (sclerotized copulatory aids), are important. In the majority of cases, males carry more taxonomically useful information than females; the latter may often be unidentifiable to the species level in the absence of males. Some studies suggest that nematodes are actually related to the arthropods and priapulids in a newly recognized group, the Ecdysozoa (1). All these nematodes belong to the superkingdom *Eukaryota,* subkingdom *Metazoa,* and phylum Nematoda (for details, see the Taxonomy Browser at http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html/index.cgi).

ASCARIS LUMBRICOIDES (ROUNDWORM)

Taxonomy

A. lumbricoides belongs to the class *Chromadorea,* order *Ascaridida,* superfamily *Ascaridoidea,* and family *Ascarididae*.

Of the three genera of ascaridid nematodes regularly occurring as parasites of humans, only *Ascaris* is a true human parasite. The others *(Toxacara* and *Toxascaris)* are common parasites of cats and dogs and occasionally infect humans. Ascaridids are related to anisakids, with three distinct lips, but differ in that their life cycle is linked to terrestrial rather than aquatic conditions.

Description

Eggs

Eggs of *A. lumbricoides* (Fig. 2A to C) are usually seen in two forms: unfertilized and fertilized. The egg shell of a fertilized egg (45 to 75 by 35 to 50 μm) consists of an inner lipid layer responsible for selective permeability, a chitin-protein layer responsible for structural strength, and an outer vitelline layer. The inner layer contains a lipoprotein, ascaroside, which helps the egg survive exposure to formaldehyde, disinfectants, and other chemicals. The fertilized egg frequently has an uneven deposit of mucopolysaccharide with adhesive properties present on its outer surface. Eggs appear brown due to staining by bile. The unfertilized eggs have thinner walls and distorted mammillations. These eggs are usually more elongated in shape, measure 85 to 95 by 43 to 47 μm, and may have a pronounced mammillated coat or an extremely minimal mammillated layer. The presence of only unfertilized eggs strongly suggests that only female worms are present in the intestine. During the development of the eggs, the parent worm must supply the zygotes with sufficient nutrients to ensure progression to the infective second-stage larvae and subsequent survival.

Larvae

First-stage larvae develop inside the eggs and molt to form the second-stage larvae. Soon after hatching in the jejunum, second-stage larvae have a typical filariform appearance (measuring approximately 250 by 14 μm), growing to approximately 560 by 28 μm just before a further molt occurs in the lungs. In the lungs, the larvae develop over 8 or 9 days to reach a length of approximately 1 mm. The larvae must either have sufficient reserves or feed directly to ensure successful migration back to the gut and more developmental molts. Growth is rapid after the fourth and final molt in the intestine.

Worms

Adult male and female *A. lumbricoides* worms (Fig. 2D) are pinkish cream, with males approximately 15 to 31 cm and

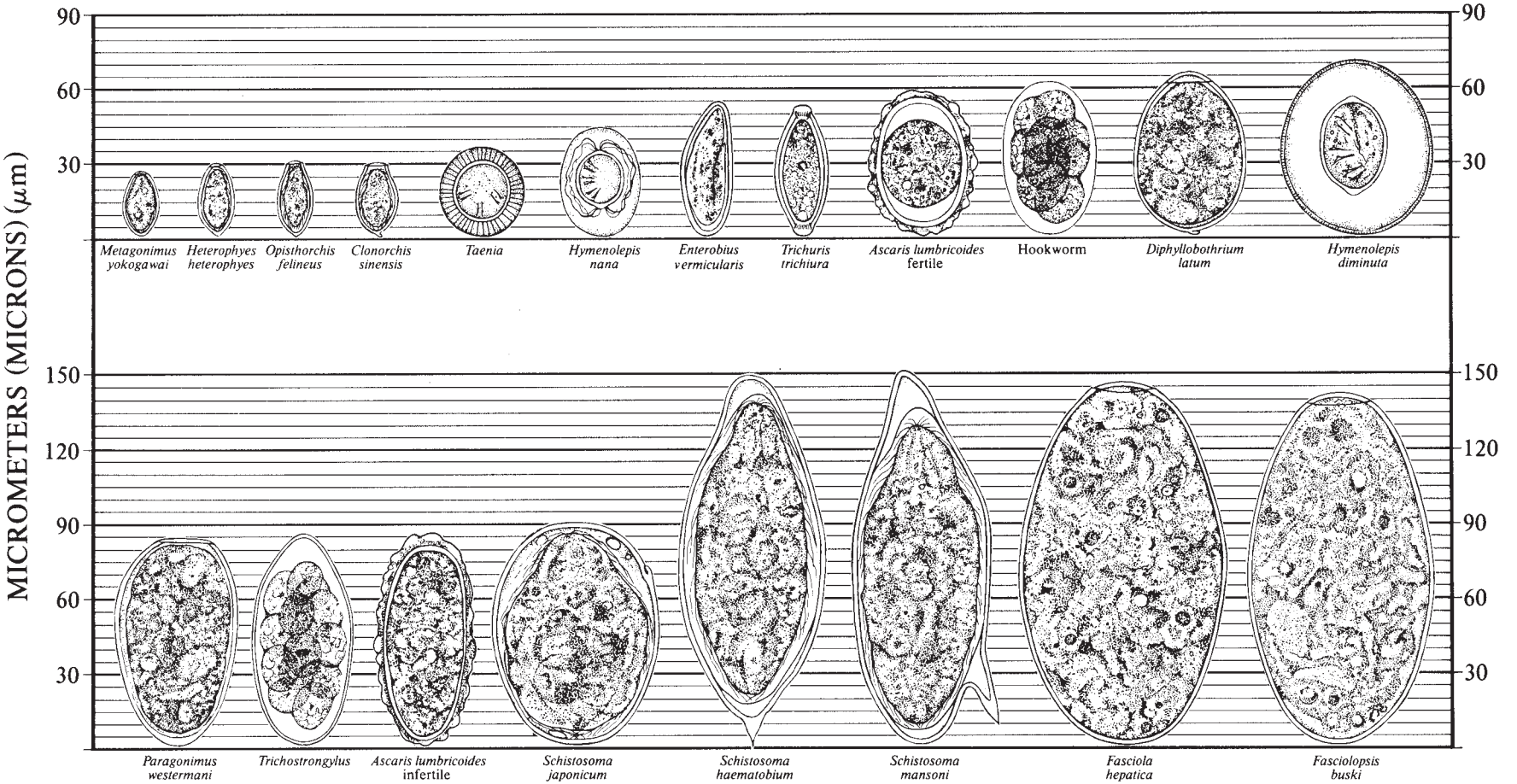
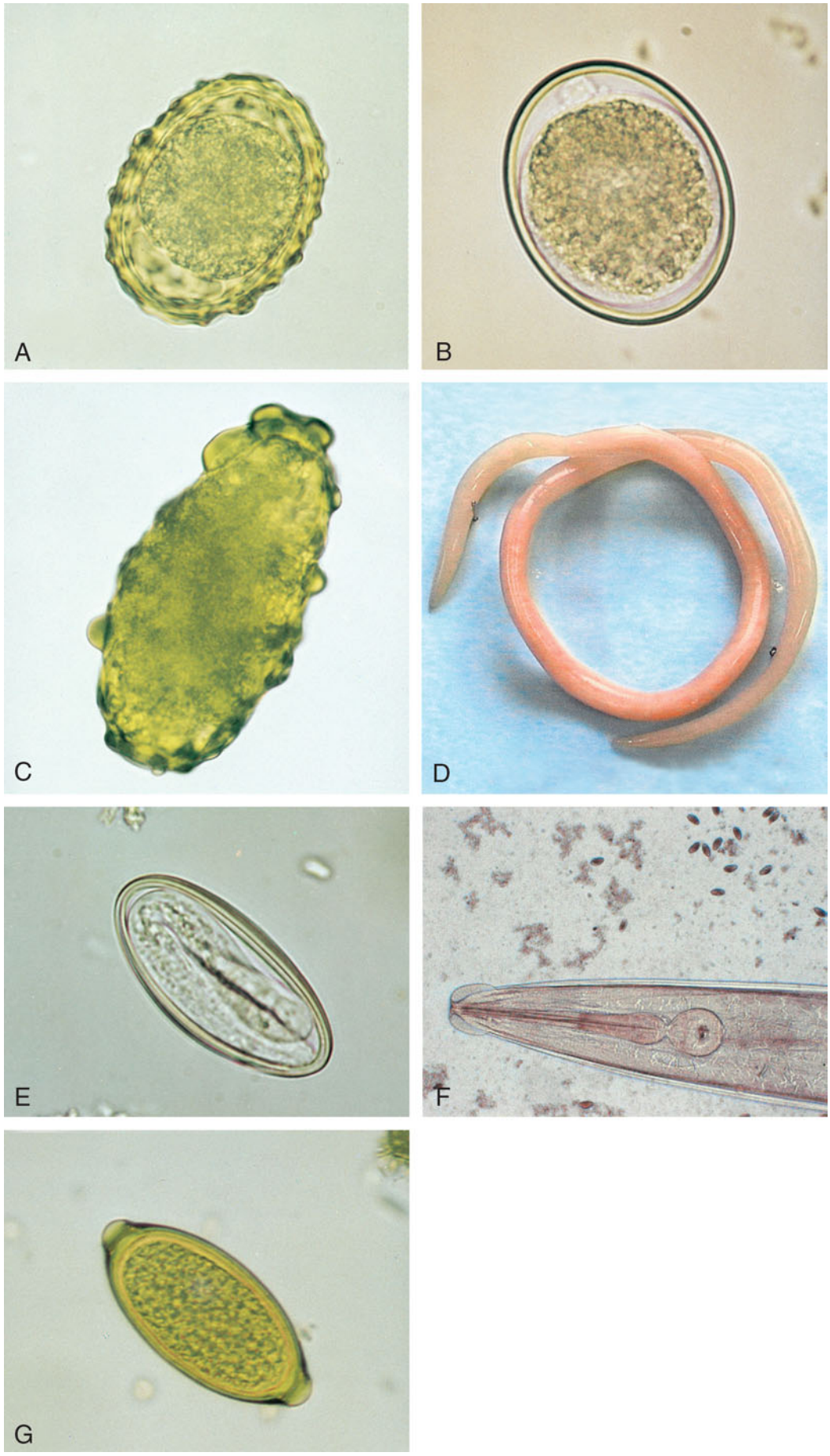


FIGURE 1 Relative sizes of helminth eggs.


A
B
C
D
E
F
G

females 20 to 35 cm in length. The males, often smaller than the females of the same age, have a curved posterior tail, which accommodates the copulatory aids. Adult worms live in the lumen of the host's small intestine and feed on digestion products.

Epidemiology and Prevention

A. lumbricoides infection is one of the most common human infections, with an estimated 1.2 billion people infected worldwide. It has a worldwide distribution and is most common in tropical and subtropical areas of the developed and underdeveloped world. It is particularly associated with crowding and poor sanitation.

Preventive measures consist of health education about personal hygiene and sanitation, and drug therapy.

Transmission and Life Cycle

Eggs passed in feces (diagnostic stage) develop in the soil over 10 to 15 days (Table 1) and may survive in this form for years. Infection follows the ingestion of embryonated eggs (infective stage) that hatch in the small intestine. Larvae penetrate the intestinal mucosa, and the venous circulation carries them to the lungs. From here they migrate to the trachea and are swallowed. They then complete their development into mature adult worms in the small intestine, with eggs produced after 60 to 70 days. A minority of individuals develop heavy infections and act as an important source of transmission.

The prepatent period (from egg ingestion to egg production) is approximately 2 months.

Clinical Significance

Initial infection is usually asymptomatic. The migration of larvae through the lungs may result in eosinophilic pneumonitis with fever, dyspnea, and bronchospasm. This may occur up to 2 weeks after infection and lasts approximately 3 weeks. Bronchospasm may be prominent and is occasionally fatal.

The clinical features in established infections relate to the worm burden. Most infections are light and asymptomatic, but nonspecific abdominal symptoms may occur. There is increasing risk of intestinal obstruction with increasing worm burden. Intestinal obstruction has a high mortality rate in some settings (2). Heavy infections are also associated with malnutrition and impaired growth in children.

Ectopic ascariasis occasionally occurs when adult worms enter hepatobiliary and pancreatic ducts or the appendix and cause obstruction. This may result in biliary colic, cholecystitis, acute cholangitis, acute pancreatitis, appendicitis, or a hepatic abscess. It occurs more commonly in countries with high rates of infection where the parasite is endemic. (For more on the clinical significance of *A. lumbricoides,* see reference 12.)

Diagnosis

The primary diagnosis of infection is by demonstration of the presence of eggs in feces (see references 4 and 5). The fertilized eggs are round to oval, bile stained, mammillated, and thick walled. Often both fertilized and unfertilized eggs are found in the same stool. Multiple specimens taken on separate days may be required. Both direct and concentration methods such as formalin-ethyl acetate sedimentation should be used for optimal yield. The flotation technique may not concentrate dense unfertilized eggs. Decorticated fertile eggs and infertile eggs may be difficult to recognize (Fig. 2A to C). Motile larvae may sometimes be seen in expectorated sputum but rarely in feces. Occasionally, an adult worm (usually female) may be passed in feces or may spontaneously migrate out of the anus, mouth, or nares, particularly in children. Rarely, multiple worms may be passed in children who have lived in areas where the worms are endemic. The female worm is easily identified by its cylindrical body and tapering ends (Fig. 2D) (males are slightly smaller but are rarely seen). Distinguishing features include a lateral white line along the entire length of the body, three lips at the anterior end, and a tough cuticle. No other human parasite is as large as *A. lumbricoides*; hence, it is usually easy to identify. However, with faulty or old plumbing, earthworms may be found in toilet bowls and these may be presented for identification.

Treatment

Albendazole or mebendazole is effective (see chapter 150; see also reference 13).

For a summary of *A. lumbricoides*, see Table 2.

ENTEROBIUS VERMICULARIS (PINWORM OR THREADWORM)

Taxonomy

E. vermicularis belongs to the class *Chromadorea,* order *Oxyurida,* superfamily *Oxyuroidae,* and family *Oxyuridae.*

Only the genus *Enterobius* of the *Oxyuridae* regularly occurs in humans and other primates.

Description

Eggs

Pinworm eggs (Fig. 2E) are ovoid, 50 to 60 by 20 to 35 μm, and asymmetrically flattened on one side and appear colorless when recovered from the perianal skin. The outer layer of the egg shell is albuminous and sticky, enabling the egg to adhere readily. When laid, the egg contains an immature first-stage larva, but this develops rapidly to become infective.

Larvae

Hatched-out larvae complete molting and development in the large bowel.

Worms

Adult female worms (Fig. 2F) measure approximately 8 to 13 by 0.3 to 0.5 mm, with typical lips at the anterior end and a long, pointed tail (hence the name pinworm). Male worms are 2.5 by 0.2 mm but are rarely seen.

FIGURE 2 Various eggs and adult worms of intestinal nematodes (magnification, ×850). (A) Fertile egg of *A. lumbricoides.* (B) Decorticated fertile egg of *A. lumbricoides.* (C) Infertile egg of *A. lumbricoides.* (D) *A. lumbricoides* adult worm with typical cylindrical body with tapering ends and thick cuticle. The *A. lumbricoides* adult worm is the largest of the human pathogenic nematodes, 15 to 35 cm in length. (E) Embryonated infective egg of *E. vermicularis.* (F) *E. vermicularis* adult female. Adult females are usually 8 to 13 by 0.3 to 0.5 mm in size. Shown is the anterior end of a female worm with the characteristic lips. Typical eggs can also usually be seen in the field. (G) Egg of *T. trichiura.*

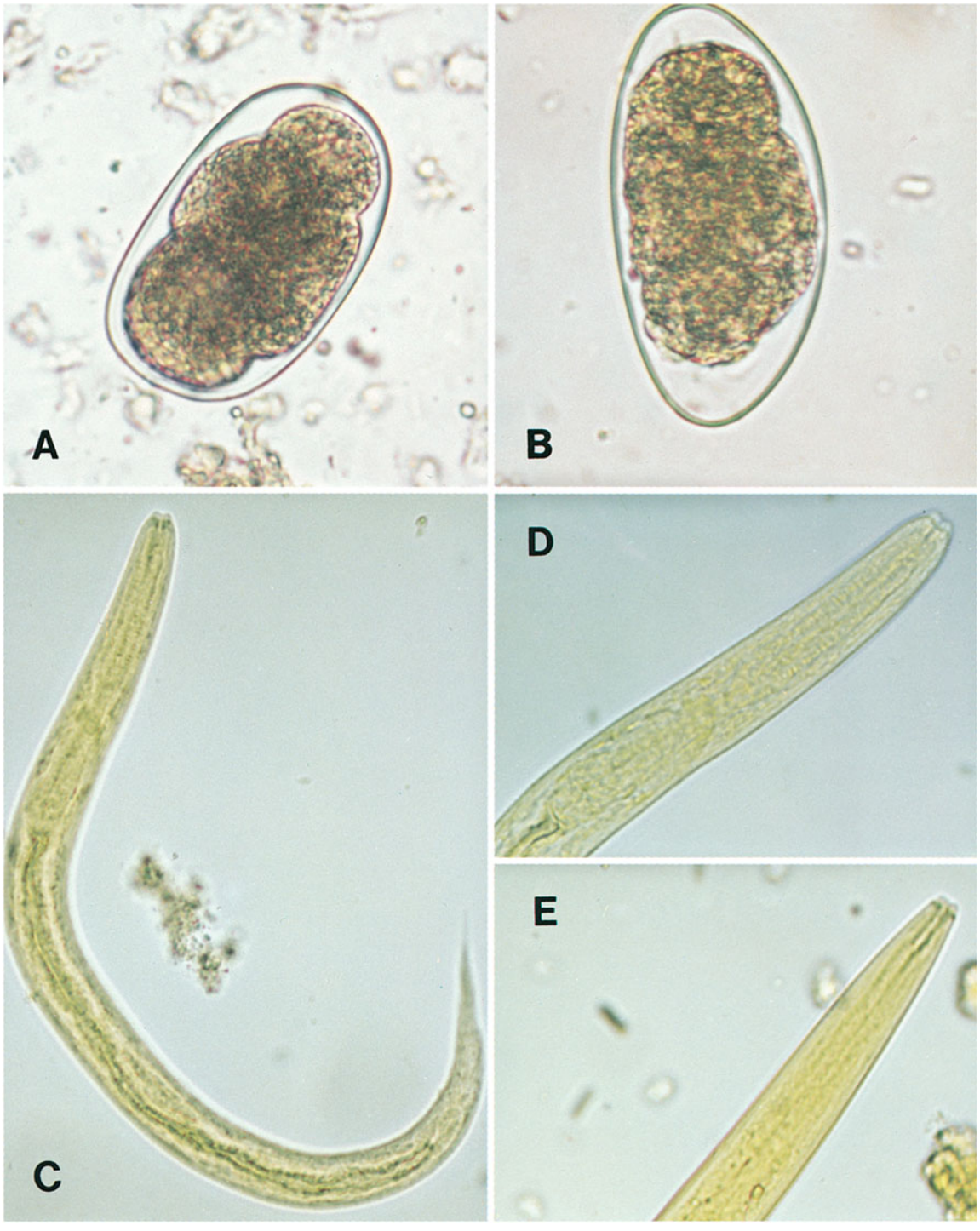


FIGURE 3 Eggs and larvae of intestinal nematodes (magnification, ×850). (A) Hookworm egg; (B) *Trichostrongylus* egg; (C) rhabditiform, or first-stage, larva of *S. stercoralis;* (D) anterior end of rhabditiform larva of *S. stercoralis* showing short buccal cavity; (E) anterior end of rhabditiform hookworm larva showing long buccal cavity; (F) tail end of filariform, or last-stage, larva of *S. stercoralis* with the notched tail (arrow); (G) tail end of filariform, or last-stage, hookworm larva with the tapering tail.

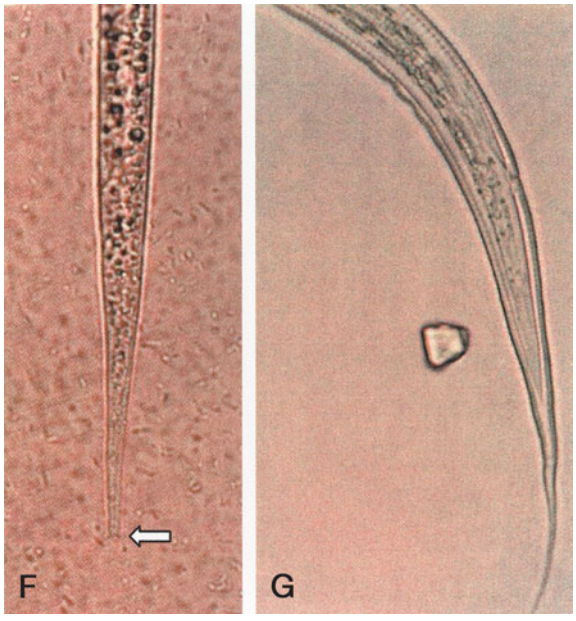


FIGURE 3 *(Continued)*

Epidemiology and Prevention

E. vermicularis infection has a worldwide distribution and is more common in children than adults.

Preventive measures consist of health education about personal hygiene and sanitation, and drug therapy.

Transmission and Life Cycle

Adult worms inhabit the cecum, appendix, and ascending colon. The female migrates down the colon when mature and deposits her eggs on perianal and perineal skin (diagnostic stage). More than 10,000 eggs may be deposited, become infective within 6 h (Table 1), and remain viable for up to 5 days.

Transmission is either direct from the anal and perianal regions to the mouth, usually by fingernail contamination (autoinfection), or from exposure to a contaminated environment. When swallowed, the embryonated eggs (infective stage) hatch in the small bowel. Larvae pass from the small bowel to the large bowel, where they mature into adult worms. Retroinfection is thought to be possible where larvae migrate from the anal skin back to the rectum.

The prepatent period (from egg ingestion to egg production) is 3 to 4 weeks.

Clinical Significance

Many patients are asymptomatic or present with pruritus ani and perineal pruritus. The pruritus may be severe and is worse at night. Excoriation from scratching and secondary bacterial infection are often evident. Children are most commonly infected. With heavy worm burdens, poor concentration, enuresis, and emotional distress are features. Occasionally, the presence of worms in the appendix contributes to inflammation and true appendicitis (3). Reinfection (autoinfection or retroinfection) is also possible.

General symptoms, including abdominal pain, weight loss, and loss of appetite, may occur.

The presence of ectopic worms and eggs in several sites, including the female genitourinary tract, the epididymis, and the peritoneum, has been reported (12). They rarely cause serious complications. (For more on the clinical significance of *E. vermicularis*, see reference 12.)

TABLE 1 Usual times for completion of life cycles under favorable conditions[a]

Nematode	Time for life cycle completion:	
	Within host	In external environment
Ascaris lumbricoides	8 wk	10–15 days
Enterobius vermicularis	4–7 wk	6 h
Hookworms	4–7 wk	5–6 days
Strongyloides stercoralis	4 wk	3–4 days (direct)
Trichuris trichiura	10–12 wk	21 days

[a] Modified from reference 15.

Diagnosis

The primary diagnosis of infection is by demonstration of the presence of eggs on the skin in the perineal area by using the "sticky tape" method (eggs adhere to cellulose tape and can then be detected microscopically) (see references 4 and 5). Repeated preparations may be required. Commercial collection kits (Evergreen Scientific or Swube [Becton Dickinson]) are available in some countries. Eggs are rarely present in feces. Eggs are non-bile stained, ovoid, asymmetrical with a characteristic "football" shape (concave on one side and flat on the other), smooth, and thick walled and may contain a partially or fully developed larva (Fig. 2E). Specimens should ideally be taken early in the morning before a bath or use of the toilet. If an opaque tape is submitted by mistake, a drop of immersion oil on the top of the tape will clear it enough for microscopy.

Occasionally, adult female worms may be seen in feces, around the anal opening, or upon colonoscopy as white or cream-colored threadlike worms (hence the name threadworm). Characteristic eggs may be seen within the distended female or extruded from the genital pore by putting a little pressure on the worm (Fig. 2F).

Treatment

Albendazole or mebendazole is the drug of choice. Pyrantel and piperazine are effective, but reinfection is common (see chapter 150; see also reference 13).

It is important to treat other family members and close contacts and to decontaminate the environment by washing bed linen and clothes. Advice should be given on adequate hygiene and hand washing.

For a summary of *E. vermicularis*, see Table 2.

HOOKWORMS

Taxonomy

Ancylostoma duodenale (Old World Hookworm)

A. duodenale belongs to the class *Chromadorea,* order *Rhabditida,* suborder *Strongylida,* superfamily *Ancylostomatoidea,* family *Ancylostomatidae,* and subfamily *Ancylostomatinae.*

Necator americanus (New World Hookworms)

N. americanus belongs to the class *Chromadorea,* order *Rhabditida,* suborder *Strongylida,* superfamily *Ancylostomatoidea,* family *Ancylostomatidae,* and subfamily *Bunostominae.*

TABLE 2 Summary of common nematodes

Parasite	Major clinical presentations of infection	Prepatent period	Laboratory findings	Treatment of choice
Ascaris lumbricoides	Symptoms relate to worm burden; most infections are light and asymptomatic; migratory phase, eosinophilic pneumonitis Symptoms of established infection include: • History of passing/vomiting worm • Mild gastrointestinal symptoms • Small-bowel obstruction in children with heavy worm burdens • Ectopic ascariasis involving appendix or hepatobiliary or pancreatic ducts • Malnutrition and growth retardation in children	2 mo	Demonstration of eggs in feces; identification of worm passed; eosinophilia, low or absent	Albendazole or mebendazole
Enterobius vermicularis	Many infections are asymptomatic; pruritis ani occurs mainly at night; excoriation from scratching and secondary infection are common; general symptoms include weight loss and loss of appetite; occasionally ectopic, involving appendix or female genital tract	3–4 wk	Demonstration of eggs in "sticky tape" preparation; eosinophilia, low or absent	Albendazole or mebendazole Prevent reinfection and treat contacts
Hookworms	Pruritic rash or "ground itch" on extremities at site of entry of larvae; migratory phase, cough and wheezing due to eosinophilic pneumonitis Symptoms of established infection include: • Iron-deficiency anemia • Hypoproteinemia leading to chronic malnutrition and growth disorders in children • Enteropathy with gastrointestinal symptoms • Light infections may be asymptomatic	4–8 wk	Demonstration of eggs or larvae in feces; culture (agar plate method or Harada-Mori technique); eosinophilia, usually moderate	Albendazole or mebendazole; iron therapy if anemia is present
Strongyloides stercoralis	Chronic infection occurs due to autoinfection; recurrent, migratory, linear rash when larvae enter perianal skin, or "larva currens"; urticarial rashes also occur; enteropathy causing intermittent or chronic diarrhea, sometimes with malabsorption; pulmonary symptoms and hypereosinophilia may occur during autoinfection; Loeffler-like syndrome; gram-negative-bacterial septicemia or meningitis due to transfer of bowel flora by migrating larvae; hyperinfection syndrome (disseminated) in immunocompromised or debilitated individuals, leading to severe enteropathy and respiratory symptoms	2–4 wk	Demonstration of larvae in feces; culture (agar plate method or Harada-Mori technique); demonstration of antibodies in serum; eosinophilia, usually moderate	Ivermectin
Trichuris trichiura	Usually asymptomatic or mild gastrointestinal symptoms; epigastric pain, vomiting, distension, anorexia, and weight loss; trichuris dysentery, sometimes with rectal prolapse, may occur in heavy infections; growth retardation in children due to chronic malnutrition and anemia	3 mo	Demonstration of eggs in feces; eosinophilia, low or absent	Albendazole or mebendazole

Ancyclostoma and *Necator* are the two genera of *Ancylostomatidae* that infect humans. Other genera may occur as rare "accidental" parasites.

Description

Eggs

Eggs (Fig. 3A) of *A. duodenale* and *Necator americanus* are indistinguishable, being characteristically oval shaped with a thin shell and measuring approximately 56 to 75 by 36 to 40 μm. Characteristically, they have a clear space between the developing embryo and the thin egg shell. Survival of eggs is maximized in moist, shady, warm soil, where larvae hatch within 1 to 2 days.

Larvae

First-stage rhabditoid larvae (Fig. 3E and G) from the hatched eggs measure about 200 μm in length. They feed on organic debris and molt, with the resulting second-stage larvae measuring up to 500 μm. After another molt, the

infective, nonfeeding third-stage larvae measure from 500 to 700 μm, those of *Ancylostoma* being generally longer than those of *Necator*. Development from first-stage to infective filariform larvae occurs within 5 to 8 days; the latter remain viable in the soil for several weeks.

Worms

For both species, male worms (5 to 11 mm) are shorter than females (7 to 13 by 0.4 to 0.5 mm), and *A. duodenale* is generally longer and more sturdily built than *N. americanus*. Adult worms are rarely seen since they remain firmly attached to the intestinal mucosa, feeding on blood obtained by puncturing the capillary network in the intestinal mucosa. It has been estimated that a single *A. duodenale* worm can withdraw as much as 0.2 ml of blood per day, whereas *N. americanus* withdraws approximately 0.05 ml.

Epidemiology and Prevention

An estimated 740 million people are infected in poor regions of the tropics and subtropics, especially in Asia and sub-Saharan Africa (8, 9).

Preventative measures consist of health education about personal hygiene and the need to wear shoes, avoidance of soil contamination, and drug therapy.

Transmission and Life Cycle

Entry of third-stage or filariform larvae (infective stage) by direct penetration of the skin initiates human infection, followed by migration to the lungs within 10 days. *A. duodenale* can also infect if swallowed, but *N. americanus* cannot. *N. americanus* requires an obligatory lung migration, whereas the ingestion of *A. duodenale* larvae can result in direct maturation to the adult stage in the intestine. Larvae leave the lungs after 3 to 5 weeks, pass through the trachea and pharynx, enter the gastrointestinal tract, and attach to the intestinal mucosa, where they mature into adults (Table 1). Attachment and the release of anticlotting factors result in blood loss, which is greater with *A. duodenale*. Hookworms may survive in the host for several years. Eggs are passed in the feces (diagnostic stage) into the environment, where they hatch into rhabditiform larvae which mature into filariform larvae and infect new hosts.

The prepatent period (from larva penetration to egg production) is 4 to 8 weeks.

Clinical Significance

The major clinical manifestation of hookworm infection is iron-deficiency anemia due to intestinal blood loss and depletion of iron stores. The degree of iron deficiency induced by hookworms depends on the intensity and duration of infection, the species of hookworm, and the iron status of the host. Young children, adolescent girls, women of reproductive age, and pregnant women are most at risk for iron-deficiency anemia because of relatively low iron stores. Anemia during pregnancy is linked to maternal mortality, impaired lactation, prematurity, and low-birth-weight infants.

Patients with light worm burdens are usually asymptomatic. Moderate and heavy worm burdens cause epigastric pain and tenderness, nausea, weight loss, and diarrhea. This may lead to protein-losing enteropathy and hypoalbuminemia. In those who develop iron-deficiency anemia, common symptoms are lethargy and fatigue, exertional dyspnea, and palpitations. In children, chronic malnutrition, impaired physical growth, and impaired cognitive development may occur.

A pruritic rash is often present at the site of penetration of larvae ("ground itch"). This must be differentiated from cercarial dermatitis and creeping eruptions from other causes. In some patients, mild cough and wheezing occur in response to larval migration through the lungs. (For more on the clinical significance of hookworms, see reference 12.)

Diagnosis

The primary diagnosis of infection is by demonstration of the presence of eggs in feces (see references 4 and 5). Multiple specimens taken on separate days may be required. For optimal yield, both direct and concentration methods such as formalin-ethyl acetate sedimentation should be used. Eggs are non-bile stained and usually have a 4- to 8-cell-stage embryo (Fig. 3A). Occasionally, a 16- to 32-cell-stage embryo or a developing larva may be seen, especially if there is delay in processing a fresh (unfixed) fecal specimen. These should be differentiated from *Strongyloides stercoralis* larvae by their morphological features (Fig. 3C to G). Hookworm eggs may be confused with *Trichostrongylus* eggs, which are generally larger and in an advanced stage of cleavage (Fig. 3B).

Although larvae are rarely seen in adequately fixed feces specimens, motile larvae may be seen occasionally in expectorated sputum. Culture for the larval stage by the Harada-Mori technique or agar plate method is useful to detect light infections (see "*Strongyloides stercoralis*" below for details) (Fig. 3C to G).

Adult worms are very rarely passed in feces. However, a colonoscopic or gastroscopic specimen may be submitted for identification. Usually, the worm is firmly attached to the mucosa. The adult worm can be identified by the structure of the buccal capsule.

When blood examination shows iron-deficiency anemia, the diagnosis of hookworm infection should be considered for people from areas where the infection is endemic.

Treatment

The two most practical and effective drugs for treating hookworm infections are albendazole and mebendazole. These benzimidazole drugs should be avoided in the first trimester of pregnancy. Pyrantel is also effective and can be used in pregnancy (see chapter 150; see also reference 13). Iron supplementation should be given to those who are iron deficient.

For a summary of hookworms, see Table 2.

STRONGYLOIDES STERCORALIS

Taxonomy

S. stercoralis belongs to the class *Chromadorea,* order *Rhabditida,* superfamily *Panagrolaimoidea,* and family *Strongyloididae.*

Members of the family *Strongyloididae* exhibit an irregular alternation of generations, with a parasitic parthenogenetic female alternating with a free-living sexual generation. Only one genus, *Strongyloides,* occurs in humans. Note that reclassification at the superfamily level has occurred; the family *Strongyloididae* previously belonged to the *Rhabditoidea* superfamily.

Description, Transmission, and Life Cycle

Eggs

Eggs of the parasitic female are deposited within the mucosa of the small intestine and usually hatch before reaching the lumen. As a result, they are rarely excreted in the feces (*Strongyloides fullerborni,* seen in Papua New Guinea, is an exception). Eggs of the free-living adult female are partially

embryonated and oval in shape and measure approximately 50 to 70 μm in length.

Larvae

First-stage, rhabditiform larvae (Fig. 3C, D, and F) measure approximately 180 to 380 by 14 to 20 μm and are characterized by a muscular esophagus comprising the anterior third of the body. A short buccal cavity and a prominent genital primordium located midbody (Fig. 3D) help to distinguish them from other nematodes such as hookworms (Fig. 3E). In the soil, first-stage larvae follow a direct or indirect course of development. In the direct cycle, larvae develop rapidly into infective third-stage, filariform larvae; in the indirect cycle, first-stage larvae develop into a free-living generation of adult male and female worms. Third-stage, or filariform, infective larvae are long and thin compared to those in the rhabditiform stages. Third-stage larvae are 500 to 600 by 16 μm, and their tails are notched (pointed in hookworm larvae) (Fig. 3F and G).

Worms

Parasitic male forms do not occur. Parasitic females are small and thin, measuring approximately 2 to 3 mm in length and 30 to 50 μm in width. The anterior portion is thicker than the posterior and contains the esophagus.

The free-living adult female is approximately half the size of its parasitic counterpart, although nearly twice as thick (approximately 80 μm). While the reproductive systems are morphologically similar, the uterus in the free-living adult female contain significantly more eggs. The free-living adult male is slightly smaller than the female, approximately 50 μm in width.

Transmission is via penetration of the skin or mucous membranes by filariform larvae (infective stage) that are carried to the lungs in the venous circulation. Larvae penetrate alveolar walls, migrate through the tracheobronchial tree, are swallowed, and finally reside in the mucosa of the upper small intestine. Here, they reach maturity and commence egg production; the eggs hatch immediately to release rhabditiform larvae, most of which are excreted in feces (diagnostic stage). In the soil, development into filariform larvae occurs, with the potential to infect new hosts (Table 1).

Some rhabditiform larvae develop into infective filariform larvae in the bowel lumen, penetrate the intestinal mucosa or perianal skin, and repeat the cycle of maturation within the same host. This process of autoinfection, albeit uncommon among intestinal nematodes, results in chronic infections that may persist for 40 years or more.

The prepatent period (from larva penetration to egg production) is 2 to 4 weeks.

Epidemiology and Prevention

Strongyloides lives in warm moist soil and is widely distributed in the tropics and subtropics, similar to hookworms. There are an estimated 30 to 100 million patients infected with *S. stercoralis* worldwide. Risk groups include those living in areas where the parasite is endemic, immigrants and refugees, indigenous peoples, and war veterans previously exposed in areas where the parasite is endemic.

Preventative measures include good personal hygiene and wearing shoes in areas where transmission may occur.

Clinical Significance

S. stercoralis infections are often chronic, lasting for several decades. Most patients have low worm burdens and are asymptomatic or have intermittent cutaneous and/or gastrointestinal symptoms.

A recurrent, pruritic, serpiginous, erythematous rash may occur due to larval migration in the skin ("larva currens"). This is most commonly seen on the buttocks, groin, and trunk and may move about under the skin over 1 to 2 days. Other, less distinctive urticarial and papular rashes also occur.

Intermittent or chronic diarrhea, abdominal pain and bloating, nausea, and anorexia are the main gastrointestinal symptoms.

Although pulmonary symptoms are uncommon in uncomplicated strongyloidiasis, passage of larvae through the lung may be associated with a cough, wheezing and dyspnea, and patchy infiltrates upon radiography (Loeffler-like syndrome).

Severe complicated strongyloidiasis may occur in immunocompromised or debilitated individuals and is due to accelerated autoinfection in the face of waning immunity. Hyperinfection syndrome is associated with the presence of many adult worms in the intestinal mucosa and penetration of the bowel wall by large numbers of filariform larvae. Severe gastrointestinal and/or respiratory symptoms and large numbers of larvae in stool and sputum are the main features. It may be complicated by malabsorption or paralytic ileus. Gram-negative bacterial septicemia and meningitis may develop if enteric bacteria are spread into blood or cerebrospinal fluid during mucosal penetration by larvae. Larvae can occasionally disseminate to the central nervous system, peritoneum, liver, and kidneys. Disseminated strongyloidiasis may mimic many other diseases, including severe bacterial sepsis and tropical pulmonary eosinophilia. It should be considered a possibility in immunosuppressed patients who have been at risk of infection in the past.

Patients on long-term steroid therapy are most at risk for severe disease. Those with impaired cellular immunity due to other causes (e.g., organ transplants, AIDS, and lymphatic malignancies) are also at risk.

Strongyloides species from animals can occasionally infect humans and produce persistent skin lesions similar to those produced by *S. stercoralis*. However, the life cycle is not completed and infection is not established (7). (For more on the clinical significance of *S. stercoralis*, see reference 12.)

Diagnosis

The laboratory diagnosis of strongyloidiasis can be difficult (see references 4 and 5). It is based on either demonstrating the presence of the larvae in feces or demonstrating antibodies to *S. stercoralis* in blood. Eggs or adult worms are very rarely detected. Microscopy of feces may detect the first-stage (rhabditiform) larvae. The sensitivity of this method is generally low, as larvae are shed sporadically and numbers depend on the stage and severity of the infection. Hence, multiple specimens collected over several days should be examined. Coproculture is a method of concentration of larvae on a freshly passed stool specimen (not refrigerated or preserved) transported without delay to the laboratory. This should be attempted for all suspected cases, especially with patients from areas where the infection is endemic who are candidates for immunosuppressive therapy (because of organ transplant or treatment for malignancies, etc.). Various methods available for culture are as follows (for details, see reference 6):

1. Agar plate culture. A small amount of feces is placed on an agar plate, and the plate is sealed. If larvae are present, they travel on the surface of the plate, carrying bacteria from the feces. Colonies of these bacteria appear on the surface as tracks with a characteristic sinusoidal pattern. The plates are examined by microscopy for confirmation of the

presence of larvae. This is the easiest culture method to perform and is now the recommended procedure (16).

2. Harada-Mori technique. Feces are placed on a filter paper dipping into a small amount of water placed in a tube. The larvae in the feces move towards the water, which can subsequently be tested by microscopy for the presence of larvae.

3. Petri dish filter paper slant method. This procedure is similar to the one previously described but is done in a petri dish.

4. Baermann technique. This requires a special apparatus and relies on the principle that larvae will actively migrate out of the feces placed on a wire mesh covered with several layers of gauze.

Occasionally, hookworm larvae may hatch out in feces which have been left at warm temperatures for long periods before processing. Hookworm larvae can be differentiated from *S. stercoralis* larvae as the hookworm larvae have a long buccal cavity and inconspicuous genital primordium (Fig. 3C to E). The presence of larvae of *Strongyloides* in expectorated sputum or duodenal aspirate may also be demonstrated via enteroscope or string test (Entero-Test capsule) (6). Occasionally, last-stage (filariform) *Strongyloides* larvae may be seen in feces or sputum in cases of hyperinfection or in cases in which the culture methods described above are used.

Although eosinophilia is common during acute infection, it does not reliably correlate with infection and may be intermittent in chronic infection.

Demonstration of anti-*Strongyloides* antibodies in blood should be used as a screening test or as an adjunct for diagnosis. Used in conjunction with eosinophil counts, it is very useful to monitor treatment. Antibody levels decline after 6 months of effective treatment (11) and may be negative by 12 to 24 months. The sensitivity of the commercially available enzyme immunoassay (immunoglobulin G enzyme-linked immunosorbent assay; IVD Research, Inc.) using the *S. stercoralis* filariform antigen test is ~90% and much higher than those of indirect hemagglutination and indirect fluorescent-antibody assays. Higher sensitivity (~95%) has been reported for the Centers for Disease Control and Prevention *Strongyloides* enzyme immunoassay (EIA) for patients with proven infection (11). Sensitivity may be lower for severely immunocompromised patients. The specificity of EIA is ~85% as cross-reactions in patients with filarial and other nematode infections may occur. If the test is positive for antibodies, efforts should be made to establish a parasitological (based on microscopy and culture) diagnosis and to exclude the possibility of infection with other parasites that could result in cross-reacting antibodies.

Treatment

S. stercoralis infection should always be treated because of the potential for development of severe complicated disease. Ivermectin is the drug of choice. Repeated cycles may be required for immunocompromised patients. Albendazole and mebendazole are less effective than ivermectin (thiabendazole, although effective, is no longer used due to frequent severe side effects) (16; see chapter 150 and reference 13).

Monitoring with stool microscopy, eosinophil counts, and serology is recommended until infection is eradicated.

For a summary of *S. stercoralis*, see Table 2.

TRICHURIS TRICHIURA (WHIPWORM)

Taxonomy

T. trichiura belongs to the class *Enoplea,* order *Trichocephalida,* superfamily *Trichonelloidea,* and family *Trichuridae.*

Description

Eggs

Eggs of *T. trichiura* (Fig. 2G) are lemon shaped with a characteristic plug at each end ("tea tray with handles"). In feces, eggs are brown and measure 50 to 55 by 20 to 24 μm.

Larvae

After hatching in the intestine, the second-stage larval form measures about 260 by 15 μm in length. The second-stage larvae burrow into the intestinal mucosa and undergo four larval molts. The adult worm is then intimately associated with the wall of the large intestine.

Worms

Adult worms are found in the large intestine, with a highly characteristic shape from which the name whipworm is derived. The long, thin anterior end lies in a burrow in the mucosa, and the thicker end, which contains the reproductive tract, extends into the intestinal lumen. Worms are whitish, and the males (30 to 45 mm) are shorter than the females (35 to 50 mm) and have a coiled posterior end. Adult females produce up to 20,000 eggs/day and live for approximately 3 years.

Epidemiology and Prevention

Trichuris has a worldwide distribution and is often associated with *Ascaris* and hookworm infections in children in tropical and subtropical areas.

Preventative measures include health education about personal hygiene, avoidance of soil contamination, and drug therapy programs in areas where infection is endemic.

Transmission and Life Cycle

Transmission is direct via oral ingestion of embryonated eggs (infective stage) from contaminated soil. Following ingestion, larvae are released and pass into the large bowel, where they reside and mature into adults in mucosal crypts. The eggs passed in feces contain an unsegmented ovum (diagnostic stage), and once the eggs are in warm, moist conditions in soil, they become infective 2 to 4 weeks after passage (Table 1).

The prepatent period (from egg ingestion to egg production) is 3 months.

Clinical Significance

The clinical features are related to the intensity of infection, as is the case with the other intestinal nematodes. Light infections are asymptomatic or present with mild gastrointestinal symptoms.

Epigastric pain, vomiting, distension, anorexia, and weight loss may occur with heavier infections, and trichuris dysentery syndrome, sometimes complicated by rectal prolapse, may be seen. Children with severe infections may develop growth retardation due to chronic malnutrition and anemia. (For more on the clinical significance of *T. trichiura*, see reference 12.)

Diagnosis

The primary diagnosis of infection is by demonstration of the presence of eggs in feces (see references 4 and 5). Multiple specimens collected on separate days may be required. Both direct and concentration methods such as formalin-ethyl acetate sedimentation should be used for optimal yield. The eggs are bile stained and thick walled with mucoid plugs at

both ends, giving them a characteristic tea tray appearance (Fig. 2G).

Adult worms are rarely passed in feces and are occasionally found upon colonoscopy (10). When they are seen, they have a cylindrical coiled body and a coiled thicker tail, giving them the appearance of a whip (hence the name whipworm).

Treatment

Albendazole or mebendazole is the drug of choice (see chapter 150; see also reference 13).

For a summary of *T. trichiura*, see Table 2.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Specimens should be collected before antibiotics or antiparasitic drugs are given (for details, refer to chapters 5 and 133). It is important to transport and process feces specimens for parasitic examination as soon as possible. Clinicians and collection staff should be encouraged to either send fresh specimens to the laboratory without delay or use commercially available preservative kits. If delay is inevitable, specimens should be refrigerated or transported in commercially available vials or kits with a preservative such as polyvinyl alcohol or sodium acetate-acetic acid-formalin. If these are not available at the point of collection, preservatives should be added as soon as the specimen is received in the laboratory. Excellent directions for proper collection are available with these kits. Discussion on which preservative to use depends on various considerations such as whether permanent-stained smears or immunoassays are required, etc. (For details, see reference 6.) Refrigeration and the use of preservatives should be avoided if *Strongyloides* larval culture is required.

Specimens that are obviously dry or drying out should be rejected, and a fresh specimen should be collected.

There is no maximum limit on the amount of specimen, but it should be reasonable for the laboratory staff to handle. Very small amounts are not acceptable as the chances of drying are high and those of finding parasites are low. Two or three specimens collected over a period of 7 to 10 days are optimal.

Worms passed or taken at colonoscopy should be transported to the laboratory without delay in a closed, clean container of appropriate size. Worms can be preserved in 50% alcohol or 5% formalin (avoid higher concentrations as dehydration can occur).

For sputum specimens, proper instructions should be given to patients emphasizing quality, avoidance of saliva, and inclusion of mouthwash before deep coughing (expectorating). Sputum and aspirates should be transported to the laboratory and processed immediately.

All specimens should be collected in clean, wide-mouthed containers with tight-fitting lids and handled using standard precautions, e.g., latex gloves should be worn by anyone handling the specimens.

LABORATORY METHODS: DIRECT EXAMINATION

The specimen should be handled in a work cabinet, and gloves and gown should be worn. Examine the specimen and note its consistency. Look for any motile worms or segments of worms. Select and sample areas that look watery, purulent, or bloody. If feces are formed, take specimens from the sides and middle for the concentration technique.

Wet preparations in saline and iodine are prepared from direct specimens or concentrates and covered with a coverslip. Scan the whole coverslip at low-power magnification (×100) with a microscope. Most helminth eggs and larvae can be identified at this magnification and can be confirmed at high power (×400) on the basis of their shapes, sizes, and characteristic features (Fig. 1 to 3). Thin smears should be prepared from the concentrate for permanent staining, which may be useful to diagnose mixed infections with protozoa.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Make note of the adequacy and quality of the specimen submitted. If it is inadequate, mention it on the report.

In developed countries, any helminth eggs or larvae found in feces are significant and treatment is recommended, even if the patient is asymptomatic. However, treatment of asymptomatic cases is not necessary in developing countries as many of these parasites are endemic and reinfection is common.

Examples of reports are as follows:

1. Larvae of *S. stercoralis* detected by fecal culture.
2. Eggs of *E. vermicularis* not detected on the sticky tape preparation. Please send properly collected repeat specimen. Contact the laboratory for instructions if required.
3. No parasites detected in current specimen. Low numbers of parasites may be missed. Please send well-collected repeat specimen. Contact the laboratory for instructions if required.

In a clinically significant case, if the first specimen is negative, ask for further specimens. The time of collection is also important, especially when *Enterobius* is suspected. If the specimen is inadequate, e.g., delayed, dry, or insufficient, a repeat specimen should be requested. A freshly collected specimen and/or use of preservative kits should be encouraged where possible.

There is no need to quantitate helminth parasites as this does not necessarily correlate with clinical illness. However, in rare instances, this may be required for epidemiological studies or for clinical assessment of children.

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Filarial Nematodes

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Filarial worms are arthropod-transmitted nematodes or roundworms that dwell in the subcutaneous tissues and the lymphatics. Although eight filarial species commonly infect humans, four are responsible for most of the pathology associated with these infections. These are (i) *Wuchereria bancrofti,* (ii) *Brugia malayi,* (iii) *Onchocerca volvulus,* and (iv) *Loa loa.* In general, each of the parasites is transmitted by biting arthropods. The distributions and vectors of all the filarial parasites of humans are given in Table 1. Each goes through a complex life cycle that includes an infective larval stage in the insects and an adult worm stage in which the parasite resides in humans, either in the lymph nodes or adjacent lymphatics or in the subcutaneous tissue. All filariae have adult female worms that produce microfilariae. These offspring either circulate in the blood or migrate through the skin. The microfilariae can then be ingested by the appropriate biting arthropod and develop into infective larvae that are capable of initiating the life cycle once more. Certain species *(W. bancrofti, Brugia* spp., and *L. loa)* circulate in the blood with a defined circadian rhythm, or "periodicity," which can be nocturnal (typical of the lymphatic filariae) or diurnal *(L. loa)*. Other species lack periodicity and are found in the peripheral blood at all hours of the day and night. When absent from the peripheral blood in lymphatic filarial infections, microfilariae are found in the deeper visceral capillaries, particularly in the pulmonary capillaries.

Microfilariae are relatively simple in their organization and structure (Fig. 1). They are vermiform and, in stained preparations, appear to be composed of a column of nuclei interrupted along its length by spaces and special cells that are the precursors of body organs or organelles. Some species of microfilariae are enveloped in a sheath, whereas others have no sheath (Table 1 and Fig. 2).

All of the filariae are transmitted by species of blood-sucking arthropods such as mosquitoes, midges, blackflies, and tabanid flies, in which the microfilariae develop into the infective stage (Table 1). Subsequent development of the infective larvae into the gravid, adult stage in the vertebrate host requires several months and in some cases a year or more.

Because the adult worms are typically sequestered in the tissues, diagnosis of infection depends on finding microfilariae in either blood or skin, depending on the species. Adult worms are long lived, whereas the life spans of microfilariae range from 3 months to 3 years. Infection is generally not established unless exposure to infective larvae is intense and prolonged. Furthermore, the clinical manifestations of these diseases develop rather slowly.

Although these parasites are not endemic in humans in nontropical or subtropical areas, they are often seen in immigrants or in individuals who have resided in or traveled to areas where filariae are endemic. There are significant differences in the clinical manifestations of filariasis, or at least in the time periods over which the infection is acquired, between patients native to the areas where the disease is endemic and those who are travelers or recent arrivals in these same areas. Characteristically, the disease in previously unexposed individuals is more acute and intense than that found in natives of the region where the disease is endemic (30); however, early removal of newly infected individuals tends to speed the end of clinical symptomatology or at least halt the progression of the disease (39).

LYMPHATIC FILARIAL PARASITES

Taxonomy

W. bancrofti, B. malayi, and *Brugia timori* each belong to the phylum Nematoda, class *Secernentea,* subclass *Spiruria,* order *Spirurida,* superfamiliy *Filarioidea,* and family *Filariidae*.

Description of the Agents

There are three lymphatic-dwelling filarial parasites of humans: *W. bancrofti, B. malayi,* and *B. timori*. The adult worms usually reside in either the afferent lymphatic channels or the lymph nodes. These adult parasites may remain viable in the human host for decades. The morphologic appearances and other characteristics of the parasites can be found in Table 1 and Fig. 1 and 2.

Epidemiology and Transmission

Wuchereria bancrofti

W. bancrofti is the most common and widespread species of filaria infecting humans. It has an extensive distribution throughout tropical and subtropical areas of the world, including Asia and the Pacific Islands, Africa, areas of South America, and the Caribbean Basin. Humans are the only definitive host for this parasite and are therefore the natural reservoir for infection. There are both periodic and subperiodic forms of the parasite. Generally, the subperiodic form is

TABLE 1 Filarial parasites of humans

Species	Distribution	Vector	Primary pathology	Location	Periodicity	Size (μm)	Tail	Sheath	
								Presence	Staining properties
Wuchereria bancrofti	Tropics	Mosquito	Lymphatic, pulmonary	Blood, hydrocele fluid	Nocturnal, subperiodic	298 by 7.5–10	Pointed tail devoid of nuclei	+	Does not stain
Brugia timori	Indonesia	Mosquito	Lymphatic	Blood	Nocturnal	300+	Nuclei in tail	+	Tends not to stain
Brugia malayi	Southeast Asia	Mosquito	Lymphatic, pulmonary	Blood	Nocturnal, subperiodic	270 by 5–6	Nuclei in tail	+	Bright pink with Giemsa
Onchocerca volvulus	Africa, Central and South America	Blackfly	Dermal, ocular, lymphatic	Skin	None	309 by 5–9	No nuclei in tail	–	
Mansonella streptocerca	Africa	Midge	Dermal	Skin	None	210 by 5–6	Crooked tail into which column of nuclei extends	–	
Loa loa	Africa	Deerfly	Allergic	Blood	Diurnal	Up to 300	Irregularly arranged nuclei extend to end of tail	+	Does not stain
Mansonella perstans	Africa, South America	Midge	Probably allergic	Blood	None	203 by 4–5	Blunt tail contains nuclei	–	
Mansonella ozzardi	Central and South America	Midge	Unknown	Blood	None	224 by 4–5	Long tail with no nuclei in it	–	

found only in the Pacific Islands (including Cook and Ellis Islands, Fiji, New Caledonia, the Marquesas, Samoa, and the Society Islands). Elsewhere, *W. bancrofti* is nocturnally periodic. The natural vectors are *Culex fatigans* mosquitoes in urban settings and usually anopheline or aedean mosquitoes in rural areas.

Brugia malayi and *Brugia timori*

The distribution of brugian filariasis is limited primarily to China, India, Indonesia, Korea, Japan, Malaysia, and the Philippines. Two forms of the parasites *B. malayi* and *B. timori* can be distinguished by the periodicities of the microfilariae. With nocturnally periodic forms, microfilariae are present in the peripheral blood primarily at night, whereas with the subperiodic forms, microfilariae are present in the blood at all times but are at maximal levels in the afternoon.

The nocturnal-parasite form of brugian filariasis is more common and is transmitted in areas of coastal rice fields (by mansonian and anopheline mosquitoes), and the subperiodic parasite form is found in the swamp forests (mansonian vector). Although humans are the common host, *B. malayi* infection can be a natural infection of cats. *B. timori* infection has been described only on two islands in Timor.

Clinical Significance (Description of Clinical Presentation)

Lymphatic filariasis (LF) causes a variety of clinical manifestations. The four most common presentations are asymptomatic (or subclinical) microfilaremia, lymphedema, hydrocele formation, and acute attacks. Less frequently, LF can present with chyluria or tropical eosinophilia (6, 19, 35). The range of clinical disease varies somewhat according to the geographic location and the species of nematode causing the infection (43). Additionally, the disease in previously unexposed individuals is more acute and intense than that found in natives of the region where the disease is endemic (30, 39). Patients with asymptomatic (or subclinical) microfilaremia rarely come to the attention of medical personnel except through the incidental finding of microfilariae in the peripheral blood. Although they may be clinically asymptomatic, virtually all persons with *W. bancrofti* or *B. malayi* microfilaremia have some degree of subclinical disease that includes dilated (and tortuous) lymphatics (visualized by imaging) and, in men with *W. bancrofti*, scrotal lymphangiectasia (detectable by ultrasound) (14). Despite these findings, the majority of individuals appear to remain clinically asymptomatic for years. Relatively few progress to either the acute or chronic stage of infection (11). Development of lymphedema may not present until long after the initial infection. Lymphatic dysfunction develops as a primary event in response to adult filarial parasites and host immune responses in virtually all infected persons. This has been shown to be progressive during infection and permanent once established (9). Lymphedema most commonly affects the lower extremities but can also affect arms, breasts in females, and the scrotum in males. Secondary effects such as thickening of the subcutaneous tissues, hyperkeratosis fissuring of the skin, and hyperplastic skin changes can occur. Recurrent infections (acute dermatolymphangioadenitis [ADLA]) in the context of chronic skin changes and lymphatic dysfunction play a major role in lymphedema disease development and progression to elephantiasis (33, 36). Acute attacks of LF include a variety of clinical entities that present with inflammation. True filarial adenolymphangitis (AFL), felt to reflect the death of an adult worm, presents with inflammation, swelling, and retrograde lymphangitis extending peripherally

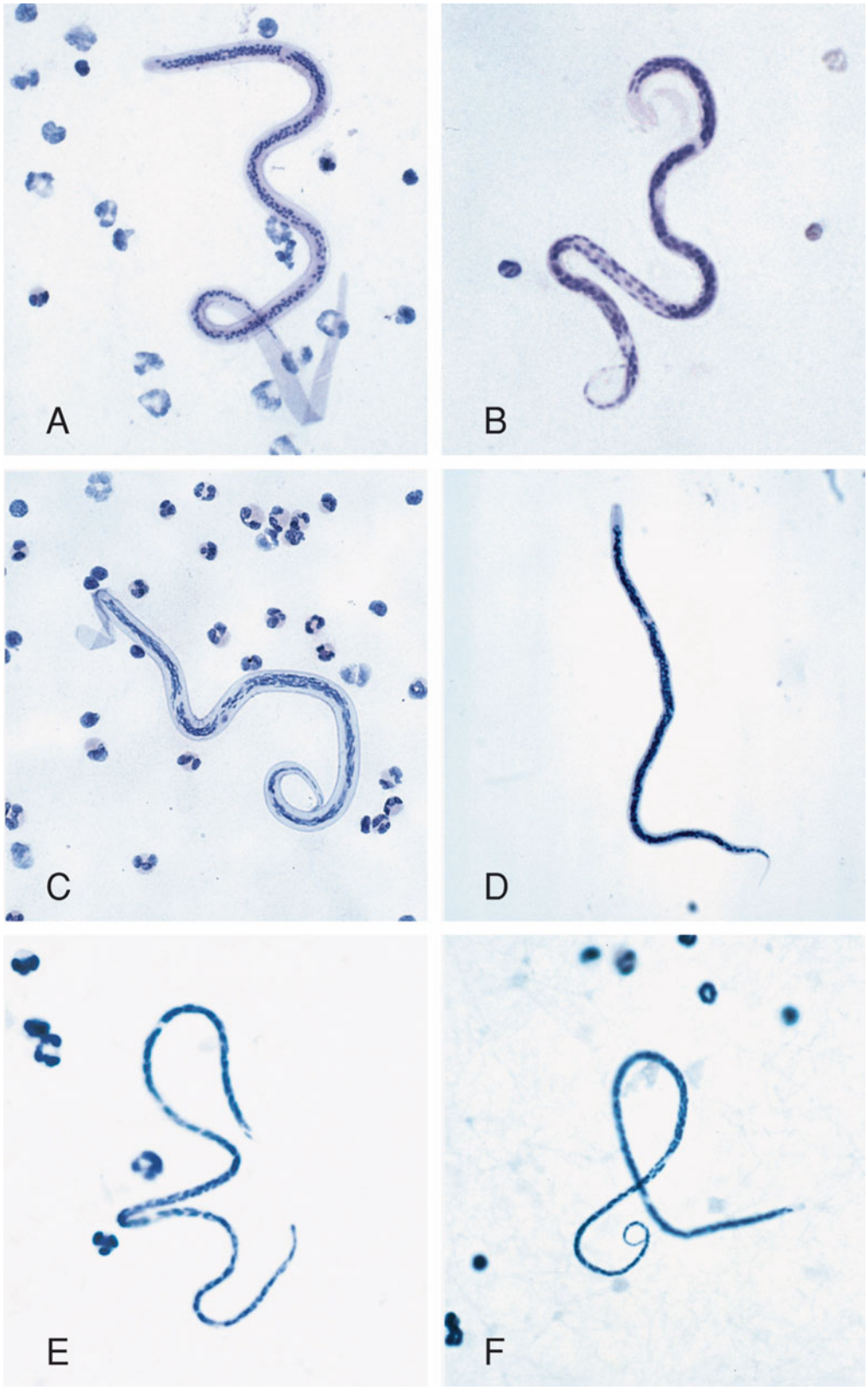


FIGURE 1 Common microfilariae found in humans. Organisms are stained with hematoxylin. Magnification, ×325. (A) *W. bancrofti;* (B) *B. malayi;* (C) *L. loa*; (D) *O. volvulus;* (E) *M. perstans;* (F) *M. ozzardi.*

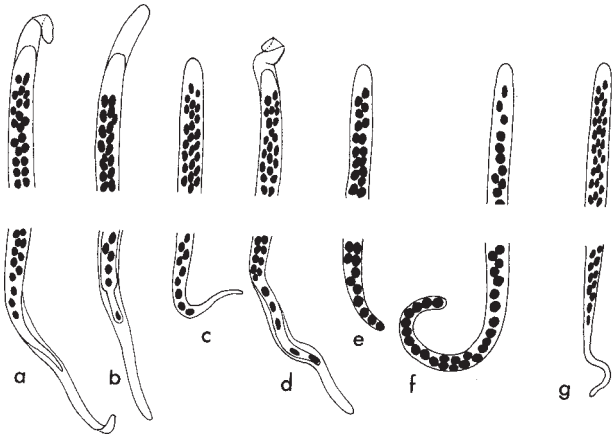


FIGURE 2 Diagrammatic representation of the anterior and posterior extremities of the common microfilariae found in humans. (a) *W. bancrofti;* (b) *B. malayi;* (c) *O. volvulus;* (d) *L. loa;* (e) *M. perstans;* (f) *M. streptocerca;* (g) *M. ozzardi*.

from the draining node where the parasites presumably reside. Regional lymph nodes are often enlarged, and the entire lymphatic channel can become indurated and inflamed. The second type of acute attack is now labeled bacterial adenolymphangitis or ADLA. Changes in the skin predispose to entry lesions, in particular in the toe webs (3, 46). For these reasons, limbs become susceptible to recurrent bacterial infections (10, 27). The clinical pattern of ADLA is distinctly different from that of AFL (10). The lymphangitis develops in a reticular rather than in a linear pattern, and the local and systemic symptoms, including edema, pain, fever, and chills, are frequently more severe (45). These cause considerable acute-disease morbidity and progression of lymphedema to elephantiasis (37). AFL and ADLA occur in both the upper and the lower extremities with both bancroftian and brugian filariasis, but involvement of the genital lymphatics occurs almost exclusively with *W. bancrofti* infection.

Hydrocele formation occurs in bancroftian filariasis when adult worms block retroperitoneal and subdiaphragmatic lymphatics. In males, this causes accumulation of straw-colored lymph either unilaterally or bilaterally between the visceral and parietal layers of the tunica vaginalis. The condition presents as a translucent mass obscuring palpation of the testis and differs from a congenital hydrocele or herniation in that the tunica is sealed at the top and peritoneal fluid is not communicated (38, 56). If there is obstruction of the retroperitoneal lymphatics, renal lymphatic pressure can increase to the point at which they rupture into the renal pelvis or tubules so that chyluria is seen. The chyluria is characteristically intermittent and is often prominent in the morning just after the patient arises.

Tropical pulmonary eosinophilia (TPE) is a distinct syndrome that develops in some individuals infected with lymphatic filarial species. This syndrome affects males more often than females, often during the third decade of life. The majority of cases have been reported from India, Pakistan, Sri Lanka, Brazil, Guyana, and Southeast Asia. The main features include a history of residence in regions where filariae are endemic, paroxysmal cough and wheezing that are usually nocturnal, weight loss, low-grade fever, and adenopathy. Patients are rarely found to have microfilariae in the blood. Chest X rays may be normal but generally show increased bronchovascular markings; diffuse miliary lesions or mottled opacities may be present in the middle and lower lung fields. Tests of pulmonary function show restrictive abnormalities in most cases and obstructive defects in half. Total serum immunoglobulin E (IgE) levels (10,000 to 100,000 ng/ml) and antifilarial antibody titers are characteristically elevated. TPE is now considered to be a form of occult filariasis in which rapid clearance of the microfilariae occurs, presumably on the basis of host immunologic hyperresponsiveness to the parasite (34). Although there is no single clinical or laboratory criterion that aids in distinguishing TPE from other pulmonary diseases associated with the tropics, the presence of high levels of antifilarial antibodies and a rapid clinical response to diethylcarbamazine (DEC) favor the diagnosis of TPE (51).

Diagnosis

Diagnosis of bancroftian and brugian filarial infection can be made noninvasively in some cases by ultrasound. This allows adult worms to be visualized in lymphatics or can identify dilated lymphatics. Definitive identification of parasites can be achieved with appropriate samples of blood or tissue. The timing of blood collection is critical and should be based on the periodicity of the microfilariae endemic in the region involved. (Table 1). Recent developments in immunodiagnostic and molecular biology techniques give further options for diagnosis.

Ultrasound

In cases of suspected LF due to *W. bancrofti,* high-frequency ultrasound of the scrotum or female breast coupled with Doppler may result in the identification of motile adult worms within dilated lymphatics. Adult worms may be visualized in the lymphatics of the spermatic cord in up to 80% of infected men with microfilaremia (11).

Direct Examination

Parasites can be identified by direct examination of blood or other fluids (such as chyle, urine, and hydrocele fluid). This examination should take advantage of the periodicity of each organism as well as the characteristic morphologic appearance (Table 1 and Fig. 2).

Microscopy

A small volume of fluid is spread onto a clean slide. The slide is then air dried, stained with Giemsa, and examined microscopically. The microfilariae of *W. bancrofti* are sheathed, lie in smooth curves in stained smears, and are 298 μm long by 7.5 to 10.0 μm in diameter. The column nuclei are dispersed; there is a short headspace, and the pointed tail is devoid of nuclei (Fig. 1A). The sheath stains faintly or not at all with Giemsa. The microfilariae of *W. bancrofti* must be distinguished from other sheathed microfilariae. The morphology of the *B. malayi* microfilariae is similar to that of the *W. bancrofti* microfilariae, with the *B. malayi* microfilariae being sheathed but somewhat smaller (279 by 5 to 6 μm). They can be differentiated from the *W. bancrofti* microfilariae by the presence of subterminal and terminal nuclei in the tails (Fig. 1A and B). *B. timori* microfilariae are similar to those of *B. malayi,* with conspicuous terminal and subterminal nuclei; however, *B. timori* microfilariae are larger (more than 300 μm long) than *B. malayi* microfilariae. Additionally, the *B. malayi* sheath stains bright pink with Giemsa, whereas the *B. timori* sheath tends not to stain and that of *W. bancrofti* does not stain (4).

Because microfilariae may be present in the blood in only small numbers, sensitive procedures such as Nuclepore

filtration and Knott's concentration technique are also used routinely to detect infections.

Nuclepore Filtration

A known volume of anticoagulated blood is passed through a polycarbonate (Nuclepore) filter with a 3-μm pore. A large volume (50 ml) of distilled water is passed through (the water will lyse or break open the red blood cells, leaving the worms intact and more easily visible). The filter is then air dried, stained with Wright's or Giemsa stain, and examined by microscopy. For studies in the field, 1 ml of anticoagulated blood can be added to 9 ml of a solution of 2% formalin–10% Teepol and stored for up to 9 months before performance of filtration.

Knott's Concentration Technique

Anticoagulated blood (1 ml) is placed in 9 ml of 2% formalin. The tube is centrifuged at 1,200 × *g* for 1 min. The sediment is spread onto a slide and dried thoroughly. The slide is then stained with Wright's or Giemsa stain and examined microscopically.

Antigen Detection

Assays for circulating antigens of *W. bancrofti* permit the diagnosis of microfilaremic and cryptic (amicrofilaremic) infection. Two tests are commercially available. One is an enzyme-linked immunosorbent assay available from Trop-Bio, and the other is a rapid-format immunochromatographic card available from Binax. Both assays have sensitivities that range from 96 to 100% and specificities that approach 100% (7, 53). Both tests can be used with blood drawn at any time of day or night, thus avoiding the need for specific bleeding times depending on the periodicity of the microfilariae. Neither of the tests is approved by the U.S. Food and Drug Administration. There are currently no tests for circulating antigens in brugian filariasis.

Nucleic Acid Detection Techniques

In appropriate laboratories, PCR can demonstrate the presence of parasite DNA and is now the most sensitive technique for definitive diagnosis of infection (55, 59). For each of the lymphatic-dwelling parasites, primers and probes have been identified that are 100% specific and provide sensitivities that are often 10-fold greater than that of detection of the parasites themselves.

Serologic Tests

Immunologically based diagnosis with measurement of IgG or IgG4 responses against crude extracts of *Brugia* or *Dirofilaria* worms suffers from poor specificity. There is extensive cross-reactivity among filarial antigens and antigens of other helminths, including the common intestinal roundworms; however, serologic tests have a role in diagnosis, as a negative test effectively excludes past or present infection. These tests are available commercially as well as from the National Institutes of Health.

Treatment and Prevention

The available chemotherapy for LF is DEC, ivermectin, and albendazole. DEC remains the treatment of choice for the individual with active LF (microfilaremia, antigen positivity, or the presence of adult worms detected upon ultrasound), although albendazole has also been shown to have some macrofilaricidal efficacy (8). If the adult parasites survive, microfilaremia along with clinical symptoms can recur within months after conclusion of the therapy. Chronic low-dose DEC (e.g., in DEC salt) may also result in cure (33). Evidence shows that the use of these drugs in combination can increase effectiveness (42, 50). The current global elimination campaign uses these three drugs in various combinations for mass treatment of communities where LF is endemic (22).

Most pathogenic filarial nematodes apart from *L. loa* harbor bacterial endosymbionts. These *Wolbachia* species are vital for parasite larval development and adult-worm fertility and viability. New approaches for treatment use antibiotics (e.g., the tetracyclines) that target the *Wolbachia*. Microfilarial levels have been seen to be reduced with these treatments (49). Once lymphedema is established, antifilarial medication is not useful if the patient does not have active infection. Management of lymphedema should concentrate on local skin care with appropriate treatment of entry lesions and on exercise and elevation (52). Antifilarial medication is also not indicated in the management of bacterial ADLA (46). This should be managed with skin care and antibiotics if indicated (45). Hydroceles can be drained repeatedly or managed surgically (56).

Avoidance of mosquito bites is usually not feasible for residents of areas where LF is endemic, but visitors should make use of insect repellent and mosquito nets. Repellent-impregnated bed nets have been shown to have a salutary effect. DEC can kill developing forms of filarial parasites and has been shown to be useful as a prophylactic agent in humans. Community-based intervention is the current approach to the elimination of LF as a public health problem (5). The underlying tenet of this approach is that mass annual distribution of antimicrofilarial chemotherapy (albendazole with either DEC [in all areas but those where onchocerciasis is coendemic] or ivermectin) will profoundly suppress microfilaremia. If the suppression is sustained, then transmission can be interrupted (29). Community education and clinical care for persons already suffering from the chronic sequelae of LF are important components of filariasis control and elimination programs (44). Vaccines may have a role in the future (41).

ONCHOCERCA VOLVULUS

Taxonomy

O. volvulus belongs to the phylum Nematoda, class *Secernentea,* subclass *Spiruria,* order *Spirurida,* superfamily *Filarioidea,* and family *Onchocercidae.*

Description of the Agent

The adult worms of *O. volvulus* typically reside in nodules comprised primarily of host tissue. These adult parasites may remain viable in the human host for decades. The morphologic appearance and other characteristics of microfilariae and adult worms can be found in Table 1 and Fig. 1 and 2.

Epidemiology and Transmission

Onchocerciasis, sometimes called river blindness, is caused by infection with *O. volvulus,* a subcutaneous tissue-dwelling filarial worm. Approximately 18 million people are infected, mostly in equatorial Africa, the Sahara, Yemen, and parts of Central and South America (Guatemala, Venezuela, Mexico, Ecuador, Colombia, and Brazil). The infection is transmitted to humans through the bites of blackflies of the genus *Simulium,* which breed along fast-flowing rivers in the above-mentioned tropical areas.

Clinical Significance (Description of Clinical Presentation)

The major disease manifestations of onchocerciasis are localized to the skin, lymph nodes, and eyes. Onchocerciasis is a cumulative infection. Intense infection leads to the disease's most severe complications and is felt to reflect repeated inoculation with infective larvae (26).

Skin

Pruritus is the most frequent manifestation of onchocercal dermatitis. This pruritus may be accompanied by the appearance of localized areas of edema and erythema. Typically, skin disease appears as papular, pruritic dermatitis. If the infection is prolonged, lichenification and pigment changes (either hypo- or hyperpigmentation) can occur; these often lead to atrophy, development of "lizard skin," and mottling of the skin.

Onchocercomata

Onchocercomata, subcutaneous nodules that can be palpable and/or visible, contain the adult worm. In African patients, they are common over bony prominences; in Latin American patients, nodules tend to develop preferentially in the upper part of the body, particularly on the head. Nodules vary in size and characteristically are firm and not tender. It has been estimated that, for every palpable nodule, there are four deeper nonpalpable ones (2).

Lymph Nodes

Lymphadenopathy is frequently found, particularly in the inguinal and femoral areas. The underlying pathology consists of scarring of the lymphoid areas (*O. volvulus* infection in Africa) or follicular hyperplasia (*O. volvulus* infection in Yemen). As the lymph nodes enlarge, they can come to lie within areas of loose skin (so-called "hanging groin") that predisposes to inguinal and femoral hernias.

Eyes

Onchocercal eye disease can take many forms and may lead to severe visual loss or blindness. Usually seen in persons with moderate or heavy infections, the ocular disease spares no part of the eye. Conjunctivitis, anterior uveitis, iridocyclitis leading to secondary glaucoma, sclerosing keratitis, optic atrophy, and chorioretinal lesions can be seen (48).

Systemic Manifestations

Some heavily infected individuals develop cachexia with loss of adipose tissue and muscle mass. Among adults who become blind, there is a three- to fourfold increase in the mortality rate.

Diagnosis

Definitive diagnosis depends on finding an adult worm in an excised nodule or, more commonly, microfilariae in a skin snip sample. Microfilariae can occasionally be found in the blood and in urine, typically after treatment. Microfilariae may also be seen in the cornea and in the anterior chamber of the eye when viewed with a slit lamp. For skin snipping, a small piece of skin is elevated by the tip of a needle or skin hook held parallel to the surface, and a scalpel blade is used to shave off the skin area stretched across the top surface of the needle. Alternatively, a sclerocorneal punch can be used to obtain a blood-free circular skin specimen.

Skin snip samples are generally obtained from an area of affected skin or from the scapular, gluteal, and calf areas (in the African form) or the scapular, deltoid, and gluteal areas (in the Central American form). Once obtained, the skin snip specimens are incubated in a physiologic solution (such as normal saline); the emergent microfilariae can be seen under a microscope typically within hours in heavy infections or after up to 24 h in light infections.

Direct Examination

Microscopy

Microfilariae lack a sheath and are approximately 309 μm long by 5 to 9 μm in diameter. The tails are tapered, usually bent or flexed, and without nuclei (Fig. 1D and 2c).

Nucleic Acid Detection Techniques

Because detection of parasites in the skin or eye is invasive and insensitive, immunodiagnostic assays may be preferable. Assays using PCR to detect onchocercal DNA in skin snip specimens are now in use in laboratories and are highly sensitive and specific (58).

Serologic Tests

IgG antifilarial antibody assays, while positive for individuals with onchocerciasis, suffer from the same lack of specificity and positive predictive value seen in the assays for bloodborne filarial infections; however, the combined use of three groups of recombinant antigens provides sensitivities and specificities that approach 100% for the diagnosis of onchocerciasis (40). Although no recombinant antigen test is available commercially, an experimental rapid card test that detects IgG4 antibodies in serum or whole blood to a recombinant protein (OV-16) has been shown to have >90% sensitivity and specificity (54).

Treatment

The major goals of therapy are to prevent irreversible lesions and to alleviate symptoms. Surgical excision of nodules is recommended when the nodules are located on the head because of the proximity of the microfilaria-producing adult worms to the eye, but chemotherapy is the mainstay of treatment. Ivermectin, a semisynthetic macrocyclic lactone, is now considered first-line therapy for onchocerciasis. It is characteristically given yearly or semiannually. With treatment, most patients have a mild reaction or no reaction. Pruritus, cutaneous edema, and/or a maculopapular rash occur in approximately 1 to 10% of treated individuals. Significant ocular complications are extremely rare, as is hypotension (1 in 10,000) (15, 16). In areas of Africa where *O. volvulus* and *L. loa* are coendemic, however, ivermectin is contraindicated (as it is for pregnant or breast-feeding women) because of severe posttreatment encephalopathy seen in patients, especially children, who are heavily microfilaremic with *L. loa*. Although ivermectin treatment results in a marked drop in microfilarial density, its effect can be short lived (<3 months in some cases). Thus, it is occasionally necessary to give ivermectin more frequently for persistent symptoms (18). A 6-week course of doxycycline that targets the *Wolbachia* endosymbiont has been demonstrated to be macrofilaristatic (rendering the female adult worms sterile for long periods of time) (21).

Prevention

Prevention of infection is being achieved by mass treatment programs using ivermectin (20). Vector control has been beneficial in those areas where infection is highly endemic in which breeding sites are vulnerable to insecticide spraying, but most areas where onchocerciasis is endemic are not

suited to this type of control. Community-based administration of ivermectin every 6 to 12 months is now being used to interrupt transmission in areas where the disease is endemic. This measure, in conjunction with vector control, has already helped reduce the prevalence of disease in foci of endemicity in Africa and Latin America. No drug has proven useful for prophylaxis for *O. volvulus* infection (57).

LOA LOA

Taxonomy

L. loa belongs to the phylum *Nematoda,* class *Secernentea,* subclass *Spiruria,* order *Spirurida,* superfamily *Filarioidea,* and family *Onchocercidae.*

Description of the Agent

The adult parasite lives in the subcutaneous tissues in humans; microfilariae circulate in the bloodstream with a diurnal periodicity that peaks between 12:00 noon and 2:00 p.m. The morphologic appearances and other characteristics of adult worms and microfilariae can be found in Table 1 and Fig. 1 and 2.

Epidemiology and Transmission

The distribution of *L. loa* is limited to the rain forests of West and Central Africa. Tabanid flies (deerflies) of the genus *Chrysops* are the intermediate hosts.

Clinical Significance (Description of Clinical Presentation)

L. loa infection may be present as asymptomatic microfilaremia, with the infection being recognized only after subconjunctival migration of an adult worm (the so-called eye worm). The classic clinical presentation is with episodic Calabar swelling (localized areas of transient angioedema) found predominantly on the extremities. If associated inflammation extends to the nearby joints or peripheral nerves, corresponding symptoms develop. Nephropathy, presumed to be immune complex-mediated, encephalopathy, and cardiomyopathy due to marked eosinophilia can occur, but rarely.

There appears to be a difference between the presentations of loiasis in those native to the area where the disease is endemic and those who are visitors (23). The latter tend to have a greater predominance of allergic symptomatology. The episodes of Calabar swelling tend to be more frequent and debilitating, and such patients rarely have microfilaremia. In addition, those who are not native to the area where the disease is endemic have extreme elevation of eosinophils in the blood, as well as marked increases in antifilarial antibody titers.

Diagnosis

Definitive diagnosis is made parasitologically, either by finding microfilariae in the peripheral blood or by isolating the adult worm from the eye or subcutaneous biopsy material following treatment. The diagnosis must often be made on clinical grounds, however, particularly in travelers (usually amicrofilaremic) to a region where the disease is endemic.

Direct Examination

Microscopy

The microfilariae are sheathed and are up to 300 μm long (females, 50 to 70 mm long and 0.5 mm wide; males, 25 to 35 mm long and 0.25 mm wide). In contrast to those of the lymphatic filariae, the nuclei extend to the end of the tail; however, they are somewhat irregularly arranged along the length of the tail (Fig. 1C and 2d). The sheath does not stain with Giemsa.

Nucleic Acid Detection Techniques

PCR-based assays for the detection of *L. loa* DNA in blood are now available in research laboratories and are highly sensitive and specific (47).

Serologic Tests

Available methods using crude antigen extracts from *Brugia* or *Dirofilaria* species do not differentiate between *L. loa* and other filarial pathogens. The utility of such testing in populations in which the disease is endemic is limited by the presence of antifilarial antibodies in up to 95% of individuals in some regions. An *L. loa*-specific recombinant protein has been tested and has good specificity but only limited (50%) sensitivity (24). Antifilarial IgG and IgG4 may be useful in confirming the diagnosis of loiasis in visitors to areas where the disease is endemic who have suggestive clinical symptoms or unexplained eosinophilia.

Treatment and Prevention

DEC is effective against both the adult and the microfilarial forms of *L. loa,* but multiple courses are frequently necessary before the disease resolves completely. In cases of heavy microfilaremia, allergic or other inflammatory reactions can take place during treatment, including central nervous system involvement with coma and encephalitis. Heavy infections can be treated initially with apheresis to remove the microfilariae and with glucocorticoids followed by small doses of DEC. If antifilarial treatment has no adverse effects, the prednisone dose can be rapidly tapered and the dose of DEC can be gradually increased. Albendazole or ivermectin (although not approved for this use by the Food and Drug Administration) has been shown to be effective in reducing microfilarial loads. DEC is an effective prophylactic regimen for loiasis (31).

MANSONELLA SPP.

Taxonomy

Mansonella perstans, Mansonella ozzardi, and *Mansonella streptocerca* each belong to the phylum Nematoda, class *Secernentea,* subclass *Spiruria,* order *Spirurida,* superfamiliy *Filarioidea,* and family *Onchocercidae.*

Description of the Agents

The adult worms of *M. perstans* reside in the body cavities (pericardial, pleural, and peritoneal) as well as in the mesentery and the perirenal and retroperitoneal tissues, whereas the location of the adult worms of *M. ozzardi* is unknown. The microfilariae of both parasites circulate in the blood without periodicity. For *M. streptocerca,* the adult parasites reside in the skin. *M. streptocerca* microfilariae are found predominantly in the skin. The morphologic appearances and other characteristics of adult worms and microfilariae can be found in Table 1 and Fig. 1 and 2.

Epidemiology and Transmission

M. perstans (formerly *Dipetalonema perstans* and *Acanthocheilonema perstans*) is distributed across the center of Africa and in northeastern South America. The infection is transmitted to humans through the bites of midges (*Culicoides* species). *M. streptocerca* (formerly *Dipetalonema streptocerca* and *Tetrapetalonema streptocerca*) is found largely in the tropical

forest belt of Africa from Ghana to Zaire. It is transmitted to the human host by biting midges (*Culicoides* species). The distribution of *M. ozzardi* is restricted to Central and South America as well as certain Caribbean islands. The parasite is transmitted to the human host by biting midges *(Culicoides furens)* and blackflies *(Simulium amazonicum)*.

Clinical Significance (Description of Clinical Presentation)

Although most patients with *M. perstans* infection appear to be asymptomatic, the clinical manifestations of this infection include transient angioedematous swellings of the arms, face, or other body parts (not unlike the Calabar swellings of *L. loa* infection); pruritus; fever; headache; arthralgias; neurologic or psychologic symptoms; and right-upper-quadrant pain. Occasionally, pericarditis and hepatitis occur.

The major clinical manifestations of *M. streptocerca* infection are related to the skin: pruritus, papular rashes, and pigmentation changes. These are thought to be secondary to inflammatory reactions around microfilariae. Most infected individuals also show inguinal lymphadenopathy. Lymph nodes of affected individuals may show chronic lymphadenitis with scarring; however, many patients are completely asymptomatic (28).

The clinical details of *M. ozzardi* infection are poorly characterized. Furthermore, many consider *M. ozzardi* to be nonpathogenic; however, headache, articular pain, fever, pulmonary symptoms, adenopathy, hepatomegaly, and pruritus have been ascribed to infection with this organism.

Diagnosis

For *M. perstans* infections, diagnosis is made parasitologically by finding the microfilariae in the blood or in other body fluids (serosal effusions). Microfilariae are small (203 by 4 to 5 μm) and have a blunt tail filled with nuclei. Perstans filariasis is often associated with peripheral blood eosinophilia and antifilarial antibody elevations (1). The diagnosis of *M. ozzardi* infection is made by demonstrating the presence of the characteristic microfilariae in the peripheral blood. These microfilariae are small (224 by 4 to 5 μm) and have long, attenuated tails devoid of nuclei (Fig. 1F and 2g). The diagnosis of streptocerciasis can be made by finding the characteristic microfilariae upon examination of skin snip specimens (see the previous section on onchocerciasis diagnosis). In areas where both *O. volvulus* and *M. streptocerca* are endemic, care must be taken to correctly identify the microfilariae. *M. streptocerca* microfilariae have no sheath, are long and slender, and measure approximately 210 by 5 to 6 μm. The most characteristic feature of *M. streptocerca* is its crooked tail (Fig. 2f), which contains nuclei.

Treatment and Prevention

Mansonella perstans

While DEC remains the treatment of choice, there is little evidence that it is efficacious. If cure is attained, usually with multiple (8 to 10) courses of therapy, patients lose their symptoms along with their eosinophilia.

Mansonella streptocerca

DEC is particularly effective in treating infection with both the microfilarial and the adult form of *M. streptocerca.* Following treatment, as in onchocerciasis, one can often see debilitating urticaria, arthralgias, myalgias, headaches, and abdominal discomfort. Ivermectin is currently the drug of choice for treatment of this infection (12, 13).

Mansonella ozzardi

DEC has little or no effect on *M. ozzardi* infection; ivermectin is again the drug of choice for treatment of this infection (17, 25, 32).

There currently are no good preventative measures for any of these infections.

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Cestodes

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Cestodes have as their key characteristic a flattened body composed of the head or scolex (bearing the fixation organs—suckers, hooks, and bothria), the neck (where the cellular reproduction occurs, to form the strobila), and the strobila, formed by numerous segments or proglottids. As new proglottids develop in the neck region, existing ones mature as they become more distal. The more-distal proglottids are gravid, almost completely occupied by a uterus full of eggs which are passed with the stools of the carrier, either inside complete proglottids or free after proglottid breakage. In some species, proglottids can actively migrate out of the anus.

Tapeworms live in the lumen of the small intestine with the head or scolex as the only fixation organ, attached to the mucosa. They absorb nutrients from the host's intestine both at the head and through their tegument. Accordingly, they have developed cephalic fixation organs like hooks, suckers, or shallow grooves as longitudinal suction sulci (bothria) (Fig. 1), and a specialized tegument.

Four species of cestode tapeworms inhabit the human intestine: *Diphyllobothrium latum, Taenia saginata, Taenia solium,* and *Hymenolepis nana*. They differ widely in size, intermediate host, and other characteristics, from the 12-m *D. latum* to the 3-cm *H. nana* (Table 1). More rarely *Dipylidium caninum* and *Hymenolepis diminuta* can also inhabit the human gut; these parasites are reviewed in "Less Common Helminths" (chapter 148). In addition, a number of cestode larvae can produce human disease if infective tapeworm eggs are ingested, mainly cysticercosis *(Taenia solium),* cystic hydatid disease *(Echinococcus granulosus),* and alveolar hydatid disease *(Echinococccus multilocularis)*. Rarer larval cestode infections affecting humans include coenurosis *(Taenia multiceps),* sparganosis *(Spirometra mansonoides),* and cysticercosis by *Taenia crassiceps.* Tapeworms and especially tapeworm larval infections still represent an important cause of morbidity and mortality, not only in most underdeveloped countries but also in industrialized countries, particularly in rural areas or among immigrants from areas of endemicity.

DIPHYLLOBOTHRIUM LATUM

Known as the fish tapeworm, *D. latum* is the longest intestinal parasite of humans. Also common in fish-eating mammals such as canids or felids (reservoir hosts), it differs from other adult tapeworms infecting humans in its morphology, biology, and epidemiology.

Taxonomy

D. latum is included in the phylum Platyhelminthes, subphylum Neodermata, class Cestoidea, subclass Eucestoda, order Pseudophyllidea, family Diphyllobothriidae, and genus *Diphyllobothrium.*

Description of Agent

Adult Tapeworm

The adult parasite can grow to 15 m in length and apparently can live for 20 years or longer in the small intestine. It is ivory in color and has a scolex which is provided with bothria on its dorsal and ventral aspects (9). *Diphyllobothrium* proglottids are much wider than they are long (~8 by 4 mm) and are easy to recognize because their genital pore is located in the center of the proglottid rather than in the lateral edges as in all other tapeworms of humans. The coiled uterus in the center of the gravid proglottids looks yellow-brown in freshly passed specimens. The uterine pore is located in the center of the proglottid near the genital pore.

Eggs

Unembryonated, operculate eggs are passed in the feces. *D. latum* eggs are oval and resemble those of trematodes but are smaller (58 to 75 μm long by 44 to 50 μm wide) and have a better defined wall. The abopercular end usually has a small knoblike protrusion. Eggs are usually numerous, and expulsion of proglottid chains is usual.

Larvae

After the eggs embryonate in a water environment for several weeks, ciliated six-hooked embryos (coracidia) hatch. Coracidia must be ingested by appropriate species of freshwater copepods (genus, *Cyclops*) for further development. In the copepod a solid-bodied larva, the procercoid, develops as a second larval stage and becomes infective for the second intermediate host (fish). In fish, the procercoid migrates to the flesh and develops in the third larval stage, the plerocercoid or sparganum, which is the infective stage for human or animal (canids or felids) hosts.

Epidemiology, Transmission, and Prevention

The geographic distribution of *D. latum* includes lake areas in Scandinavia, other areas of northern Europe, the former USSR, Finland, northern Japan, and North America, principally the

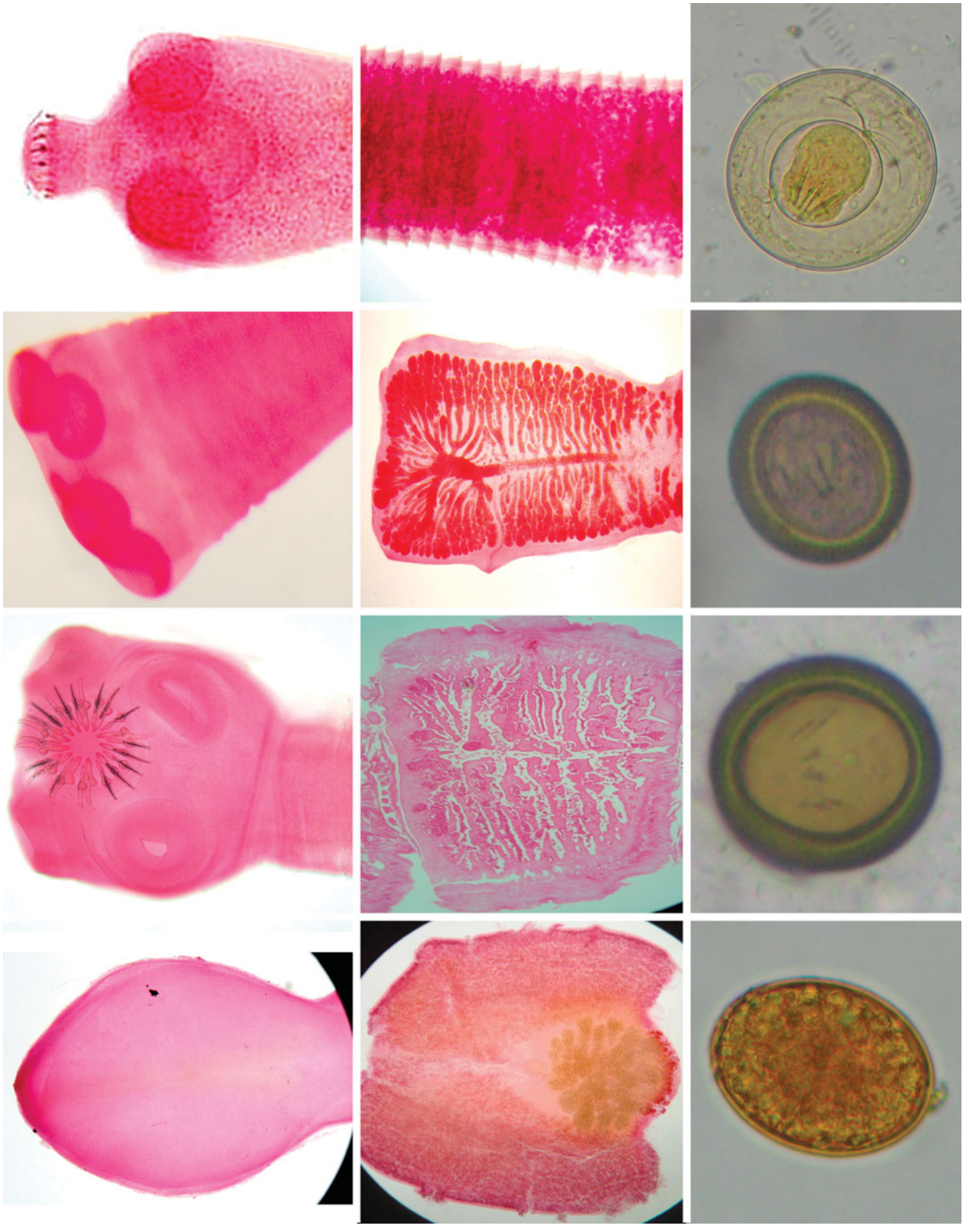


FIGURE 1 Scolex, gravid proglottids, and eggs of *H. nana*, *T. saginata*, *T. solium*, and *D. latum*. Note the coiled, central uterus in *D. latum*, the absence of hooks in the scolex of *T. saginata*, and the similar appearances of the eggs of *T. saginata* and *T. solium*.

TABLE 1 Some characteristics of main cestodes infecting humans

Organism	Length (cm)	Scolex	Gravid proglottids	Intermediate host(s)
D. latum	1,200	Spatulate, two bothria	Rosette-shaped central uterus	Copepods, fish
T. saginata	600	Squared, four suckers, no hooks	>15 main lateral uterine branches	Cattle
T. solium	300	Squared, four suckers, hooks (double crown)	<12 main lateral uterine branches	Pigs, humans
H. nana	3	Knoblike, four suckers, hooks (single crown)	Bag-shaped uterus	Insects, rodents, humans

upper Midwest, Alaska, Canada, and the southwestern coast of South America. Several other *Diphyllobothrium* species *(D. pacificum, D. cordatum, D. ursi, D. dendriticum, D. lanceolatum, D. dalliae,* and *D. yonagoensis)* have also been reported to infect humans but less frequently (3, 28). *Diphyllobothrium pacificum,* identified by Nybellin in 1931, is a parasite of seawater found along the western coast of South America, specifically in Peru and Chile. *D. pacificum* is much smaller than *D. latum* and usually measures 50 to 200 cm long, although it can occasionally reach 3 to 4 m.

The most common sources of human *Diphyllobothrium* infection are the pike burbot, perch, ruff, and turbot (28). Infected fish (undercooked, raw, or insufficiently treated flesh) transmit plerocercoids to humans or fish-eating mammals. Infection with *Diphyllobothrium* is preventable by eating well-cooked fish or fish that has been deep-frozen (at least –10°C for 24 h).

Clinical Significance (Description of Clinical Presentation)

Infected individuals notice passing segment chains with their stools. The parasite may produce no clinical symptoms in some people, but when it reaches a large size it may cause mechanical bowel obstruction, diarrhea, abdominal pain, and, particularly in northern European countries, pernicious anemia resulting from B12 vitamin deficiency because the tapeworm competes with the intestinal epithelium for the uptake of the vitamin. This condition is rare outside Scandinavian countries, and some authors postulate a genetic predisposition.

Collection, Transport, and Storage of Specimens

For identification purposes eggs are well preserved in 5 to 10% formalin solutions. For DNA recovery, 95% ethanol would be a better option. Electron microscopy may require cacodylate buffer or other glutaraldehyde media. Adult tapeworm material is better defined if it is washed in saline, relaxed for better visualization of its internal structures by warming the saline at 55°C for a short period (5 min), and then placed between two glass slides and stored in a fixative solution. Fixatives could be 10% formalin, acetic acid-formaldehyde-alcohol, or sodium acetate-acetic acid-formaldehyde. Fixed pieces can be stained by injecting Semichon's carmine or India ink. The proglottids can also be sectioned and stained using hematoxylin and eosin; however, morphological characteristics can be more easily seen in whole mounts.

Direct Examination

Microscopy

Eggs can be easily seen by microscopical examination of stools. Either flotation or sedimentation techniques may be used. However, since the eggs are operculated, they generally do not float using the flotation concentration method; both the surface film and sediment need to be examined if this concentration method is used. For that reason, most laboratories routinely use the sedimentation concentration method. Low-magnification microscopy should easily permit identification of the characteristic scolex or proglottids when available (Fig. 1). Neither culture nor antigen detection is relevant for the detection and identification of *D. latum*.

Nucleic Acid Detection Techniques

Although several groups have described genus variation in *Diphyllobothrium* by using nucleic acid detection methods, the information has no clinical relevance in terms of routine tapeworm recovery and identification (19).

Serologic Tests

Serologic tests are not available.

Treatment

Both praziquantel and niclosamide are effective drugs. At recommended doses both are associated with only mild side effects, mostly gastrointestinal.

Evaluation, Interpretation, and Reporting of Results

Both stool microscopy and parasite identification are unambiguous. Eggs or tapeworm pieces should be reported as *D. latum* eggs (except in South America, where *D. pacificum* is more frequently found).

TAENIA SAGINATA

Known as the beef tapeworm, *Taenia saginata* is still endemic to most of the world. Humans are its only definitive host. While *T. saginata* infections do not carry major risks for the host, differential diagnosis with *Taenia solium* is important because the latter can cause neurocysticercosis.

Taxonomy

T. saginata is included in the phylum Platyhelminthes, subphylum Neodermata, class Cestoidea, subclass Eucestoda, order Cyclophyllidea, family Taeniidae, and genus *Taenia*.

Description of Agent

Adult Tapeworm

The adult *T. saginata* tapeworm attains lengths of 4 to 8 m and has a scolex provided with four suckers and an unarmed

(no hooks) rostellum. Gravid proglottids are longer than they are wide (18 to 20 mm by 5 to 7 mm). Each proglottid has a genital pore at the midlateral margin. In mature proglottids, the ovary has only two lobes and presents a vaginal sphincter. Gravid proglottids, which are highly muscular and active, break off from the strobila and actively migrate out of the anus (a pathognomic characteristic of this species).

Eggs

Eggs from *T. saginata* and *T. solium* are indistinguishable by morphological characteristics. They are spherical, measure 30 to 40 μm in diameter, and have a quite characteristic thick, yellow-brown, radiate shell (embryophore) composed of collagen subunits, which gets thicker as the eggs mature. Eggs are frequently surrounded by a thin layer of vitelum (Fig. 1). Within the egg is a six-hooked embryo, the oncosphere.

Larvae

The unarmed scolex is invaginated into a fluid-filled bladder, the cysticercus. Larval cysts are 4 to 6 mm long by 7 to 10 mm wide and have a pearl-like appearance in tissues.

Epidemiology, Transmission, and Prevention

T. saginata is distributed worldwide, although it is especially prevalent in some parts of Africa, Central and South America, eastern and western Asia, and some countries in Europe. Cattle serve as the intermediate host, and ingestion of eggs from contaminated pasturelands by grazing cattle results in development in cattle tissues of the infective cysticercus stage. After ingestion of the cysticercus in raw or inadequately cooked beef, it takes approximately 2 to 3 months for the infection to become patent in the human host.

In Southeast Asia there is a human tapeworm morphologically very similar to *Taenia saginata* (*Taenia saginata asiatica, Taenia asiatica,* or Taiwan taenia). In this tapeworm, the cysticercus stage occurs in the liver of pigs and less frequently in cattle. The adult tapeworm infects the human host, and its appearance is very similar to that of *T. saginata* (6, 7).

Clinical Significance (Description of Clinical Presentation)

Although patients may exhibit no symptoms with this infection, they usually notice passing proglottids or find them in their underwear. The mature worm can also cause abdominal discomfort, diarrhea, and occasionally intestinal obstruction as a result of its large size.

Collection, Transport, and Storage of Specimens

For collection, transport, and storage of specimens, see *Diphyllobothrium* above.

Direct Examination

Microscopy

Typical *Taenia* eggs can be found in feces. Sedimentation or the less used Kato-Katz method is apparently more sensitive for the detection of *Taenia* eggs in stools than other concentration techniques. Finding of *Taenia* eggs does not allow a diagnosis of infection by this species; it is usually made by identification of gravid proglottids that have been passed in feces or have actively migrated out of the anus. Identification of the proglottids is based on shape and size and mainly on the morphology of the uterus, which can be demonstrated after injection with India ink or staining with carmine or hematoxylin stains. In *T. saginata* there are 15 to 20 primary lateral branches on each side of the central uterine stem (Fig. 1).

Antigens in stools (coproantigen) have been detected by enzyme-linked immunosorbent assay (ELISA) since 1990, but this assay is used mainly in research settings because of scarce availability.

Nucleic Acid Detection Techniques

Species-specific PCR techniques have been described to detect parasite DNA and differentiate *T. saginata* from *T. solium*. Most of these assays require actual parasite material, although some are apparently able to establish the difference with DNA from eggs in feces (13, 21).

Serologic Tests

Serum antigen detection ELISAs for *T. saginata* cysticercosis in cattle have been developed using monoclonal antibodies to *T. saginata.* Although these assays can detect parasite burdens of <50 cysts per animal, they have not yet been routinely applied except in research settings (4, 12, 26).

Treatment

Both praziquantel and niclosamide are effective drugs. At recommended doses both are associated with only mild side effects, mostly gastrointestinal. In regions where *T. solium* is endemic, there is a possibility that latent neurocysticercosis may respond to praziquantel and cause severe headaches or seizures. Niclosamide is not absorbed from the gastrointestinal tract and thus does not carry this risk (15).

Evaluation, Interpretation, and Reporting of Results

Eggs should be reported as "*Taenia* sp." because direct observation does not confirm the species. The finding of *Taenia* sp. eggs should be notified to the attending physician to ensure prompt treatment. The presence of the scolex in the parasite material expelled (spontaneously or posttreatment) should be reported both because it allows species diagnosis and because if it is not found, the chances of treatment failure increase.

TAENIA SOLIUM

Known as the pork tapeworm, *T. solium* has an extensive geographic distribution. This infection has a huge impact on human health because of its association with seizure disorders caused by infection of the human brain with its larval stage (neurocysticercosis) (11).

Taxonomy

T. solium is included in the phylum Platyhelminthes, subphylum Neodermata, class Cestoidea, subclass Eucestoda, order Cyclophyllidea, family Taeniidae, and genus *Taenia.*

Description of Agent

Adult Tapeworm

The adult *T. solium* tapeworm measures 2 to 4 m and has a scolex provided with four suckers and a rostellum armed with two crowns of hooks. Gravid proglottids have similar length and width (approximately 1 cm). Each proglottid has a genital pore at the midlateral margin. In mature proglottids, the ovary has two main lobes and one accessory lobe (lacking in *T. saginata*), and a vaginal sphincter muscle is lacking (present in *T. saginata*). Gravid proglottids have few (<12) lateral

branches on the central uterine stem (Fig. 1). Since the eggs of *T. solium* are infective to humans and can cause cysticercosis, extreme caution in the handling of these proglottids or infective stools is recommended.

Eggs

Eggs from *T. saginata* and *T. solium* are indistinguishable by morphological characteristics. They are spherical, measure 30 to 40 μm in diameter, and have a quite characteristic thick, yellow-brown, radiate shell (embryophore) composed of collagen subunits, which gets thicker as the eggs mature. Eggs are frequently surrounded by a thin layer of vitelum (Fig. 1). Within the egg is a six-hooked embryo, the oncosphere.

Larvae

The fluid-filled bladder (cysticercus) larvae are bigger than those of *T. saginata,* measuring approximately 8 to 10 mm in diameter. They lodge in the pig's tissues, mostly in muscle and brain.

Epidemiology, Transmission, and Prevention

T. solium taeniasis and cysticercosis are highly endemic to all parts of the developing world where pigs are raised as a food source, including Latin America, most of Asia, sub-Saharan Africa, and parts of Oceania. The infection is now also increasingly diagnosed in industrialized countries due to immigration of tapeworm carriers from zones of endemicity (7, 11).

As for *T. saginata,* humans are the only definitive host. Ingestion of contaminated pork containing *T. solium* cysticerci causes human taeniasis. Conversely, *T. solium* eggs cause cysticercosis in pigs (the usual intermediate host) and humans. Pigs acquire cysticercosis by eating stools contaminated with infective eggs in places where deficient sanitation exists. Humans get infected by fecal-oral contamination from a tapeworm carrier, commonly in the household or another close environment.

Clinical Significance (Description of Clinical Presentation)

Human infection is acquired by ingestion of infective cysticerci in inadequately cooked pork or pork products. Taeniasis seems mostly asymptomatic, and most patients do not even notice passing proglottids in stools. The clinical significance of *T. solium* infections relates to the risk of neurocysticercosis (see below), which is high for tapeworm carriers and their close contacts (11).

Collection, Transport, and Storage of Specimens

For collection, transport, and storage of specimens, see the corresponding section in "*Diphyllobothrium*" above. Handling of *T. solium* proglottids or contaminated stools should be done with appropriate biosafety conditions to avoid cysticercosis.

Direct Examination

Microscopy

Typical *Taenia* eggs can be found in feces. Sedimentation or the less used Kato-Katz method is apparently more sensitive for the detection of *Taenia* eggs in stools than other concentration techniques. Finding of *Taenia* eggs does not allow a diagnosis of infection by the species, which is usually made by identification of gravid proglottids that have been passed in feces. Identification of the proglottids is based on shape and size and mainly on the morphology of the uterus, which can be demonstrated after injection with India ink or staining with carmine or hematoxylin stains. In *T. solium* there are few primary branches on each side of the central uterine stem (Fig. 1).

Antigens in stools (coproantigen) have been detected by ELISA since 1990. Coproantigen detection ELISA is much more sensitive than microscopy and thus highly recommended for the diagnosis of human taeniasis (specifically in the case of *T. solium* because of the risks of cysticercosis transmission), but its availability is still limited.

Nucleic Acid Detection Techniques

Species-specific PCR techniques that differentiate *T. saginata* from *T. solium* have been described. Most of these assays require actual parasite material, although some are apparently able to establish the difference with DNA from eggs in feces (13, 21).

Serologic Tests

Recently, stage-specific serological assays directed to the adult tapeworm have been developed, with high sensitivity and specificity. Mostly, serology is directed to the detection of *T. solium* antibodies in relation to the diagnosis of neurocysticercosis. Antibody detection by enzyme-linked immunoelectrotransfer blot assay is the method of choice, with a sensitivity of 98% in cases with more than one viable larval cyst and specificity of 100%. *T. solium* antigen detection in serum or cerebrospinal fluid has been performed in cases of human cysticercosis, based on a known genus-specific cross-reaction in ELISAs for *T. saginata*. Although these assays can detect parasite burdens of <50 cysts in infected animals, they have not yet been routinely applied except in research settings.

Treatment

Both praziquantel and niclosamide are effective drugs. At recommended doses both are associated with only mild side effects, mostly gastrointestinal. In regions where *T. solium* is endemic, there is a possibility that latent neurocysticercosis may respond to praziquantel and cause severe headaches or seizures. Niclosamide is not absorbed from the gastrointestinal tract and thus does not carry this risk (15).

Evaluation, Interpretation, and Reporting of Results

Eggs should be reported as "*Taenia* sp." because direct observation does not confirm the species. The finding of *Taenia* sp. eggs should be notified to the attending physician to ensure prompt treatment and minimize the chances of cysticercosis in the patient or his/her contacts. The presence of the scolex in the parasite material expelled (spontaneously or posttreatment) should be reported both because it allows species diagnosis and because if it is not found, the chances of treatment failure increase.

HYMENOLEPIS NANA

H. nana is the smallest of the intestinal tapeworms of humans and also the most common tapeworm infection throughout the world. It can be transmitted from person to person (an intermediate host is not necessarily required) (28).

Taxonomy

H. nana is included in the phylum Platyhelminthes, subphylum Neodermata, class Cestoidea, subclass Eucestoda, order Cyclophyllidea, family Hymenolepididae, and genus *Hymenolepis.*

Description of Agent

Adult Tapeworm

The adult parasite measures 2 to 4 cm and seems to live approximately 1 year. The scolex has four suckers and one crown of hooks.

Eggs

The eggs are 30 to 50 μm in diameter and thin shelled, and they contain a six-hooked oncosphere that lies in the center of the egg and is separated from the outer shell by considerable space. The oncosphere is surrounded by a membrane that has two polar thickenings from which arise four to eight filaments extending into the space between it and the outer shell (Fig. 1). These filaments are not seen in *H. diminuta.* Eggs may hatch inside the host's intestine, and the embryos (oncospheres) invade the mucosa to develop into larval stages.

Larvae

The cysticercoid larvae have an invaginated scolex but no fluid-filled bladder. They lodge in the intestinal mucosa and emerge to the intestinal lumen as young tapeworms after a few days.

Epidemiology, Transmission, and Prevention

H. nana is normally a parasite of mice, and its life cycle characteristically involves various beetles as intermediate hosts. In humans, transmission is usually accomplished by direct ingestion of infective eggs containing oncospheres. When eggs are ingested, a solid-bodied larva, a cysticercoid, first develops in the wall of the small intestine. Subsequently, the larva migrates back into the intestinal lumen, where it reaches maturity as an adult tapeworm in 2 to 3 weeks. In beetles that ingest eggs of *H. nana,* the cysticercoids develop in the body cavity and have thick protective walls around them. Although humans may acquire the infection by accidental ingestion of infected beetles (often occurring in dry cereals), direct infection is far more common and is the primary reason why *H. nana* usually occurs in institutional and familial settings in which hygiene is substandard. A feature of human *H. nana* infection is the opportunity for internal autoinfection with the parasite, which may result in large worm burdens. Autoinfection occurs when eggs discharged by adult tapeworms in the lumen of the small intestine hatch rapidly and invade the wall of the intestine; here, cysticercoids are formed, and they subsequently reenter the intestine to mature as adult worms.

Clinical Significance (Description of Clinical Presentation)

Most infections cause no symptoms. However, hymenolepiasis can be associated with abdominal pain, diarrhea, headaches, or irritability, probably in infections with heavier worm burdens (23, 30).

Collection, Transport, and Storage of Specimens

For collection, transport, and storage of specimens, see the corresponding section in "*Diphyllobothrium*" above.

Direct Examination

Microscopy

Diagnosis of the infection rests on finding the spherical eggs in feces by microscopy. Either flotation or sedimentation techniques are of help. Proglottids (Fig. 1) are rarely seen, since they disintegrate after breaking off from the main strobila. Neither culture, antigen detection, nor nucleic acid detection techniques are relevant for the detection and identification of *H. nana.*

Serologic Tests

Antibody detection ELISAs have been used in research settings but are of no clinical use (1).

Treatment

Both praziquantel and niclosamide are effective drugs. At recommended doses both are associated only with mild side effects, mostly gastrointestinal. A second dose of praziquantel after 10 to 15 days may decrease the likelihood of relapses. Niclosamide needs to be administered for 7 days because it is not absorbed and thus does not affect the cysticercoid larvae in the intestinal mucosa. Nitazoxanide has been reported to be useful as a therapeutic alternative (29).

Evaluation, Interpretation, and Reporting of Results

Eggs are characteristic and should be reported as *H. nana* eggs. The closest differential diagnosis is with *H. diminuta,* which rarely infects humans. The eggs of *H. diminuta* are bigger and have a wider interior space and thus can be differentiated by microscopy.

LARVAL CESTODES INFECTING THE HUMAN HOST

The larval stages of *T. solium, Echinococcus granulosus, Echinococcus multilocularis,* and, less frequently, *Sparganus* sp., *Taenia multiceps,* and *Taenia crassiceps* can invade the human tissues. These are briefly described below.

Cysticercosis *(Taenia solium)*

In the normal life cycle of *T. solium,* humans are the definitive host and pigs act as the intermediate host, hosting the larval stage or cysticercus. Porcine cysticercosis is a serious economic problem for pig farmers. However, the most serious manifestations are found in human cysticercosis (11). Larval vesicles located in the central nervous system (Fig. 2) cause seizures or other neurological symptoms. Indeed, neurocysticercosis is associated with a significant proportion of seizure cases in areas of endemicity (22, 24). Clinical manifestations of neurocysticercosis are related to individual differences in the number, size, and topography of lesions and in the severity of the host's immune response to the parasites. Symptoms and signs are varied and nonspecific, and recognition of a typical syndrome is not possible. Parasites in the brain parenchyma usually cause seizures and headache, whereas those located in the cerebral ventricles or in the subarachnoid space ("racemose" cysticercosis) cause intracranial hypertension and hydrocephalus. Diagnosis is made using brain imaging, either computed tomography (CT) scan or magnetic resonance imaging (MRI), and confirmed by serology. CT has a lower cost (quite important for poor areas where the disease is endemic), is more available, and has better sensitivity for the detection of calcified parasites. Conversely, MRI has better sensitivity for small lesions, those located close to the skull, and intraventricular parasites. The serological assay of choice is an immunoblot using seven purified glycoprotein larval antigens, which has 98% sensitivity and 100% specificity except in cases with a single lesion, for which sensitivity drops to approximately 70% (31). Antigen detection assays have been described, but no controlled data on sensitivity and specificity are yet available. Treatment of

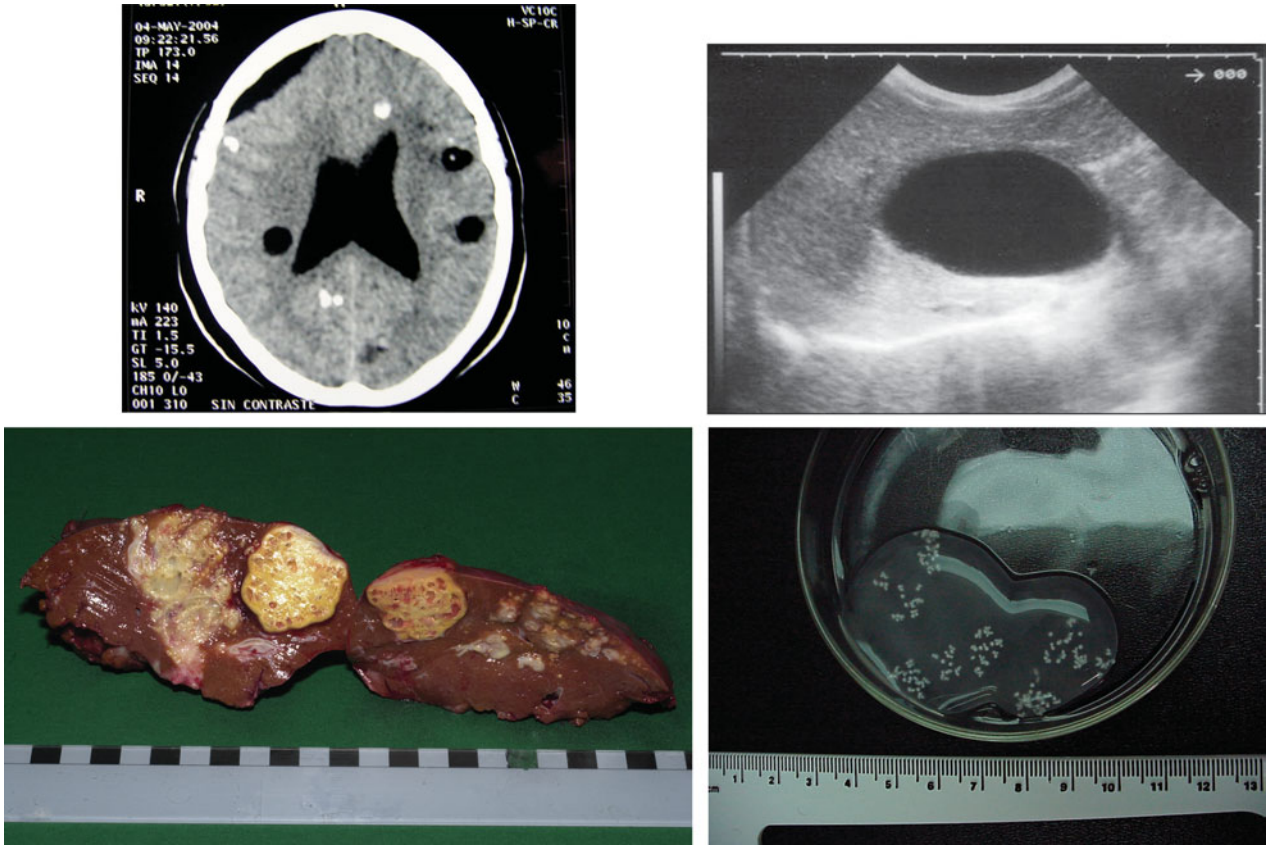


FIGURE 2 Larval cestodes infecting the human host. *Taenia solium* cysticerci in the human brain (neurocysticercosis), shown in a noncontrasted MRI of the brain. *Echinococcus granulosus* hydatid in the human liver (hydatid disease) as seen on liver ultrasound (kind contribution of Enrico Brunetti, Università di Pavia, Pavia, Italy). *Echinococcus multilocularis* alveolar hydatid disease in human liver (kind courtesy of K. Buttenschoen and P. Kern, University Hospital Ulm, Ulm, Germany). *Taenia multiceps* coenurus showing multiple scolices in the cystic wall.

neurocysticercosis uses antiparasitic agents (albendazole or praziquantel) for viable parasites, usually given with steroids to ameliorate the inflammation produced by the death of the cyst. Surgery is limited to excision of single, big lesions or implantation of ventricle-peritoneal shunts. Antigen detection assays permit patient monitoring and follow-up of antiparasitic treatment (10).

Cystic Hydatid Disease *(Echinococcus granulosus)*

In the normal life cycle of *Echinococcus granulosus,* the dog is the definitive host and herbivores (mainly sheep) act as the intermediate host. These become infected with the larval stage (cystic hydatid) by ingesting infective eggs dispersed from the feces of the infected dog. Human cystic hydatid disease is an important cause of human morbidity, requiring costly surgical and medical treatment. This cestodiasis is still endemic to most of the Old World, particularly in Greece, Cyprus, Bulgaria, Lebanon, and Turkey; some other European countries; South America; and Africa. Sporadic autochthonous transmission is currently recognized in Alaska and other states in the United States (5). The affected organs are most commonly liver and lungs and more rarely heart, brain, bone, or other locations. Diagnosis is made using ultrasound or CT scan for liver infection or chest X rays or CT for lung infections (Fig. 2). Antibody detection by serology is of much help, although sensitivity is lower than for other infections, reaching 80 to 85%. It is more sensitive for hepatic than for pulmonary cases. Treatment uses antiparasitic agents (albendazole or albendazole plus praziquantel) for small cysts or presurgery; PAIR (puncture, injection, aspiration, and reinjection), which is a technique of ultrasound- or CT-guided aspiration and sterilization of the cyst's contents; or either laparoscopic or open surgery (18, 33). Spillage of cyst contents could lead to acute anaphylactic reactions or dissemination of infection in the surroundings. Cystic lesions may resolve without therapy in a proportion of patients (25).

Alveolar Hydatid Disease *(Echinococcus multilocularis)*

The adult stage of *E. multilocularis* lives in the small intestine of the definitive host, commonly wild predators in the northern hemisphere, occurring in parts of Europe, Asia, Japan, and North America, including Alaska (2, 16). The larval stages infect microtine rodents that usually serve as the common intermediate host. Human infections (causing alveolar hydatid disease) occur by accidental ingestion of the oncosphere by contamination with the feces of the definitive host. The manifestation of alveolar hydatid disease resembles a slowly developing "malignant tumor" of the liver, with subsequent invasion

of the blood vessels and bile ducts and metastatic dissemination. The lesions vary in size and can produce minor foci up to large infiltrating structures in the host's tissue. Thus, alveolar hydatid disease differs greatly in the pathology and clinical course from cystic hydatid disease. This disease often affects persons aged over 50 years and is characterized by a chronic course lasting for months or years. Clinical manifestations relate to the extent of tumor-like lesions of the cyst. Beside physical examination, diagnosis usually is based on imaging techniques including ultrasound, CT, and MRI supported by serology. Treatment is mainly surgical. Chemotherapy with benzimidazole agents is restricted to residual, postsurgical, or inoperable lesions (17).

Sparganosis *(Spirometra mansonoides)*

Mainly found in Southeast Asian countries, the metacestode larvae of *Spirometra* species can invade the human tissues either by ingestion of contaminated crustaceans in drinking water or of infected meat (frog or snake) or by direct contact via a poultice. The most commonly affected sites are subcutaneous tissues and the eye. The diagnosis rests on the pathological demonstration of the larvae after excision by biopsy (32).

Coenurosis *(Taenia multiceps* or *Taenia serialis)*

Taenia multiceps and *Taenia serialis* have canids as definitive hosts and sheep as their normal intermediate host, harboring the larvae or coenurus. The coenurus is a vesicle containing a transparent fluid, with a fine membrane in which multiple (500 to 700) scolices can be seen. Infected sheep lose their balance and rotate around their site, become dizzy, and fall (screw disease). It infrequently causes coenuriasis in humans. Human infections have largely been confined to the African continent, but a few cases have been described from France, England, and North and South America. The space-occupying coenurus usually invades the brain, producing lethal lesions. Diagnosis is based on pathological demonstration of the typical larval membrane and multiple scolices (14, 27).

Cysticercosis *(Taenia crassiceps)*

T. crassiceps is a common tapeworm of the red fox. Larval forms are generally found in subcutaneous tissues and body cavity of rodents. Human cases are quite rare, mostly in immunocompromised patients (subcutaneous, muscular, or ocular infections) (8, 20).

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Trematodes

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At least 70 species of digenean trematode have been recorded as adult parasites from humans. All of these species are endoparasitic, occupying a variety of tissue sites (see Tables 1 through 3). Adult trematodes have a distinctive morphology, often with a leaf-like body plan. The most prominent morphological features in most species are two rounded suckers. One of these, the oral sucker, surrounds the mouth, while the other, the ventral sucker, lies approximately one-third of the way along the body and serves as a primary attachment organ (28). Adults of trematodes infecting humans are rarely encountered because of their endoparasitic nature and are seen usually only after anthelmintic purging or at autopsy. Hence, diagnosis of the diseases caused by these helminths relies to a large extent on direct observations of excreted ova.

Detailed descriptions of the life cycles of digenean parasites of humans are shown (Fig. 1 and 2). There are many subtle variations in life cycle patterns, but there are two predominant life cycle strategies among these trematodes (Fig. 1 and 2). The first strategy, exemplified by the schistosomes, is one in which humans are infected by direct invasion of the skin by cercariae. The second strategy is seen among the so-called foodborne digeneans (21), a diverse assemblage of species which enter humans with ingested food (Fig. 2).

Two features of the digenean life cycle are noteworthy. First, digeneans often display high specificity in their choice of first intermediate host. So intimate are these host-parasite associations that the geographical distribution of a digenean is determined largely by that of its snail host. For this reason, many digeneans show a focal distribution in countries where they are endemic. Second, most human parasites are zoonotic, requiring the cooccurrence of other mammalian or avian hosts in an area of endemicity to maintain human infection (26).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Observation of eggs in human excreta is diagnostic for all digeneans of humans. With few exceptions (17, 33), the eggs have a distinct morphology and their presence is pathognomonic of specific infection. Despite the diverse range of body sites infected by adult trematodes, the eggs of most species are voided with feces. Exceptions to this include *Schistosoma haematobium,* and rarely other schistosomes, whose eggs are excreted with urine, and *Paragonimus* species, whose eggs may be observed in sputum.

Detailed instructions on handling of human fecal material are provided in chapter 133. Digenean eggs range in size from 20 to 150 μm and can be identified with low-power objective lenses (i.e., 10× and 40× lens objectives). There is great variation in egg sizes among different species and, in areas where fasciolids are endemic, for example, it is important to scan slides at lower magnification. Whereas the eggs of most digeneans are unembryonated when excreted, the eggs of schistosomes and some other groups contain fully differentiated larvae in feces or urine. The eggs of schistosomes hatch spontaneously upon exposure to freshwater. Although observations of viable schistosome miracidia may be advantageous for species identification, spontaneous hatching may hinder direct observations of eggs. Fresh stool samples or stained preparations can be used for direct examination. Sedimentation techniques (chapter 135) may be useful for diagnosis in light, early, or chronic infections, when egg excretion is low. Among currently available diagnostic techniques, the Kato-Katz thick smear is a standard method for qualitative and quantitative field diagnosis of intestinal schistosomiasis and geohelminth infections (2, 6, 27).

The eggs of human parasitic digeneans are ovoid, usually with a yellow to brown, translucent shell (see Fig. 4). The eggs usually are without adorning features, although diagnostic spines and polar ridges and an operculum ("lid") may be present. Diagnostic features, therefore, are the shape and size of the egg, texture and adornments of the eggshell, the presence or absence of an operculum, and the extent of postzygotic development of the embryo.

DIGENEANS OF THE CIRCULATORY SYSTEM—SCHISTOSOMES

Schistosome species responsible for human disease are shown in Table 1, along with their distribution, snail hosts, and treatment. Currently, 200 million people in 74 countries have schistosomiasis, although 85% of cases occur in sub-Saharan Africa (10). Of these, some 120 million people are symptomatic and 20 million have severe illness.

Taxonomy

Schistosomes are characterized by dioecious adults, cercariae with a forked tail and a two-host life cycle (Fig. 1). Adult schistosomes occupy an intravascular site, either in the mesenteric vessels or the vesical plexus. Five species

TABLE 1 Geographical distribution, intermediate hosts, and egg morphology of the major schistosomes infecting humans

Species	Disease and geographic area	Snail hosts	Drug	Eggs (specimen and size)
S. mansoni	Intestinal schistosomiasis, infecting humans and sometimes other mammals: Angola, Benin, Botswana, Burkina Faso, Burundi, Cameroon, Central African Republic, Chad, Republic of the Congo, Cote d'Ivoire, Democratic Republic of Congo, Equatorial Guinea, Eritrea, Ethiopia, Gabon, The Gambia, Ghana, Guinea, Guinea-Bissau, Kenya, Liberia, Madagascar, Malawi, Mali, Mauritania, Mozambique, Namibia, Nigeria, Rwanda, Senegal, Sierra Leone, South Africa, Swaziland, Togo, Uganda, Tanzania, Zambia, Zimbabwe, Egypt, Libya, Oman, Saudi Arabia, Somalia, Sudan, Yemen, Antigua, Brazil, Dominican Republic, Guadeloupe, Martinique, Montserrat, Puerto Rico, St. Lucia, Suriname, Venezuela	*Biomphalaria* species. In Africa and Asia: many species in *B. alexandrina, B. choanomphala, B. pfeifferi, B. sudanica* species groups. In Americas: *B. glabrata* and 2 other species.	P,[a] 20 mg/kg, 2–3 doses. Community programs usually give 40 mg/kg in single dose.	Feces (rarely urinary). Size: 140 by 61 μm
S. japonicum	Intestinal schistosomiasis, infecting humans and bovines: China, Indonesia, Philippines	*Oncomelania hupensis, O. nosophora, O. formasona, O. hupensis quadrasi,* and *O. lindoensis*	As above, but 60 mg/kg in single dose in community programs	Feces. Size: 85 by 60 μm
S. haematobium	Genitourinary schistosomiasis: Algeria, Angola, Benin, Botswana, Burkina Faso, Burundi, Cameroon, Central African Republic, Chad, Republic of the Congo, Cote d'Ivoire, Democratic Republic of Congo, Egypt, Ethiopia, Gabon, The Gambia, Ghana, Guinea, Guinea-Bissau, Iran, Iraq, Jordan, Kenya, Lebanon, Liberia, Libya, Madagascar, Malawi, Mali, Mauritania, Mauritius, Morocco, Mozambique, Namibia, Niger, Nigeria, Oman, Saudi Arabia, Senegal, Sierra Leone, Somalia, South Africa, Sudan, Swaziland, Syria, Togo, Tunisia, Uganda, Tanzania, Yemen, Zambia, Zimbabwe	*Bulinus* species. Many species in the *B. (Physopsis)* and *B. (Bulinus)* subgenera of *Bulinus*.	As for *S. mansoni*	Urine (rarely feces). Size: 150 by 62 μm
S. mekongi	Intestinal schistosomiasis: Cambodia, Laos, Thailand	*Neotricula aperta*	As for *S. japonicum*	Feces. Size: 57 by 66 μm
S. intercalatum	Rectal schistosomiasis: Cameroon, Central African Republic, Chad, Republic of the Congo, Democratic Republic of Congo, Equatorial Guinea, Gabon, Mali, Nigeria, Sao Tome, and Principe	*Bulinus (Physopsis) africanus, Bulinus (B.) camerunensis*		Feces. Size: 176 by 66 μm
S. bovis, S. mattheei, S. margrebowiei	African schistosomes infecting bovines and wildlife. Rare human infections.	*Bulinus* spp.		
S. sinesium, S. malayensis	Asian schistosomes infecting animals. Rare human infections.			
Species of *Austrobilharzia, Gigantobilharzia,* and *Trichobilharzia*	Swimmers' itch, cercarial dermatitis, worldwide distribution	*Bulineus, Lymnaea, Nassarius, Physa, Planorbis, Stagnicola*		Eggs not observed in humans

[a] P, praziquantel.

TABLE 2 Geographical distribution, hosts, and life histories of lung and hepatic digeneans of humans

Family	Species	Location in human host	Disease and geographic area	Snail hosts	Source of metacercariae	Human infection	Drug[a]	Eggs (specimen and size)
Fasciolidae	*F. hepatica* and *F. gigantica*	Bile ducts	Fasciolosis, worldwide as disease of livestock; humans: Australia, Bolivia, China, Cuba, Egypt, Ecuador, Iran, North Africa, Peru, Portugal, Turkey, Vietnam	*Lymnea truncatulata, L. tomentosa, L. viatrix*	Vegetation: wet grass, watercress, water mint, semiaquatic vegetables	Emerging disease, 2.4 million infections worldwide	T, 10 mg/kg	Feces. Size: *F. hepatica,* 130–150 by 60–85 μm; *F. gigantica,* 160–190 by 70–90 μm.
Opisthorchiidae	*C. sinensis, O. viverrini,* and *O. felineus*	Bile ducts	Opistorchiasis/clonorchiasis, China, former Soviet Union, Cambodia, Korea, Laos, Taiwan, Thailand, Vietnam	*C. sinensis:* various freshwater snails *(Alocinma, Bulinus, Parafossarulus) O. viverrini* and *O. filineus:* freshwater snails from genus *Bithynia* and related genera	Many species (>100) of freshwater fish	Focal but major disease in Thailand. Strong association with cholangiocarcinoma for *O. viverrini.*	P, 25 mg/kg for 2 days	Feces. Size: *C. sinensis,* 23–35 by 10–20 μm, *O. viverrini* and *O. felineus,* 30 by 12 μm.
Opisthorchiidae	*O. guayaquilensis, Metorchis albidus, M. comjunctus, Pseudamphistomum aethiopicum, P. truncatum*	Bile ducts	Opisthorchiasis: Asia	Not listed	Fish	Rare cases		
Troglotrematidae	*Paragonimus africanus, P. heterotremus, P. mexicanus, P. miyazakii, P. skrijabini, P. uterobilateralis, P. westermani*	Pulmonary cysts, also abdominal cavity, brain	Paragonimiasis: Cameroon, China, Colombia, Costa Rica, Cote d'Ivoire, Ecuador, Equatorial Guinea, Gabon, Guatemala, Honduras, India, Indonesia, Japan, Laos, Liberia, Malaysia, Mexico, Nepal, Nicaragua, Nigeria, North Korea, Panama, Papua New Guinea, Peru, Philippines, Poland, Russian Federation, Samoa, South Korea, Sri Lanka, Taiwan, Thailand, Venezuela, Vietnam	Operculate snail: *Semisulcospira, Thiara, Oncomelania*	Freshwater crabs, crayfish	Focal, but high prevalence in areas of endemicity, major cause of human disease. Pulmonary disease and more rarely extrapulmonary infection in brain and body cavity	T, 20 mg/kg; P, 35 mg/kg for 3 days	Feces, sputum. Size: varies with species.
Dicrocoeliidae	*Dicrocoelium* species	Bile ducts	Dicrocoeliasis: human cases in Czech Republic, Kenya, Nigeria, Russian Federation, Saudi Arabia, Somalia, Spain, United States	Land snails: Order Stylommatophora	Insects (ants)	Rare disease in humans. Spurious infections brought about by ingestion of infected animal livers	P?	Fecal, bile. Size: 38–45 by 22–30 μm; brown, thick-walled, embryonated, operculate

[a] T, triclabendazole; P, praziquantel.

TABLE 3 Digeneans of the human intestine[a]

Family	Genera	Disease and geographic area	Snail hosts	Source of metacercariae	Human infection	Drug[b]	Eggs (specimen and size)
Echinostomatidae	*Echinochasmus, Echinoparyphium, Echinostoma, Episthmium, Euparpyhium, Himasthla, Hypoderaeum, Paryphostomum*	Echinostomiasis: China, Egypt, Hungary, India, Indonesia, Italy, Japan, Korea, North and South America, Philippines, Romania, Singapore, Taiwan, Thailand	Families Viviparidae, Planorbidae	Fish (loach), molluscs (snails, clams), amphibians (tadpoles, frogs)	Focal, prevalence 5–44%	P	Feces. 100 by 65–70 μm.
Fasciolidae	*Fasciolopsis*	Fasciolopsiasis: Bangladesh, Cambodia, China, India, Indonesia, Korea, Laos, Pakistan, Taiwan, Thailand, Vietnam	Family Planorbidae	Water plants (water chestnut, caltrop, lotus roots, bamboo), other aquatic vegetation	Widespread but focal; prevalence rates up to 60% in children	P, 15–40 mg/kg; MB, 100 mg/kg	Feces. 130–140 by 80–85 μm.
Gastrodiscidae	*Gastrodiscoides, Gastrodiscus*	Gastrodiscoidiasis: China, India, Myanmar, Philippines, Russia, Thailand, Vietnam	*Helicorbus*	Squid, plants, crustaceans (crayfish), amphibians (frogs, tadpoles)	Rare, focal	NR	Feces. 127–169 by 62–75 μm.
Heterophyidae	*Appophalus, Centrocestus, Cryptocotyle, Diorchitrema, Haplorchis, Heterophyes, Heterophyopsis, Metagonimus, Phagicola, Procercovum, Pygidiopis, Stellantchasmus, Stichodora*	Metagonimiasis or heterophyiasis: Balkans, Brazil, China, Egypt, Greenland, Indonesia, Israel, Japan, Korea, Philippines, Russia, Spain, Sudan, Taiwan, Tunisia, Turkey, United States	Families Thiaridae, Littoriniidae	Fish (freshwater or brackish, carp, mullet, cyprinoids), crustaceans (shrimp)	Low prevalence, but common; cases with heavy infections of clinical significance	P, 10–20 mg/kg	Feces
Nanophyetidae	*Nanophyetus salmincola*	Nanophyetiasis: Russia, United States	*Oxytrema silicula*	Fish (salmon, trout)	Rare	P	64–97 by 34–55 μm

[a] Modified from Fried et al. (13).
[b] Abbreviations: P, praziquantel; MB, mebendazole, NR, not recorded.

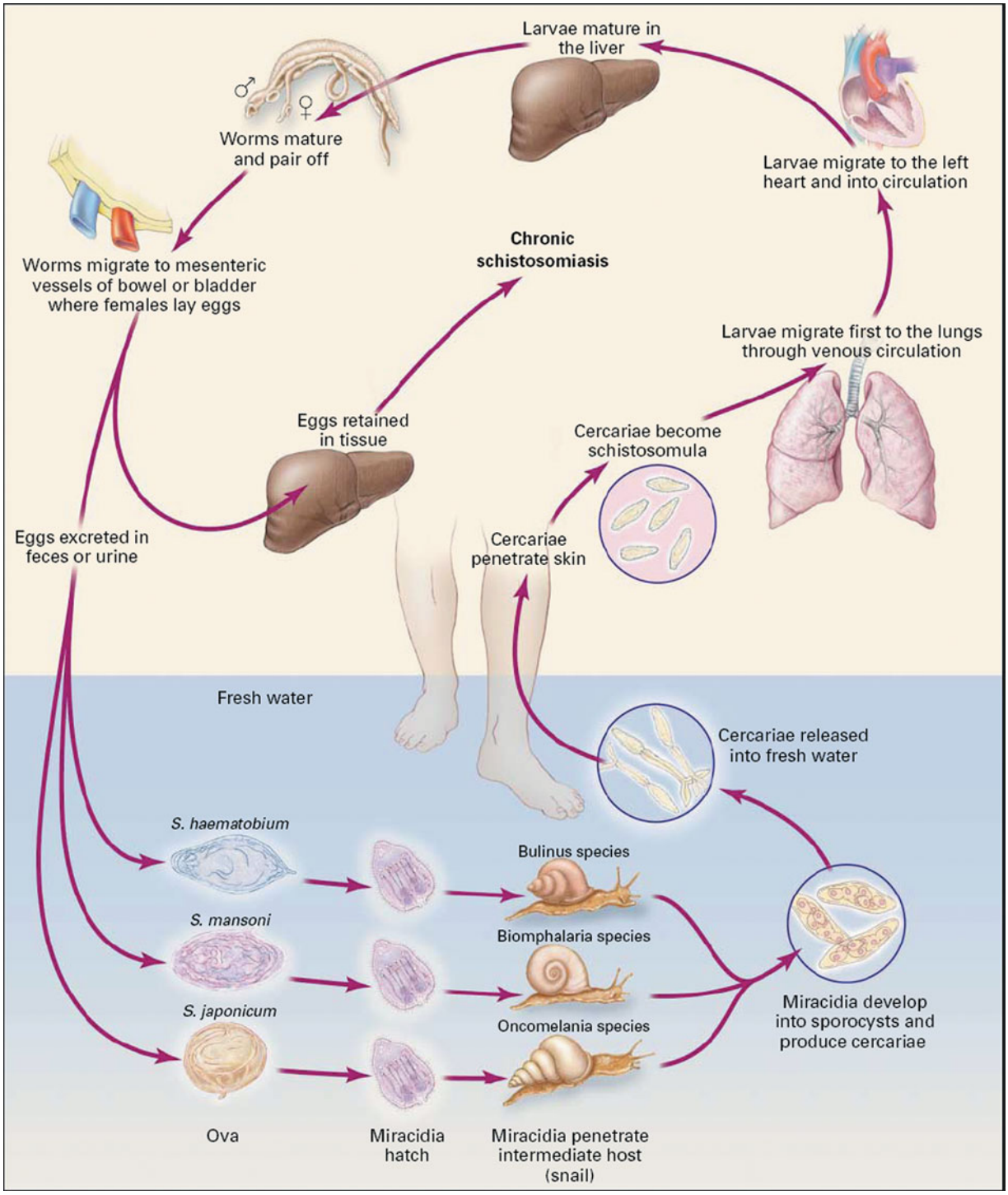


FIGURE 1 Five species of *Schistosoma* are known to infect humans. Infection with *Schistosoma mansoni, S. japonicum, S. mekongi,* or *S. intercalatum* adults occurs in mesenteric veins; *S. haematobium* adults occur in the vesical plexus. Humans are infected after cercarial penetration of the skin. After penetration, the cercariae shed their bifurcated tails, and the resulting schistosomula enter capillaries and lymphatic vessels en route to the lungs. After several days, the worms migrate to the portal venous system, where they mature and unite. Pairs of worms then migrate to the site of patent infection. Egg production commences 4 to 6 weeks after infection. Eggs pass from the lumen of blood vessels into adjacent tissues, and many then pass through the intestinal or bladder mucosa and are shed in the feces or urine (see the text). In freshwater, the eggs hatch, releasing miracidia that, in turn, infect specific freshwater snails (Table 1). Reprinted from the *New England Journal of Medicine* (32), used with permission.

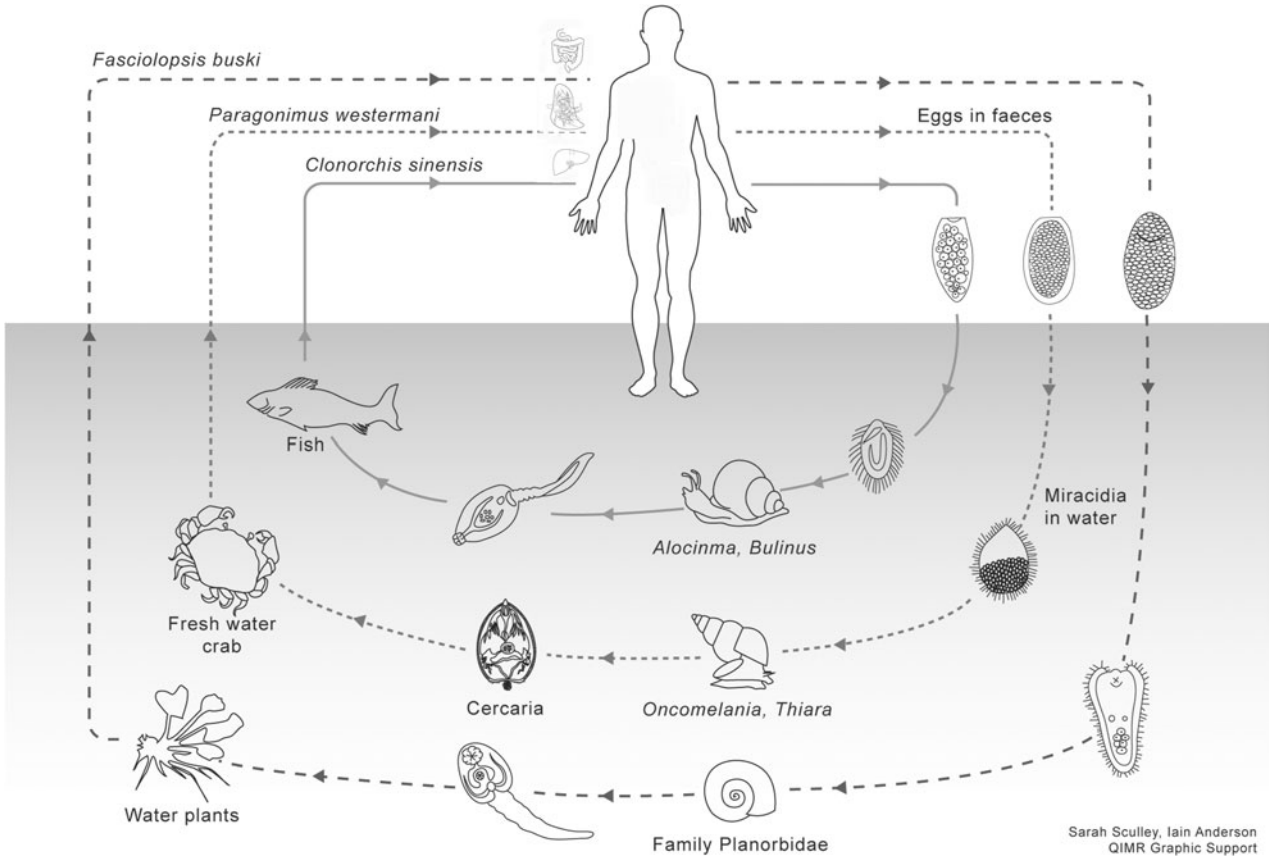


FIGURE 2 Life cycles of some foodborne trematodes, exemplified by *C. sinensis,* a parasite of human liver and bile ducts, *P. westermani,* a parasite of human lungs, and *F. buski,* an intestinal parasite. The life cycles of all species involve a snail as first intermediate host. After reproductive generations in the snail, a cercaria emerges which encysts in or on the second intermediate host or on vegetation. The second intermediate host for each species is listed in Table 2. Adapted from an illustration from the *Atlas of Medical Parasitology* (http://www.cdfound.to.it/HTML/atlas.htm), used with permission.

commonly infect humans, of which three (*S. mansoni, S. japonicum,* and *S. haematobium*) are the most important (Table 1). Morphological descriptions of adult worms, which are not encountered in diagnostic samples, are summarized elsewhere (28).

Hepatosplenic Schistosomiasis

The most extensively studied schistosome, *S. mansoni,* occurs throughout sub-Saharan Africa, Egypt, the Middle East, Madagascar, eastern countries of South America, and some islands of the Caribbean (Table 1). It is believed that the species was carried to South America with the slave trade. The distribution of this parasite overlaps that of *S. haematobium* in many parts of Africa. *Schistosoma japonicum* is responsible for significant disease in foci in Asia (10) (Table 1) and has been eradicated from Japan. Schistosomiasis in the Mekong Basin, once attributed to *S. japonicum,* is now known to be caused by *Schistosoma mekongi.* Sustained control efforts in China have reduced the number of infected people from approximately 12 million to 1 to 2 million by the late 1980s and less than 1 million in 1998 (9, 10). Unlike most other human schistosomes, *S. japonicum* is a zoonosis, and large bovines, especially water buffalo, are of primary concern in perpetuating the life cycle (26). Control strategies in China are directed at mass or targeted chemotherapy of humans and zoonotic hosts, thereby minimizing human morbidity due to schistosomiasis.

S. mekongi has a highly focal distribution in the Mekong Basin, with foci of endemicity in Laos and Cambodia (4). Although originally regarded as a subspecies of *S. japonicum,* the species was recognized as distinct based on morphological characteristics and life cycle patterns. In 1990, some 80,000 people of an at-risk population of over 150,000 people were infected, but the number of infected individuals was reduced to 7,000 by mass chemotherapy (34). After cessation of chemotherapy campaigns, prevalence has increased again in Laos (9).

Schistosoma intercalatum is responsible for rectal schistosomiasis in regions of Africa. The species occurs as two geographically isolated strains (now considered as distinct species [19]), the Cameroon and the Democratic Republic of Congo strains, which display highly focal distribution (10). This parasite belongs to the *S. haematobium* group of species, characterized by, among other features, eggs with a terminal spine (compare with Fig. 3).

Genitourinary Schistosomiasis

S. haematobium, the sole agent of urinary schistosomiasis, occurs in much of the African continent as well as Madagascar and the Middle East (10, 28) (Table 1). Adult

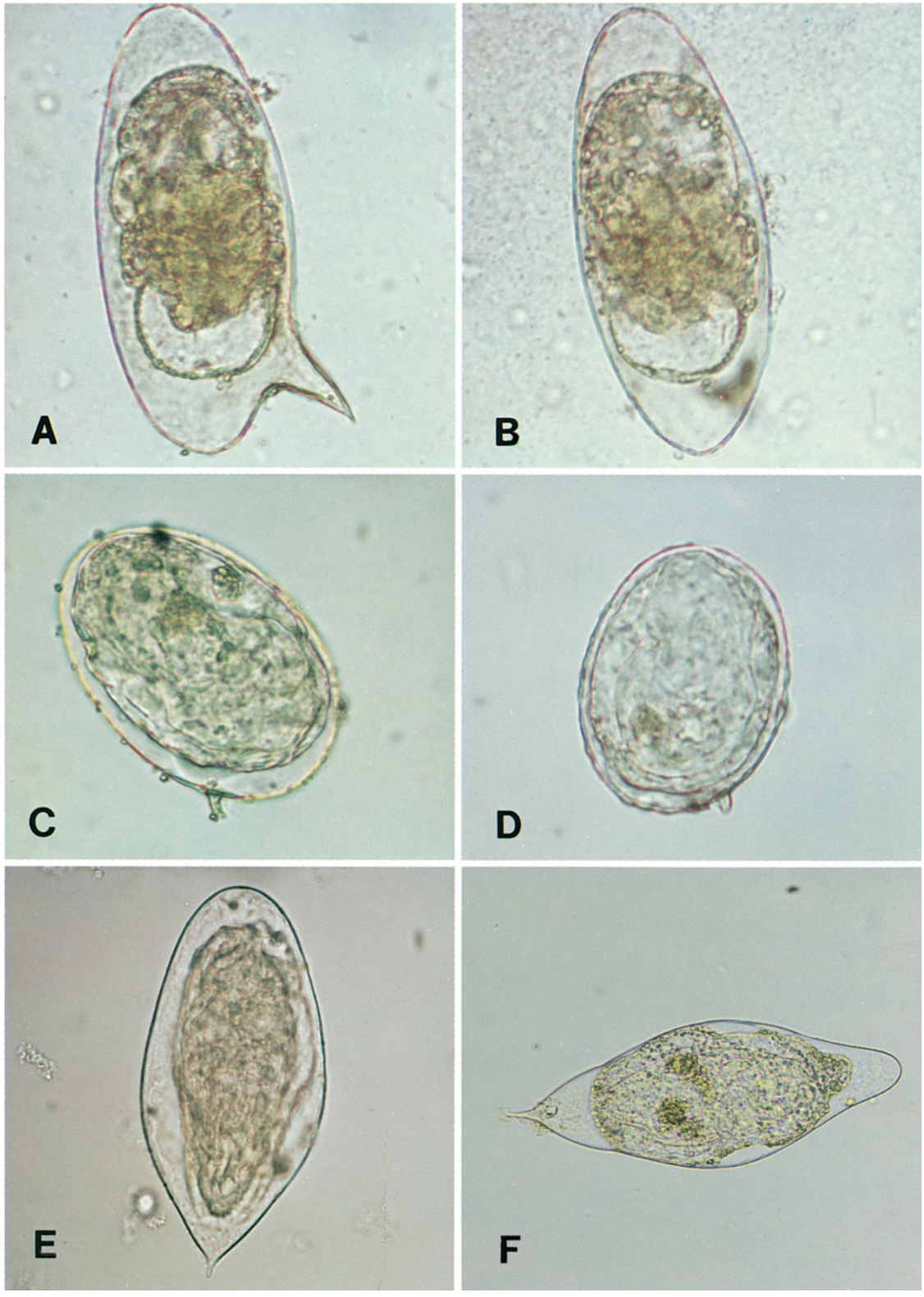


FIGURE 3 Eggs of schistosome species (magnification, ×600). (A) *S. mansoni;* (B) *S. mansoni* egg with typical lateral spine not in view; (C) *S. japonicum;* (D) *S. mekongi;* (E) *S. haematobium;* (F) *S. intercalatum.* (Panels C through E are from reference 3; used with permission.)

worms live in the vesical plexus, and eggs pass from the host across the bladder wall and are excreted with urine.

Epidemiology, Transmission, and Prevention

The life cycles of schistosomes are shown in Fig. 1. Humans are infected through direct penetration of the skin by cercariae. Endemicity is governed by the geographic distribution of snail hosts. The recent development of large-scale dams in countries of endemicity has led to changes in transmission dynamics (10). The impact of the Three Gorges Dam in China on the transmission of *S. japonicum* is uncertain but may lead to increased geographical distribution of the parasite in that country (26). Within regions of endemicity, factors contributing to schistosome transmission include the distribution biology and population dynamics of the snail hosts, extent of contamination of freshwater with feces or urine, and degree of exposure of humans to contaminated water.

Clinical Significance

Cercarial dermatitis occurs in schistosomiasis but is more commonly reported after infection with avian schistosomes (Table 1) and *Schistosoma spindale.* Acute toxemic schistosomiasis (Katayama fever) can occur with any schistosome species but is more apparent in nonimmune individuals and may be characterized by fever, headache, generalized myalgias, right-upper-quadrant pain, and bloody diarrhea (32).

Eggs (Fig. 3) are laid by female worms in the vasculature and must traverse endothelia and gut or bladder mucosa to escape the body. In those schistosomes infecting mesenteric veins, eggs pass through the wall of the intestine or rectum, while for *S. haematobium,* the eggs escape across the bladder wall. For all species, however, many ova become entrapped in tissues. The chronic effects of schistosomiasis, therefore, relate to the site of adult infections and granulomatous and fibrotic responses to entrapped parasite eggs (32). Granulomas occur in many tissues in response to entrapped eggs; however, most accumulate in tissues fed by vasculature leading from the site of infection. Eggs retained in the gut wall induce inflammation, hyperplasia, and ulceration, and occult blood occurs in the feces. A suggested relationship between colorectal cancer and schistosomiasis remains controversial (31). Eggs entrapped in the liver lead to portal hypertension and splenic and hepatic enlargement, potentially giving rise to the formation of fragile esophageal varices. Ascites also is common.

In urinary schistosomiasis, granulomatous inflammatory response to embolized eggs gives rise to dysuria, hematuria, and proteinuria, calcifications in the bladder, obstruction of the ureter, renal colic, hydronephrosis, and renal failure. Secondary bacterial infection of the bladder and other affected tissues may occur. There is consistent association between *S. haematobium* infection and squamous-cell carcinoma of the bladder (32). *S. haematobium* infection causes genital disease in approximately one-third of infected women, leading to vulval and perineal disease and tubal infertility, and may facilitate the transmission of human immunodeficiency virus.

Direct Examination

The presence of schistosome eggs (Fig. 3) (3) in feces or urine is diagnostic of schistosomiasis. Eggs of hepatosplenic schistosomes may be observed by light microscopy in stool specimens with or without suspension in saline. Formalin-based techniques for sedimentation and concentration are particularly useful, especially for patients releasing few eggs. Hatching tests, in which fecal matter is suspended in nonchlorinated water in darkened vessels with directed surface light, have been used to detect motile miracidia. It is often important to observe larval activity (flame cell beating or miracidial swimming) in eggs for diagnosis of active infection. In chronically infected patients with a typical clinical presentation but with negative urine and feces specimens, a biopsy of bladder or rectal mucosa must be used for diagnosis. However, parasitological examination remains the most sensitive diagnostic procedure available.

Examination of stools with the Kato-Katz technique is used in field studies for quantification of fecal egg burdens. The Kato-Katz method gives a theoretical sensitivity cutoff of 20 eggs per g of feces (12), but large daily variation in egg shedding and the uneven distribution of eggs in feces may lead to inconsistent counts. It has been recommended for *S. mansoni* infections that either five replicate slides or sets of triplicate slides made from stools collected on two successive days be used for assessment of infection status (6).

Sedimentation or concentration methods are most useful for diagnosis of *S. haematobium* from urine samples. In addition, the use of tests for blood, protein, and eosinophils in urine, while not specific, may be indicative of infection (1). Serological detection of schistosome infections is useful, but its use is limited because antibodies persist after parasitologic cure and because of problems of cross-reactivity (32). A positive serologic test may be diagnostic for patients for whom there are no excreted eggs. Enzyme-linked immunosorbent assays (ELISA) and immunoblot tests using soluble egg antigen and recombinant antigens are used (1), as are tests for evidence of peripheral-blood eosinophilia, anemia, hypoalbuminemia, elevated urea and creatinine levels, and hypergammaglobulinemia (1, 32). Portable ultrasound is useful for assessment of pathologic damage to tissues. The use of questionnaires for populations in which the disease is endemic is helpful in revealing infections caused by *S. japonicum* and *S. haematobium,* but for *S. mansoni* this method may lead to underestimation of prevalence (36).

PCR tests for schistosomes in stools use high-repeat nuclear genomic (30) or mitochondrial sequences (14). Both tests report high sensitivity, at times exceeding that of direct coprological examination. The mitochondrial sequence probes, which amplify sequences spanning the *cox2/nad6* genes for *S. japonicum* and *S. mansoni, nad1-2* for *S. japonicum,* and *nad5* for *S. mansoni,* show absolute specificity and an absence of false-positive reactions (14).

Treatment

The drug of choice for schistosomiasis, and effectively the only drug available for use, praziquantel, has seen widespread application in many regions of endemicity. There has been spectacular success in mass treatment campaigns with praziquantel in many countries, and its use has become more widespread because the cost of drug production has reduced dramatically in recent years. Despite this, the anthelmintic has some limitations, being effective against only adult forms in which it induces spastic muscle contractions and tegument degradation (15). Praziquantel has been suggested as a compound therapy with oxamniquine, and there have been reports of efficacy with triclabendazole (35). The development of praziquantel-resistant schistosomes cannot be discounted.

The effects of artemesinin derivatives against schistosomes have been studied in experimental model and clinical field settings. Artemether acts against schistosomules and may provide protection against reinfection (5). The use of artemether is contraindicated in areas in which malaria is coendemic, since widespread use may lead to drug resistance in *Plasmodium.*

Schistosome Dermatitis (Swimmer's Itch)

This minor ailment arises from infection with a number of avian and mammal schistosomes (Table 1). The avian schistosome cercariae cannot migrate from the dermal layers of human skin and induce local dermatitis accompanied by itching, pruritus, and papule formation.

FOODBORNE DIGENEANS

All other digeneans we will consider are a diverse assemblage of taxa, some of which infect humans through ingestion of uncooked food (Fig. 2; Tables 2 and 3). The vast majority of these species are zoonotic. The primary epidemiological features governing human infection include the distribution of suitable snail intermediate hosts, human food consumption behaviors, the presence of suitable zoonotic hosts, and the potential for water contamination with human or animal excreta.

Trematodes of the Respiratory System—*Paragonimus* (Troglotrematidae)

Taxonomy

Paragonimiasis is caused by a number of species distributed throughout Asia, Africa, and the Americas (Table 2). The most common species infecting humans is *Paragonimus westermani,* which occurs in China and other Southeast Asian nations. Adult *Paragonimus* species inhabit the lungs, where they induce formation of encapsulating cysts. The flukes are large and fleshy, measuring 8 to 16 by 4 to 8 mm. Taxonomic information on the species is presented elsewhere (7).

Epidemiology, Transmission, and Prevention

The life cycle of *Paragonimus* is shown in Fig. 2 and Table 2. Of the more than 40 species, 9 have been recorded as human parasites. Eggs are passed in the lungs and are transferred up the bronchial tree with sputum. Eggs may be spat with sputum or swallowed and passed with feces. The first intermediate hosts are freshwater snails. Human infection arises after ingestion of uncooked or marinated freshwater crabs or crayfish (1), but unwashed hands of food preparers and contaminated utensils also may be a source of human infection. After ingestion, the immature flukes penetrate the intestinal wall and migrate to the lungs through the body cavity. All species are zoonotic, infecting carnivores, pigs, and rodents.

Clinical Significance

Some 20.7 million people have paragonimiasis, and a further 195 million are at risk of infection. Signs of infection include fever with dry cough, sometimes blood-stained sputum containing eggs, chest pain, dyspnea, and bronchitis, and symptoms sometimes resemble those of pulmonary tuberculosis. Peripheral blood eosinophilia is common. The flukes induce the formation of an epithelial cyst, measuring approximately 1 cm in diameter, and which may calcify over time. Parasites may also occur in extrapulmonary locations, and serious complications occur when parasites are found in the brain. The necrosis of brain tissues and extensive granulomatous inflammatory response may induce symptoms similar to those of cerebral cysticercosis, caused by the cestode *Taenia solium* (1).

Diagnosis

Diagnosis of paragonimiasis is largely dependent on observation of the eggs (3) (Table 2 and Fig. 4) in sputum, feces, or pleural effusions. A cough with brown sputum is indicative of lung infection, and the sputum should be examined for eggs. The cysts formed around adult worms appear in X rays as characteristic rings or nodules, but direct observation of eggs is required to differentiate the disease from pulmonary tuberculosis. Serological tests include ELISAs using either 32- and 35-kDa antigens or parasite yolk ferritin as antigen (1). Pleurisy with eosinophilia and a dominant immunoglobulin M antibody titer may be indicative of paragonimiasis (29). A PCR-based method to detect a 500-bp sequence of nuclear genomic DNA has been used for molecular diagnosis in experimental models of *Paragonimus heterotremus* (16).

Treatment

The current WHO recommendation for treatment is administration of triclabendazole at 20 mg/kg of body weight in two divided doses (20). Praziquantel, administered at 25 mg/kg for 2 to 3 consecutive days, has been used (20).

Trematodes of the Liver

At least 13 species of flukes, belonging to the families Fasciolidae, Opisthorchiidae, and Dicrocoeliidae (18) (Table 2), have been recorded as adult worms from the liver and bile ducts of humans. Most human liver fluke species belong to the family Opisthorchiidae. Infections with members of the family Dicrocoeliidae are not considered further here. Brief information on the family is provided (Table 2), and the reader is referred to other reviews (28).

Family Opisthorchiidae

Taxonomy

Of the opisthorchiid parasites infecting humans (Table 2), only three species are of major significance, namely, *Opisthorchis viverrini, Opisthorchis felineus,* and *Clonorchis sinensis.* Adult opisthorchiids of humans are macroscopic, flattened flukes, approximately 5 to 10 mm in length.

Epidemiology, Transmission, and Prevention

The life cycle information is presented in Fig. 2 and Table 2. In opisthorchiids, the metacercariae are found encysted in the muscles of cyprinid fish (18). In regions of high endemicity, infection rates can be as high as 90% in humans and up to 97% in fish hosts. Individuals are infected by eating undercooked fish. In Thailand, consumption of traditional foods, including marinated fish, leads to infection with *O. viverrini* (33).

Clinical Significance

Disease severity varies among the different species (33) and with the intensity of infection. *C. sinensis* infects approximately 7 million people in China, Hong Kong, India, North Korea, Siberia, Taiwan, and Vietnam. *O. viverrini* infects about 9 million people in Thailand, Laos, and Cambodia, and *O. felineus* is widespread throughout Northern Europe and Asia, infecting about 1.5 million people. All three species live in the bile duct and are thought to feed on biliary epithelia. Light infections are usually asymptomatic, but heavy infections can induce disease. Symptoms most commonly are associated with an acute phase of infection and may include fever, abdominal pain, hepatitis-like symptoms, and eosinophilia. A number of asymptomatic hepatobiliary abnormalities are associated with infection (24). A strong correlation exists between chronic *O. viverrini* infections and cholangiocarcinoma.

Diagnosis

Eggs of opisthorchiids (Fig. 4) are embryonated when laid, are oval and yellowish brown, and are operculate, with

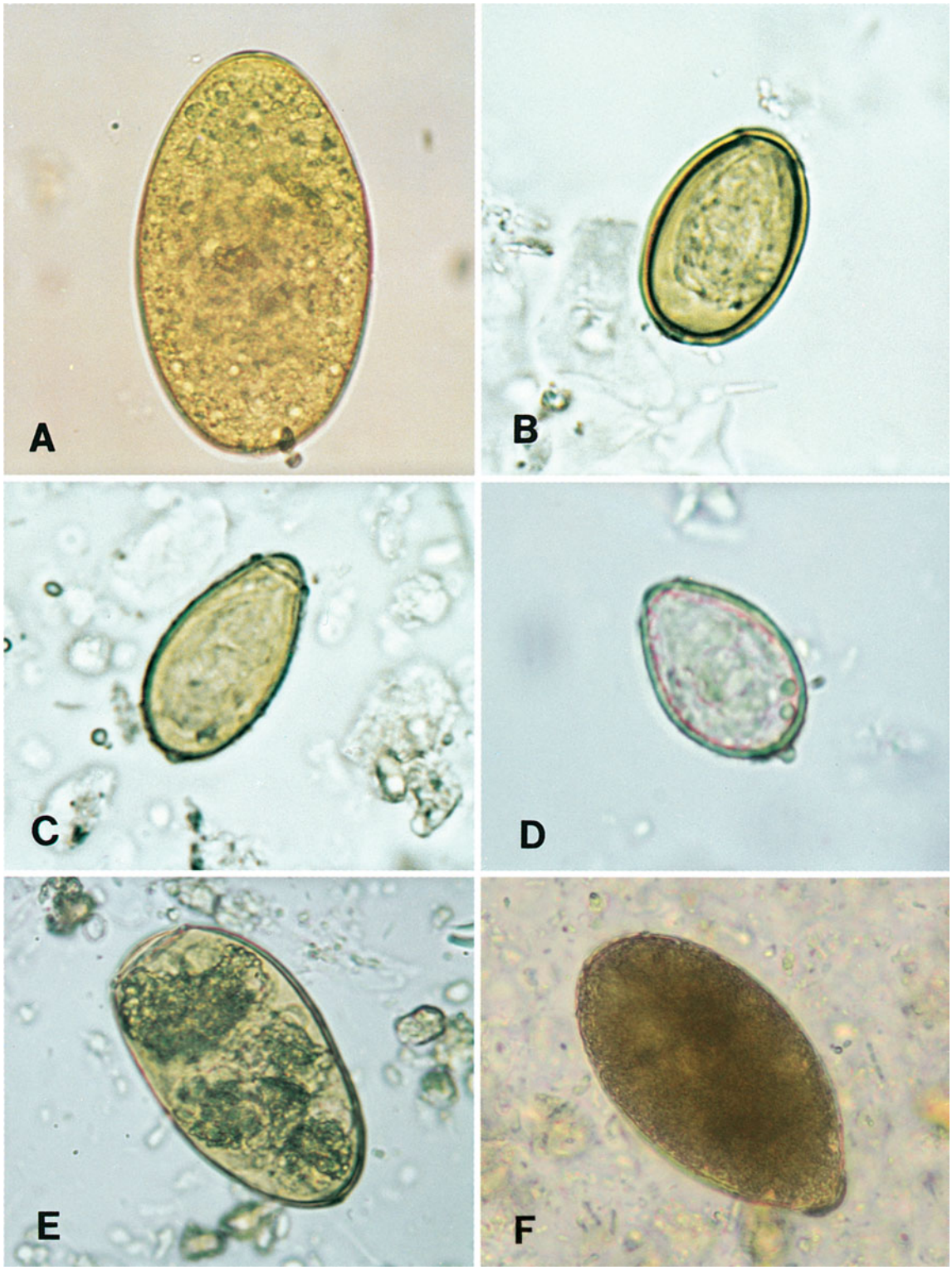


FIGURE 4 Eggs of trematode parasites. (A) *F. buski* (magnification, ×500); (B) *H. heterophyes* (magnification, ×1,500); (C) *C. sinensis* (magnification, ×1,500); (D) *O. viverrini* (magnification, ×1,500); (E) *P. westermani* (magnification, ×600); (F) *N. salmincola* (magnification, ×750). (Panels B through D are from reference 3; used with permission.)

a shoulder or thickened region of eggshell surrounding the operculum. The shell surface may appear rough and may have a small knob at the abopercular pole of the egg (18). The eggs are smaller than those of many other digeneans of humans, ranging from 20 to 35 μm in length. Opisthorchiid eggs may be confused with those of other taxa, especially the heterophyids (see below). Differential diagnosis may be arrived at by patient history and by examination of purged worms in feces after anthelmintic treatment.

Many antigen assays for *O. viverrini* have been tested, but most are plagued by cross-reactivity problems and persistence of antibodies after parasitologic cure (37). A coproantigen ELISA has been developed using monoclonal antibodies raised against adult *O. viverrini* E/S antigen and has displayed high specificity and sensitivity, whereas ELISAs for circulating antibodies show high sensitivity but low specificity (1). A PCR test, based on a 334-bp molecular probe amplifying a highly repetitive nuclear genomic sequence, also shows promise (37). Recently, a multiplex PCR assay using mitochondrial sequences has been developed for opisthorchioids of humans. For this assay, the single reaction uses two sets of primers designed for a 612-bp product corresponding to the *nad2-V-A-D-nad1* region of the mitochondrial genome of *C. sinensis* and for a 1,357-bp product spanning *nad1-N-P-I-K-nad3* for *O. viverrini* (23).

Treatment

Treatment for clonorchiasis or opisthorchiasis relies on oral administration of praziquantel. The benzimidazole drugs, albendazole and mebendazole, have been tested in animal models and clinical trials, with encouraging efficacy, as has artemether (20).

Family Fasciolidae

Taxonomy

Fascioliasis, caused by flukes of the genus *Fasciola,* is primarily a disease of herbivorous mammals. Until recently, fascioliasis was considered a sporadic infection of humans, but estimates indicate that some 2.4 million persons in 61 countries are infected and there are 180 million individuals at risk throughout the world (25). The two causative species, *Fasciola hepatica* and *Fasciola gigantica,* have a worldwide distribution in domesticated animals; human disease is focal and regions where human fascioliasis is endemic are recognized (1, 25, 28).

Epidemiology, Transmission, and Prevention

Fasciolid metacercariae encyst on semiaquatic or moistened vegetation, predominantly watercress, grass, water mint, or salad vegetables (Fig. 2 and Table 2). The frequency of the parasites in domesticated animals does not necessarily correlate with human disease. Areas of low human endemicity include regions of France and Chile; regions of high endemicity are in Peru and Bolivia (25). Most of the areas with high endemicity are regions where *F. hepatica* is present. While common zoonotic hosts are cattle and sheep, other hosts, such as pigs, equines, and rats, may serve as reservoir hosts for human infection. Children more commonly are infected than adults, suggestive of the development of an age-dependent immunity (38).

Clinical Significance

Many infections with fasciolids remain asymptomatic. Acute disease arises because of extensive tissue damage as parasites migrate through the hepatic parenchyma to gain access to the bile ducts. Parasite activity in the bile ducts leads to proliferation of ductal epithelium, inflammation, and fibrosis. Heavy infections can lead to cholestasis and result in hepatic atrophy and periportal cirrhosis (1). Common symptoms in chronic infection include biliary colic and cholangitis. Eosinophilia is common in all stages of disease.

Diagnosis

The eggs of fasciolids are large compared with those of other digeneans (Table 2). *Fasciola* eggs cannot be distinguished from those of the related intestinal parasite *Fasciolopsis buski* (Fig. 4) and also resemble those of *Gastrodiscoides* (Table 3). Liver fasciolids have a long (2-month) prepatent period, and because of this, fascioliasis is one disease where serological diagnosis is valuable. Immunological tests, particularly ELISA, based on a cysteine protease, display high sensitivity and specificity (11).

Treatment

Fasciola species appear to be insensitive to praziquantel, and triclabendazole is the drug of choice (Table 2). Recently, there have been reports of triclabendazole-insensitive isolates in livestock and humans (20). New derivatives of triclabendazole are being tested in clinical trials for use in humans (20).

Trematodes of the Intestine

Most trematodes infecting humans are parasites of the intestine and, of these, most belong to two families, the Echinostomatidae and Heterophyidae. Fried et al. (13) list the digenean families with species parasitizing humans, of which we will mention the two families above and the Fasciolidae and Nanophyetidae. Summary information on some families of intestinal trematodes is presented in Table 3. Families Brachylaimidae, Diplostomidae, Gymnophallidae, Lecithodendriidae, Microphallidae, Paramphistomatidae, Plagiorchiidae, and Strigeidae are rarely encountered in humans and are not considered further in this chapter. For further information on intestinal trematodes not provided here, the reader is referred to chapter 148 and to Fried et al.

Echinostomatidae

Echinostomatid flukes are small, typically 3 to 10 mm in length and 1 to 3 mm in width, with a large ventral sucker and distinctive collared spines. At least 24 species are known to parasitize humans, mostly in Asia (Table 3). All species are zoonotic and focal in distribution, and foci are often in the vicinity of fresh- or brackish water habitats. Humans are infected by eating a range of raw or undercooked vertebrate and invertebrate foods (Table 3), including snails. In most cases, infection is asymptomatic. Heavy infections can lead to a range of symptoms including flatulence, colic, and diarrhea. Some heavy infections in children have been fatal. Eggs of echinostomatids are similar in shape to those of fasciolids but are smaller (Table 3). Interspecific variation in egg size occurs among the echinostomes, and species identification is not possible unless adult worms are obtained by purgation with anthelmintics. A single dose of praziquantel is recommended for treatment (20).

Fasciolidae

Fasciolopsis buski is the largest fluke parasitizing humans, measuring 8 to 10 cm in length and 1 to 3 cm in width (22). Adult worms inhabit the duodenum and jejunum of humans and a small range of other hosts that include pigs, horses, cattle, goats, and sheep. The species is distributed focally in many countries (Table 3). Adults attach and feed on the

intestinal wall. Human disease relates to the severity of infection, and symptoms vary from intestinal disturbance and pain, associated with eosinophilia, to severe diarrhea, gastric pain, bowel obstructions, and nausea. Feces are often profuse, are yellow-green, and may contain undigested food particles. The intermediate hosts of *F. buski* are shown in Table 3. Humans are infected by eating aquatic plants on which metacercariae are encysted or by drinking metacercariae that have encysted on the water surface (13).

The eggs of *F. buski* are large, operculate, nonembryonated, ellipsoidal, and yellow (Table 3 and Fig. 4). The eggs are very similar to those of other fasciolids from which species may be distinguished by observation of purged adults. The current drug of choice is praziquantel, but albendazole and mebendazole have also been tested (13, 20).

Heterophyidae

Many species of heterophyid trematodes are known to infect humans (8, 13). Heterophyids are mostly small parasites, less than 0.5 mm in length. Some species are marine. The most commonly encountered heterophyids of humans are *Heterophyes heterophyes* and *Metagonimus yokogawai.* The former species has been reported in humans from Egypt, Sudan, Iran, Turkey, Tunisia, China, Taiwan, the Philippines, Indonesia, and India (13). *M. yokogawai* is common in China, Indonesia, Japan, Korea, Taiwan, and Siberia.

The adult flukes are intestinal inhabitants of a wide range of piscivorous birds and mammals. Eggs (Fig. 4) are embryonated when passed from the host but do not hatch until ingested by a snail intermediate host (28). Cercariae will encyst in a range of fish including mugilids, cyprinids, and gobiids. Humans are infected by consumption of raw, freshly salted, or undercooked fish (Table 3).

Disease symptoms in humans relate to infection intensity and arise because of parasite irritation of the intestinal mucosa. Eggs may on occasion pass into the bloodstream and lodge in tissues. These eggs have been known to cause fatal myocarditis in the Philippines (1). Diagnosis of heterophyids is facilitated by observation of eggs in feces (Table 3). Heterophyid eggs are similar in size to those of opisthorchiid liver flukes, and care must be taken to differentiate the infections. Often this can be achieved only by examination of purged adult worms. The recommended treatment for heterophyid infection is a single dose of praziquantel (Table 3).

Nanophyetidae

This family of small oval flukes parasitizes mammals, including humans. One species, *Nanophyetus salmincola,* is found along the coastal U.S. Pacific Northwest as metacercariae of salmonid fish. The species may be the most commonly encountered human trematode endemic to North America. Praziquantel is efficacious for nanophyetiasis.

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Less Common Helminths

GARY W. PROCOP AND RONALD C. NEAFIE

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This chapter covers the less common causes of helminthic parasitic infections, particularly those caused by the less common nematodes and cestodes. The trematodes are covered in entirety in chapter 147. This chapter is not inclusive, given the wide variety of helminthic parasites that have been reported to cause human disease, but includes some of the most interesting and challenging of parasitic diseases. The reader who desires further information about these and other helminths of medical importance is directed to *Pathology of Infectious Diseases,* volume 1, *Helminthiasis,* by the Armed Forces Institute of Pathology (AFIP) (2). The Centers for Disease Control and Prevention's (CDC) parasitology website (http://www.dpd.cdc.gov/dpdx) is another excellent resource for information regarding parasitic infections.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

The collection, transport, and storage of specimens are similar regardless of the helminth present, so these guidelines are consolidated here to reduce duplication. Worms present in the lumen of the gastrointestinal tract, such as *Anisakis,* can be retrieved by endoscopy. Surgical resection specimens may contain a worm or larvae. The intact worm and surgical specimens may be preserved and transported in 10% neutral buffered formalin. Stool is an important diagnostic specimen for parasites that reproduce within the intestinal tract, e.g., *Capillaria philippinensis,* and should be collected and fixed in a standard manner for the detection of eggs and parasites (i.e., formalin and polyvinyl alcohol), if available. Blood for serology should be handled in a standard manner. Additional information about serologic tests for the diagnosis of parasites covered in this chapter is available through the CDC at http://www.dpd.cdc.gov/dpdx/HTML/DiagnosticProcedures.htm.

LESS COMMON NEMATODES

Anisakis and Related Species

Description of Agent(s)

Anisakis species (of which *Anisakis simplex* is the type species), *Pseudoterranova (Phocanema) decipiens,* and *Contracaecum* species are nematodes of the family Anisakidae.

Epidemiology, Transmission, and Prevention

The larvae of anisakids, which are present in many varieties of fish, cause human infections if raw or poorly cooked fish is consumed (11). Fish that have been salted or pickled may still contain viable anisakid larvae. Disease may be prevented by consuming only thoroughly cooked fish.

Clinical Significance

The clinical features vary depending on the stage of disease. Early, within 12 h of eating infected fish, nonspecific gastric discomfort is experienced while the larvae are associated with only the superficial aspects of the mucosa. Superficial larvae may migrate up the esophagus and cause coughing, potentially with expectoration of the worm. More severe symptoms occur if the worm penetrates through the mucosa into the submucosal and deeper tissues (Fig. 1). This evokes hypersensitivity, leukocytosis, and eosinophilia (13, 14). Sudden, intense abdominal pain may be thought to represent appendicitis, acute gastritis, a gastric ulcer, or Crohn's disease (30). Diarrhea and constipation may occur, and the stool contains occult blood.

Direct Examination/Microscopy

The intact worms are white to cream, nonsegmented larvae that measure 10 to 50 mm by 0.3 to 1.2 mm. One dorsal and two subventral reduced lips and a triangular boring tooth are useful for identification. Histopathologic analysis of infected tissues often demonstrates cross-sections of the worm and the host inflammatory response, which contains numerous eosinophils. Cross-sectional studies are useful for identifying the type of anisakid present.

Serologic Tests

Serologic tests to detect the immunologic response to *Anisakis* and related species are available at commercial reference laboratories in the United States. Although this test is not currently performed at the CDC, they may be contacted for assistance in locating a commercial vendor, if necessary. A variety of serologic assays have been developed, which vary in sensitivity and specificity. These identify most infected individuals (85 to 95%), with a demonstrable serologic response occurring from 10 to 35 days postinfection. Cross-reactivity with other ascarids may occur. This may be desirable to detect the related species, e.g., *Pseudoterranova,* but would limit the utility of this assay in certain populations, such as those likely

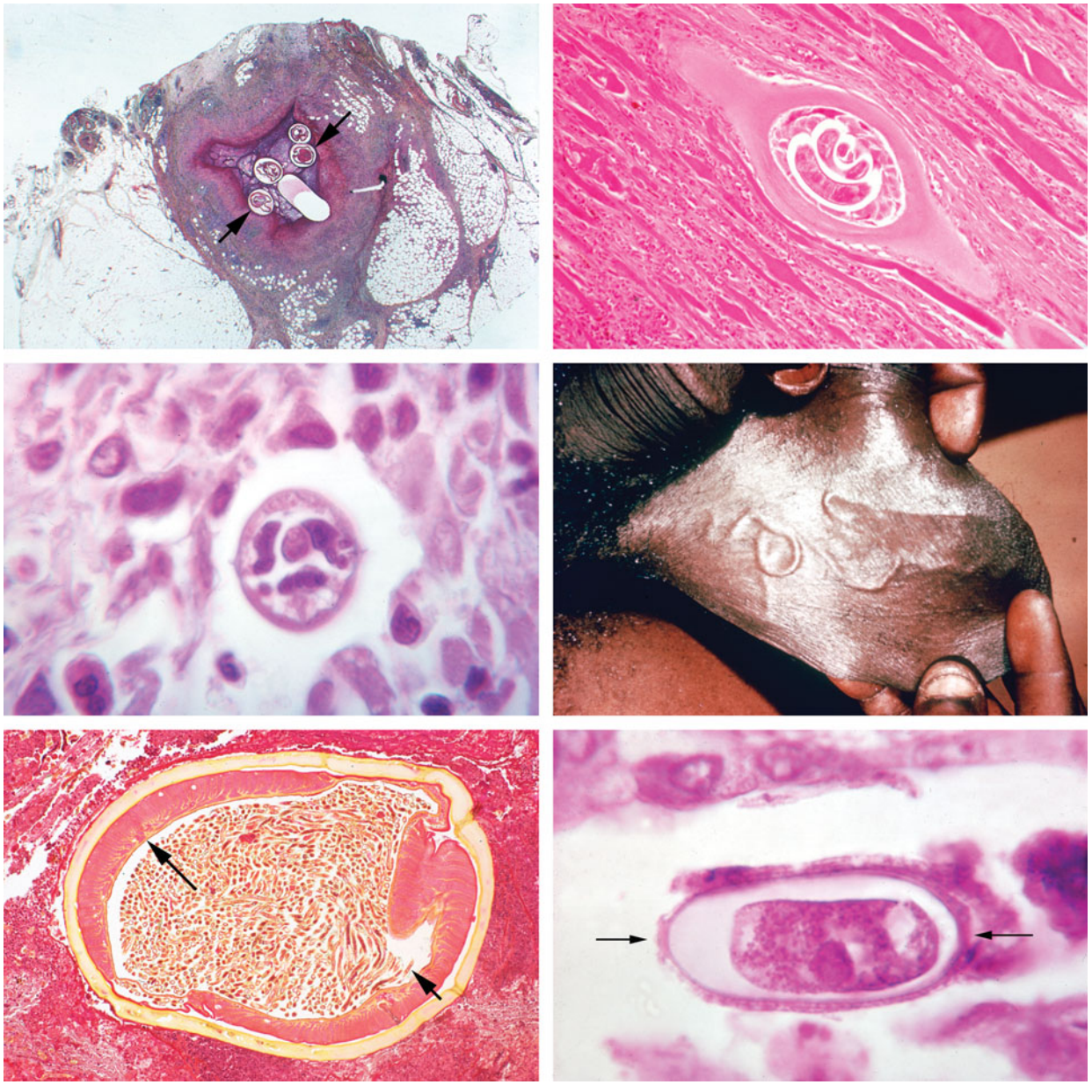


FIGURE 1 (top row, left) This *Anisakis* species (arrows) has penetrated into the deep tissues of the abdomen. Multiple cross-sections of the worm, which is 300 μm in diameter, are seen in the omentum. Movat stain; original magnification, ×2.5 (AFIP negative no. 96-5760).

FIGURE 2 (top row right) This coiled first-stage *T. spiralis* larva is in a "nurse cell." Note the hyaline, amorphous appearance of the external aspect of the nurse cell and the surrounding chronic inflammatory infiltrate. The worm diameter is 35 μm. Hematoxylin and eosin stain; original magnification, ×30 (AFIP negative no. 70-6004).

FIGURE 3 (middle row, left) The minute lateral alae are useful in the identification of *Toxocara* species. The worm diameter is 18 μm. Hematoxylin and eosin stain; original magnification, ×500 (AFIP negative no. 69-4372).

FIGURE 4 (middle row, right) The serpiginous tract of a female *D. medinensis* worm is demonstrated in the scrotum of this patient (AFIP negative no. 74-9011).

FIGURE 5 (bottom row, left) Rhabditiform larvae (short arrow) fill the body cavity of this gravid *D. medinensis* worm. Also note the presence of the two prominent bands of somatic muscle (long arrow). The worm diameter is 1.1 mm. Movat stain; original magnification, ×25 (AFIP negative no. 92-8429).

FIGURE 6 (bottom row, right) The bipolar plugs (arrows), pitted egg shell, and rectangular shape are characteristic of *Capillaria* species. This photomicrograph is from human small intestine and demonstrates an egg 40 μm long. Hematoxylin and eosin stain; original magnification, ×490 (AFIP negative no. 69-1287).

to harbor intestinal ascarids. Conversely, more specific assays have been devised to detect *Anisakis,* but these unfortunately do not detect the related species that may cause anisakiasis.

Treatment

Removal of the larva via endoscopy while it is only associated with the superficial mucosa is the most efficacious treatment, whereas surgical resection may be needed for more deeply insinuated larvae (47). Antihelminthic drugs do not appear to be useful, but corticosteroids may be used to decrease the associated inflammation.

Trichinella Species

Description of Agent(s)

Trichinella spiralis is the most important cause of human disease in this genus, but other species, such as *Trichinella pseudospiralis* and *Trichinella britovi,* may also cause trichinosis (23).

Epidemiology, Transmission, and Prevention

Trichinosis occurs worldwide. The life cycle of *T. spiralis* is simple in that the infected mammal that first serves as the definitive host also harbors infective larvae encysted in its muscle (i.e., the intermediate host) (Fig. 2) (4). The domestic pig is the most important host for the transmission of this roundworm to humans, but the meat of bear, walrus, and wild pigs may also contain infective larvae. The parasitic cycle is propagated in the barnyard between pigs and rodents. Disease may be prevented by eating only thoroughly cooked meat products of potential hosts and by attempting to control trichinosis in these hosts through good animal husbandry practices.

Clinical Significance

Infections vary from mild, subclinical disease to a severe illness, depending upon the parasite load. The clinical manifestations vary with the stage of infection. Gastrointestinal symptoms, associated with adult worms, last only about a week and include nausea, vomiting, abdominal pain, and diarrhea and/or constipation. Fever, facial edema that is particularly predominant around the eyes, myalgia, and marked peripheral eosinophilia are the four cardinal features of trichinosis. These are caused by the penetrating, migrating larvae. Light infections may only result in peripheral eosinophilia. The migration of larvae may cause organ-specific features. If the brain and meninges are primarily affected, neurologic symptoms predominate, whereas involvement of the myocardium causes myocarditis, and possibly arrhythmias causing sudden death. Larvae encyst not in these tissues, but rather in skeletal muscle.

Direct Examination/Microscopy

The direct microscopic examination of a muscle biopsy sample, performed by simply compressing the fresh muscle fibers between glass slides and observing microscopically, may disclose larvae. More commonly, larvae will be detected in the histopathologic analysis of the formalin-fixed, paraffin-embedded tissue sections (Fig. 2).

Serology

Antibody response begins 3 to 5 weeks after infection in most patients (80 to 100%) and follows the acute phase of disease. Therefore, the conversion from a negative serologic test during acute illness to a positive test is strong evidence of disease. The tests are highly sensitive, and although they are less than 100% specific, the cross-reactivity usually results in test values in the equivocal range. This test is available at the CDC and from several commercial vendors.

Treatment

Therapy varies according to the stage of disease. Thiabendazole is active against the intestinal worm, but not against the larvae. There is no proven method to kill the encysted larvae, but albendazole or another similar agent is used for therapy. Well-controlled therapeutic studies, however, are lacking. Corticosteroids, salicylates, and antihistamines may lessen symptoms associated with inflammation.

Toxocara Species

Description of Agent(s)

Toxocara canis is the intestinal ascarid of dogs and other canids, whereas *Toxocara cati* is the intestinal ascarid of cats. Although larvae of both species can cause toxocariasis in humans, most infections are caused by *T. canis* (20).

Epidemiology, Transmission, and Prevention

Toxocariasis has a worldwide distribution, but the prevalence of zoonotic disease varies widely by geographic area. The seroprevalence of human disease reflects helminthic control in dogs and cats. The highest infection rates occur among the poor, often in rural settings (19). The natural cycle of *Toxocara* begins with the passage of unembryonated eggs in the feces of the definitive host. The adult female worm may release up to 85,000 eggs per day. Therefore, one stool passage into an environment such as a sandbox can cause significant contamination. Between 10 and 20 days are required for the L2 (i.e., second-stage) larvae to develop in the eggs; the eggs at this stage are infective. If the eggs are ingested by a suitable definitive host, then the larvae penetrate the intestinal tract and migrate through the liver, bloodstream, and lungs, until they are swallowed to mature into adult worms in the intestinal tract. When the eggs are ingested by a nonpermissive host, such as a child, the resulting *Toxocara* larvae cannot complete their life cycle, but rather wander aimlessly in the aberrant host, causing visceral larva migrans. Another means of contracting disease is by ingesting raw or undercooked meat from a paratenic host (i.e., another aberrant host, such as a pig, that contains wandering larvae). Prevention is achieved foremost through zoonotic control by deworming dogs and cats, most importantly puppies, which are particularly permissive for infection and are often associated with children. Other preventive measures include avoidance of animal feces, thorough washing of fruits and vegetables, and thorough cooking of meats from potential paratenic hosts (42).

Clinical Significance

Toxocara infections are one of the most common helminthic infections of humans. Fortunately, most infections are subclinical, producing only mild disease for which a definitive diagnosis is not sought. The most severe forms of disease occur with involvement of the eyes, or significant involvement of the heart, brain, or other vital organs (3, 15, 34, 41). Pregnant women with toxocariasis may have miscarriages.

Direct Examination/Microscopy

Eggs are not produced in the human, so a stool evaluation is not helpful. However, occasionally the eggs of other geohelminths such as *Ascaris,* hookworm, or *Trichuris* may be present because of common risk factors. The detection of the migrating larvae in surgically excised tissues by histopathologic evaluation provides the definitive morphological diagnosis.

The single minute lateral alae and the cross-sectional diameter are useful criteria for identifying these parasites in histologic sections (Fig. 3) (35).

Serology Tests

Serologic studies for *Toxocara* should be interpreted with caution, since a positive result could represent previous, asymptomatic infection. The sensitivity and specificity of these assays are high, but less than 100%. These parameters cannot be determined with confidence, since there is not a better parasitological test to confirm the presence of a true infection. These tests, however, are useful in the appropriate clinical context and have been successfully used in seroepidemiologic studies. Tests are available at CDC and from commercial reference laboratories.

Treatment

There is no proven therapy. Anthelminthic therapy with albendazole or a similar therapeutic is often used. Corticosteroids may be necessary to control the inflammatory response in patients with a large parasite burden. Eye infections require a combined medical and surgical approach (21).

Dracunculus medinensis

Description of Agent

D. medinensis, also known as the guinea worm, is not a filarial worm, but rather a member of the family Dracunculidae (23). Other *Dracunculus* species exist that infect a variety of other animals.

Epidemiology, Transmission, and Prevention

Dracunculiasis is found only in the rural parts of Africa. Disease occurs more commonly in the dry season, affects men more commonly than women, and is most common in individuals from 10 to 60 years old, which taxes the productivity of affected communities. An intensive effort by the World Health Organization (WHO), local governments, and numerous other humanitarian organizations has significantly decreased the annual incidence of disease (1, 7, 45). At the beginning of this program (1986), an estimated 3.5 million people in 20 countries were affected. The annual incidence of worldwide disease diminished by an astounding 98% by December 2001, and seven countries completely eradicated the disease.

Infection follows drinking contaminated freshwater that contains copepods, which harbor the infective larvae. After ingestion, the larvae migrate to the retroperitoneum, where they mature and mate. The female worm eventually migrates to a subcutaneous location where a blister forms, which bursts upon contact with water and releases numerous larvae. The larvae are ingested by a copepod, wherein they become infective. Preventive measures are centered on education and providing clean drinking water (25, 26).

Clinical Significance

Patients are asymptomatic during larval penetration of the gastrointestinal tract and retroperitoneal maturation and mating of the worms. Symptoms are secondary to inflammation and tissue damage caused by worm migration. Although lesions are most common in the lower extremities, they may occur anywhere in the body, producing a serpiginous path of the migrating worm under the skin (Fig. 4). The large blister that is formed may become secondarily infected. Dead worms may be absorbed, calcify, or produce symptoms secondary to location (e.g., a joint).

Direct Examination/Microscopy

The presence of the end of a worm protruding from a burst blister or ulcer in the appropriate setting is diagnostic. The microscopic analysis of these worms in cross-section demonstrates a cuticle 30 to 50 μm thick, indistinct lateral cords, prominent dorsal and ventral bands of smooth muscle, and a large uterus filled with rhabditoid larvae that fills the body cavity (Fig. 5).

Serologic Tests

Serologic tests are usually not necessary for the diagnosis, given the obvious findings in people at risk for disease, but they are useful for seroprevalence studies and to identify infected individuals prior to the partial emergence of the adult worm.

Treatment

The oldest and traditional treatment, as depicted in the ancient Egyptian medical text the *Papyrus Ebers,* consists of removing the gravid worm by wrapping the exposed end around a stick and applying gentle pressure for days. Treatment with thiabendazole and metronidazole, although not lethal for the worm, facilitates removal by this process (9). Although often effective, risks include rupture of or breaking the worm prior to full removal. Currently, surgical excision is preferred. Anti-inflammatory agents and antihistamines are important for symptomatic relief. Antibiotics may be necessary to curtail secondary bacterial infections, and tetanus vaccination is important.

C. philippinensis

Description of Agent

C. philippinensis is a trichurid nematode responsible for intestinal capillariasis (12, 31).

Epidemiology, Transmission, and Prevention

The natural parasitic cycle of *C. philippinensis* likely involves marine, fish-eating birds as the definitive hosts and fish as the intermediate hosts. Humans become infected by ingesting raw or undercooked fish that harbor the infective larvae. Larvae at various stages of maturation may be found in the lumen of the intestine of the definitive host or the patient. Therefore, the population of parasites within the lumen of the bowel may increase (hyperinfection) without the consumption of additional contaminated fish. Thorough cooking of fish is protective.

Clinical Significance

Infections with *C. philippinensis* are rare, but because intraintestinal hyperinfection is possible, they may be fatal. Nonspecific gastrointestinal complaints predominate early in the course of disease. As the disease progresses and the number of worms increases, there is continued diarrhea with malabsorption leading to cachexia (18). Endoscopy may be useful for establishing the diagnosis (46). Death occurs secondarily to malnutrition, or because of secondary bacterial infections (enteritis and/or septicemia).

Direct Examination/Microscopy

The microscopic examination of the stool may demonstrate a mixture of eggs and larvae. The eggs of *C. philippinensis* (Fig. 6), which also have bipolar plugs, are superficially reminiscent of the more commonly recognized trichurid worm, *Trichuris trichiuria*. The plugs of *C. philippinensis,* however, are

nonprotruding, the egg is more rectangular, and there is a distinctive pitting of the egg shell (6). The adults superficially resemble *Strongyloides stercoralis* but may be differentiated by the presence of stichocytes and three bacillary bands, the latter being most easily recognized in cross-section.

Serologic Tests

Serologic tests are not available from the CDC or from commercial reference laboratories. Experimental serologic tests have been developed which, like the coproantigen, hold promise as future diagnostic tools (17).

Treatment

Mebendazole is the drug of choice, but albendazole and thiabendazole may also be used. Relapsing disease requires prolonged antihelminthic therapy. Aggressive electrolyte replacement and monitoring are critical, as are control of diarrhea and administration of nutrients.

Gnathostoma Species

Description of Agent(s)

Gnathostoma species are gastric spirurid nematode parasites for which the definitive hosts are a variety of mammals (29). Infective L3 (i.e., third-stage) larvae cause disease in humans, and gnathostomiasis may be considered a subtype of visceral larva migrans. *Gnathostoma spinigerum* is the most common cause of human disease, but *Gnathostoma hispidum, Gnathostoma nipponicum,* and *Gnathostoma doloresi* may also cause human disease.

Epidemiology, Transmission, and Prevention

G. spinigerum has essentially a worldwide distribution. Dogs and cats are the primary definitive host for this gnathostome, whereas *G. hispidum* and *G. doloresi* infect wild and domestic pigs and *G. nipponicum* is a parasite of weasels.

The adult male and female gnathostomes live in the stomach of the definitive host, where they mate, and eggs are passed in the stool. After development in water, the eggs hatch and release first-stage larvae that infect the *Cyclops* copepod, wherein the larvae mature. When a second intermediate host, such as fish, snakes, or frogs, eats the copepod, the larvae penetrate the gastric wall of the new host, wherein they migrate to the musculature and encyst. If an appropriate definitive host eats the secondary intermediate host, the advanced L3 larvae excyst and penetrate the gastric wall. After penetration, the L3 larvae migrate through the liver, muscles, and connective tissues, only to return to the stomach and mature into adults. Humans are accidental hosts in which the nematode larvae cannot mature to adulthood and continue to migrate aimlessly. The parasite is transmitted to humans through the ingestion of raw or undercooked meat from a secondary intermediate host. Disease can be prevented by thoroughly cooking potentially infected foods.

Clinical Significance

The clinical presentations of gnathostomiasis are protean, since the wandering larvae may be present in any tissue or organ system (22, 38, 39). Although the infection is not commonly fatal, significant morbidity may be associated with infection. Early after the ingestion of food that contains infective larvae, the patient may experience nonspecific gastrointestinal symptoms, such as nausea, vomiting, diarrhea, and epigastric pain.

Direct Examination/Microscopy

Mature (adult) gnathostomes are not present in humans, but L3 larvae are. This form is morphologically similar to the adult form but is smaller, measuring 3 to 4 mm in length by 630 μm in diameter (*G. spinigerum*). The head bulb contains four rows of cephalic hooklets, with approximately 45 hooklets per row. The body of the larva, like that of the adult, is covered with transverse rows of sharply pointed spines that diminish toward the posterior end of the worm. An entire worm may be spontaneously expelled or may require surgical excision.

Serologic Tests

Serologic assays have been developed predominantly in areas of endemicity. These are not available in the United States, but the CDC can assist in identifying a source for testing, if necessary. The possibility of cross-reactivity should be considered with this and several other serologic assays for helminthic parasites, and should be used only in the appropriate clinical context.

Treatment

Surgical removal of the parasite is the foremost effective treatment, which may be aided by advanced imaging tools (5). There have been some reports claiming successful treatment with albendazole and diethylcarbamazine, but the efficacy of these agents remains to be determined. Symptomatic relief with antihistamines and corticosteroids may be considered.

Angiostrongylus cantonensis

Description of Agent

A. cantonensis is a filariform worm and the most important cause of angiostrongyliasis (37).

Epidemiology, Transmission, and Prevention

A. cantonenesis is widely distributed throughout the world but represents an important public health threat in Southeast Asia and the Asian Pacific Islands. This parasite persists in a wide variety of rodents, which serve as the definitive host, wherein the adult worms reside in the pulmonary artery and the right side of the heart. Eggs released from the adults lodge in pulmonary capillaries, where they hatch and release larvae. The larvae subsequently migrate up the trachea and are swallowed and passed in the feces. These larvae infect mollusks, the intermediate host, wherein maturation to the infective L3 larvae occurs. A number of animals, including shrimp, crabs, fish, and frogs, may eat the infected mollusks and serve as important paratenic hosts. Rodents that ingest either the infected mollusk or tissues from the paratenic host thereby become infected. In the definitive host, the infective larvae penetrate the intestine, become blood borne, and migrate to the central nervous system, where they molt twice. Thereafter, the worms reenter the systemic circulation and finally reside in the right ventricle and pulmonary artery to mature and complete the cycle. Prevention of disease can be achieved through control of the local rat population and thorough cooking of mollusks and potential paratenic hosts.

Clinical Significance

The clinical manifestations reflect the worm burden and the site of worm residence, the central nervous system. The worms tend to remain associated with the brain and meninges in human infections (Fig. 7). Patients demonstrate signs and symptoms typical of meningitis or meningoencephalitis, with headache, fever, possibly eosinophilia (10 to

60%), and any of a number of neurologic disturbances, depending on the location of the worms. The cerebrospinal fluid (CSF) demonstrates pleocytosis, eosinophilia (26 to 75%), and elevated protein and occasionally will contain immature worms. Alternatively, patients may have infections of the eye, with retinal detachment and blindness. Less commonly, human infections may result in pulmonary disease.

Direct Examination/Microscopy

Demonstration of the worms in histologic sections, or intact in clinical specimens from the CSF or eye, definitively establishes the diagnosis. Eosinophilia in the CSF may be the first indicator of a possible parasitic infection of the central nervous system. The differential diagnosis of the causes of CSF eosinophilia, however, is broad and includes other parasitic infections, e.g., *Gnathosoma* or *Toxocara* spp., allergic reactions, and coccidioidomycosis. The gross appearance of an adult female worm is distinctive, with the spiral winding of the uteri and ovarian branches imparting a "barber pole" appearance. Cross-section of the female demonstrates a large intestine and two uteri, whereas cross-section of the male reveals a large intestine and a single reproductive tube. Eggs are not produced in human tissues.

Serologic Tests

Serologic assays are powerful tools for the diagnosis of angiostrongylosis, particularly since the parasite is usually located in a difficult-to-access location, the central nervous system. The evolution of these assays has resulted in a highly sensitive and specific assay, particularly when purified, non-cross-reacting antigens are used. Although intrathecal antibody is not always produced, the presence of intrathecal antibody synthesis provides strong evidence of infection. This assay is available from some commercial vendors in the United States.

Treatment

The optimal treatment has not been established. Fortunately, the disease is often self-limited. Removal of CSF and the administration of corticosteroids and nonsteroidal anti-inflammatory agents have been used to control pain and inflammation. A variety of antihelminthic medications have been used, with mebendazole the current drug of choice. Careful monitoring of the patient is important, since antihelminthic therapy may sometimes exacerbate the symptoms (24).

Dirofilaria immitis

Description of Agent

D. immitis, the filarial dog heartworm, causes human pulmonary dirofilariasis (10, 44).

Epidemiology, Transmission, and Prevention

Although dogs are the most important host for *D. immitis,* other mammals, such as fox and bear, are also suitable hosts. The blood of infected dogs or other suitable hosts contains microfilariae that are released from the adult worm, which resides in the right ventricle of the heart. These are taken into the mosquito during a blood meal, wherein they mature into infective L3 larvae and are capable of being transferred to another mammal during a blood meal. The larvae migrate through subcutaneous tissues and eventually enter the bloodstream and the right side of the heart, wherein they mature into adults if the host is suitable. Humans, however, are unsuitable hosts, and the worm dies before reaching maturity, is swept into the pulmonary arterial circulation, and becomes lodged in the subsegmental pulmonary arteries and arterioles, causing thrombosis, infarction, inflammation, and eventually a granulomatous reaction surrounded by a wall of fibrous tissue (Fig. 8).

The geographic distribution of disease reflects the prevalence of canine dirofilariasis. The areas of highest prevalence in the United States are in the south, particularly along the Gulf and Atlantic coasts and along the Mississippi River (40). Dog ownership is not a risk factor for disease. Prevention is centered around control of zoonotic disease. The use of mosquito repellents, particularly in areas of high endemicity, should also help interrupt transmission.

Clinical Significance

Slightly more than half of the patients with dirofilariasis are asymptomatic. The remainder have nonspecific symptoms, such as cough, chest pain, hemoptysis, low-grade fever, chills, and malaise (10). Only 5 to 10% have peripheral blood eosinophilia. In addition to the pulmonary manifestation, *D. immitis* has also rarely been identified in subcutaneous abscesses, the abdominal cavity, the eyes, and the testes. *Dirofilaria* species other than *D. immitis* are more likely to be found in a subcutaneous location (Table 1). The nodules caused by *D. immitis* are often discovered by a routine chest radiograph (40). These are usually single, but occasionally two or three nodules may be present.

Direct Examination/Microscopy

The pulmonary nodules produced by *D. immitis* are characteristically small (0.8 to 4.5 cm; mean, 1.9 cm), subpleural, spherical, and well circumscribed. *D. immitis* has a smooth, thick cuticle (5 to 25 μm) with the three distinct layers characteristic of the genus *Dirofilaria;* other *Dirofilaria* species that infect humans have a similar cuticle, but with external longitudinal ridges (Fig. 9 and 10). The thick, multilayered cuticle projects inwardly at the lateral chords, forming two prominent, opposing internal longitudinal ridges. The somatic musculature is typically prominent, but the lateral chords are usually poorly preserved. Transverse sections may reveal two large uteri and a much smaller intestine in female worms (Fig. 9); a single reproductive tube and intestine are present in the males. The definitive identification of the worm based on internal structures may be difficult to impossible, given the advanced stage of parasite degeneration in many of these specimens. The presence of a parasitic worm in a pulmonary artery and associated with a pulmonary infarct, however, is sufficient for a diagnosis.

Serologic Tests

Serologic tests to detect antibody to *Dirofilaria* are commercially available from several reference laboratories. Although advances have been made to increase specificity without sacrificing sensitivity, cross-reactivity may occur in patients infected with other nematodes, particularly other filarial worms. As with many serologic assays, a positive test is useful, whereas a negative test does not exclude the possibility of infection.

Treatment

The excision of the nodule is curative. Additional antiparasitic therapy is not necessary.

Other Less Common Nematodes

There are a number of additional less commonly occurring nematodes than those described. Some of these and associated important features are included in Table 1.





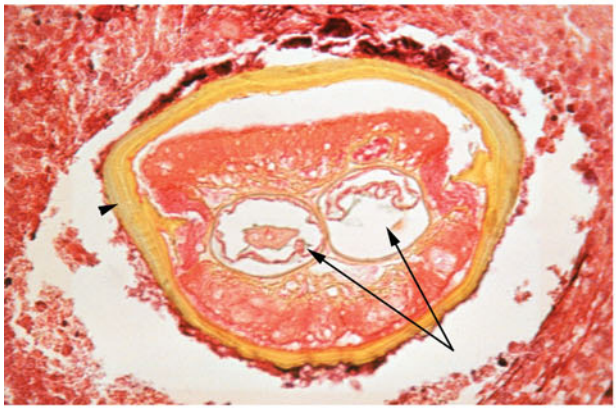










TABLE 1 Other less common nematodes

Organism	Definitive host	Intermediate host(s)/ vectors	Disease(s) produced	Method of diagnosis
Ancylostoma species other than *Ancylostoma duodenale, A. ceylanicum, A. caninum,* and *A. braziliense*	Cats, dogs, and hamsters	Not applicable	Ancylostomiasis, eosinophilic enteritis, cutaneous larva migrans (creeping eruption)	Detection of eggs in stool or adults in histologic sections; rarely, larvae in biopsy sample of skin
Angiostrongylus costaricensis	Rodents, less commonly dogs and monkeys	Mollusks	Infection of the intestinal walls and mesenteric arteries	Identification of parasite in surgically excised tissues; eggs not present in stool. Serologic studies are useful.
Dirofilaria species other than *D. immitis, D. tenuis, D. repens, D. ursi, D. subdermata, D. striata*	Raccoons *(D. tenuis)*, dogs and cats *(D. repens)*, bears *(D. ursi)*, porcupines *(D. subdermata)*, wild cats *(D. striata)*	Mosquitoes, except for *D. ursi,* which is transmitted by blackflies	Usually subcutaneous nodules that contain mature, immature, or degenerated worms	Morphological features of the worm in excised tissues (Fig. 10)

LESS COMMON CESTODES (TABLE 2)

Dipylidium caninum

Description of Agent

D. caninum, a common tapeworm of dogs and cats, also commonly infects children (8, 43).

Epidemiology, Transmission, and Prevention

Dipylidiasis occurs throughout the world (16, 28). This disease, like hymenolepiasis, is transmitted primarily through the ingestion of an infected flea. In the natural cycle, the dog or cat contains the intestinal adult parasite that releases gravid proglottids filled with eggs. The eggs are ingested by fleas or lice, which are the intermediate hosts. The eggs hatch

FIGURE 7 (row 1, left) The immature *A. cantonensis* worm (arrows) in the meninges of this patient is eliciting a marked eosinophilic response. The worm is 200 μm in diameter. Hematoxylin and eosin stain; original magnification, ×50 (AFIP negative no. 73-6862).

FIGURE 8 (row 1, right) The coiled remnants of an immature male *D. immitis* worm are present in this branch of the pulmonary artery. The maximum worm diameter is 250 μm. Movat stain; original magnification, ×15 (AFIP negative no. 71-11563).

FIGURE 9 (row 2, left) The two uteri (arrows), muscle, and trilaminar (arrowhead), smooth cuticle are characteristic of an immature female *D. immitis* worm. The worm diameter is 250 μm. Movat stain; original magnification, ×80 (AFIP negative no. 72-2732).

FIGURE 10 (row 2, right) The *Dirofilaria* species other than *D. immitis* have external longitudinal cuticular ridges, whereas the cuticle of *D. immitis* is smooth. *Dirofilaria tenuis* is pictured here in cross-section; it is 270 μm in diameter and has obvious cuticular ridges (arrows). *Dirofilaria* species other than *D. immitis* are often found in a subcutaneous location rather than in the pulmonary arterial vasculature. Movat stain; original magnification, ×80 (AFIP negative no. 94-5122).

FIGURE 11 (row 3, left) An egg packet of *D. caninum,* which was obtained from a crushed gravid proglottid, is 150 μm in diameter. The eggs within the packet are 40 μm in diameter. Unstained (AFIP negative no. 86-7369).

FIGURE 12 (row 3, right) The thick inner membrane of the egg of *Hymenolepis diminuta* is surrounded by a gelatinous matrix and then by an outer striated shell. The eggs of *H. diminuta* are spherical, whereas those of *Hymenolepis nana* are ovoid. The egg pictured here is 80 μm in diameter. Unstained; original magnification, ×250 (AFIP negative no. 96-5119).

FIGURE 13 (row 4, left) A sparganum superficially resembles an adult tapeworm. Close inspection, however, clarifies its immature form, with a head with only a ventral groove or bothrium (arrow) and a lack of proglottids. The maximum width is 6 mm. Unstained; original magnification, ×0.5 (AFIP negative no. 70-15303).

TABLE 2 Other less common cestodes[a]

Organism	Definitive hosts	Intermediate hosts	Disease produced	Diagnostic method
Hymenolepis diminuta, the rat tapeworm	Rats	Insects	Usually asymptomatic; heavy infections resemble heavy *H. nana* infections	Eggs in the feces (Fig. 12); proglottids disintegrate before fecal passage.
Hymenolepis nana, the dwarf tapeworm[b]	Rats; human-to-human transmission possible	Fleas	This worm, the smallest adult tapeworm that infects humans, produces disease that is usually mild, and patients may be asymptomatic. Massive infections may produce abdominal pain, allergic reactions, anorexia, nausea, diarrhea or constipation, and flatulence.	Identification of characteristic eggs in the stool; proglottids disintegrate and release eggs before they are passed in the stool. The eggs are ovoid, in contrast to those of *H. diminuta,* which are spherical, and have an inner hyaline membrane, a thin egg shell, polar thickenings from which polar filaments arise, and a distinct oncosphere that contains six hooklets.
Less common *Taenia* species				
T. taeniaeformis	Cats	Rodents	Larval stage may infect liver, like cysticercosis.	Demonstration of strobilocercus in histologic sections
T. multiceps	Canids	Sheep, rabbits	Coenurosis, an infection with the coenurus in the CNS,[c] eyes, or subcutaneous tissues (like cysticercosis)	Demonstration of the coenurus in histologic sections
Mesocestoides species	Foxes, dogs, cats, and other mammals	Unknown, possibly reptile or arthropod vectors	Asymptomatic to mild abdominal symptoms	Detection of gravid proglottid with characteristic parauterine organ. Eggs are usually not present in the stool.
Bertia species	Primates	Mites and possibly other insects	Asymptomatic to mild abdominal symptoms	Motile proglottids in the stool, similar to *D. caninum* infection. Eggs within proglottids are not within packets.
Inermicapsifer madagascariensis		Raw sugar cane ingestion has been suggested.	Asymptomatic to symptoms similar to *D. caninum* infections	Motile proglottids in the stool, similar to *D. caninum* infection. Proglottid and egg morphology and number of eggs per packet are used for identification.
Raillietina species	Rodents	Insects	Asymptomatic to symptoms similar to *D. caninum* infections	Motile proglottids in the stool, similar to *D. caninum* infection. Proglottid and egg morphology and number of eggs per packet are used for identification.

[a] Praziquantel is the treatment of choice for infections with adult tapeworms; it is also effective in coenurosis. Effective preventive measures center around controlling disease in the zoonotic host (e.g., cats or dogs) or controlling the zoonotic hosts themselves (e.g., rats). Controlling intermediate hosts is also effective but may prove more difficult.

[b] This parasite is covered in detail in chapter 146.

[c] CNS, central nervous system.

within the intermediate host, releasing larvae that penetrate the body cavity, where they mature into infective cysticercoid larvae. When an infected intermediate host is ingested, the cysticercoid metacestode larva attaches to the small intestine and matures into an adult tapeworm, completing the cycle. Prevention is achieved by deworming animals and controlling fleas and lice.

Clinical Significance

Dipylidiasis is usually an innocuous infection. Larger worm burdens may cause weight loss, abdominal pain, failure to thrive, or the appearance of colic. Disease usually comes to the attention of parents and pediatricians when motile, gravid proglottids are seen in the stool.

Direct Examination/Microscopy

The proglottids of *D. caninum* differ from those of most other cestodes in that they have two genital pores (*dipylos* means two gates), which can be appreciated with the use of a dissecting microscope and compressing the proglottid between two glass slides. The identification may also be achieved by demonstrating the characteristic egg packets (Fig. 11) and/or

characteristic eggs that have distinct morphological features (i.e., four envelopes). A fecal examination will likely be negative for eggs or egg packets, since intact proglottids are usually passed in the stool. Microdissection or histologic examination of the proglottids will reveal the eggs and egg packets. In addition to the egg packets, the histologic examination of the proglottid will reveal other features common to cestodes, such as a tegument, smooth muscle, and calcareous corpuscles.

Serologic Tests

Because the disease is usually subclinical and the diagnosis is achieved when the proglottids are discovered and examined, serologic tests are not necessary.

Treatment

Both praziquantel and niclosamide are effective against *D. caninum*. Upon discovery, examination and treatment of household pets should proceed, as should aggressive flea control.

Spirometra Species

Description of Agent(s)

Sparganosis is the infection of humans by L3 plerocercoid larvae of a pseudophyllidean tapeworm (33). The plerocercoid larva in a human host does not reach maturity and is known as a sparganum. The tapeworms that cause sparganosis are *Spirometra mansoni, Spirometra mansonoides, Spirometra ranarum,* and *Spirometra erinacei*. The precise taxonomic relationship of a cestode known as *Sparganum proliferum* is unclear, but this organism may simply represent a variant of *S. mansonoides* or *S. erinacei*.

Epidemiology, Transmission, and Prevention

Adult *Spirometra* species are widely distributed and are tapeworms of animals, particularly dogs and cats. The parasitic cycle for these worms begins with the passage of eggs from an infected suitable host. The coracidium that emerges from the egg infects the first intermediate host, a copepod. The second intermediate host, which includes snakes, frogs, and fish, becomes infected by ingesting the infected copepod. The cycle is completed when a suitable definite host ingests the second intermediate host. Humans are unsuitable hosts and may become infected by ingesting raw or undercooked meat from a second intermediate host or by drinking contaminated water that contains the infected copepod. Humans have also become infected by using infected animal flesh (e.g., frog flesh) as a poultice.

Clinical Significance

The results of ingesting a plerocercoid larva depend on both the species of the host and the species of the larva. For example, the ingestion of a plerocercoid larva of *Diphyllobothrium* by a human will result in the development of an adult tapeworm, whereas the ingestion of a plerocercoid larva of a *Spirometra* species will result only in the continued existence of the larva in the new host (i.e., the sparganum). This is a situation wherein the human is biologically behaving like a second intermediate or paratenic host. The clinical features of disease are influenced by worm burden (most patients harbor only a single worm), worm location, and worm viability. Spargana migrate, but this migration usually does not cause symptoms. Migration to a subcutaneous location, however, may result in a nodule. This nodule may be excised to exclude the possibility of malignancy. Ocular sparganosis, particularly involving the conjunctiva, may result following the application of a natural poultice that contains raw snake or frog tissues. Inflammation and sometimes calcification ensue following death of the sparganum, and when these occur in the brain they may cause obstructive hydrocephalus (32).

Direct Examination/Microscopy

The spargana of *Spirometra* species are flat, ribbon-like worms that superficially resemble adult tapeworms (Fig. 13). Closer inspection demonstrates an immature anterior end without hooklets; a cleft or ventral groove, termed a bothrium, is present (Fig. 13) (36). Mature proglottids are not produced. Histopathologic examination of the sparganum demonstrates calcareous corpuscles characteristic of a cestode. Developed internal organs are not seen, but rather irregularly scattered muscle fibers and excretory ducts in a loose stroma.

Serologic Tests

If the diagnosis is not suspected, it is usually made on the basis of the unsuspected findings at surgery of a viable sparganum and the findings of the subsequent histopathologic examination. In such instances, serology is not useful. However, if this disease is clinically suspected, the diagnosis may be achieved through the combination of radiology and serology (27).

Treatment

Medical therapy is currently deemed unsuitable for the treatment of sparganosis; the plerocercoid larva is resistant to praziquantel. Complete surgical excision is recommended. An incomplete excision, particularly if the anterior end of the larva remains in the tissue, may result in continued growth of the organism (2).

SUMMARY

There are a wide variety of less commonly encountered helminthic parasites, which may be nematodes, cestodes, or trematodes. The diseases caused by these parasites are interesting and demonstrate their highly evolved life cycles and the complex interactions with their hosts. These diseases range from subclinical, e.g., dipylidiasis, to possibly life-threatening, e.g., angiostrongylosis. In many instances, the disease occurs only in a limited geographic area, which is largely determined by the biological ranges of the definitive and intermediate hosts. The treatment of these parasites varies depending on the infectious agent, but common preventive measures may significantly diminish the transmission of many of these parasitic diseases. These measures include the zoonotic control of parasitic disease in animal hosts and the vectors of transmission, washing of fruits and vegetables, access to clean drinking water, and thorough cooking of meats before consumption.

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Arthropods of Medical Importance*

SAM R. TELFORD III

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Arthropods comprise a diverse group of invertebrate animals (Fig. 1), united in a common body theme (bauplan) of a jointed, chitinous exoskeleton. Four major groupings have classically been recognized: insects, arachnids, crustacea, and millipedes/centipedes; a fifth group contains only the living fossils the horseshoe crabs, which have existed unchanged for hundreds of millions of years. All arthropod classes were extant hundreds of millions of years ago, thereby providing ample opportunity for life history traits such as parasitism to independently evolve, and evolve multiple times, in each class.

Medically important arthropods have long been considered to mainly comprise ectoparasites, parasites that limit their activities to the skin. Parasitism, however, is only one of several associations that comprise the interaction of arthropods of medical importance with humans. Arthropods may actively defend themselves against predation (crushing or swatting) by biting, stinging, piercing, or secreting noxious chemicals. Such defenses would operate regardless of the attacker, be it human or other arthropod. Passive modes of defense may inadvertently affect humans, such as irritation after brushing the urticarial hairs of certain caterpillars. Arthropods may also be medically important due to indirect effects: fear of insects, delusional parasitosis, or allergy due to dust mites. The various modes by which arthropods may affect human health thus reflect the diversity of these animals, but there are very few instances where it may be argued that natural selection favored the offspring of those that focused on causing misery. Accordingly, arthropods should be viewed as a normal part of the environment that under individual circumstances may cause pathology. In addition, because of their ubiquity, spurious associations with pathology are common.

ARTHROPODS AS VECTORS

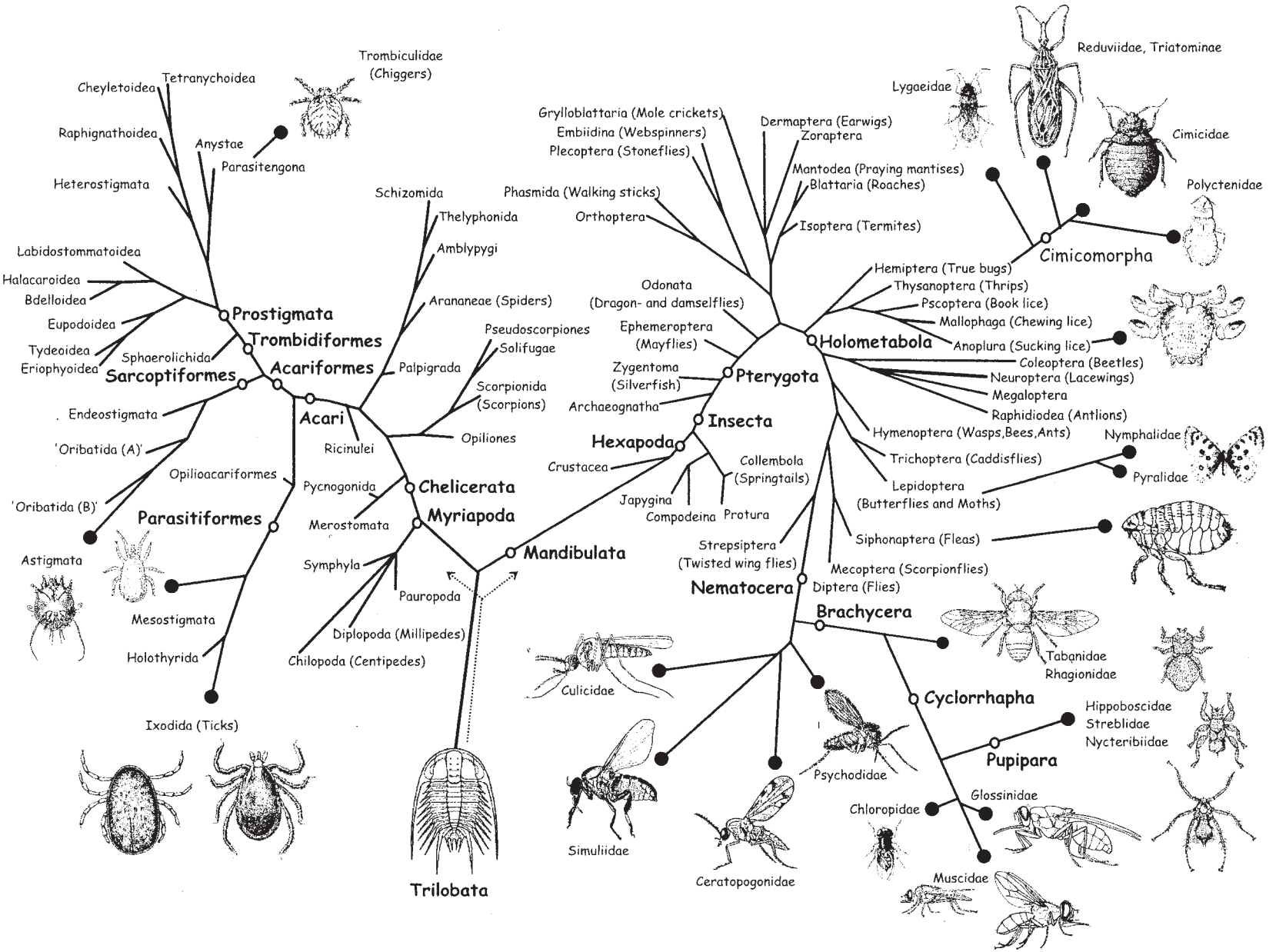
Arthropods are thought of by many in clinical settings with respect to their role as vectors, transmitters of infectious agents including viruses, bacteria, protozoa, and helminths. Infectious agents may have an obligate relationship with an arthropod (biological transmission) or may simply contaminate an arthropod (mechanical transmission). Malaria parasites undergo a complex developmental cycle within certain mosquitoes and could not perpetuate without them. In contrast, the agent of trachoma *(Chlamydia trachomatis)* is found on the external surfaces of eye gnats and flies (17) and may be transferred between hosts by the act of landing and crawling, but *C. trachomatis* more commonly perpetuates by direct contact of hosts. Mechanical transmission of an infectious agent is dependent on its stability and quantum of infection. Human immunodeficiency virus (HIV), for example, does not survive long outside of the body, and the femtoliter or so of material that may contaminate the mouthparts of a mosquito would not contain enough viable lymphocytes with HIV to initiate infection; thus, mosquitoes have never been epidemiologically linked with HIV transmission even though mosquitoes and other hematophagous arthropods obviously feed on viremic individuals.

There are five major groups of vectors: the diptera (flies and mosquitoes), hemiptera (kissing bugs), siphonaptera (fleas), anoplura (lice), and acarines (ticks and mites). The general life history strategies for each group provide the basis for understanding vectorial capacity, which is the sum of physiological and ecological attributes that allow transmission. Specific vector-pathogen relationships are discussed in detail in chapters focusing on the respective agents but are succinctly summarized here in Table 1.

Diptera

The dipteran vectors are winged insects that include mosquitoes, sand flies, blackflies, gnats, horse/deerflies, and tsetse flies. These range in size from minute (ceratopogonid midges less than 2 mm in length) to large (horseflies more than 2 cm in length). Unlike other winged insects, dipterans have only one pair of wings. Those that take blood meals as adult females may serve as vectors. Blood meals are used as nutrient to produce eggs (anautogeny); once those eggs are laid, another blood meal may be taken and more eggs produced. Thus, unless a mosquito (as an example of a dipteran) inherits its infection (transovarial or vertical transmission), the first blood meal infects it and the second allows the agent to be transmitted; under favorable environmental circumstances, a mosquito may survive for several weeks and take more than two blood meals. Both male and female flies and mosquitoes also require sugar meals (usually from plant nectar), but sugar meals can result in reproduction only in certain species (autogeny).

*This chapter contains information presented in chapter 138 by Thomas R. Fritsche in the eighth edition of this Manual.


Trombiculidae (Chiggers)
Cheyletoidea
Tetranychoidea
Raphignathoidea
Anystae
Parasitengona
Heterostigmata
Labidostommatoidea
Halacaroidea
Bdelloidea
Eupodoidea
Tydeoidea
Eriophyoidea
Prostigmata
Sphaerolichida
Trombidiformes
Acariformes
Sarcoptiformes
Endeostigmata
'Oribatida (A)'
'Oribatida (B)'
Opilioacariformes
Parasitiformes
Astigmata
Mesostigmata
Holothyrida
Ixodida (Ticks)
Schizomida
Thelyphonida
Amblypygi
Arananeae (Spiders)
Palpigrada
Pseudoscorpiones
Solifugae
Scorpionida (Scorpions)
Opiliones
Acari
Ricinulei
Pycnogonida
Chelicerata
Merostomata
Myriapoda
Symphyla
Pauropoda
Diplopoda (Millipedes)
Chilopoda (Centipedes)
Trilobata
Mandibulata
Grylloblattaria (Mole crickets)
Embiidina (Webspinners)
Plecoptera (Stoneflies)
Phasmida (Walking sticks)
Orthoptera
Dermaptera (Earwigs)
Zoraptera
Mantodea (Praying mantises)
Blattaria (Roaches)
Isoptera (Termites)
Odonata (Dragon- and damselflies)
Ephemeroptera (Mayflies)
Zygentoma (Silverfish)
Archaeognatha
Pterygota
Insecta
Hexapoda
Crustacea
Collembola (Springtails)
Japygina
Compodeina
Protura
Strepsiptera (Twisted wing flies)
Nematocera
Diptera (Flies)
Culicidae
Simuliidae
Psychodidae
Ceratopogonidae
Brachycera
Cyclorrhapha
Pupipara
Chloropidae
Glossinidae
Muscidae
Tabanidae
Rhagionidae
Hippoboscidae
Streblidae
Nycteribiidae
Lygaeidae
Reduviidae, Triatominae
Cimicidae
Polyctenidae
Cimicomorpha
Holometabola
Hemiptera (True bugs)
Thysanoptera (Thrips)
Pscoptera (Book lice)
Mallophaga (Chewing lice)
Anoplura (Sucking lice)
Coleoptera (Beetles)
Neuroptera (Lacewings)
Megaloptera
Raphidiodea (Antlions)
Hymenoptera (Wasps,Bees,Ants)
Trichoptera (Caddisflies)
Lepidoptera (Butterflies and Moths)
Nymphalidae
Pyralidae
Siphonaptera (Fleas)
Mecoptera (Scorpionflies)

Eggs are laid in water or within detritus. Vermiform larvae emerge from the eggs and develop through several stages (instars) in water or detritus, feeding on organic material or bacteria, culminating in pupae from which emerge new adults, thereby undergoing complete metamorphosis (holometabolous development). Depending on the species and the ambient temperature, the duration of the dipteran life cycle may be as short as a week or so. Tsetse flies are an unusual exception to this pattern and produce only one advanced larva for each blood meal, with the larva nourished internally from milk gland analogs during a gestation of several weeks (8). This large maternal investment in offspring makes adulticiding (trapping and spraying) very effective in reducing tsetse fly populations and thereby reducing the transmission of the agents of sleeping sickness.

The diptera are only transiently associated with a blood meal source. Host-seeking cues include body heat, carbon dioxide (exhaled by the host), mechanical vibrations, and lactic acid or other skin-associated compounds. The flies visually seek hosts with their compound eyes, mainly larger, dark-colored objects with movement, but proximal cues associated with a host seem required to initiate feeding (32). Repellants such as DEET (diethyltoluamide) work by disrupting the airborne gradients of chemical cues that allow diptera to home in to their hosts. Once a host has been identified, feeding is initiated and completed within minutes. Sand flies, blackflies, and mosquitoes have a diverse salivary armamentarium of pharmacologically active substances that promote finding and removing blood. During probing, antihemostatic and anti-inflammatory chemicals are secreted into the feeding channel created in the epidermis. Probing lacerates capillaries, and the ADP that is released from damaged endothelial cells would serve to promote platelet activation, but because diptera secrete an apyrase that cleaves ADP into a monophosphate, platelet aggregation is greatly reduced during blood feeding. In addition, local inflammatory processes are temporarily diminished due to secretion of chemicals such as prostaglandin E_2 (29). Thus, the first few bites from a given species remain unnoticed by the host, although factors in the saliva of blackflies may leave an egg-sized lump that is hot to the touch at the site of their bites. Such lumps may persist for several days and may be accompanied by low-grade fever. Such "blackfly fever" should not be misinterpreted as infection; it will resolve on its own without treatment.

The variety of proteins that are deposited within skin during vector feeding are the basis for hypersensitivity reactions, mainly itch, in hosts that are exposed to bites more than once over the course of weeks. Repeated exposure over months may lead to tolerance and a return to failing to react to bites. Although much work remains to be done, there appears to be some cross-reactivity between salivary products from different groups of vectors so that some individuals may equally react to the bites of mosquitoes, bugs, or ticks.

The blood-feeding flies have cruder salivary tools, reflecting their less elegant way of feeding, which usually involves scraping the skin with a roughened maxilla and feeding from the pooled blood or lymph. Accordingly, fly bites are more painful regardless of prior exposure, and few flies successfully engorge to repletion on humans.

Freshly blood-fed diptera seek a resting place for diuresis and digestion, usually the nearest vertical surface. This behavior renders those species that bite within houses susceptible to control by indoor residual insecticide spraying.

The transient nature of infestation by blood-feeding diptera means that few specimens are submitted to clinical laboratories. Other than analyses related to confirming the diagnosis of a vector-borne infection, dipterans would come to the attention of laboratorians for issues related to hypersensitivity, or for myiasis (see below).

Hemiptera

The only hemiptera that serve as vectors are the kissing or reduviid bugs, belonging to a diverse and speciose order of minute to large insects with compound eyes, antennae, sucking mouthparts, two pairs of wings (one delicate pair hidden under an outer pair of more robust ones), and a segmented abdomen. All are easily seen without magnification, adult bedbugs being roughly 1 cm in length and adult triatomines ranging in size from 1 cm to more than 5 cm. Small numbers of eggs are produced by females, from which emerge miniature versions of the adults. These nymphs undergo an incomplete metamorphosis (hemimetabolous development), with development through five nymphal stages, each one requiring a blood meal to proceed. The full duration of the life cycle can be as little as 3 months or as great as 2 years. Reduviid bugs are cryptic, living within cracks of mud walls or other narrow, confined spaces. They serve as vectors of trypanosomes *(Trypanosoma cruzi,* the agent of Chagas' disease, and *Trypanosoma rangeli,* an apparently nonpathogenic trypanosome that is often cotransmitted with *T. cruzi)* in the Nearctic. *Triatoma rubrofasciata* transmits a trypanosome that infects monkeys in Southeast Asia (40), but to date there is no known bug-transmitted trypanosomiasis of humans in the Palearctic. Interestingly, there are good natural cycles of *T. cruzi* transmission within the United States from California through virtually all of the southern-central states, all the way over to Maryland, but there are only rare human cases of autochthonous Chagas' disease reported. Two factors account for this paradox: people live in quality houses that are less likely to have infestations of bugs and, more importantly, the main southern U.S. vector, *Triatoma sanguisuga,* does not defecate on the host while feeding (27). Transmission of *T. cruzi* to humans requires contamination of the site of the bite or mucosa by trypanosomes that are excreted in the bug feces.

As with mosquitoes, salivary products from reduviids contain a variety of pharmacologically active compounds. Unlike those of mosquitoes, repeated reduviid feedings may cause a dangerous anaphylactoid reaction in residents of houses where the bugs are common. Scientists working with reduviid bugs often need to carry an epinephrine injector with them due to their propensity to feeding their colonies upon themselves, thereby receiving large doses of salivary antigens.

Although it is possible that a true reduviid bug may be presented by a patient for identification in clinical settings outside of Latin America, it is more likely that such specimens are related heteropterans such as assassin bugs, which are insect predators, or the plant-feeding stinkbugs, chinch bugs, harlequin bugs, or squash bugs. Assassin bugs may

FIGURE 1 Phylogeny of arthropods. (Reprinted with permission from W. C. Marquardt, W. C. Black IV, J. E. Freier, H. H. Hagedorn, J. Heminway, S. Higgs, A. A. James, B. Kondratieff, and C. G. Moore, *Biology of Disease Vectors*, 2nd ed., Elsevier Academic Press, Burlington, Mass., 2005.)

TABLE 1 Summary of the major arthropod genera involved in the biological transmission of infectious diseases

Arthropod(s)	Etiologic agent(s)	Disease
Crustacea		
Decapods	*Paragonimus* spp.	Paragonimiasis
Copepods	*Diphyllobothrium* spp.	Diphyllobothriasis
	Dracunculus medinensis	Guinea worm disease
	Gnathostoma spinigerum	Gnathostomiasis
Insecta		
Anopleura		
Pediculus	*Rickettsia prowazekii*	Epidemic typhus
	Bartonella quintana	Trench fever
	Borrelia recurrentis	Epidemic relapsing fever
Siphonaptera		
Xenopsylla, Nosopsyllus	*Yersinia pestis*	Plague
	Rickettsia typhi	Murine typhus
	Hymenolepis diminuta	Rat tapeworm
Pulex, Oropsylla	*Yersinia pestis*	Plague
Ctenocephalides	*Dipylidium caninum*	Dog tapeworm disease
Hemiptera		
Panstrongylus, Rhodnius, Triatoma	*Trypanosoma cruzi*	Chagas' disease
Diptera		
Aedes	*Wuchereria, Brugia*	Filariasis
	Flaviviruses	Dengue, yellow fever
	Other arboviruses	Encephalitis
Anopheles	*Plasmodium* spp.	Malaria
	Wuchereria, Brugia	Filariasis
	Arboviruses	Encephalitis
Culex	*Wuchereria*	Filariasis
	Arboviruses	Encephalitis
Culicoides	*Mansonella* spp.	Filariasis
Glossina	*Trypanosoma brucei*	African sleeping sickness
Chrysops	*Loa loa*	Loiasis
	Francisella tularensis	Tularemia
Simulium	*Onchocerca volvulus*	Onchocerciasis
	Mansonella ozzardi	Filariasis
Phlebotomus, Lutzomyia	*Leishmania* spp.	Leishmaniasis
	Bartonella bacilliformis	Bartonellosis
	Phlebovirus	Sandfly fever
Arachnida		
Acari (ticks)		
Ixodes	*Borrelia burgdorferi*	Lyme disease
	Anaplasma phagocytophilum	Human granulocytic ehrlichiosis
	Rickettsia conorii	Boutonneuse fever
	Babesia spp.	Babesiosis
Dermacentor	*Rickettsia rickettsii*	Rocky Mountain spotted fever
	Francisella tularensis	Tularemia
	Coltivirus	Colorado tick fever
	Flavivirus	Omsk hemorrhagic fever
Amblyomma	*Rickettsia rickettsii*	Rocky Mountain spotted fever
	Francisella tularensis	Tularemia
	Ehrlichia chaffeensis	Human monocytic ehrlichiosis
Hyalomma	Nairovirus	Crimean-Congo hemorrhagic fever
Rhipicephalus	*Rickettsia conorii*	Boutonneuse fever
	Rickettsia rickettsii	Rocky Mountain spotted fever
Ornithodoros	*Borrelia* spp.	Relapsing fever
Acari (mites)		
Leptotrombidium	*Orientia tsutsugamushi*	Scrub typhus
Liponyssoides	*Rickettsia akari*	Rickettsialpox

inflict extremely painful wounds by their piercing mouthparts, but these would require only typical first aid measures and perhaps a booster of tetanus toxoid given the depth of the puncture wound (proboscises range in length from 3 to 8 mm). The plant feeders may issue noxious secretions, which may taste bad if accidentally ingested (from a bug that infested a fruit or vegetable that was eaten directly from the vine) but would otherwise not be a cause for concern.

Bedbugs *(Cimex lectularius* and *Cimex hemipterus)* are hemipterans but are not known to serve as vectors for any pathogen. These bugs with short broad heads, oval bodies, and four-jointed antennae undergo incomplete metamorphosis, with four nymphal stages, each taking a blood meal in order to develop. The duration of the life cycle is roughly 6 to 8 weeks but may be as long as 11 months, depending on temperature and humidity. These bugs are small, 5 mm or less in length as adults (Fig. 2). They are cryptic and require hiding places such as cracks in walls, mattress foundations, or rattan furniture. At night, bedbugs will emerge and infest sleeping people, taking 10 to 20 min for engorgement. Feeding is often interrupted by the movements of people during their sleep, and so multiple bites may result from a single bedbug (Fig. 2). Blood meals may be taken every week or so, depending on the stage, with batches of 10 to 50 eggs laid; one female may lay 200 to 500 eggs in her life. Various laboratory studies have reported the survival of diverse agents such as HIV (18), hepatitis B virus, *Francisella tularensis,* and West Nile virus within artificially infected bedbugs. Epidemiologically, bedbugs have never been associated with any of these infections. Although one can never say never, the likelihood that a bedbug has served as a vector is infinitesimally small, and a physician may want to question a patient more closely on known risky practices such as intravenous drug abuse.

Siphonaptera

Fleas are bilaterally compressed, heavily chitinized insects with greatly modified hind legs for their characteristic jumping mode of locomotion; they lack wings. They are generally small, no larger than 5 mm in length. Fleas undergo complete metamorphosis, starting from a wormlike larva feeding on organic debris and blood pellets expelled, often with remarkable force and over a long distance, from the anus of the adult flea. The larva develops through three molts (has three instars) and then secretes a silk to form a cocoon, in which it pupates. The female flea requires blood for egg production; one blood meal may serve for the production of several dozen eggs, which are laid on the fur of the host. Eggs become detached from the host's fur, coming to rest within its nest. Most fleas are what are known as "nest parasites." Individual fleas may live as long as a year but usually only for a couple of months, laying eggs daily. As with most arthropods, the duration of the life cycle is affected by temperature and relative humidity, but on average most species take 30 to 75 days to develop from egg to adult.

Of the 2,000 or so flea species that have been described, the vast majority are from rodents and have various degrees of host specificity. Because fleas are generally nest parasites of rodents (and most rodent species are not human commensals), are relatively host specific, and cannot travel long distances (crawling or jumping on the order of a few meters, implying that humans must be directly associated with their habitat for infestation to occur), only a few species are of medical importance. These include the "human" flea *(Pulex irritans),* the dog and cat fleas (*Ctenocephalides canis* and *Ctenocephalides felis),* the main plague vector *(Xenopsylla cheopis),* and the sticktight flea *(Echidnophaga gallinacea).* Of these, *C. felis* is the most notorious pest, feeding voraciously and rapidly developing dense infestations. Chronic infestations of homes are largely due to the presence of a cat or dog (despite its name, this flea will feed on either animal), their bedding, wall-to-wall carpeting, and relatively great humidity within the home. (Cold climates rarely have self-sustaining infestations because winter heating tends to dry out the carpets and molding and other places where the larval fleas tend to be hidden.) Although bites sustained over several weeks will usually induce a typical delayed-type hypersensitivity reaction, with an intensely itching red spot developing at the site of the bite (usually around the ankles), note that not all members of a household will react in the same manner. Some people are more attractive to arthropods than others; others react differently to bites. It is quite possible for only one person to have itchy bites and others in the same house to have none, and thus a diagnosis of flea bite should not depend on the perception that if a household is infested, everyone should demonstrate similar lesions.

The human flea actually feeds on a variety of mammals, including domestic livestock. In tropical sites where homes



FIGURE 2 Bedbugs. (Left) *Cimex lectularius* (bedbug). Scale = 0.5 mm. (Department of Tropical Public Health, Harvard School of Public Health.) (Right) Immediate-type hypersensitivity reaction to bedbug bites acquired in a four-star hotel in Dupont Circle, Washington, D.C., December 2005.

have dirt floors, and livestock share living accommodations, extremely dense infestations may develop. Although this flea is cosmopolitan in its distribution, the cat flea appears to have supplanted it as the major flea pest for humans in many countries.

An unusual flea, the chigoe or jigger *(Tunga penetrans),* will attach to a host and maintain a feeding site. Originally found in Latin America, the chigoe has been carried across to sub-Saharan Africa by humans and may be found anywhere there. This flea will often penetrate under toenails or burrow into skin between toes, or in the soles of feet, an infestation known as tungiasis (Fig. 3). The female *T. penetrans* will swell to 10 times its size, and the host's immune response causes skin to swell up and cover her, with only the end of the abdomen exposed. Through this opening, she will deposit eggs and feces. When the flea dies, the lesion becomes secondarily infected, causing great irritation and pain. Tourists will often become infested by walking barefoot in shady spots around beaches.

Perhaps the most famous flea is the plague vector, the oriental rat flea, *X. cheopis.* Although it prefers rats *(Rattus rattus* and *Rattus norvegicus)* as hosts, in their absence (or when they die) this flea will feed on humans and other animals. These fleas may be found on rats in virtually every tropical or warm temperate port city around the world, having been transported there with their hosts by trade ships. Plague is usually maintained in enzootic foci by wild rodents and their more specific fleas, but in Vietnam, Madagascar, and India, rats and *X. cheopis* appear to be important for perpetuation. Although enzootic plague usually results in sporadic cases, the great fecundity of rats under the right circumstances means that explosive outbreaks of plague may occur in urban areas, with hundreds or thousands of cases. Human exposure in the western United States, where plague is enzootic, may be due to chance contact with ground squirrels or their fleas (often *Diamanus montanus*) in sheds, in disused cabins, in crawl spaces, or by digging around burrows. Domestic cats often serve as an intermediary, hunting moribund rodents and subsequently exposing their owners.

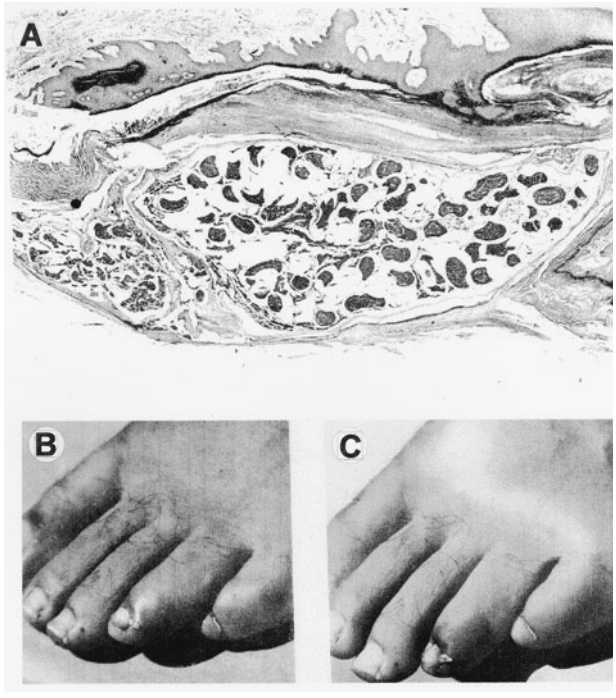


FIGURE 3 Tungiasis. (A) Low-power section of dermal lesion showing a flea uterus filled with developing eggs. (B and C) Progressive edema and secondary bacterial infection of *Tunga* lesion. (Reprinted from J. E. Ash and S. Spitz, *Pathology of Tropical Diseases,* W. B. Saunders, Philadelphia, Pa., 1945.)

Phthiraptera

Infestation by lice is called pediculosis. Lice are wingless, flat (dorsoventrally), elongate, small (0.4 to 3 mm) insects that are generally characterized by strong host specificity. Classically, two orders were recognized, the Mallophaga (chewing lice) and the Anoplura (the sucking lice); the former now comprises three suborders, and the latter remains as a suborder, all within the order Phthiraptera, which contains about 4,000 species. Of these, the sucking lice are clinically relevant, although the chewing lice may be presented as spurious ectoparasites, or associated with double-pored tapeworm *(Dipylidium caninum)* infection. Most chewing lice are commensals of birds, whereas the sucking lice feed mainly on mammals.

Lice undergo incomplete (hemimetabolous) development, with nymphal forms resembling adult lice, and are often found concurrently with the adults. Thus, size may appear to greatly vary within a single collection of specimens from one host. All lice are delicate and very sensitive to temperature and humidity requirements; all die within days without the host.

Chewing lice usually are found in the feathers of birds, feeding on skin fragments. Virtually all chewing lice lack piercing mouthparts and therefore do not feed on blood. The dog-biting louse *(Trichodectes canis)* may be presented as a specimen because it is a common commensal of dogs and may be spuriously associated with "bites." Dogs serve as definitive hosts for *D. caninum,* the eggs of which are shed in feces; the feces dry on the fur and the louse may then ingest the eggs. The tapeworm eggs hatch and a cysticercoid becomes localized in the hemocoel of the louse. Transmission is effected when the louse is accidentally ingested, usually when the dog is grooming; humans (usually children) are incidentally infected if their hands become contaminated and then touch food or drink or are placed in their mouths.

Book lice (order Psocoptera, also known as psocids) resemble chewing lice and live on molds infesting old books. They are frequently presented as specimens in diagnostic workups for itches of unknown etiology, but do not infest humans or any other animal.

The lice of greatest clinical importance (Fig. 4) are the head louse *(Pediculus humanus capitis),* body louse *(Pediculus humanus corporis),* and pubic louse *(Pthirus pubis).* All of these sucking lice have prominent claws attached to each of their legs, morphologically adapted for grasping the hairs of their host. They feed at least daily and deposit 1 to 10 eggs from each blood meal, gluing one egg at a time onto the shafts of hairs (or in the case of the body louse, onto threads within clothing). The eggs, or nits, are almost cylindrical in shape, and have an anterior operculum. Nits hatch within 4 to 15 days, and each of the three nymphal stages lasts 3 to 8 days. Lice are transferred between hosts by close physical contact, or by sharing clothing in the case of body lice. Schools are excellent sites for the spread of a louse infestation, not only through the sharing of hats and scarves, but by children deliberately infesting others during play. It is possible that transient circumstances, such as a subway headrest recently used by an infested head, could serve as the means

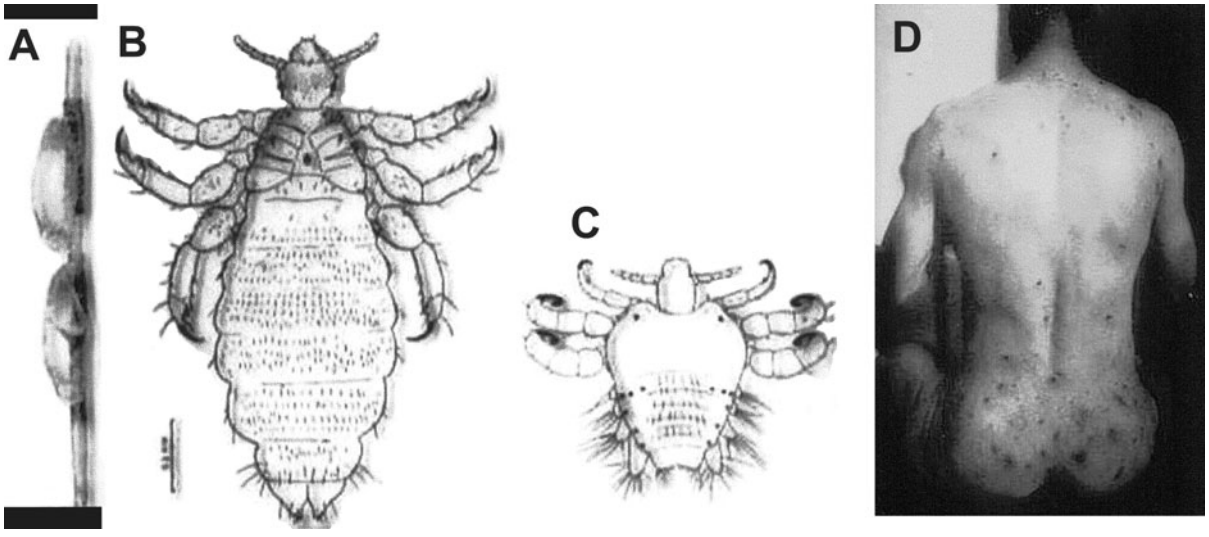


FIGURE 4 Lice. (A) Head louse nits; (B) body louse; (C) pubic louse; (D) vagabond's disease. (Department of Tropical Public Health, Harvard School of Public Health.)

for transfer. The oft-tendered excuse "I got it from a toilet seat" with reference to pubic lice should not be dismissed out of hand, but the probability of acquiring a louse in this manner is far less than by skin-to-skin contact. On the other hand, the presence of pubic lice on the eyebrows usually denotes a sexually transmitted infestation, and pubic louse infestation of a child is cause for an inquiry into the possibility of child abuse.

Body or pubic louse infestation may result in intense irritation for several days, with each bite generating a red papule. Chronically infested individuals may become desensitized, or may develop a nonspecific febrile illness with lymphadenopathy, edema, and arthropathy (although such signs and symptoms should prompt a search for the agent of trench fever). A very few chronically infested individuals may develop "morbus errorum" or vagabond's disease (Fig. 4), with a thickening and dark pigmentation of the skin (14).

Although body louse or pubic louse infestation may be considered evidence of poor hygiene or poor judgement, infestation by head lice should not be a stigma. Nor should head louse infestation be considered to be a public health menace, or even a clinical problem. Very few infestations are dense enough to cause signs or symptoms, and head lice are not vectors. Nonetheless, draconian measures are taken by many school districts, banning an infested child until treatment is thorough enough to remove all nits. Many such schools fail to discriminate between live and dead nits, insisting that all traces of them be absent. Complete removal of all nit remnants can be very difficult, relying mainly on fine-toothed combs, a tool that has been found in the tombs of the Egyptian pharaohs (25).

The sucking lice are both important vectors as well as pests. Among the most notorious vectors in history are the body lice, which may serve as vectors for the agents of epidemic typhus, trench fever, and louse-borne relapsing fever. Napoleon's invasion of Russia in 1812 was probably thwarted by epidemic typhus decimating his troops. It is said that at the end of World War I, a typhus epidemic in Russia and Romania killed 800,000 people (43). Body lice are the product of poor hygiene, with clothes never or rarely being changed, such as in cold weather, by the homeless, or in refugee camps. Trench fever is not uncommon in the homeless, and the most recent large outbreak of typhus was in Burundi, among Rwandan refugees. Oddly, even though the head louse is conspecific and appears vector competent in laboratory experiments, it has not been epidemiologically associated with any of these infections, nor has the pubic louse.

Acarina

The acarines are a subclass within the class Arachnida, which also contains the spiders. Acarina comprise the mites and ticks, tiny to small arthropods with eight legs as nymphs and adults (as opposed to six for insects) and with fused main body segments as opposed to three discrete ones for insects. Acarine bauplane are characterized by two functional body parts, the gnathosoma (or capitulum), which comprises the "head," and the idiosoma, which contains all the remaining functions (reproductive, motility, and digestion). They undergo incomplete metamorphosis, passing through a larval stage and one to several nymphal stages before attaining sexual maturity. The mites are one of the most speciose groups of animals, with 45,000 recognized species. They are among the oldest terrestrial animals, dating in the fossil record to the Devonian (nearly 400 million years ago), and are found in every habitat on earth. There are two major orders of the acarines, the Acariformes and the Parasitiformes. In the former, there are two main groups, the Sarcoptiformes (astigmata) and the Trombidiformes (prostigmata), both of which contain species of clinical significance. The latter comprises three orders, the Holothyrida, the Ixodida, and the Mesostigmata. Of these, only the last two are clinically significant; the holothyrids consist of about 20 species that are found only in Australasia and some neotropical forests, and are unlikely to be encountered (although they are known to secrete a toxin that may incapacitate a human that has ingested such a mite). The mesostigmata (also known as gamasida or dermanyssoidea) include some that infest birds or rodents and that under certain circumstances will infest humans and cause itch. The ixodida consist of the ticks, which are essentially very large mites.

Other than the ticks and some mesostigmatids, mites are generally not considered vectors of agents that infect humans. They are, however, important for their pest potential, causing itch, dermatitis, and allergy.

The house mouse mite *(Liponyssoides sanguineus)* is the vector for rickettsialpox due to infection by *Rickettsia akari*. The relatively mild disease, characterized by fever and exanthema, was first described after a garbage strike in Kew Gardens in the Bronx, New York, N.Y., during the 1940s (16). Garbage piled up, and house mouse populations became dense. When the strike was resolved and the garbage was removed, the mice died or emigrated, leaving behind dense infestations of hungry mites. The prostigmatid trombiculid mites (chiggers) are the vectors for the agent of scrub typhus *(Orientia tsutsugamushi)*, a rickettsiosis of Eurasia and northeastern Australia. Nearly 3 billion people live in countries where scrub typhus is endemic. Scrub typhus is acquired from infestation by tiny (0.2-mm) larval trombiculid mites such as *Leptotrombidium deliense* or *Leptotrombidium akamushi;* an eschar forms at the site of the chigger bite, with proximal lymphadenopathy, fever, headache, exanthema, and myalgia. Case fatality rates can range from 5 to 35% (3). Interestingly, the nymphal and adult stages of this mite feed on detritus or other arthropods and do not take vertebrate blood. Therefore, *O. tsutsugamushi* relies mainly on transovarial or vertical transmission (passage through the egg) for perpetuation, although rodent hosts that are infected may feed noninfected larvae and generate new matrilineages of infected mites. All other vector-borne agents have greater opportunities for horizontal transmission, that is, amplification by infecting a vertebrate host and having multiple blood meals during development.

Mites are extremely difficult to identify, particularly given the likely confusion with dust mites or other ubiquitous free-living forms. All require clearing and mounting on a slide and examination under a compound microscope.

Ticks are prolific vectors, with more recognized transmitted agents than any arthropod other than mosquitoes. All of the nearly 900 known species of ticks require blood for their development and reproduction. Clinically relevant ticks belong to either the Ixodidae (the hard ticks) or the Argasidae (the soft ticks); a third family, the Nuttallielidae, comprises a monotypic genus of soft ticks found in southern Africa and for which an association with a pathogenic agent has yet to be described. Hard ticks are so named because of the hardened sclerotized idiosomal shield, or scutum. In female hard ticks, the scutum is on the anterior third of the idiosoma, with the remainder of the idiosoma consisting of a pleated, leathery cuticle that allows for tremendous expansion during blood feeding. In male hard ticks, which may or may not feed at all, the scutum extends the length of the idiosoma. In contrast, soft ticks have no scutum; their entire idiosoma is leathery.

The "head," or capitulum, consists of the holdfast (hypostome), the chelicerae (which are homologs of insect mandibles), and the palps, which cover the mouthparts (hypostome and chelicerae) and serve a sensory function. Chelicerae act as cutting organs, the two sides sliding past each other, with the cutting teeth at the end gaining a purchase into a host's skin. The hypostome is thereby inserted, allowing anchoring of the entire tick due to recurved, backward-facing teeth or denticles (Fig. 5). Many hard ticks also secrete a cement around the hypostome. Often, when removing an attached dog tick *(Dermacentor variabilis)* from a host, a large piece of skin comes with the hypostome, mostly cement and surrounding epidermis. Thus, by virtue of the cement and denticles, tick hypostomes rarely emerge intact when a tick is removed. "Leaving the head in" is not critical, and most times the remnant will be walled off as a foreign body, or will work itself out, perhaps by the act of scratching. Treatment, therefore, should simply be disinfection of the site of the bite and certainly not excavation of the epidermis looking for the "head." Soft ticks are transient feeders and will only rarely be found attached.

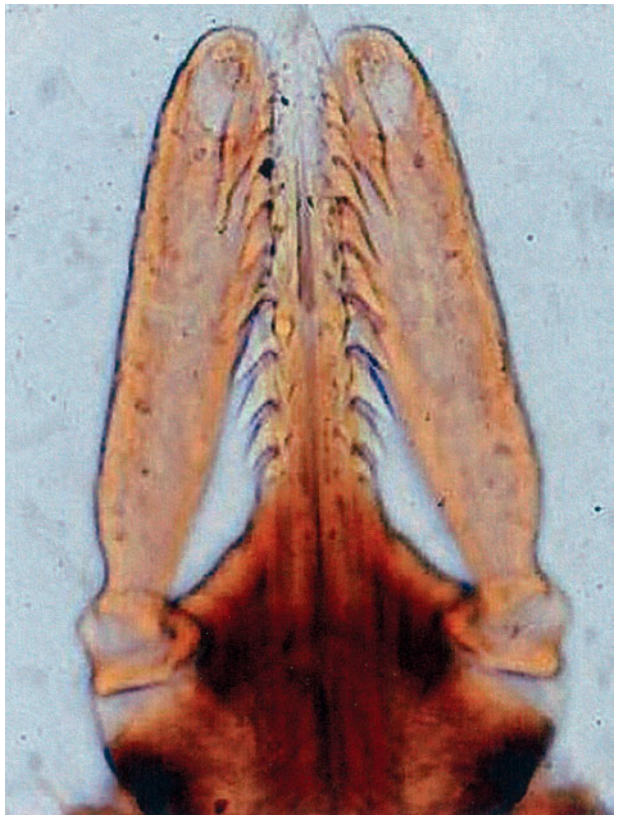


FIGURE 5 Tick hypostome, showing recurved denticles.

Hard ticks require several days to complete their blood meal; the number of days depends on the species and stage of the tick. North American deer ticks (*Ixodes dammini* [30, 35]) will feed for 3 days as a larva, 4 days as a nymph, and 7 days as the female. The closely related European sheep or castor bean tick, *Ixodes ricinus,* feeds for 2 days as a larva, 3 days as a nymph, and 7 days as the female. The duration of feeding depends also on host immune status (previous exposure may induce immediate-type hypersensitivity, which slows down the feeding process) and temperature (*I. dammini* and *I. ricinus* feed twice as long on cold reptiles as on ones that are held at 37°C). The extended duration of feeding is required for the cuticle to soften so that the idiosoma may accommodate 10 to 100 times its weight in blood; also, the site of the bite is prepared so that a pool of lymph and blood is available for removal. During the first 70% of the feeding process, very little blood or lymph appears to be present within ticks, which remain dorsoventrally flat. Hemoglobin is excreted from the anus, lipids are retained, and water from the blood is recycled back into the host as saliva (4). In the last day, usually in the last 3 or 4 h of the blood meal, the tick takes what has been termed "the big sip," removing a large volume of whole blood, and then detaches and drops from the host.

Because they must remain attached for days, hard ticks have evolved means of temporarily disabling a host's local inflammatory response, which might inhibit its feeding. Hard tick saliva is an extremely complex mixture of anticoagulant, anti-inflammatory, and antihemostatic agents (28) that act mainly at the site of the feeding lesion. Tick saliva will also neutralize Th2 responses systemically (42). Hosts that have never been exposed to ticks will not realize that a tick is attached. Indeed, most patients with Lyme disease or spotted fever never knew that they had been "bitten" (34, 41). The most dangerous tick is not necessarily the one that a patient finds and removes, aborting the transmission process, but the one that he or she never knew was there and which was able to complete its feeding.

In contrast, soft ticks are more like mosquitoes in their feeding, spending tens of minutes to no more than a few hours feeding, usually as their host is sleeping. Soft tick saliva does not "need" to be as clever, and in fact some species have painful bites. The pajuello (pajaroello), *Ornithodoros coriaceus,* of California and Mexico is renowned for its "toxic bite," which causes local pain and burning (14).

Tick life cycles have an extended duration, usually months or years. Deer ticks, for example, take 2 years to go from egg to egg. For this reason, there is generally no risk associated with hard ticks engorging and dropping off of a companion animal within a person's home. The engorged tick will not feed again, and will take weeks to molt or lay eggs, and in the interim, usually the relative humidity within the house is too low for extended survival of the tick. On the other hand, cats as opposed to dogs appear to be a risk factor for acquiring Lyme disease, perhaps because deer ticks feed well on dogs but poorly on cats; ticks, particularly nymphs, will detach in mid-feed and readily reattach to the cat's owner (10). The exceptions to the lack of risk associated with ticks engorging and detaching in the household is with the brown dog tick *(Rhipicephalus sanguineus),* which is the vector of Marseille fever (Mediterranean spotted fever, boutonneuse fever) and *Ehrlichia canis* and has also recently been documented as a vector of Rocky Mountain spotted fever. These ticks will hide behind wall molding to molt and indeed are known for dense infestations covering the interior walls of dog kennels.

Ticks can be difficult to identify, depending on their state of engorgement and whether mouthparts are intact. Although tick systematists have recently altered some of the generic epithets, in general the classic Centers for Disease Control and Prevention (CDC) diagrams of the mouthparts and idiosoma (Fig. 6) can be used to at least classify a tick to the genus level. Often, simply knowing the country in which a tick may have been acquired will significantly narrow down the list of possibilities (Table 2).

ARTHROPODS AS "SCALARS"

Vectors impart directionality to a pathogen. In contrast, there are arthropod-pathogen relationships that are not characterized by directionality, and analogous to mathematical terminology, arthropods that inadvertently serve as a source of infection are called scalars (33). Helminths may use an arthropod as an intermediate host, but that arthropod does not deliver the infectious stage of the helminth during an obligate behavior such as blood feeding. Drinking water with copepods (crustacea) containing third-stage larvae of the filarial nematode *Dracunculus medinensis* will initiate infection when the copepods are digested by stomach acids, thereby liberating the nematode larvae, but the copepods do not swim towards a vessel scooping water out for drinking. Accordingly, the patient's history needs to specifically address the possibility of exposure via drinking from natural bodies of water (copepods and *Dracunculus*); the presence of flour beetles, fleas, or roaches (hymenolepidid cestodes, *Dipylidium caninum,* and *Gongylonema* spp.); eating of crabs or crayfish (*Paragonimus* spp.); or being in an environment with dense infestations of houseflies (trachoma). With the exception of houseflies and trachoma (which perpetuates in the absence of flies, spreading by direct contact with ocular exudates), all these are obligate relationships.

Muscoid Flies

The muscoid diptera include the muscids (houseflies and stable flies), the Calliphoridae (blowflies), and the Sarcophagidae (the flesh flies). Egg deposition and larval development occur in characteristic materials, viz., fecal material for houseflies, decaying plant material for stable flies, live flesh for blowflies, and carrion for flesh flies. These flies can be remarkably prolific within short periods: a female housefly, for example, deposits 100 to 150 eggs in moist, decaying organic material, usually excrement, at one time, but may do so 20 or more times (15). Larvae emerge from the eggs within 12 h. Three larval stages develop during a week, and a puparium is formed. The adult fly emerges from the pupa within 4 days, and copulation may occur within 1 day. Thus, a full life cycle may take as few as 12 days.

Houseflies have received much attention for their potential as scalars because of their association with poor hygiene. Flies are strong fliers and move readily from outdoors to indoors. A large fleshy structure at the apex of the proboscis provides a surface for contamination, as do the hairy body and legs of the fly. In addition, flies may regurgitate while feeding, and the vomitus may contain organisms that were acquired in a previous landing. Houseflies commonly feed on human excrement, and virtually every possible enteric pathogen (those causing amebiasis, cholera, typhoid, hepatitis A, and poliomyelitis; even roundworms and *Helicobacter pylori*) has been detected within or upon them. With few exceptions, such findings are epidemiologically irrelevant inasmuch as all of the agents perpetuate in their absence. Poliovirus, for example, was recovered in flies captured from sites where polio was actively being transmitted (38), but use of DDT failed to curtail the epidemic. In contrast, residual DDT treatment of army camps reduced the incidence of shigellosis (39). It is likely that individual cases of enteric disease may derive from fly contamination of food, but whether the risk of such an event merits worry by patients or their health care providers remains unclear. Dense infestations of flies should be reduced (by source reduction, i.e., by preventing flies from getting access to garbage and excrement); a few flies in the house do not warrant setting off a bug bomb.

Cockroaches

Roaches are dorsoventrally flattened, smooth-bodied, winged insects with long antennae, biting-type mouthparts, and abdominal projections (cerci). The outer pair of wings is thick and leathery, and the inner pair is membranous. Roaches may fly, but they usually scuttle about on long, spiny legs. They undergo incomplete metamorphosis, with the immature forms looking like miniature adults, although without wings. Eggs are laid within a hard capsule, the ootheca, which is deposited in a dark crevice. Development is slow, taking about 4 weeks between molts; many roaches have only one generation each year. Roaches can live for months without food, but water seems critical. They are

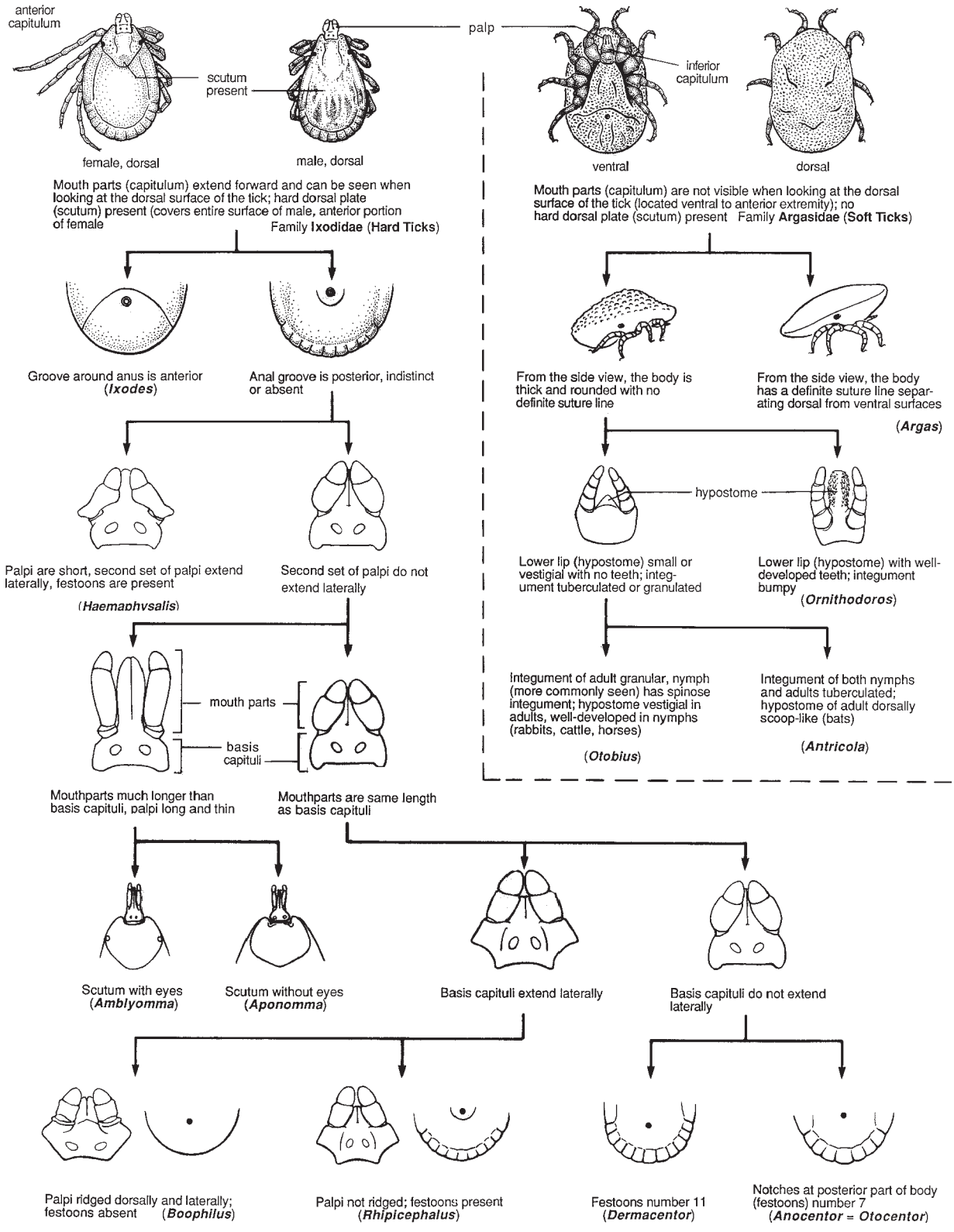


FIGURE 6 Key to major tick genera in the United States. (Reprinted with permission from L. S. Garcia, *Diagnostic Medical Parasitology*, 5th ed., ASM Press, Washington, D.C., 2007.)

TABLE 2 Likely human-biting ticks and possible tick-borne infections by global region

Region	Likely tick(s) infesting humans	Possible zoonosis[a]
North America	*Ixodes dammini, Ixodes scapularis, Ixodes pacificus, Ixodes dentatus, Ixodes cookei*	Lyme disease, babesiosis, HGE, Powassan fever
	Dermacentor variabilis, Dermacentor andersoni, Dermacentor occidentalis	RMSF, CTF, tularemia
	Amblyomma americanum, Amblyomma maculatum	Masters' disease/STARI, RMSF, tularemia, HME
	Ornithodoros hermsi, Ornithodoros turicata	Relapsing fever
South America	*Amblyomma cajennense*	RMSF
	Ornithodoros spp.	Relapsing fever
Europe, Russia	*Ixodes ricinus, Ixodes persulcatus*	Lyme disease, babesiosis, HGE, TBE
	Dermacentor marginatus	TIBOLA
	Rhipicephalus sanguineus	Marseille fever, HME?
Japan, China, Korea	*Ixodes ovatus, I. persulcatus*	Lyme disease, babesiosis, HGE, TBE
	Dermacentor spp.	Tick typhus, JSF?
	Haemaphysalis spp.	JSF?
South Asia	*Haemaphysalis* spp.	KFD
	Hyalomma spp.	CCHF
	R. sanguineus	Tick typhus
Southeast Asia	*Ixodes granulatus*	Lyme disease?
	R. sanguineus	Tick typhus? HME?
Australia	*Ixodes holocyclus*	Tick typhus
North Africa	*Hyalomma* spp.	CCHF
	R. sanguineus	Marseille fever, HME?
	I. ricinus	Lyme disease
West Africa	*Hyalomma* spp.	CCHF
	Ornithodoros erraticus	Relapsing fever
	Amblyomma variegatum	ATBF
Sub-Saharan Africa	*A. variegatum, Amblyomma hebraeum, Haemaphysalis leachi*	ATBF
	Hyalomma spp.	CCHF
	Ornithodoros moubata	Relapsing fever

[a] HGE, human granulocytic ehrlichiosis (*Anaplasma phagocytophilum*); HME, human monocytic ehrlichiosis (due to either *Ehrlichia chaffeensis* or *Ehrlichia canis*); RMSF, Rocky Mountain spotted fever; STARI, southern tick-associated rash illness (etiology unknown, possible borrelia); CTF, Colorado tick fever; TIBOLA, tick-borne lymphadenopathy (*Rickettsia slovaca*); JSF, Japanese spotted fever (*Rickettsia japonica*); TBE, tick-borne encephalitis; CCHF, Crimean-Congo hemorrhagic fever; KFD, Kyasanur Forest disease; ATBF, African tickbite fever (*Rickettsia africae*).

omnivorous, feeding on the finest of foods to the vilest of waste, usually at night. Secretions deposited by scent glands (including trail and aggregation pheromones) give rise to a characteristic disagreeable odor that confirms an infestation even when live roaches cannot be found. Common roaches range in size from the small German cockroach (*Blatella germanica*), about half an inch in length, to the American cockroach, nearly 2 in. Much work has been done attempting to incriminate roaches as scalars, even estimating nearly 14,000 CFU of bacteria (mainly *Staphylococcus* spp.) on the surface of the body of one German cockroach (14). Feces laden with *Vibrio,* the agent of cholera, were fed to American cockroaches, with ingestion of as much as 200 μl of material and recovery of live vibrios 30 to 80 h later from roach feces (5). A roundworm (*Gongylonema* spp.) normally infecting ungulates may encyst within roaches and be transmitted to humans when ingested, usually as a contaminant of food. As with flies, the presence of roaches suggests poor environmental hygiene, but only rare instances of enteric disease might be associated with them. Dense infestations may produce large amounts of antigenic material (feces, molted cuticle, or parts from carcasses), which may be implicated in allergic or asthmatic reactions.

Control of roach infestations can be difficult. Boric acid, deposited along walls, behind moldings, and around other sites where they may hide, is effective in killing adults and nymphs by abrading cuticle between abdominal segments, rendering the roach prone to desiccation. Removing standing water (wiping up and getting rid of clutter around sinks) can also reduce infestations by preventing access to water. Roach infestations are most common in apartment buildings serving transient student or immigrant populations, who bring the insects in with their household goods.

DIRECT INJURY DUE TO ARTHROPODS

Arthropods Typically Thought of as Vectors

Vectors such as lice, ticks, bugs, fleas, mosquitoes, and blackflies may directly cause injury by their bites, either by hypersensitivity reactions or toxic effects of their salivary products. Hypersensitivity reactions mainly manifest as itch, with the concomitant potential for secondary infection due to scratching. Bedbug and flea bites may cause immediate-type hypersensitivity reactions with itchy erythema greater than 3 cm in diameter (Fig. 2). Ticks may induce a chronic local granulomatous lesion, persisting for months, perhaps due to remnants of the mouthparts (denticles) left within the epidermis. This phenomenon is particularly pronounced with "seed tick" infestation (stepping into a newly emerged batch of larval *Amblyomma* ticks, often thousands), where dozens or hundreds of ticks may attach at the belt line. Itch

may be immediately relieved by calamine (or Caladryl with Benadryl) lotion, or even holding the affected part under very hot running water, which will induce mast cells to degranulate. Over-the-counter hydrocortisone creams may help mild cases of itch, but severe cases may require prescription strength steroid cream (e.g., betamethasone, 0.05%). Daily application of hydrocortisone should promote a resolution of itch within a week. Tick granulomas may be treated with Retin-A gel (0.05%), which may promote the turnover of epidermis and ejection of remaining antigenic material (personal observation).

As mentioned in the introduction to diptera, blackfly bites may produce blackfly fever, usually as a dose-dependent reaction. Usually, the site of the bite become edematous, with a golf ball-sized lump and an oozing punctate lesion. Fever and myalgia will manifest that night and disappear within 24 h. Such symptoms should not be construed as infection; few pathogens, if any, will have such a short prepatent period. Treatment is symptomatic. Similarly, soft tick bites due to pajaroello or the African tampan *(Ornithodoros moubata)* may immediately cause pain, swelling, and irritation at the site of the bite, with raised hard wheals (24). The effects are said to last several days, with "irritability" of the affected part. Anecdotally, hunters in northern New England and the upper Great Lakes may complain of bites of *Dermacentor albopictus* larvae (a species that usually feeds only on ungulates such as deer or moose) during the early winter; apocrypha indicates that Native Americans called these larvae "bite all same as a piece of fire" (6). The condition has not been studied.

An unusual toxicosis due to tick bite is tick paralysis. The presence of certain feeding ticks induces an acute ascending paralysis. First described for sheep and cattle in Australia in 1843, a similar disease was reported for a child in Oregon in 1912. The Australian *Ixodes holocyclus* attacks cattle, sheep, and dogs but rarely humans, and thus tick paralysis is not a common clinical condition there. However, in the western United States, bites of *Dermacentor andersoni* commonly produce cases of "staggers" in cattle or sheep, which may terminate fatally. Children are the usual victims of tick paralysis, with ticks attached at the nape of the neck. The illness is characterized by fatigue, irritability, distal paresthesias, leg weakness with reduced tendon reflexes, ataxia, and lethargy. Unless the tick is removed, quadriplegia and respiratory failure may result; the case fatality rate without treatment can be 10%. Removal of the tick induces a miraculous recovery within 48 h. (Tick biologists unromantically suggest such an etiology for the tale of Snow White, who awakens after the Prince bends over her and kisses her, probably removing a tick from behind her ear.) A 40- to 60-kDa toxin has been isolated from *I. holocyclus* and has been named holocyclotoxin (21); an antitoxin has been produced for veterinary use. Others with a much lower molecular weight have also been isolated. The toxin has not been isolated from the American tick paralysis ticks. Other ticks (*Amblyomma americanum* and *Ixodes* spp.) have also been reported to induce tick paralysis.

Endoparasitic Arthropods

Arthropods that invade a host's body and cause disease include mites (scabies), fleas (tungiasis), fly larvae (myiasis), and pentastomes (halzoun). Tungiasis was discussed above as part of the general flea section. Pentastomes, or tongue worms, are primitive, elongate arthropods that live as adults in the lungs and air passages of hosts, including fish, amphibians, reptiles, and some mammals. The body, like that of acarines, consists of a cephalothorax and indistinct abdomen, with no legs; often, the cuticle appears ringed or annulated. Chitinous hooks protrude from the head. Their size ranges from 1 to 10 cm. Although long thought to be in its own phylum, recent cladistic analyses based on morphological and molecular characters place it within the Crustacea and most closely related to the branchiurid fish lice (20). Pentastomes undergo a complex life cycle with incomplete development, requiring intermediate hosts in which the nymphal stages may encyst.

Human infestation by *Linguatula serrata* may be due to ingestion of eggs (from contamination by nasal discharges from the dog intermediate host) or ingestion of encysted nymphs within the raw or poorly cooked liver, lungs, or mesentery of an intermediate host such as rabbits, cattle, or sheep (31). A nasopharyngeal syndrome results, known as halzoun in the Middle East and marrara in Sudan. Facial edema, nasal discharge, coughing, and sneezing are due to the migration of nymphal forms into the nasopharynx. Removal of the offending nymphs (by visual inspection and forceps) and symptomatic treatment with antihistamines to reduce edema may be helpful. *Armillifer armillatus* and *Porocephalus crotali* of snakes also cause infections in humans, probably as a result of contaminating drinking water with eggs from their feces and not because of ingestion of poorly cooked snake meat. Usually, human infection is noted only at autopsy or by the presence of calcified abdominal or lung objects in radiographs. Treatment is symptomatic.

The most common of the ectoparasite-caused direct injury is scabies, caused by infestation with the human scabies mite *(Sarcoptes scabiei)*. A number of different populations of *S. scabiei* have been treated as full species based on their tropism for other animals (including dogs, pigs, sheep, cattle, and goats), but all are morphologically identical. Infestation may occur anywhere in the world. Canine sarcoptic mange is commonly associated with scabies infestations in the owners of the dogs. In either animal or human scabies, transmission is by direct personal contact, and infestations often cluster among groups of people, particularly families. There is little evidence that environments become contaminated; fomites for this organism have not been identified.

The female scabies mite burrows beneath the stratum corneum (Fig. 7), leaving behind eggs and feces within a track-like trail. A few dozen eggs are deposited, and these hatch within a week. Larvae form new burrows, but they may also emerge from the skin and move freely about. Nymphs develop from fed larvae, and they in turn develop into the adult male and female. Normal infestations consist of a dozen or two female mites. Nocturnal itching begins within a month of the first infestation but may begin within a day in previously exposed individuals. Thus, newly exposed individuals, prior to their recognizing an infestation by the presence of itching, may contaminate other individuals. Erythematous papules and vesicles first appear on the webs of fingers and spread to the arms, trunk, and buttocks. The burrows contain granular, highly antigenic feces, which cause both delayed- and immediate-type hypersensitivity reactions. Interestingly, in individuals who are immunocompromised, hundreds of female mites may be found, itching is minimal, but a hyperkeratosis is prominent. Such "crusted" or Norwegian scabies are highly infectious to other people.

Scabies infestations can be easily diagnosed by scraping a newly developed papule (not one that has been scratched) with a scalpel coated with mineral oil. The scrapings in oil may be transferred to a slide and examined by bright-field microscopy at ×100 or ×400 for 300- to 400-μm mites or the smaller black fecal granules. Scabies may be treated with 5% permethrin cream or 1% permethrin rinse (Nix; same as is used for head lice). The method of choice until recently

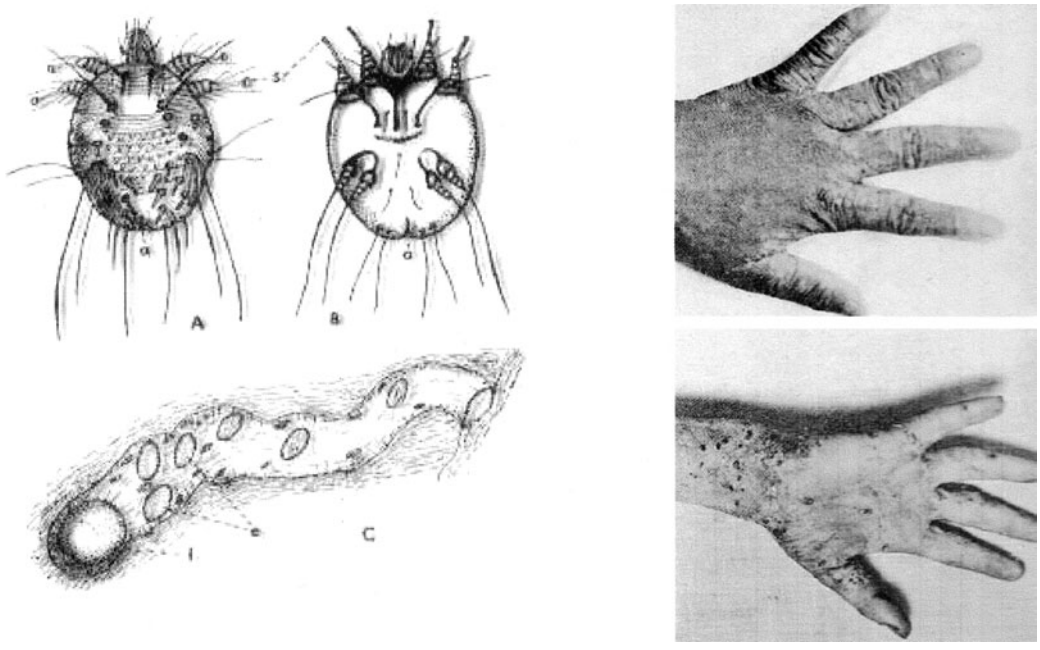


FIGURE 7 Scabies. Diagram of feeding lesion and adult female mite. Chronic scabies affecting the hands. (Reprinted from J. E. Ash and S. Spitz, *Pathology of Tropical Diseases,* W. B. Saunders, Philadelphia, Pa., 1945.)

was topical lindane (Kwell), but the U.S. Food and Drug Administration now suggests the use of permethrin first and lindane only if that fails. Lindane can be neurotoxic to children and small adults.

Demodex folliculorum, the follicle mite, infests virtually everybody. This elongate 500-μm-long mite may be found within follicles on the face, ears, and breast, and it is often brought to the attention of a physician because a tweezed eyelash may have a few mites at the base of the hair shaft. Although they appear to be largely nonpathogenic, the same species causes demodectic mange in dogs and cattle. Pityriasis folliculorum, with small pustules appearing on the forehead, has been attributed to them.

Dust mite allergies (one of many causes of asthma, rhinitis, and atopic dermatitis) are due to inhalation of feces excreted by *Dermatophagoides farinae* or related pyroglyphid mites, which are human commensals that feed on flakes of skin shed from a person. The mites themselves do not infest a person but remain in the environment (usually within bedding or carpets) to feed and develop. About a half gram of skin may be shed from a person each day; one female mite lays one egg a day, for about 2 months; thus, large accumulations of mites may readily develop. The 300- to 400-μm-long mites may be presented by patients as suspects for other nonspecific lesions or sets of signs and symptoms because they may be found in virtually all houses and can be detected if dust is allowed to settle on standing water; the mites float and will move and can be seen at ×20. Humidity less than 60% will greatly reduce dust mite infestations, as will periodic vacuuming and washing of bedding and carpets.

Myiasis is the infestation of human or animal tissue by fly larvae, deposited as eggs or first-stage larvae; the larvae develop by feeding on the surrounding tissue, emerge as third-stage larvae, and pupate in the environment. There are three kinds of myiasis with respect to life cycle patterns: obligate, facultative, and accidental. Obligate myiasis reflects the need for larvae to feed well during development because adult flies do not feed, or they feed poorly. Botflies comprise the main examples of obligate myiasis. Most botflies normally infest animals, and thus human botfly infestation by these species is considered zoonotic. Two flies, *Dermatobia hominis* (human botfly) and *Cordylobia anthropophaga* (tumbu fly), are more adapted to humans, as illustrated by their life cycles. The former fly lays its eggs on a transport (phoretic) host such as a mosquito, and when the mosquito feeds on a human, the eggs hatch during the course of the blood meal and the larvae penetrate the skin at the site of the mosquito bite or burrow in on their own. The latter fly is attracted to sweat, urine, or feces and will oviposit on clothing that has been spread out to dry on the ground or hung up to dry, on areas of cloth that are redolent with such odors, which may remain when primitive clothes washing practices are used. The eggs hatch when placed close to the body and the larvae burrow into the skin. Thus, any clothing washed in tropical countries without the aid of modern soap powders and dryers should be ironed before being worn.

Facultative myiasis is usually due to infestation by blowflies *(Phormia regina),* green bottle flies *(Lucilia sericata)* and related calliphorids, flesh flies (*Wohlfahrtia* spp.), or common houseflies *(Musca domestica).* These flies normally deposit eggs into fecal or other rotting organic material; they will also directly lay eggs into wounds or necrotic tissue, but the larvae may not confine themselves to such resources and move into healthy tissue.

Accidental myiasis includes the incidental findings of fly larvae, often housefly, under wound dressings or within unusual sites such as the gastrointestinal tract. The rat-tailed maggot *(Eristalis tenax)* is actually a hoverfly (syrphid) that breeds in sewage or dirty water. Eggs might be deposited around the anus during defecation, and the larvae may find

their way into the lower gastrointestinal tract or urethra. Drinking unfiltered dirty water might cause temporary infestation of the gut, with larvae surviving into the lower intestine. More often, larvae are found in containers of stool samples that are intended for the clinical laboratory, and are most likely due to oviposition after the sample is taken.

A variety of clinical presentations are evident depending on the site where the larvae are present. Botflies cause furuncular lesions or migratory integumomyiasis (a serpiginous track may be produced in the skin). Wound myiasis comprises shallow or pocketlike initial lesions that become more deeply invasive. Maggots may invade the nose and accompanying structures, causing nasal or oral myiasis. Maggots may get into the ears, producing aural myiasis. Ophthalmomyiasis is due to external or internal infestation. Enteric, vaginal, or vesicomyiasis is due to invasion of the gut or genitalia. In all presentations, pathology may be due to tissue trauma or local destruction, but it is more often associated with secondary bacterial infection. On the other hand, many maggots do not promote bacterial infection, but rather secrete bacteriolytic compounds and have been used as a surgical intervention to debride wounds (9). Sterile *L. sericata* maggots are available by prescription in the United Kingdom, with recommendations for how many "pots" of maggots may be needed for wounds of specific dimensions (http://www.larve.com). Thus, the development of secondary bacterial infection in myiasis may suggest the death of the maggot.

In all cases, treatment is by removal of the maggots, laboriously picking them out using forceps. The maggots causing furuncular myiasis or migratory integumomyiasis will have their posterior end visible within the lesion, exposing the spiracular plates that cover their tracheolar breathing apparatus. Although much lore exists on how to best remove such maggots, including "luring" the maggots with bacon or pork fat, the simplest method is to cover the lesion with petroleum jelly (e.g., Vaseline), thereby preventing the maggot from breathing. (Obstruction of the spiracles is probably the mode of action for bacon or pork rind, not a preference for tasty fat.) It will eventually move out enough in an attempt to get air and so may be grasped with forceps.

Identification of maggots can be difficult. The spiracular plates can be diagnostic (Fig. 8) and should be removed from the posterior end, mounted on slides in an appropriate mounting medium, and compared with pictorial keys. Often, a live maggot can be reared to pupation on raw meat, and the resulting adult fly can be identified. On the other hand, other than academic curiosity, most myiasis cases presenting in developed countries are the result of tourism, and not knowing the specific identity of the fly would not influence further preventive measures; treatment would be sufficient. One important reason for accurately identifying a maggot is for forensic purposes: the developmental rates of the various obligate or facultative myiases have been described as a great aid in assigning time of death for cadavers. The American Board of Forensic Entomology (http://www.research.missouri.edu/entomology/) provides certification for experts in such tasks, and their membership may be consulted should a need for precise identification arise.

Stinging and Biting Resulting in Envenomation

Hymenoptera

Wasps, bees, and ants all belong to the order Hymenoptera, which contains well over 100,000 recognized species. Hymenoptera are minute to medium-sized insects with compound eyes, mandibles, and two pairs of transparent wings (although for the ants, wings may be seen on males or females only at certain times of the year). They undergo complete metamorphosis from a grub-like larva to a pupa. Those of clinical importance are within the Aculeata, particularly the Vespidea (social and solitary wasps), Apoidea (social and solitary bees), and Formicoidea (ants). Most of the Aculeata have a characteristic constriction between the first and second abdominal segments. These insects have a complex social behavior, with males, females, and worker castes. Workers have a stinging organ, which is used for defending the colony or capturing prey.

Most stings by hymenoptera cause localized reactions, sometimes with extreme pain and resulting in a transient induration with hyperemia. By virtue of their living in large colonies, bees and ants may swarm an intruder, and dozens, if not hundreds, of stings may be sustained. Airway obstruction may result should multiple stings be received on the face or neck. Although the honeybee's agricultural value in pollinating plants greatly exceeds any slight risk due to their stings, African honeybees (*Apis mellifera adansoni,* a subspecies of the regular honeybee; also known as "killer bees") which were imported into Brazil in the hope that they would be better pollinators can be dangerous because they are more aggressive than the typical honeybee. Bees differ from wasps and ants in that their stinging apparatus is forcibly torn out during the act of stinging, thereby ensuring the death of the individual bee as a result. Wasps and ants may sting multiple times; some fire ants may hang on by their mandibles and repeatedly insert their posteriorly located stinger.

Bee venom has been well characterized and consists of a large amount of a polypeptide (melittin), phospholipase A2, histamine, hyaluronidase, mast cell discharging peptide, and apamin. Histamine appears to be the cause of the acute pain of bee stings.

Bumblebees, paper wasps, yellow jackets, and hornets may all sting, usually because the victim intrudes too near a nest. The most important clinical manifestation of bee or wasp stings is anaphylaxis. Chest tightness, nausea, vertigo, cyanosis, and urticaria may be seen even in individuals who apparently had never previously been exposed. Dozens of people die each year in the United States due to bee sting anaphylaxis.

Ants, on the other hand, rarely pose a risk for anaphylaxis but produce a reaction that may persist for a longer duration. An induration or wheal may be observed immediately after the sting, and a papule may develop that itches or remains irritated for several days. Secondary bacterial infection may ensue. Fire ants *(Solenopsis invicta)* may bite or sting, both modes accompanied by the injection of a venom, an ethyl ketone for the former and various 2,6-dialkypiperidines in the latter. Other ants (the formicinae) have a venom that is mainly formic acid.

Urticating Caterpillars

Lepidoptera (moths and butterflies) undergo complete development, with the well-known caterpillar and cocoon stages. More than 100,000 species of these familiar insects have been described. Although certain adult moths (in four superfamilies) may imbibe blood or tears, no signs or symptoms are associated with what is evidently an independent evolution of hematophagy. At least eight families of Lepidoptera, however, have caterpillars with urticating hairs, and some of the neotropical species can produce surprisingly severe reactions, including a hemorrhagic syndrome (*Lonomia achelous,* a saturniid moth of the Amazon) due to a fibrinolytic toxin exuding from poison spines (13). More typical are rashes caused by contact with hairs (erucism, or

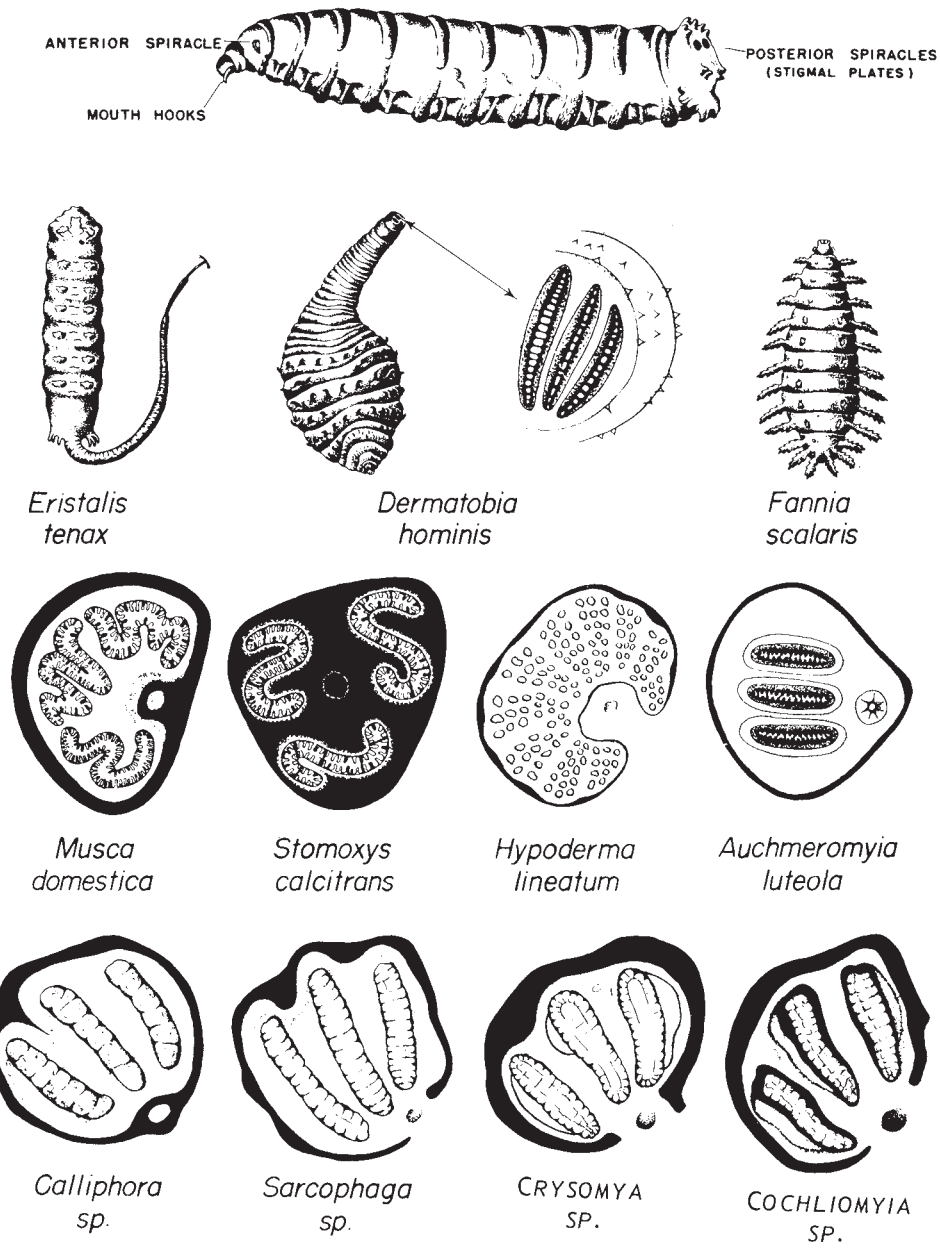


FIGURE 8 Key characters of some myiasis-producing fly larvae. Row 1, mature larva of a muscoid fly (from R. Hegner et al., copyright 1938 by Appleton Century Inc., New York, N.Y.). Row 2, larva of *Eristalis tenax,* the rat-tailed maggot; *Dermatobia hominis,* the human botfly (with an enlarged view of a posterior spiracle); and *Fannia scalaris,* the latrine fly. Rows 3 and 4, appearance of posterior spiracles of some species that produce accidental, facultative, or obligatory myiasis. (CDC, Atlanta, Ga.)

erucic rash). A common shade tree pest, the brown-tailed moth *(Nygmia phaeorrhoea),* in Europe and northeastern North America, liberates tiny barbed hairs when the caterpillar molts. These hairs are blown about by the wind and when skin is exposed, a severe dermatitis results; ingestion or inhalation can also cause significant irritation of the mucosa or bronchospasm. Contact with the eye may induce conjunctivitis. Dermatitis produced by urticating hairs typically comprises itchy, erythematous patches associated with small vesicles and edema. These lesions are only where the hairs have free access to the skin or where contact is made. Use of a masking tape-type lint roller can be very effective in removing urticating hairs, which may or may not be visible.

Scorpions

Scorpions are arachnids with a characteristic crablike appearance. The body consists of the cephalothorax and a segmented abdomen with a segmented tail, which terminates in a prominent stinging apparatus (aculeus). The four pairs of legs include well-defined pincers on the first pair of legs. Scorpions may range in size from 2 to 10 cm. Scorpions undergo incomplete development. Of the 1,000 or so species that are known,

fewer than 50 have been reported to cause illness with their sting. Scorpions are problems mainly in the warmer climates. The bulbous end of the tail contains muscles that force venom through the stinger. All scorpions are predatory on other arthropods, immobilizing their prey with their venom. Humans are stung by walking barefoot at night, by not shaking their shoes out in the morning in an area of endemicity, by lifting rocks or logs, or in bedding that is on the floor. The stings cause local pain (probably due to the great biogenic amine content of the venom), edema, discoloration, and hypesthesia. Systemic signs can include shock, salivation, confusion or anxiety, nausea, tachycardia, and tetany. Venom characteristics differ depending on the genus of scorpion: some stimulate parasympathetic nerves and can lead to secondary stimulation of catecholamines, resulting in sympathetic stimulation, which in turn may contribute to respiratory failure (1). Others affect the central nervous system, are hemolytic, or cause local necrosis. Hypersensitivity and anaphylaxis may occur in individuals who are repeatedly exposed.

Treatment is usually symptomatic, although in areas with known dangerous species (the Middle East [*Leiurus* spp., *Buthacus* spp., and *Buthus* spp.], southern Africa [*Parabuthus* spp., *Buthus* spp., and *Uroplectes* spp.], South and Central America [*Tityus* spp.], and the southern United States [*Centruroides* spp.]), quickly getting a pressure bandage on over the sting and immobilizing the limb (if that is where the sting was) would help to prevent venom from traveling via the lympatics. Lidocaine may be injected directly into the sting to reduce pain. Medical attention should be sought as soon as possible because antivenin, when administered promptly, reduces morbidity. However, antivenin is usually species specific, and without bringing the culprit in for identification, antivenin use would be on a presumptive basis.

Centipedes and Millipedes

The centipedes (class Chilopoda, 2,800 species) and millipedes (class Diplopoda, 8,000 species) are elongate, vermiform arthropods with dozens of segments, each of which bears a pair of legs. Both undergo an incomplete development, with larvae looking like miniature adults. "Centipedes" would suggest having 100 segments (and pairs of legs) or fewer; "millipedes" would suggest more than 100 and up to 1,000. This simplification is not quite correct, but in general, many legs indicate a millipede, and fewer legs (but more than for insects or ticks) indicate a centipede. Other differences are apparent: millipedes are rounded in cross-section, but centipedes are dorsoventrally flattened; millipedes have mouthparts that are ventral and nonpiercing, whereas centipedes have mouthparts that protrude anteriorly and are clearly capable of piercing. Millipedes move slowly, reflecting their mode of life as feeders on detritus. Centipedes are very rapid predators on other arthropods.

The diversity of both millipedes and centipedes is greatest in the tropics, and virtually all those of medical importance are found in warm climates. Millipedes may squirt a noxious, corrosive fluid from pores on their segments. Such fluid may contain benzoquinone, aldehydes, and hydrocyanic acid and cause an immediate burning sensation followed by erythema and edema, even progressing to blistering (2). Most millipedes also have a repugnant smell; both the corrosive fluid and smell tend to protect them from predation. People become exposed when they step on or sleep on millipedes, or provoke them (children playing with them are often victims). Treatment consists of washing the affected site as soon as possible to dilute and remove the corrosive fluids, and is symptomatic for the skin lesions and pain. Centipedes have powerful biting mandibles and small fang-like structures (forcipules) situated between them and derived from the first pair of legs that may inject a venom. This venom is used to immobilize prey, but will be used in defense. Centipede bites occur when people step on or sleep on them or play with them. Envenomation is manifested by local pain and swelling, with proximal lymphadenopathy. Headache, nausea, and anxiety are common. Skin lesions may ulcerate and become necrotic. Death due to centipede bite has been confirmed for only one case, a Filipino child who was bitten on the head by a large *Scolopendra* sp. The common house centipede, *Scutigera coleoptrata* of the eastern United States, is commonly found in bathtubs. This hairy looking, small (5 to 8 cm long) centipede is actually beneficial, preying on roaches and other potential pests within houses. Its strong mandibles can, however, inflict a painful pinch, and it does have a mild venom that produces a bite similar in quality to a bee sting.

Spiders

The Order Araneae comprises nearly 30,000 species, but only a fraction of them have any medical importance. Most are very small (0.5 cm or smaller in length), but a few may be as large as a man's hand. Two major suborders are recognized, the Mygalomorphae, in which the fang-tipped chelicerae operate in parallel using an up-and-down strike, and the Aranaeomorphae, in which the chelicerae operate like insect mandibles, with a side-to-side motion. Spiders have an unsegmented cephalothorax, an abdomen, four pairs of legs, the prominent chelicerae, and spinnerets, specialized organs that secrete silk for making webs. All spiders are predatory on other arthropods and use their venom to immobilize prey, which are stored live. Spiders rend their prey with their chelicerae, and bathe them with a digestion fluid for hours prior to ingestion. Thus, although all spiders have stout chelicerae and can bite, and all spiders have a venom with which to immobilize their prey, most are too small to be noticed by humans even if they were bitten. The spiders of main medical interest belong to four groups (7, 12): the funnel web or trapdoor spiders of Australia (*Atrax* spp., and *Hadronyche* spp.), New World and southern African recluse spiders (*Loxosceles* spp.), South American armed spiders (*Phoneutria* spp.), and the cosmopolitan widow spiders (*Latrodectus* spp.). The recluse and widows have been widely transported by humans.

The clinical manifestations and complications of envenomation may differ among the four main spiders, and the syndromes caused by each have been given names that reflect the identity of the spider. With spider bites in general there are local pain and erythema at the site of the bite, and these may be accompanied by fever, chills, nausea, and joint pains. In loxoscelism, the site of the bite will ulcerate and become necrotic. Skin may slough, and there may be destruction of the adjacent tissues. Hemolysis, thrombocytopenia, and renal failure may ensue. With latrodectism, phoneutrism, and funnel web spider neurotoxicity, the venoms have a strong neurotoxic action. Muscle rigidity and cramping (similar to acute abdomen) are seen with latrodectism; complications include electrocardiogram abnormalities and hypotension. With phoneutrism, visual disturbances, vertigo, and prostration may occur; complications include hypotension and respiratory paralysis (7). Funnel web spiders induce autonomic nervous system excitation, with muscular twitching, salivation, and lachrimation, nausea and vomiting, and diarrhea; fatal respiratory arrest may result from apnea or laryngospasm (23).

Funnel web spider bites require prompt first aid, and the same recommendations could be used for latrodectism or phoneutrism. A compression bandage should be applied over

the site of the bite, and the affected limb should be immobilized by splinting with a compression bandage if a person is bitten on an extremity (standard procedures for snakebite). This may help prevent the venom from moving from the local lymphatics. The patient should seek medical attention as soon as possible for antivenin treatment. Otherwise, treatment is symptomatic with analgesics and antipyretics.

Tarantulas are popular pets and appear fearsome. Fortunately, they are docile and most injuries associated with them are due to their urticating hairs, particularly with respect to conjunctivitis. They do, however, have robust mandibles and venom and should not be provoked; bites are similar in quality to a bee sting.

Necrotic dermal lesions are often classified as loxoscelism even if the appropriate spiders are not known to be present in the area: only 35 of 216 diagnoses of "brown recluse spider bites" proved to be supported by the minimum evidence for assigning such a specific etiology, viz., the known presence of *Loxosceles reclusa* within the geographic area of exposure (37). Severe reactions to tick bite, or even the erythema migrans of Lyme disease, may be confused with recluse spider bites (22).

Miscellaneous Injury Due to Arthropods

Beetles (coleoptera) comprise 40% of all insect species, but even with this great diversity, very few may be harmful. Most injury is related to vesication by secretions containing cantharidin. Hemolymph (blood) of some of these beetles, generally called the blister beetles, also contains this irritant, and thus exposure may be by direct contact with live or dead (crushed) beetles. Cantharidin is the active ingredient of Spanish fly, the alleged aphrodisiac made from pulverized *Lytta vesicatoria.* Ingestion of Spanish fly would irritate the ureter and urethra and cause painful priapism. Overdose or chronic use causes renal tubular necrosis. It is likely that Spanish fly is no longer in demand given the availability of nitric oxide inhibitors.

An unusual group of carabeids, the bombardier beetles, will spray a boiling hot jet of benzoquinone as a defense (11), causing burns and blistering. Blistering is also produced by crushing the staphylinid beetle *Paederus fusca* of Southeast Asia, which contains a toxic alkaloid, pederin.

More commonly, carpet beetles (dermestids), which feed on wool rugs and other animal fur products, are associated with a papulovesicular eruption. In particular, larval dermestids and their hairs or shed skins (exuviae) cause a contact dermatitis.

Water beetles (actually members of the Hemiptera), also known as water skimmers, can inflict strong pinches on unsuspecting toes wading through water.

OTHER INJURY

Delusional or Illusion of Parasitosis

Illusory parasitosis is a condition in which a patient who has a real itch has the mistaken belief that the current itch is due to active infestation by irrelevant arthropods. Often the itch is due to a drug reaction, sunburn, detergents, irritating dusts, poison ivy, or even bona fide arthropod bites (among many other potential sources of itch) that were sustained months in the past. Once a patient has been helped to identify the actual cause of the itch, he or she will not persist in blaming an arthropod. In contrast, in delusory parasitosis the patient cannot be dissuaded that the source of the discomfort is probably not an arthropod. In some patients, there are no objective lesions but simply an insistence that they are infested. Many such cases involve highly educated people who can describe their infestation with great detail. However, they rarely produce a specimen, and when they do, it is usually not an arthropod or part thereof. Delusory parasitosis can be a serious illness which can be treated by experienced mental health professionals.

IDENTIFYING SUBMITTED SPECIMENS

For most purposes, identification to the species level ("this is *Eutrombicula alfreddugesi*") may not provide any more clinically relevant information than identification to a higher taxonomic level ("this is a chigger"). In some cases, simply being able to say "this is definitely an arthropod part, perhaps beetle" may be all the information that is required (in this example, a beetle part may suggest dwarf tapeworm infection or be consistent with urticaria due to the hairs of dermestid beetle larvae, or vesication due to the hot spray of the bombardier beetle). A whole arthropod may be compared with the bauplan diagram to place it within a general group. A classic pictorial key published by the CDC (available from http://www.dpd.cdc.gov/dpdx/HTML/CDProducts.htm) or a simple dichotomous key (Table 3) may be used to narrow the identification down to a known genus of medically important arthropod. Anything more specific will usually require consulting a taxonomic reference or finding an entomologist who will help. Agricultural extension services, university entomology departments, local mosquito or pest control organizations, parasitologists, or local bug-collecting enthusiast clubs may all be of help in identifying a specimen, and usually will not charge out of professional courtesy. (Note that few such entities will be Clinical Laboratory Improvement Amendments certified, and thus fee for service is problematic.)

In some cases, a patient will insist that an arthropod specimen be tested for the presence of an infectious agent. This is particularly common in areas where Lyme disease is endemic. The value of such a practice is dubious, other than for psychologically satisfying the patient's demand. Some commercial laboratories will test for the presence of Lyme disease spirochetes using a PCR assay, but given that the prevalence of infection in the vector may range from 15 to 70%, a positive test is likely. Furthermore, at least with deer ticks, other agents such as those causing granulocytic ehrlichiosis and babesiosis may be present but not revealed by tick testing for Lyme disease spirochetes. Other tick species also are likely to have guilds of microbes as opposed to a sole agent (36). In any event, by the time a PCR result is rendered, the patient may already be experiencing signs and symptoms. For Lyme disease, and likely for ehrlichiosis, postexposure prophylaxis may be provided in the form of two doses of a tetracycline, if the patient remains unconvinced.

The most important variable in determining the riskiness of a tick bite is how long the tick has fed (Fig. 9). If a deer tick is attached for no more than 24 h, regardless of whether it is demonstrated to be infected with Lyme disease spirochetes, *Babesia microti,* or the agent of granulocytic ehrlichiosis, the likelihood of an infectious dose of organisms being transmitted is very small. The biological basis for this "grace period" is a phenomenon known as reactivation, wherein pathogens within ticks require a period of replication after emerging from a period of dormancy that they enter during the long interstadial period between blood meals (19). Reactivation was first described for the agent of Rocky Mountain spotted fever, which attains infectivity within tick salivary glands only after 12 to 18 h of exposure

TABLE 3 Key to the common arthropod classes, subclasses, and orders of medical importance, adult stages[a]

1. Three or four pairs of legs [2]
   Five or more pairs of legs [22]
2. Three pairs of legs with antennae (**insects: class Insecta**) [3]
   Four pairs of legs without antennae (**spiders, ticks, mites, scorpions: class Arachnida**) [20]
3. Wings present, well developed [4]
   Wings absent or rudimentary [12]
4. One pair of wings (**flies, mosquitoes, midges: order Diptera**) [5]
   Two pairs of wings [6]
5. Wings with scales (**mosquitoes: order Diptera**)
   Wings without scales (**other flies: order Diptera**)
6. Mouthparts adapted for sucking, with an elongate proboscis [7]
   Mouthparts adapted for chewing, without an elongate proboscis [8]
7. Wings densely covered with scales, proboscis coiled (**butterflies and moths: order Lepidoptera**)
   Wings not covered with scales, proboscis not coiled, but directed backward (**bedbugs and kissing bugs: order Hemiptera**)
8. Both pairs of wings membranous, similar in structure, although size may vary [9]
   Front pair of wings leathery or shell-like, serving as covers for the second pair [10]
9. Both pairs of wings similar in size (**termites: order Isoptera**)
   Hind wing much smaller than front wing (**wasps, hornets, and bees: order Hymenoptera**)
10. Front wings horny or leathery without distinct veins, meeting in a straight line down the middle [11]
    Front wings leathery or paperlike, with distinct veins, usually overlapping in the middle (**cockroaches: order Dictyoptera**)
11. Abdomen with prominent cerci or forceps; wings shorter than abdomen (**earwigs: order Dermaptera**)
    Abdomen without prominent cerci or forceps; wings covering abdomen (**beetles: order Coleoptera**)
12. Abdomen with three long terminal tails (**silverfish and firebrats: order Thysanura**)
    Abdomen without three long terminal tails [13]
13. Abdomen with narrow waist (**ants: order Hymenoptera**)
    Abdomen without narrow waist [14]
14. Abdomen with prominent pair of cerci or forceps (**earwigs: order Dermaptera**)
    Abdomen without cerci or forceps [15]
15. Body flattened laterally, antennae small, fitting into grooves in side of head (**fleas: order Siphonaptera**)
    Body flattened dorsoventrally, antennae projecting from side of head, not fitting into grooves [16]
16. Antennae with nine or more segments [17]
    Antennae with three to five segments [18]
17. Pronotum covering head (**cockroaches: order Dictyoptera**)
    Pronotum not covering head (**termites: order Isoptera**)
18. Mouthparts consisting of tubular joined beak; tarsi three- to five-segmented (**bedbugs: order Hemiptera**)
    Mouthparts retracted into head or of the chewing type; one- or two-segmented tarsi [19]
19. Mouthparts retracted into the head; adapted for sucking blood (**sucking lice: order Anopleura**)
    Mouthparts of the chewing type (**chewing lice: order Mallophaga**)
20. Body oval, consisting of a single saclike region (**ticks and mites: subclass Acari**)
    Body divided into two distinct regions, a cephalothorax and an abdomen [21]
21. Abdomen joined to the cephalothorax by a slender waist; abdomen with segmentation indistinct or absent; stinger absent (**spiders: subclass Araneae**)
    Abdomen broadly joined to the cephalothorax; abdomen distinctly segmented, ending with a stinger (**scorpions: subclass Scorpiones**)
22. Five to nine pairs of legs or swimmerets; one or two pairs of antennae; principally aquatic organisms (**copepods, crabs, and crayfish: class Crustacea**)
    Ten or more pairs of legs or swimmerets absent; one pair of antennae present; terrestrial organisms [23]
23. Body segments each with only one pair of legs (**centipedes: class Chilopoda**)
    Body segments each with two pairs of legs (**millipedes: class Diplopoda**)

[a] Data from J. Goddard, *Physician's Guide to Arthropods of Medical Importance,* 2nd ed., CRC Press, Inc., Boca Raton, Fla., 1996, and National Communicable Disease Center, *Pictorial Keys: Arthropods, Reptiles, Birds, and Mammals of Public Health Significance,* Communicable Disease Center, Atlanta, Ga., 1969.

to a host. Reactivation is probably a general phenomenon with most ixodid (hard) tick-transmitted agents, the only known exceptions being the viruses causing tick-borne encephalitis and the related Powassan fever, in which transmission is thought to be instantaneous with attachment.

Estimating the degree of engorgement may provide more of an index of individual risk than actual presence or absence of infection. A simple measurement may be made with deer ticks and might be tried for other species of ticks. The scutal index is the ratio of the length of the scutum, the dark shield on the dorsum of the tick, to the length of the tick from tip of the mouthparts to the caudal edge of the tick. A scutal index of >2.5 suggests that a deer tick has been attached for more than 24 h, and therefore transmission is likely (26). Such a finding might prompt a physician to provide prophylactic antibiotics, which would effectively abort Lyme disease or human granulocytic ehlichiosis. Although such prophylaxis would not prevent babesiosis, note that of the agents transmitted by deer ticks, *B. microti* requires the greatest duration of attachment for infectivity, probably relating to the requirement for sporogony during attachment.

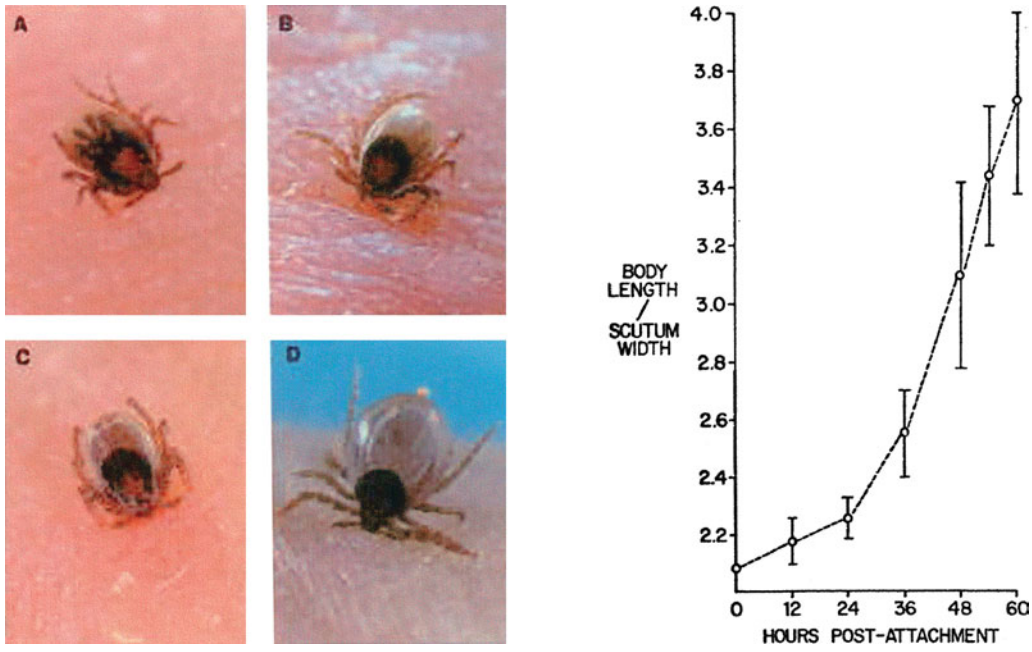


FIGURE 9 Gradual engorgement of feeding ixodid ticks. (Left [A to D]) Four-day feeding sequence of nymphal *Ixodes ricinus*. (Reprinted with permission from F. R. Matuschka and A. Spielman, Risk of infection from and treatment of tick bite, *Lancet* **342:**529, 1993.) (Right) Scutal index of engorgement for *Ixodes dammini*. (Reprinted with permission from J. Piesman and A. Spielman, Human babesiosis on Nantucket Island: prevalence of Babesia microti in ticks, *Am. J. Trop. Med. Hyg.* **29:**742–746, 1980.)

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ANTIPARASITIC AGENTS AND SUSCEPTIBILITY TEST METHODS

XI

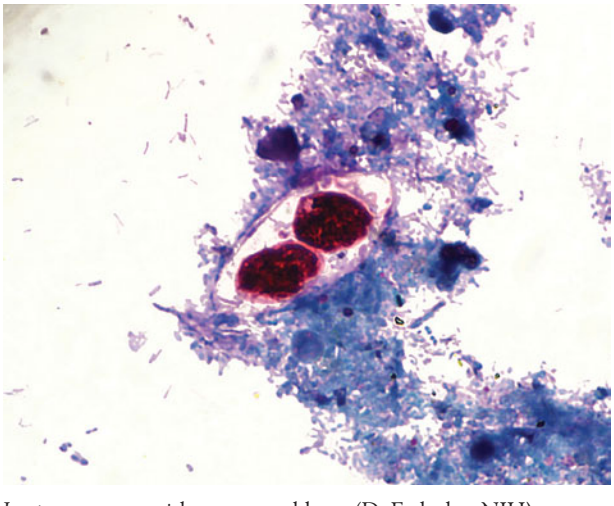
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Isospora oocyst with two sporoblasts (D. Fedorko, NIH).

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Antiparasitic Agents

KARIN LEDER AND PETER F. WELLER

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There are a number of effective antiprotozoal and anthelminthic drugs currently available. These antiparasitic agents are important both for therapy of individual patients and for control of parasitic infections at a community level. Large-scale chemotherapy is reducing transmission of and morbidity and mortality from certain infections including lymphatic filariasis, onchocerciasis, schistosomiasis, and intestinal nematodes. However, the lack of financial incentives to manufacture existing drugs or to develop new agents is a major limitation to the range of current and future antiparasitic chemotherapies. Emerging resistance among parasites, lack of effective antiparasitic vaccines, and the enormous burden of disease worldwide also pose challenges to the effective management of parasitic infections.

This chapter will focus on the mechanisms of action, pharmacology, clinical utility, and adverse effects of common first-line antiparasitic therapies and newer drug alternatives. Most helminth infections in humans can be treated with one of five drugs, namely, albendazole, mebendazole, praziquantel, ivermectin, and diethylcarbamazine (DEC), so these five drugs will be reviewed in detail. A new agent, nitazoxanide, which has both anthelminthic and antiprotozoal activities, will also be discussed. Other major antiprotozoal drugs, including those used for malaria, gastrointestinal protozoa, leishmaniasis, and trypanosomiasis, will also be reviewed. However, an exhaustive list of all antiparasitic drugs is not included. Specifically, we have excluded discussion regarding agents that do not have a first-line indication or that are recommended only in special situations, such as furazolidone in children or paromomycin in pregnant women. Additionally, most antibacterial and antifungal agents that can also be used for treatment of protozoal infections, such as the 5-nitroimidazoles, trimethoprim-sulfamethoxazole, azithromycin, and amphotericin, will not be discussed in detail here, but their general indications for parasitic infections are shown in Tables 1 and 2. Resistance to antiparasitic agents and drug susceptibility testing are dealt with in chapters 151 and 152.

ANTHELMINTIC AGENTS

Benzimidazoles

The benzimidazoles are antiparasitic agents with a broad spectrum of activity against many helminthic and certain protozoal infections. All members of the benzimidazole class have in common a bicyclic ring system in which benzene has been inserted. Mebendazole and albendazole, both of which are synthetic agents, are the most widely used drugs of this class. Mebendazole is 5-benzoyl-2-benzimidazole carbamic acid, and albendazole is methyl 5-(propylthio)-2-benzimidazole carbamate. The low cost, high efficacy, and ease of administration of these two agents have led to their widespread use for many human parasitic infections. Major indications for their use are shown in Tables 3 and 4. Whereas mebendazole has been approved for treatment of multiple nematode infections by the U.S. Food and Drug Administration (FDA), albendazole is considered investigational for all uses except as treatment for hydatid infections and neurocysticercosis (59b). Other members of this drug class include flubendazole, thiabendazole, and triclabendazole. Flubendazole, a parafluoro analogue of mebendazole, has the same mechanism of action as mebendazole and albendazole and will not be considered separately. It has been licensed in Europe but not in the United States. Thiabendazole is 2-(4-thiazolyl)-1*H*-benzimidazole. Administration of thiabendazole is frequently associated with side effects, and it has now been largely replaced by other anthelmintic agents. Triclabendazole is a new imidazole derivative that has been used as a veterinary agent for many years. It is now considered the drug of choice for fascioliasis (13), but it has not been approved by the FDA and is not widely available.

Mechanism of action. The antiparasitic activity of albendazole and mebendazole results mainly from their ability to bind to a cytoskeletal protein of parasites called β-tubulin, thereby inhibiting the polymerization of tubulin into microtubules (51). The disruption of microtubule synthesis within parasitic intestinal cells results in decreased absorptive function. In addition, mebendazole and albendazole directly inhibit glucose absorption by parasites, leading to a depletion of parasite glycogen stores (32, 34). Parasites therefore have insufficient energy sources for formation of ATP, without which they are unable to reproduce or survive. Although tubulin is also present in mammalian hosts, the benzimidazoles bind to parasite tubulin with an affinity that is hundreds of times greater than the affinity of binding to mammalian tubulin, thereby causing minimal mammalian toxicity.

Thiabendazole has mechanisms of action similar to those of the other benzimidazoles. It blocks glucose uptake, depletes glucose stores, and interferes with microtubule aggregates (32).

TABLE 1 Treatment of major protozoal infections[a]

Organism or disease	Primary agent used for treatment	Alternative agent(s)
Intestinal protozoa		
Entamoeba histolytica	Invasive trophozoites: metronidazole Luminal agent: iodoquinol	Tinidazole Alternative luminal agents: diloxanide furoate, paromomycin
Babesia species	Clindamycin plus quinine	Atovaquone plus azithromycin
Balantidium coli	Tetracycline	Metronidazole, iodoquinol
Crytosporidium parvum	Nitazoxanide	
Cyclospora cayatanensis	Trimethoprim-sulfamethoxazole	
Dientamoeba fragilis	Tetracycline	Iodoquinol, paromomycin, metronidazole
Giardia lamblia	Metronidazole	Tinidazole, nitazoxanide, quinacrine, albendazole, furazolidone, paromomycin
Isospora belli	Trimethoprim-sulfamethoxazole	
Microsporidium species	Albendazole *(Encephalitozoon intestinalis* responds better than *Enterocytozoon bieneusi)*	
Other protozoa		
Amebic meningoencephalitis	Amphotericin B	
Leishmania species	Pentavalent antimonial	Miltefosine, pentamidine, amphotericin B, liposomal amphotericin B
Malaria		
Plasmodium vivax and *P. ovale*	Chloroquine plus primaquine	
Plasmodium falciparum	Quinine sulfate plus doxycycline or plus pyrimethamine-sulfadoxine or quinine plus clindamycin	Mefloquine plus artesunate, Malarone
Plasmodium malariae	Chloroquine	
Toxoplasma gondii	Pyrimethamine plus sulfadiazine	Pyrimethamine plus clindamycin
Trichomonas vaginalis	Metronidazole	Tinidazole
Trypanosoma brucei species (African trypanosomiasis)	Early: Suramin or eflornithine Late: Melarsoprol	Pentamidine
Trypanosoma cruzi (American trypanosomiasis)	Nifurtimox	Benznidazole

[a] Adapted from reference 59b.

In addition, it inhibits the fumarate reductase system of helminths, thereby depriving parasites of their energy source and ultimately resulting in their death (34). The exact mechanism of action of triclabendazole is unknown, but it is thought to act on microtubuli, causing decreased parasite motility.

Pharmacology. Some benzimidazoles are poorly soluble in water and therefore are not well absorbed following oral administration. Although this limits their activity against tissue-dwelling parasites, it contributes to their minimal toxicity and to their efficacy in the treatment of many intestinal helminthic infections (20). None of the benzimidazoles are available as intravenous formulations.

Less than 10% of mebendazole is absorbed after oral administration. It is metabolized in the liver to inactive compounds, eliminated in the bile, and excreted predominantly in the feces. It is 95% protein bound in plasma, and its half-life in serum is 2.5 to 5.5 h. Serum levels are markedly variable between individuals, but tissue and echinococcal cyst concentrations tend to be low (27).

The oral bioavailability of albendazole is also poor, with less than 10% absorption following an oral dose (58). Administration of albendazole with a fatty meal markedly improves bioavailability. It is rapidly metabolized in the liver, and concentrations of the parent drug in plasma are negligible. However, its primary metabolite, albendazole sulfoxide, also has anthelmintic activity (7). This results in a higher efficacy for albendazole than mebendazole for most indications. Albendazole sulfoxide is 70% protein bound and is widely distributed throughout the body. Peak concentrations of albendazole and albendazole sulfoxide in plasma show great intersubject variability but range from 0.24 to 2.96 μg/ml (64). Albendazole induces enzymes of the cytochrome P-450 system which are responsible for its metabolism. Cimetidine and dexamethasone both raise drug levels (44). Albendazole sulfoxide has been detected in urine, bile, liver, cyst fluid, and cerebrospinal fluid (CSF). Plasma levels are 3- to 10-fold and 2- to 4-fold higher than in cyst fluid and CSF, respectively (64, 65). Albendazole sulfoxide has a half-life of approximately 9 h. It is oxidized further to inactive compounds such as albendazole sulfone and is excreted in the urine.

Neither mebendazole nor albendazole is dialyzable. No dosage adjustment is required for individuals with renal impairment, but a reduction in dose should be considered if there is significant hepatic insufficiency.

Thiabendazole is rapidly absorbed from the gastrointestinal tract after oral administration, is metabolized in the liver, and is excreted predominantly in the urine. Its half-life is 1.2 h. It is generally administered after meals to minimize side effects.

Triclabendazole is also rapidly absorbed from the gastrointestinal tract, and absorption is improved further if the drug is given postprandially (52). Its half-life is 12 to 18 h.

TABLE 2 Treatment of major helminthic infections[a]

Organism or disease[b]	Primary agent used for treatment	Alternative agent(s) or treatment
Nematodes		
Ancylostoma caninum (eosinophilic enterocolitis)	Albendazole	Mebendazole, pyrantel pamoate
Angiostrongylus species	Mebendazole	
Ascaris lumbricoides	Albendazole	Mebendazole, pyrantel pamoate, ivermectin, nitazoxanide
Capillaria species	Mebendazole	Albendazole
Cutaneous larva migrans	Albendazole	Ivermectin, topical thiabendazole
Enterobius vermicularis	Albendazole	Mebendazole, pyrantel pamoate
Filariasis (*Wuchereria bancrofti, Brugia malayi*)	Diethylcarbamazine with or without albendazole or ivermectin	
Gnathostoma species	Albendazole	Ivermectin, surgical removal
Hookworm	Albendazole	Mebendazole, pyrantel pamoate
Loa loa	Diethylcarbamazine with or without albendazole or ivermectin	
Onchocerca volvulus	Ivermectin	
Strongyloides stercoralis	Ivermectin	Thiabendazole, albendazole
Toxocara species (visceral larva migrans)	Albendazole	Mebendazole
Trichinella spiralis	Mebendazole	Albendazole
Trichostrongylus species	Albendazole	Mebendazole, pyrantel pamoate
Trichuris trichiura	Mebendazole	Albendazole, ivermectin, nitazoxanide
Cestodes		
Cysticercosis	Albendazole	Praziquantel
Dipylidium caninum	Praziquantel	Niclosamide
Diphyllobothrium latum	Praziquantel	Niclosamide
Echinococcus species	Albendazole	Praziquantel
Hymenolepis nana	Praziquantel	Nitazoxanide
Taenia saginata	Praziquantel	Niclosamide
Taenia solium	Praziquantel	Niclosamide
Trematodes		
Clonorchis sinensis	Praziquantel	Albendazole
Fasciola hepatica	Triclabendazole	Nitazoxanide, bithionol
Intestinal flukes	Praziquantel	
Metorchis conjunctus	Praziquantel	
Opisthorchis viverrini	Praziquantel	
Paragonimus species	Praziquantel	Bithionol
Schistosoma species	Praziquantel	*Schistosoma mansoni:* oxamniquine[c]

[a] Adapted from reference 59b.
[b] This is not an exhaustive list of all possible parasitic infections, but commonly encountered parasites are included.
[c] No longer manufactured and/or problems with availability.

It is metabolized in the liver into an active sulfoxide metabolite and into an inactive sulfone metabolite, both of which are highly protein bound and excreted in the urine.

Spectrum of activity. The benzimidazoles are effective against adult worms and developing helminthic embryos. Mebendazole and albendazole have similar and broad ranges of activity. Both drugs have good efficacy against many common intestinal nematode infections, including ascariasis, trichuriasis, enterobiasis, and hookworm infections. Three-day regimens of both drugs are generally recommended for therapy of individual patients, but a single dose is used for mass or targeted community treatment of intestinal nematodes in areas of endemicity. Albendazole is preferred in most instances, but its efficacy against very heavy infections with *Trichuris trichiura* remains questionable, and mebendazole may be preferable in this circumstance. The reported curative efficacies of the two drugs in different parasitic infections vary according to the baseline intensity of infection in the patient, geographical location, diagnostic tests employed, and duration of follow-up posttreatment.

Mebendazole and albendazole also have activity against other nematode infections, including trichostrongyliasis, capillariasis, trichinellosis, gnathostomiasis, toxocariasis, and cutaneous larva migrans. Additionally, albendazole is being increasingly used in combination with either DEC or ivermectin for treatment of Bancroftian or Brugian filariasis and loiasis; it is particularly useful for these infections as part of a single-dose regimen for mass chemotherapy programs. Although albendazole displays some efficacy against *Strongyloides stercoralis*, ivermectin shows higher cure rates and is the recommended treatment of choice.

TABLE 3 Major indications for albendazole[a]

Indication	Usual dose	Reported efficacy
Echinococcus granulosus	400 mg twice daily, usually minimum of 3–6 mo	Clinical cure, as evidenced by cyst disappearance, in one-third, and improvement in radiological appearance in an additional 30–50%
Cysticercosis	400 mg twice daily, usually for 8 days (8–30 days)	75–95% of parenchymal cysts destroyed and 40–70% of patients show resolution of all active cysts, but unclear if beneficial over placebo
Ascaris lumbricoides	Single 400-mg dose	Median cure rates of 95–97% and egg reduction rates of 99–100%
Cutaneous larva migrans	400 mg daily for 3 days	No large clinical trials, and generally reserved for those with severe or disseminated infection
Enterobius vermicularis	400-mg dose, repeat in 2 wk	Cure rate close to 100%
Hookworm	Single 400-mg dose	70–95% cure rate and 85–100% egg reduction rate
Microsporidiosis	400 mg twice daily, usually for 2–4 wk	Variable efficacy (related to species of pathogen and immune function of host)
Trichuris trichiura	Single 400-mg dose	Cure rate of 35–70% and egg reduction rate of 70–90% (3 days of therapy gives higher cure rates in heavy infections, but mebendazole is the preferred agent for treatment)
Toxocariasis	400 mg twice daily for 5 days	No large clinical trials
Lymphatic filariasis	Single 400-mg dose	Microfilaremia reduced by 98–99% for prolonged periods when administered in combination with DEC or ivermectin; prolonged high doses also have a macrofilaricidal effect
Loa loa	Single 400-mg dose	No microfilaricidal effect, but partial macrofilaricidal effect with sterilization and/or death of adult worms
Giardia lamblia	400 mg daily for 5 days	Cure rate of 97%

[a] From reference 59b.

Both mebendazole and albendazole also show activity in certain cestode infections. Albendazole is again preferred because of its more favorable pharmacokinetics, and it is now considered the drug of choice for medical management or adjunctive treatment of hydatid disease due to *Echinococcus granulosis*. Prolonged albendazole therapy (minimum of 10 years) can also be used in inoperable alveolar echinococcosis or as adjuvant therapy for patients with *Echinococcus multilocularis* infection. In addition, albendazole is used for treatment of parenchymal neurocysticercosis. Note that, despite its anticysticercal activity, not all studies have shown a clear benefit of any form of antiparasitic therapy in this condition, and controversy still exists about the exact indications for therapy.

Neither mebendazole nor albendazole is commonly used in trematode infections, although some activity against opisthorchiasis, chlonorchiasis, and *Echinostoma* species has been reported (41, 54, 82).

Although considered primarily an anthelmintic agent, albendazole is also an alternative agent for giardiasis. Additionally, albendazole has activity in microsporidial infections, particularly those caused by *Encephalitozoon hellem, E. cuniculi,* and *E. intestinalis*.

Thiabendazole kills the adult worms of *Strongyloides stercoralis* and used to be the drug of choice in uncomplicated strongyloidiasis. It also has activity in other nematode infections, including ascariasis, enterobiasis, hookworm, trichuriasis, toxocariasis, trichinellosis, capillariasis, and trichostrongyliasis. It is no longer recommended as first-line therapy for any of these indications because of the high frequency of associated side effects. It is still used topically for treatment of cutaneous larva migrans.

TABLE 4 Major indications for mebendazole[a]

Indication	Usual dose	Reported efficacy
Ascaris lumbricoides	Single 500-mg dose or 100 mg twice daily for 3 days	Median cure rates of 95–98% and egg reduction rates of 99–100%
Enterobius vermicularis	Single 100-mg dose, repeated after 2–3 wk	Mean cure rate of 95%
Hookworm	Single 500-mg dose	Cure rates of 20–80% and egg reduction rates of 60–95% (>95% cure achieved with 3 days of mebendazole, 100 mg twice daily)
Trichuris trichiura	Single 500-mg dose or 100 mg twice daily for 3 days	Cure rate of 40–75% and egg reduction rates of 90–95%; higher cure rates (70–90%) achieved following 3 days of mebendazole (100 mg twice daily)
Toxocariasis	100–200 mg twice daily for 5 days	No large clinical trials

[a] From reference 59b.

Triclabendazole is recommended as the treatment of choice for fascioliasis. It is also considered an alternative to praziquantel for therapy of paragonimiasis and intestinal flukes, although experience with these infections in human trials is more limited.

Adverse effects. Adverse effects following short courses of the benzimidazoles mebendazole and albendazole are infrequent and generally mild. Transient abdominal pain, nausea, and diarrhea may develop. Headache, dizziness, insomnia, and allergic phenomena are also reported. With prolonged high-dose therapy, such as for echinococcosis, transient and reversible elevations of serum transaminases occur in 1 to 5% of recipients. Occasionally, alopecia (<1%) and reversible leukopenia (<1%) are seen. Discontinuation of therapy is infrequently needed, but a recent death related to albendazole-induced pancytopenia has been described (73).

When mebendazole has been used by pregnant women, the incidence of spontaneous abortions or fetal malformations has not been greater than the background population rate. There are also no reports of teratogenicity from albendazole, and women who have received it inadvertently during pregnancy have not experienced adverse fetal outcomes (3). However, studies of benzimidazole drugs performed in animals suggest a possible teratogenic and embryotoxic effect. Since there are no good prospective safety data on either of these agents in humans, it is recommended that neither albendazole nor mebendazole be administered during pregnancy, particularly during the first trimester, unless the potential benefits justify the possible risk to the fetus (110). The amount of albendazole or mebendazole excreted in breast milk has not been well studied, and these drugs should be used with caution in lactating women.

Thiabendazole is associated with side effects in approximately 50% of recipients. The most common adverse reactions are nausea, vomiting, diarrhea, dizziness, anorexia, and malodorous urine. It can also cause rash, pruritis, headache, drowsiness, visual disturbances, hypoglycemia, bradycardia, and hypotension. Severe neuropsychiatric reactions including delirium and disorientation can also occur. It is contraindicated for use during pregnancy.

Triclabendazole is well tolerated, and few significant adverse effects have been described. Occasional side effects include headache, dizziness, and abdominal pain. It is not recommended for use during pregnancy because of insufficient safety data.

Praziquantel

Praziquantel is a synthetic heterocyclic isoquinolone-pyrazine derivative. Major indications for its use are shown in Table 5. It has the unique characteristic of being active against almost all trematodes and cestodes, but it is not useful for treatment of nematode infections. It has been approved for use by the FDA (59b).

Mechanism of action. Praziquantel induces ultrastructural changes in the teguments of parasites, resulting in increased permeability to calcium ions. Calcium ions accumulate in the parasite cytosol, leading to muscular contractions and ultimately to paralysis of adult worms. By damaging the tegument membrane, praziquantel also exposes parasite antigens to host immune responses (8). These effects lead to dislodgment of worms from their intestinal sites and subsequent expulsion by peristalsis.

Pharmacology. Praziquantel is available for oral administration, and over 80% of the drug is absorbed after an oral dose. Coadministration with a high-carbohydrate meal increases serum drug concentrations (90). The drug is biotransformed in the liver, and metabolites are excreted mainly in the urine. The cytochrome P-450 hepatic metabolism of praziquantel is induced by corticosteroids, phenytoin, and phenobarbital. Levels of praziquantel in serum are therefore lowered when any of these drugs are coadministered. Cimetidine, which inhibits P-450-mediated metabolism, can be given concurrently to increase plasma praziquantel levels. Levels in plasma after a single 40-mg/kg dose have been reported as 1.007 to 1.625 μg/ml (45). Praziquantel does not cross the blood-brain barrier well, so levels in CSF are only approximately 20% of plasma levels (20, 90). It is 80% protein bound, and its half-life in serum is 1 to 3 h. It is not dialyzable, and no adjustment in dose is recommended in either renal or hepatic insufficiency.

Spectrum of activity. Praziquantel is active against the larval and adult stages of many trematodes. It is the drug of choice for schistosomiasis and is effective as a single oral dose for all *Schistosoma* species that infect humans. It is used both for treatment of individuals and for mass community chemotherapy programs and leads to decreased transmission and prevalence of infection. Praziquantel is also used for treatment of opisthorchiasis, clonorchiasis, paragonimiasis, and intestinal fluke infections including fasciolopsiasis, heterophyiasis, and metagonimiasis. In contrast to its effect on

TABLE 5 Major indications for praziquantel[a]

Indication	Usual dose	Reported efficacy
Clonorchiasis	75 mg/kg/day in 3 doses for 1 day	Cure rates of 85–100%
Cysticercosis	50–100 mg/kg/day in 3 doses for 1–30 days	Eliminates 50–60% of cysts, but unclear if beneficial over placebo
Opisthorchiasis	75 mg/kg/day in 3 doses for 1 day	Cure rates of >95%
Intestinal flukes	75 mg/kg/day in 3 doses for 1 day	No large clinical trials
Paragonimiasis	75 mg/kg/day in 3 doses for 2 days	>95% cure for pulmonary infections (may be lower in ectopic sites)
Schistosomiasis	40–60 mg/kg/day in 2–3 doses for 1 day	Cure rates of 75–100% and egg reduction rates of 90–95% in those not cured
Tapeworms	Single 10–25-mg/kg dose	Cure rates >95% for *Taenia, Diphyllobothrium,* and *Hymenolepis* species infections

[a] From reference 59b.

other human trematode infections, praziquantel has not proven to be effective in the treatment of *Fasciola hepatica*.

Many cestode infections can also be treated with praziquantel. Most tapeworm infections respond, including those caused by *Taenia*, *Diphyllobothrium*, and *Hymenolepis* species. Because praziquantel does not kill eggs, precautions should be taken to prevent autoinfection, laboratory-acquired infection, or dissemination to others, particularly for *Taenia solium*. Praziquantel is also used in the treatment of neurocysticercosis as an alternative to albendazole, although the overall benefit of therapy in many cases remains unclear.

Praziquantel has also been used in combination with albendazole for treatment of echinococcal infections. Praziquantel has high protoscolicidal activity in vitro, and some reports have suggested that the combination has superior efficacy compared to either drug alone (17, 62).

Adverse effects. Adverse effects of praziquantel are generally mild, but many series suggest that some side effects occur in over 30% of patients. Common reactions include dizziness, lethargy, headache, nausea, and abdominal pain. Severe adverse reactions are uncommon, although administration to individuals with neurocysticercosis can result in seizures and neurological sequelae related to precipitation of an inflammatory response.

Animal studies do not suggest a teratogenic effect of praziquantel, but an increased abortion rate has been seen in rats. There are minimal data on its safety in humans, so it should be avoided during pregnancy when possible (20). No adverse effects of praziquantel administration during lactation have been reported, but it is excreted in human breast milk, and discontinuation of breast-feeding on the day of therapy and for the following 72 h is recommended.

Ivermectin

Ivermectin is a semisynthetic macrocyclic lactone derivative of avermectins, which are natural substances derived from the actinomycete *Streptomyces avermitilis*. Major indications for its use are shown in Table 6. It was initially developed as an agent for veterinary use but is now used widely in humans. Ivermectin is a potent oral agent with relatively broad-spectrum anthelmintic activity. It has been approved by the FDA for therapy of onchocerciasis and uncomplicated strongyloidiasis (59b).

Mechanism of action. The exact mechanism of action of ivermectin is unclear. It causes an influx of chloride ions across glutamate-gated chloride channels in nerve and muscle cell membranes, resulting in hyperpolarization of the affected cells and consequent paralysis and death of parasites (14, 98). It was postulated that ivermectin may act as an antagonist of the neurotransmitter γ-aminobutyric acid, but this has now been disproven (76). Although specific ivermectin binding sites have been identified in mammalian brain tissue, the affinity of ivermectin for sites within parasites is about 100 times greater than for mammalian tissue.

Pharmacology. Ivermectin is available only as an oral preparation and is rapidly absorbed after oral administration. The effect of food on the bioavailability of the drug has not been well studied. Ivermectin is metabolized in the liver and excreted almost entirely in the feces. Peak levels in serum of approximately 46 ng/ml have been reported after a single 12-mg dose (27). It is highly protein bound in plasma, and it has a half-life of 10 to 16 h. The drug accumulates in fat tissue and does not readily cross the blood-brain barrier (4).

Spectrum of activity. Ivermectin is the drug of choice for onchocerciasis and nondisseminated strongyloidiasis. In onchocercal infections, ivermectin does not have a significant effect on the viability of adult worms, but it is thought to impair release of microfilariae. In addition, it is a potent microfilaricide and leads to a sustained reduction in microfilaremia for many months (14). It can be used both for the treatment of individual patients and for mass chemotherapy programs in areas where onchocerciasis is endemic. In uncomplicated strongyloidiasis, ivermectin has excellent efficacy in normal and immunocompromised patients, although experience with its use in disseminated infection is more limited (76).

In addition to the above indications, ivermectin also has activity against microfilariae of *Wuchereria bancrofti*, *Brugia malayi*, and *Loa loa*. It does not have a significant effect on adult worm viability in these infections, so reduced microfilaremia is sustained only with repeated doses, and it has not replaced DEC as first-line therapy for these infections. Ivermectin also has some activity against *Mansonella ozzardi* and *Mansonella streptocerca* microfilariae.

Ivermectin also has efficacy against many intestinal helminths, including *Ascaris lumbricoides*, *Trichuris trichiura*, *Enterobius vermicularis*, and cutaneous larva migrans (67).

TABLE 6 Major indications for ivermectin[a]

Indication	Usual dose	Reported efficacy
Ascaris lumbricoides	Single 200-μg/kg dose	Cure rates of 78–99%
Cutaneous larva migrans	200 μg/kg daily (usually 12 mg) for 1–2 days	Cure rates of 77–100%
Gnathostoma spinigerum	200 μg/kg daily for 2 days	Cure rates of 76–95%
Onchocerca volvulus	Single 150-μg/kg dose; repeat every 6–12 mo until asymptomatic	Skin microfilarial counts reduced by 85–95%, and levels remain suppressed by >90% at 1 yr
Strongyloides stercoralis	200 μg/kg daily for 1–2 doses	Cure rates of 85–97% in uncomplicated infection (normal or immunocompromised hosts)
Trichuris trichiura	200 μg/kg daily for 3 days	Cure rates of 35–84%
Ectoparasites: scabies and lice	Single 200-μg/kg dose	Almost 100% efficacy, particularly if repeat dose given in severe infestations after 2 wk

[a] From reference 59b.

However, it is not generally used as first-line treatment for these indications due to the widespread availability and excellent efficacy of albendazole. It also can be used for treatment of gnathostomiasis (49). It is not active against trematodes or cestodes.

In addition to its anthelmintic activity, ivermectin is the drug of choice for ectoparasitic infestations, including scabies and lice.

Adverse effects. Ivermectin is generally well tolerated. Most of the adverse effects that occur following its administration are a result of the host's immune response to destruction of parasites rather than toxic effects of the drug per se. Adverse effects include fever, rash, dizziness, pruritis, myalgia, arthralgia, and tender lymphadenopathy, but the severity of these symptoms relates to the pretreatment intensity of infection rather than to serum ivermectin concentrations (69). Severe reactions occasionally occur following its use, including a hypersensitivity response to dying microfilarial parasites known as the Mazzotti reaction. This anaphylactoid response is characterized by allergic manifestations including pruritis, edema, fever, and systemic hypotension. However, these severe reactions are primarily restricted to individuals with high parasite loads. In patients infected with *Loa loa* who have elevated levels of microfilaremia, ivermectin has been associated with the development of fatal encephalopathy (101).

Ivermectin has not been reported to be teratogenic in rats or rabbits, but it has been associated with cleft palate and occasional unexplained maternal deaths in mice (14, 20). There are insufficient data to recommend its use in pregnant women, although the risk of fetal damage in 203 pregnant women inadvertently treated with ivermectin was no greater than in controls, and it has been suggested that ivermectin can be given safely after the first trimester (35, 77, 110). It is excreted in breast milk in low concentrations, so its administration to lactating women should be avoided when possible.

DEC

DEC is a piperazine derivative. Its main use is in filarial infections. Major indications for its use are shown in Table 7. DEC is not licensed for use in the United States but can be obtained from the Centers for Disease Control and Prevention (CDC) under an Investigational New Drug protocol (phone no., 404-639-3311; website, http://www.cdc.gov/).

Mechanism of action. The mode of action of DEC is uncertain. It is predominantly a microfilaricidal agent, and it is thought that its main effect is to alter the surface membrane of microfilariae, thereby enhancing destruction via host immune responses (32, 34). It also has some macrofilaricidal activity in certain conditions, likely via hyperpolarization and immobilization of adult worms (48).

Pharmacology. DEC is available only in tablet form. It is freely soluble in water and is almost completely absorbed after oral administration. The drug is metabolized in the liver, although over 50% is excreted unchanged in the urine. There is negligible protein binding, and it is widely distributed in tissues. It readily crosses the blood-brain barrier. Peak levels in plasma of 100 to 150 ng/ml have been reported after a single 0.5-mg/kg dose (26). The half-life of DEC is 2 to 17 h. Renal excretion is reduced in the presence of an alkaline urinary pH, and dose reductions are required in patients with renal impairment.

Spectrum of activity. DEC is an effective microfilaricidal drug against *W. bancrofti, B. malayi, Brugia timori, Onchocerca volvulus, L. loa,* and *M. streptocerca*, but it has little or no effect on *Mansonella perstans* or *M. ozzardi* microfilariae. It has been the drug of choice for lymphatic filariasis for the last 50 years. It is used both for individual therapy for filarial infections and for mass community chemotherapy programs, either alone or in combination with ivermectin or albendazole. It is predominantly a microfilaricidal agent, although it has some macrofilaricidal activity in *W. bancrofti, B. malayi, B. timori,* and *L. loa* infections (68, 70, 75). DEC has also been used in the treatment of toxocaral visceral larva migrans, but albendazole is now the preferred agent because of its better safety profile.

Adverse effects. The side effects of DEC include mild headache, dizziness, anorexia, nausea, and arthralgias. Administration of the drug to individuals with filarial infections can induce adverse results that are not due to the drug itself but instead are related to host responses, in part to release of *Wolbachia* endosymbionts from filariae, following damage to adult worms (local reactions) and death of microfilariae (systemic reactions). These allergic reactions tend to be relatively mild with lymphatic filariasis and infrequent with loiasis but can be severe with onchocerciasis or with heavy *L. loa* infections. Reactions include intense pruritis, rash, fever, hypotension, and encephalopathy. The potentially fatal Mazzotti reaction and serious ophthalmic adverse effects can occur following treatment of *O. volvulus* with DEC, and this has led to DEC being contraindicated for onchocercal infections.

Animal studies have not shown DEC to be teratogenic, but it may increase the risk of abortion, so its administration should be avoided in pregnancy when possible (20, 43). It is not excreted in breast milk and is safe during lactation.

Pyrantel Pamoate

Pyrantel, a tetrahydropyrimidine, is a relatively broad-spectrum agent against nematodes. It is associated with more side effects and lower efficacy than the benzimidazoles, so it is largely being replaced. It is approved by the FDA but is considered investigational for most indications except enterobiasis.

TABLE 7 Major indications for DEC[a]

Indication	Usual dose	Reported efficacy
Loa loa	Up to 9 mg/kg/day in 3 doses for 21 days	Few large trials, but single course is curative in <50% of patients
Wuchereria bancrofti and *Brugia malayi*	Up to 6 mg/kg/day in 3 doses for 14 days or single dose	>90–99% reduction in microfilaremia, but may need additional courses to eradicate adult worms

[a] From reference 59b.

Mechanism of action. Pyrantel is a depolarizing neuromuscular blocking agent. It exerts its anthelmintic effect via release of acetylcholine and inhibition of helminthic acetylcholinesterase. This results in stimulation of ganglionic receptors and spastic paralysis of adult worms. The worms become dislodged from the intestinal wall and are expelled in the feces by normal peristalsis.

Pharmacology. Pyrantel is administered orally but is almost insoluble in water and is therefore poorly absorbed from the gastrointestinal tract. More than 50% is excreted unchanged in the feces. The absorbed drug is partially metabolized in the liver. There is no significant interaction with food.

Spectrum of activity. Pyrantel has excellent efficacy in the treatment of ascariasis and hookworm and pinworm infections. It also has some activity against hookworm and *Trichostrongylus*. It is not active against *Trichuris trichiura*.

Adverse effects. Although pyrantel is generally well tolerated, it can lead to adverse reactions including anorexia, nausea, vomiting, abdominal cramps, and diarrhea. It has also been associated with neurotoxic effects including headache, dizziness, drowsiness, and insomnia. Transient increases in hepatic enzymes have also been reported.

Animal studies have not shown adverse effects in the fetus, and it has been used during pregnancy in humans without harmful fetal effects (100). It is not recommended for children under 2 years of age.

NEW AGENT WITH ANTHELMINTIC AND ANTIPROTOZOAL ACTIVITY: NITAZOXANIDE

Nitazoxanide is a new 5-nitrothiazole derivative with broad-spectrum activity against numerous intestinal protozoa, helminths, and anaerobic bacteria. Major indications for its use are shown in Table 8. It was initially developed as a veterinary anthelmintic with activity against intestinal nematodes, cestodes, and liver trematodes (30). In 2002, the FDA approved oral-suspension nitazoxanide for the treatment of diarrhea caused by *Cryptosporidium* species and *Giardia lamblia* in pediatric patients 1 to 11 years of age, and in 2004, nitazoxanide was approved for treatment of diarrhea caused by *G. lamblia* in adults (30, 59a). At present, it is pending licensure for treatment of infection due to *Cryptosporidium* species in adults and for use in treating immunocompromised hosts.

Mechanism of action. Nitazoxanide inhibits pyruvate ferredoxin oxidoreductase, an enzyme essential to anaerobic energy metabolism. This is its mechanism of action against anaerobic bacteria (e.g., *Trichomonas vaginalis*, *Entamoeba histolytica,* and *Clostridium perfringens*) and protozoa, although for protozoa additional mechanisms may also be involved (33). The mechanism of nitazoxanide's activity against helminths has not yet been determined.

Pharmacology. Nitazoxanide is given by the oral route and is available as a suspension or a tablet. Bioavailability is nearly doubled by administration with food (94). It is absorbed from the gastrointestinal tract, with approximately

TABLE 8 Major indications for nitazoxanide[a]

Indication	Usual dose[b]	Reported efficacy
Cryptosporidium parvum	Immunocompetent children 1–11 yr[c] and adults: b.i.d. treatment for 3 days	Cure rates of 52–80%
	Immunocompromised patients: 1,000 mg b.i.d. for 2–8 wk	Cure rates of 18–67% (efficacy varies with CD4 count in human immunodeficiency virus-positive patients)
Giardia lamblia	b.i.d. treatment for 3 days[c]	Cure rates of 71–94%
Ancylostoma duodenale	b.i.d. treatment for 3 days	Cure rate of 96%
Ascaris lumbricoides	b.i.d. treatment for 3 days	Cure rates of 69–100% (in heavy infections of >10,000 eggs/g, lower egg clearance rates of 48%, but egg reduction rates of >99%)
Balantidum coli	b.i.d. treatment for 3 days	Cure rate of 77%
Blastocystis hominis	b.i.d. treatment for 3 days	Cure rates of 97–100%
Cyclospora cayetanensis	b.i.d. treatment for 3 days	Cure rate of 71%
Entamoeba histolytica	b.i.d. treatment for 3 days	Cure rates of 69–96%
Enterobiasis vermicularis	b.i.d. treatment for 3 days	Cure rates of 80–100%
Fasciola hepatica	b.i.d. treatment for 7 days (daily dose as shown below)	Cure rates of 40–97%
Hymenolepis nana	Single oral dose of 50 mg/kg or b.i.d. treatment for 3 days	Cure rates of 80–97%
Isospora belli	b.i.d. treatment for 3 days	Cure rate of 100%
Strongyloides stercoralis	b.i.d. treatment for 3 days	Cure rate of 94%
Taenia saginata	Single 25-mg/kg dose or b.i.d. treatment for 3 days	Cure rates of 95–100%
Trichuris trichiura	b.i.d. treatment for 3 days	Cure rates of 76–100% (in moderate infections with 2,000–5,000 eggs/g, lower egg clearance rates of 56%, but egg reduction rates of >99%)

[a] Adapted from reference 30.

[b] The dosing schedule of nitazoxanide when given twice a day (b.i.d.) for 3 days is as follows: children 1 to 3 years old, 100 mg b.i.d. for 3 days; children 4 to 11 years, 200 mg b.i.d. for 3 days; and adults, 500 mg b.i.d. for 3 days.

[c] Licensed for this use by the FDA.

one-third of the oral dose excreted in urine and two-thirds excreted in feces (9, 74). In blood, nitazoxanide is rapidly hydrolyzed to form an active metabolite, tizoxanide. The maximum concentration of tizoxanide in plasma following an oral dose of 500 mg of nitazoxanide is 2 mg/liter (6.5 μM), and its half-life in plasma is 1 to 2 h (1). Tizoxanide is extensively bound to plasma proteins (>99%), and its urinary elimination half-life is 7.3 h (95). Tizoxanide then undergoes glucuronidation to form tizoxanide glucuronide. Nitazoxanide is not detected in plasma, urine, bile, or feces, but tizoxanide is found in plasma, urine, bile, and feces and tizoxanide glucuronide is found in plasma, bile, and urine (74).

The pharmacokinetics of nitazoxanide in patients with impaired liver or renal function has not been studied, and the drug must be administered to these patients with caution (30).

Spectrum of activity. Nitazoxanide is a broad-spectrum antimicrobial agent with activity against protozoa, nematodes, cestodes, trematodes, and bacteria. Although it is currently licensed in the United States only for treatment of *Cryptosporidium parvum* in immunocompetent children and *Giardia lamblia* in children and adults, it has been shown to be effective in vitro and/or in vivo against infections caused by many enteric protozoa and helminths, including *Ascaris lumbricoides, Balantidium coli, Blastocystis hominis, Cyclospora cayetanensis, Echinococcus multilocularis, Echinococcus granulosus, Entamoeba histolytica, Fasciola hepatica,* hookworms, *Hymenolepis nana, Isospora belli*, microsporidial species (*Enterocytozoon bieneusi* and *Encephalitozoon intestinalis*), *Taenia saginata, Trichomonas vaginalis, Trichuris trichiura,* and *Vittaforma corneae* (30). Nitazoxanide also has in vitro and in vivo activity against *Bacteroides* species, *Clostridium difficile,* and both metronidazole-susceptible and metronidazole-resistant strains of *Helicobacter pylori* (30, 74).

Adverse effects. Nitazoxanide is very well tolerated, with adverse effects similar to those of a placebo. Mild and transient side effects have been seen in only 3 to 4% of patients, principally related to the gastrointestinal tract (abdominal pain, diarrhea, and nausea) (22). No significant adverse effects on electrocardiography, hematology, clinical chemistry, or urinalysis parameters have been noted (33).

There are minimal data on the safety of nitazoxanide in pregnant or lactating women (30).

ANTIMALARIALS

Quinoline Derivatives

The quinoline derivatives can be divided into four groups: the 4-aminoquinolines, the cinchona alkaloids, synthetic compounds such as mefloquine and halofantrine, and the 8-aminoquinolines.

4-Aminoquinolines

The 4-aminoquinolines include chloroquine, hydroxychloroquine, and amodiaquine. Chloroquine is the most widely used of these agents. It is a cheap, safe drug that has been used extensively for treatment and prophylaxis of all four *Plasmodium* species that infect humans. Hydroxychloroquine is a related synthetic compound with an identical clinical spectrum, similar pharmacokinetics, and similar adverse effects. Amodiaquine is another related agent which also has the same mechanism of action and spectrum of activity. It is reported to be more effective than chloroquine for parasite clearance, but its utility is limited by toxicity. It has not been approved by the FDA (59b).

Mechanism of action. The main mechanism of action of the 4-aminoquinolines is thought to be via nonenzymatic inhibition of heme polymerization. The 4-aminoquinolines act specifically against intraerythrocytic stages of malaria parasites. Asexual intraerythrocytic malaria parasites actively concentrate quinoline ring compounds within hemoglobin-containing vesicles. In the absence of drug, *Plasmodium* organisms degrade host erythrocyte hemoglobin to provide amino acid nutrients essential for parasite growth. The degradation of hemoglobin produces free heme, which is stored as ferriporoporphyrin IX within the red blood cell. Ferriporoporphyrin IX is toxic to the parasite and is usually polymerized into nontoxic malaria pigment (hemozoin). In the presence of drug, there is inhibition of the conversion of heme into hemozoin, leading to the accumulation of products toxic to the parasite and resulting in parasite death (89). These agents also inhibit protein synthesis by inhibiting incorporation of phosphate into DNA and RNA and by inhibiting DNA and RNA polymerases (32).

Pharmacology. The 4-aminoquinolines are extensively distributed in tissues and are characterized by a long elimination half-life. Despite similarities in their chemical structures, these drugs show differences in their biotransformation and routes of elimination (83).

Chloroquine is available in oral and parenteral forms. Many different formulations of chloroquine are manufactured worldwide. Chloroquine is rapidly absorbed from the gastrointestinal tract after oral administration and has oral bioavailability exceeding 75%. Food has variable effects on absorption. The drug is extensively distributed in body tissues and reaches high levels within the brain (50). Chloroquine binds to melanin-containing cells in the skin and eye, so it can also reach high levels in these sites. There is marked variability in peak concentrations in plasma between individuals, but oral administration at standard treatment doses results in blood concentrations above 1 mmol/liter for at least 4 days (85). It is approximately 60% protein bound and has a half-life of about 4 to 6 days. Approximately 30 to 50% of the drug is metabolized in the liver to inactive compounds, and the remainder is excreted in the urine. Treatment reduction (usually 50% of the normal dose) is required in patients with severe renal or hepatic failure. It is not dialyzable.

In contrast, amodiaquine is a prodrug and is almost entirely metabolized to a biologically active metabolite, desethylamodiaquine, following oral administration. Otherwise, it has pharmacokinetic properties similar to those of chloroquine but has a smaller volume of distribution.

Spectrum of activity. The 4-aminoquinolines are efficient and rapidly acting blood schizonticides. They can be used in both the treatment and the prophylaxis of susceptible strains of all four *Plasmodium* species. They have no effect on tissue schizonts or exoerythrocytic stages. They are gametocytocidal for *Plasmodium vivax* and *P. malariae* but have minimal effect on *P. falciparum* gametocytes. Following infections with *P. vivax* or *P. ovale*, primaquine is also needed to eradicate liver hypnozoites and prevent relapses of infection.

Despite emerging resistance among *P. vivax* parasites, chloroquine remains first-line therapy for *P. vivax, P. malariae,* and *P. ovale* infections. It also remains the treatment of choice for susceptible *P. falciparum* strains, although *P. falciparum* strains from most areas of the world are now resistant to it.

Because of its potential toxicity, amodiaquine is not recommended for prophylaxis of malaria and is generally not used as first-line treatment. However, it results in faster parasite clearance and more rapid resolution of symptoms than chloroquine, and it may be effective in some cases of chloroquine-resistant malaria, so it is used as an alternative treatment regimen in some areas.

Chloroquine is also active against *E. histolytica* trophozoites but is rarely used for this indication since the nitro-5-imidazoles are the drugs of choice.

Adverse effects. Chloroquine has a bitter taste. It is generally well tolerated at the doses required for malaria prevention or treatment, even when taken for prolonged periods. However, it can lead to nausea, abdominal discomfort, dizziness, retinal pigmentation, blurred vision, electrocardiographic changes, muscular weakness, and, rarely, transient psychiatric symptoms. It can also cause severe pruritis, particularly in African blacks. Irreversible neuroretinitis can result if it is taken at high doses for prolonged periods. If an overdose is taken, it can cause shock, arrhythmia, and death.

At the doses used for malaria treatment or prophylaxis, chloroquine has rarely been reported to cause adverse congenital effects (53). However, affinity for melanin-containing tissues such as the retina, iris, and choroid of the eye has been reported, and definitive delineation of fetal risk remains undefined. It is used commonly for treatment and prophylaxis of malaria in pregnant women without evidence of teratogenicity, and it is generally agreed that the benefits of preventing and treating malaria in pregnant women outweigh the potential fetal risks. Chloroquine is excreted in breast milk in small amounts.

Amodiaquine is more palatable than chloroquine and seems to cause less itching. However, serious adverse events including agranulocytosis, aplastic anemia, and drug-induced hepatitis have been reported. These have occurred predominantly following long-term amodiaquine use (mean, 7 to 8 weeks) for malaria prophylaxis, and short-term treatment regimens are thought to be safe (72).

Cinchona Alkaloids

The cinchona alkaloids, quinine and quinidine, contain a quinoline ring. Quinidine is the diastereoisomer of quinine. Quinine was originally extracted from the bark of the South American cinchona tree, but a synthetic form is now available, usually as a quinine sulfate salt. Quinidine is more active than quinine, but it is also more cardiotoxic.

Mechanism of action. The exact target of cinchona alkaloids is unknown. They are thought to act by forming complexes with ferriprotoporphyrin IX, thereby interfering with hemoglobin digestion and resulting in cell lysis and death of schizonts (29). They are also thought to interfere with the function of plasmodial DNA and inhibit the synthesis of parasite nucleic acids and proteins. Quinine also interacts with certain fatty acids present in parasitized erythrocytes, preventing red blood cell lysis and interrupting schizont maturation (32). Additionally, it increases intracellular pH, resulting in lethal effects on the parasite.

Pharmacokinetics. Quinine is available for oral administration as a sulfate salt and for parenteral administration as quinine dihydrochloride. It is >80% absorbed from the gastrointestinal tract following oral doses. It is widely distributed in body tissues, but the concentrations in CSF are <10% of concurrent levels in plasma. It is over 90% protein bound. Quinine is metabolized in the liver, and the native drug and its metabolites are excreted in the urine (50). It has a short half-life, 8 to 12 h, necessitating multiple daily doses. After daily dosing, average concentrations in serum are approximately 7 μg/ml. Intravenous quinine is used in many countries when oral therapy cannot be tolerated, but quinidine gluconate is considered the parenteral drug of choice in the United States (59b). Quinidine has similar pharmacokinetic properties.

The pharmacokinetic properties of the cinchona alkaloids are considerably altered in malaria, with a reduction in clearance that is proportional to the severity of disease. Consequently, doses should be decreased by 30 to 50% after the third day of treatment to avoid accumulation of the drug in seriously ill patients (108). Drug levels may also be increased by administration with foods that alkalinize the urine, because increased tubular reabsorption results. Caution is recommended for use in patients with significant renal or liver impairment, but there are no specific dose adjustment guidelines. Both agents are partially dialyzable.

Spectrum of activity. The cinchona alkaloids can be used in the treatment of all four species of *Plasmodium* that infect humans. Their main indication is in chloroquine-resistant *P. falciparum*. Oral therapy is indicated in uncomplicated malaria, but intravenous formulations of quinine dihydrochloride or quinidine gluconate are used in severe infections.

Quinine and quinidine are blood schizonticides but have little effect on sporozoites or preerythrocytic forms of the parasite. Consequently, they do not eradicate *P. vivax* or *P. ovale* hypnozoites in the liver. They also are not gametocytocidal against *P. falciparum*. They are generally given in combination with other agents. Although relative resistance to these agents has emerged, they remain useful drugs for the treatment of malaria worldwide.

Quinine is also used for the treatment of babesiosis. It is ineffective when used as a single agent but can be given together with clindamycin or azithromycin (36).

Adverse effects. Quinine has an extremely bitter taste and can be associated with nausea, vomiting, and epigastric pain. It also often leads to the symptom complex of cinchonism (nausea, tinnitus, dysphoria, and reversible high-tone deafness). Quinine can also cause hyperinsulinemic hypoglycemia, especially in children and pregnant women with severe malaria, as it increases release of insulin from the pancreas. It has also been associated with massive hemolysis in patients with heavy *P. falciparum* infections. Agranulocytosis, thrombocytopenia, retinopathy, and tongue discoloration are additional adverse effects described. Overdose of quinine can lead to ataxia, convulsions, and coma.

When used as treatment for severe malaria, intravenous quinidine is associated with cardiac arrhythmias. It prolongs the QT interval, widens the QRS complex, and prolongs the PR interval. It can therefore lead to hypotension and ventricular arrhythmias including torsade de pointes. Consequently, it should be administered only in an intensive care setting with cardiac monitoring. As with quinine, administration can also result in blood dyscrasias and cinchonism.

Despite reports of congenital defects following administration of quinine during pregnancy, it can be administered during pregnancy when the benefits of maternal treatment outweigh the potential fetal risks (56, 87). Quinidine has not been reported to be teratogenic. Quinine can have an abortificient effect and lead to induction of labor. It is excreted in small amounts in breast milk but can be administered during breast-feeding when necessary.

Synthetic Quinoline Compounds

Mefloquine

Mefloquine is a synthetic 4-quinoline methanol compound structurally related to quinine.

Pharmacokinetics. Mefloquine is available for oral administration only. Food enhances bioavailability, and it should not be taken on an empty stomach. It is >85% absorbed following oral administration and is concentrated within red blood cells (46). It is >95% protein bound and has a half-life of 2 to 3 weeks. Because of its long half-life, mefloquine is frequently used for prophylaxis of malaria as a once-weekly dose. However, when mefloquine is administered weekly, it requires about 8 weeks before steady drug levels are reached, so a loading dose is often recommended. Peak concentrations in plasma following 500- and 1,000-mg oral doses are 430 and 965 ng/ml, respectively. It is highly lipophilic and widely distributed throughout the body and can cross the blood-brain barrier. Mefloquine is metabolized in the liver and excreted in the bile and feces. There are no specific recommendations regarding the need for dosage adjustment in patients with renal or hepatic failure. It is not dialyzable.

Mechanism of action. Mefloquine interacts both with host cell phospholipids and with the ferriprotoporphyrin IX of the parasitized erythrocyte (32). The precise mechanism of action is not known, but it is thought to act by interfering with the digestion of hemoglobin during the blood stages of the malaria life cycle, likely via a mechanism similar to that of quinine (29). It does not inhibit protein synthesis.

Spectrum of activity. Mefloquine is active against the erythrocytic schizonts of all four species of human malaria parasites, and it is used for both chemoprophylaxis and therapy. Since weekly administration is sufficient for chemoprophylaxis, it is convenient for use in travelers to malaria-endemic areas. Its main utility in malaria treatment results from its activity against most chloroquine-resistant *P. falciparum* strains, although resistance has been recognized, particularly in some areas of Southeast Asia. When used for therapy, it is frequently combined with another agent, usually an artemisinin derivative. Mefloquine does not kill tissue schizonts, so patients with *P. vivax* should be subsequently treated with an 8-aminoquinoline. It also has no effect on gametocytes.

Adverse reactions. Adverse reactions to mefloquine include nausea and vomiting, as well as central nervous system effects such as dysphoria, dizziness, disturbed sleep, nightmares, and ataxia. Severe neuropsychiatric reactions including delirium and seizures have been reported occasionally and are thought to occur in approximately 1:200 to 1:1,300 patients treated for acute falciparum malaria (5, 39, 103). Mefloquine can also potentiate dysrhythmias in individuals on beta blockers. It is quite expensive and is unaffordable for general use throughout areas such as tropical Africa.

Mefloquine is teratogenic in high doses in animals, but reports of studies in humans do not support teratogenic effects (102). A possible higher rate of spontaneous abortion has been suggested, so it is generally avoided in the first trimester of pregnancy. It can be used in later stages of pregnancy if the benefits outweigh the potential risks (71). It is excreted in low concentrations in breast milk but can be used in lactating women when necessary.

Halofantrine

Halofantrine is a synthetic phenanthrene-methanol compound. It has not been approved by the FDA (59b).

Pharmacokinetics. Halofantrine is available only for oral administration but has variable bioavailability. Absorption is enhanced by administration with fatty food, but because high levels in blood enhance toxicity, it is recommended for administration on an empty stomach. It is metabolized in the liver to an active metabolite, *N*-desbutylhalofantrine. The half-lives of halofantrine and its metabolite are 6 to 10 and 3 to 4 days, respectively. It is excreted mainly in the feces.

Mechanism of action. Halofantrine has activity against the asexual erythrocytic stages of malaria parasites, although the exact mechanism of action is unclear.

Spectrum of activity. Halofantrine is efficacious in the treatment of *P. vivax* and *P. falciparum* malaria, but data concerning *P. ovale* and *P. malariae* are limited (11). It is not recommended for prophylaxis of malaria because of toxicity. It is active against blood schizonts only and appears to have no effect against sporozoites, gametocytes, or tissue stages. Halofantrine is more active than mefloquine; however, cross-resistance between these drugs occurs. Its expense and potential toxicity also limit its use, so it is not recommended as a first-line agent.

Adverse effects. Halofantrine leads to gastrointestinal adverse effects including nausea, vomiting, diarrhea, and abdominal pain. It also has potential cardiovascular toxicity and causes concentration-dependent prolongation of the QT interval. It is therefore contraindicated in patients with long-QT syndrome as it can lead to cardiac arrest. It can also lead to pruritis and hepatic enzyme elevations.

Halofantrine is contraindicated in pregnancy. The degree of excretion in breast milk is unknown, and it is not advised for use in lactating women.

8-Aminoquinolines

The 8-aminoquinolines are primaquine and tafenoquine (WR 238,605). Tafenoquine is not yet licensed for malaria prophylaxis or treatment and has not been approved by the FDA (59b).

Mechanism of action. The 8-aminoquinolines interfere with parasite mitochondrial enzymes involved in energy production. They also have an inhibitory action against DNA, although the exact mechanism by which this occurs is unclear (32). An active metabolite of the drug is thought to interrupt the mitochondrial transport system and pyrimidine synthesis in hypnozoites (107).

Pharmacokinetics. The 8-aminoquinolines are well absorbed after oral administration with >90% bioavailability. Primaquine is rapidly metabolized in the liver and its half-life is 4 to 7 h, so it needs to be administered daily. Following a 15- or 45-mg oral dose, peak levels in serum are 53 and 107 ng/ml, respectively (60). Detailed information regarding distribution in body tissues is not available, but primaquine is found at relatively low concentrations in most body sites.

Tafenoquine has a longer half-life, 2 to 4 weeks. Weekly or possibly monthly doses seem to be sufficient when used for prophylaxis, thus making it better tolerated than primaquine. A single dose of tafenoquine may be sufficient for

prevention of relapse following *P. vivax* infections (106). Optimal dose-finding studies are being performed.

Clinical indications. Primaquine and tafenoquine are less active against blood-stage malarial forms than most other antimalarial agents but instead are very active against preerythrocytic sporozoites and exoerythrocytic tissue schizonts of all four malarial species. Their main use is to prevent relapse of *P. vivax* and *P. ovale* infections from latent hypnozoites following treatment with chloroquine. Additionally, they are gametocytocidal against *Plasmodium* organisms, especially *P. falciparum*, and can interrupt transmission of malaria. They are also effective causal prophylactic agents but are not generally recommended for routine use in travelers. Tafenoquine is reported to be more active than primaquine and has higher schizonticidal activity (10).

Adverse effects. Mild gastrointestinal side effects including nausea and abdominal pain are common following 8-aminoquinoline administration. These drugs should not be used in people with glucose-6-phosphate dehydrogenase deficiency as they can induce hemolysis. Patients with NADH methemoglobin reductase deficiency are at risk of developing methemoglobinemia. Primaquine also occasionally causes arrhythmias. These agents should also not be used during pregnancy or lactation because of the potential risk of hemolytic effects in the fetus.

Artemisinin (Qinghasou) Derivatives

Artemisinin is an extract from the Chinese herbal plant *Artemisia annua*, also known as qinghasou. It is a sesquiterpene lactone peroxide. Synthetic derivatives include artemether, dihydroartemisinin, arteether, and artesunate. They have not yet been approved for use by the FDA (59b).

Mechanism of action. Artemisinin and its derivatives act mainly against the asexual erythrocytic stages of malaria. They have an antiparasitic effect, particularly on young, ring-form parasites, leading to their clearance and preventing development of more mature pathogenic forms. They bind to the parasite membrane and to ferriprotoporphyrin IX, so the drugs are highly concentrated within parasites and reach 100 to 300 times higher concentrations in *P. falciparum*-infected red blood cells than in uninfected cells (37). By binding iron in the malarial pigment, they lead to production of toxic oxidative free radicals which damage parasite organelles and alkylate parasitic proteins. This leads to the inhibition of protein synthesis and ultimately to parasite death.

Pharmacology. The derivatives of artemisinin have greater solubility and consequently have been developed for easier administration by a variety of routes. Artesunate is water soluble and can be given intravenously, intramuscularly, orally, or by suppository. Artemether and arteether are oil soluble and available in tablet, capsule, or intramuscular injection forms. Although artesunate is the most potent in vitro, there is no apparent clinical difference in efficacy between the formulations. The artemisinin derivatives have a short half-life, less than 1 to 2 h. They are usually administered once daily for a minimum of 3 days. All are metabolized to the active compound dihydroartemisinin. They should be used with caution in individuals with hepatic or renal impairment.

Spectrum of activity. The artemisinin derivatives are effective against *P. falciparum* and *P. vivax* and are the most potent and rapidly acting parasiticidal drugs. They act specifically against the erythrocytic stages of *Plasmodium*. They are also active against gametocytes, reducing gametocyte carriage by about 90%, and therefore decrease malaria transmission in areas where they are widely used (15, 81). They are not effective against the intrahepatic stage of *P. vivax* or *P. ovale* infections. These drugs are effective against multidrug-resistant *P. falciparum* and are now the drugs of choice against mefloquine- and/or quinine-resistant isolates. No clinically significant resistance has been reported. However, late recrudescence is common unless these agents are combined with another drug, so they are usually administered with a second agent such as mefloquine or doxycycline. Despite the quicker clearance of parasitemia with artemisinin derivatives, randomized trials comparing them with quinine have not shown a benefit in terms of mortality.

There is minimal information available regarding the efficacy of the artemisinin derivatives in *P. ovale* and *P. malariae* infections. The artemisinin derivatives also have activity against schistosomiasis but have not been widely used for this indication (2).

Adverse effects. The artemisinin derivatives are very well tolerated with no serious toxicity or subjective adverse effects. They have been associated with adverse neurological effects in animal models, but there is no evidence that this occurs in humans. It is recommended that they be avoided in pregnancy because of insufficient safety data. The amount excreted in breast milk is unknown.

Antifolates

Pyrimethamine-sulfadoxine, also known as Fansidar, is still commonly used for treatment of malaria in some countries. Pyrimethamine is a synthetic aminopyrimidine antimalarial agent, and sulfadoxine is a long-acting sulfonamide agent.

Pharmacology. Fansidar tablets are composed of 25 mg of pyrimethamine and 500 mg of sulfadoxine. Both drugs are well absorbed orally. After oral administration of a single tablet, peak concentrations of pyrimethamine and sulfadoxine in plasma are 0.13 to 0.4 and 51 to 76 μg/ml, respectively. The half-life of pyrimethamine is 80 to 95 h, and the half-life of sulfadoxine is 150 to 200 h (108). Both components are approximately 90% protein bound. Sulfadoxine is metabolized in the liver, and both agents are excreted mainly in the urine.

Mechanism of action. Pyrimethamine acts against the asexual erythrocytic stage of *Plasmodium* by inhibiting the plasmodial enzyme dihydrofolate reductase. Although it is active against *P. falciparum*, rapid development of resistance occurs and is a major factor limiting its use as a single agent. Combining pyrimethamine with a sulfonamide or sulfone provides sequential, synergistic inhibition of the folate biosynthesis pathway. Malaria parasites are unable to utilize host-derived folic acid, so inhibition of folic acid biosynthesis prevents malarial DNA replication, ultimately leading to cell death.

Spectrum of activity. Pyrimethamine-sulfadoxine is effective for both treatment and chemoprophylaxis of *P. falciparum* malaria. It is no longer recommended for routine prophylaxis because of the potential for severe adverse effects. It acts mainly against blood schizonts and does not have significant gametocytocidal activity. Emerging resistance is limiting its use in treatment; however, its low cost means that it remains first-line therapy for *P. falciparum* in many African countries. Pyrimethamine-sulfadoxine also has some efficacy in the treatment of *P. vivax*, but it has

longer parasite and fever clearance times than chloroquine and higher failure rates (30 to 40%), so it is not recommended. The efficacy of Fansidar against *P. ovale* and *P. malariae* has not been adequately evaluated.

Pyrimethamine is also used in combination with sulfadiazine for the treatment of toxoplasmosis.

Adverse effects. Pyrimethamine-sulfadoxine can result in adverse effects including rash, nausea, vomiting, headache, and peripheral neuritis. It can also be associated with more serious and occasionally fatal reactions, including the Stevens-Johnson syndrome and blood dyscrasias (particularly agranulocytosis and megaloblastic anemia). Other reported adverse effects include hepatitis, toxic nephrosis, exfoliative dermatitis, and erythema multiforme. The long half-life of the sulfa component means that sensitivity reactions can be sustained for prolonged periods even after the drug is discontinued.

Pyrimethamine has been associated with teratogenic effects in animals. There are no controlled human studies; however, it has been used frequently during pregnancy, particularly in African countries, and has been associated with good fetal outcome. It is officially recommended for use during pregnancy only when potential benefits outweigh the possible risks to the fetus. Fansidar can cause kernicterus in infants, so it should be used with caution in pregnant women late in the third trimester. Both pyrimethamine and sulfadoxine are excreted into the breast milk and are preferably avoided during lactation.

Atovaquone-Proguanil (Malarone)

Malarone is a tablet combination of 250 mg of atovaquone and 100 mg of proguanil. Atovaquone is a hydroxynaphthoquinone, and proguanil is an antifolate. Malarone has been approved for use by the FDA for prophylaxis and treatment of malaria.

Pharmacokinetics. Atovaquone is a highly lipophilic compound with low aqueous solubility and poor and variable oral availability. Its absorption is increased if it is administered with fatty foods or a milky drink. It is not metabolized and is excreted almost exclusively in the feces. It is 99% plasma protein bound, and its half-life is 2 to 3 days. Proguanil is rapidly and extensively absorbed after oral administration. It is metabolized by the cytochrome P-450 in the liver to the active cyclic triazine metabolite, cycloguanil. It is 75% protein bound and is excreted mainly in the urine. Its half-life is 12 to 21 h. After two malarone tablets twice daily for 3 days, mean levels of proguanil and atovaquone in plasma have been reported as 170 and 908 ng/ml, respectively (25).

There are no official guidelines regarding dose reductions for malarone in hepatic or renal insufficiency.

Mechanism of action. Atovaquone has a novel mechanism of action. *Plasmodium* species are dependent on de novo pyrimidine biosynthesis, which is selectively coupled with electron transport. Atovaquone inhibits the electron transport system in the mitochondria of parasites, thereby blocking nucleic acid synthesis and inhibiting replication (55). When used as monotherapy, atovaquone is associated with high recrudescence rates. Proguanil also acts against the asexual erythrocytic stage of the parasite by selectively inhibiting plasmodial dihydrofolate reductase. However, in combination with atovaquone, it acts via a different mechanism and directly lowers the effective concentration at which atovaquone causes collapse of the mitochondrial membrane potential (93).

Spectrum of activity. Malarone is effective against asexual and sexual forms of the *P. falciparum* parasite and is recommended for treatment and prophylaxis of falciparum malaria. It is becoming an increasingly utilized alternative to mefloquine for chemoprophylaxis in travelers to malaria-endemic areas. Malarone is also effective for treating *P. vivax* and *P. ovale*, but neither drug is effective against hypnozoites so primaquine is additionally required to prevent relapses after drug discontinuation. Malarone also shows good efficacy against *P. malariae* in limited studies (84). Reports of clinical failures and resistance of *P. falciparum* isolates to malarone via a single mutation are emerging.

Adverse effects. Malarone is generally very well tolerated. Side effects are mild and include anorexia, nausea, vomiting, abdominal pain, diarrhea, pruritis, and headache. Between 5 and 10% of recipients develop transient asymptomatic elevations in transaminase and amylase levels. Because of inadequate safety data, it is not recommended for use during pregnancy or lactation.

OTHER ANTIPROTOZOAL AGENTS

Diloxanide Furoate

Diloxanide furoate, also known as Furamide, is a substituted acetanilide. Its main use is as a luminal amebicidal agent. It is not widely available in the United States and can be obtained only from specific pharmacies (59b).

Mechanism of action. The mechanism of action of diloxanide furoate is unknown.

Pharmacology. Diloxanide furoate is available in tablet form. The parent drug is poorly absorbed following oral administration, but it is hydrolyzed in the bowel lumen to an active compound, diloxanide, which is >90% absorbed. Metabolites are excreted primarily in the urine.

Spectrum of activity. Diloxanide furoate acts primarily as a luminal agent and helps clear the bowel of *E. histolytica* cysts, thereby preventing relapse in cyst carriers (59). It is not effective for amebas in the bowel wall or in other tissues such as the liver, so it is generally given with a 5-nitroimidazole.

Adverse effects. Side effects are generally not severe but include rash, nausea, abdominal pain, diarrhea, and flatulence. It is not recommended for use in pregnancy or during lactation.

Iodoquinol

Iodoquinol is a halogenated 8-hydroxyquinoline derivative, diodohydroxyquin.

Mechanism of action. Iodoquinol is thought to act by inactivating essential parasitic enzymes and inhibiting parasite multiplication (32).

Pharmacology. Iodoquinol is available for oral administration but is very poorly absorbed, with 8% reaching the systemic circulation. It is excreted primarily in the feces, but small quantities of glucuronic acid metabolites are excreted in the urine. It should not be used in individuals with renal or hepatic insufficiency and should be used with caution in patients with thyroid or neurologic disease.

Spectrum of activity. Iodoquinol is a potent amebicidal drug. It is effective against trophozoites and cysts of *E. histolytica* located within the lumen of the intestine and is used to eradicate amebic cysts to help prevent relapse of infection (59). Because it is poorly absorbed systemically, it is not effective for invasive intestinal or extraintestinal *E. histolytica* infections and is therefore frequently combined with a 5-nitroimidazole.

Iodoquinol also has activity against *Balantidum coli*, *Dientamoeba fragilis,* and *Giardia lamblia*. It has also been used for *Blastocystis hominis*, although the significance of this agent as a true pathogen remains controversial (57).

Adverse effects. The main side effects of iodoquinol include nausea, abdominal cramps, diarrhea, headache, pruritis, and rash. Skin and hair may be temporarily stained yellow-brown following exposure. In high doses or with prolonged use, it can cause optic neuritis, optic atrophy, peripheral neuropathy, ataxia, and seizures. It is also associated with nephrotoxicity. It should be avoided in individuals who are sensitive to iodine. The degree of safety associated with its use in pregnancy or lactation is uncertain, so it is recommended that it be avoided.

Pentavalent Antimonial Compounds

The pentavalent antimony derivatives are used for treatment of leishmaniasis. They include sodium antimonylgluconate (or stibogluconate), also known as Pentostam, and *N*-methylglucamine antimoniate (or meglumine antimoniate), also known as Glucantime. Selection of one drug over the other is based primarily on cost and availability. Neither drug is licensed for use in the United States, but sodium stibogluconate is available from the CDC for individual patient use (59b).

Mechanism of action. The precise mechanism of action of the pentavalent antimony derivatives remains unclear. They are thought to inhibit enzymes of glycolysis within parasites. Because glycolysis is the major source of parasitic ATP, the blockade of this source of energy is fatal to parasites (32).

Pharmacology. The pentavalent antimonials are administered parenterally (intramuscularly or intravenously) or via intralesional injection. They remain in plasma and are excreted predominantly by the kidneys. Small amounts are metabolized in the liver to trivalent antimony, which contributes to the toxicity associated with their use. Following intramuscular administration, mean peak antimony concentrations in blood of 10.5 to 12 μg/ml have been reported (16). There are no specific guidelines regarding dose adjustment in renal impairment.

Spectrum of activity. Antimony preparations are efficient in killing many protozoan and helminthic parasites but are no longer recommended for most parasitic infections because of their toxicity. However, they are still used for the treatment of visceral, mucocutaneous, and cutaneous leishmaniasis, as few effective alternatives exist. Various treatment regimens are used, but the exact duration and efficacy vary depending on the type of leishmaniasis, the severity of the lesion, and the area of endemic infection.

Adverse effects. Minor adverse effects from the pentavalent antimonials are common and include nausea, vomiting, headache, and malaise. More severe reactions such as leukopenia, agranulocytosis, and electrocardiographic changes (prolongation of the QT interval and ventricular arrhythmias) can also occur. Renal insufficiency, proteinuria, and elevation of hepatic and pancreatic enzyme levels have also been described.

Miltefosine

Miltefosine is a phosphocholine analogue that was originally developed as an anticancer compound. It is the first effective oral agent for visceral leishmaniasis and is becoming increasingly important because of growing resistance of leishmaniasis strains to pentavalent antimonials. Miltefosine was registered in 2002 for the oral treatment of visceral leishmaniasis in India and has recently been approved in Germany, but it is not available in the United States.

Mechanism of action. The mechanism of action of miltefosine is not well understood. The drug interferes with cell signaling pathways and membrane synthesis (42). Miltefosine has a direct antiparasitic activity and appears to act on key enzymes involved in the metabolism of ether lipids present on the surface of parasites, but the exact mechanisms of its cytotoxicity are unknown (42, 63). Miltefosine does not appear to have a direct immunostimulatory effect, but it does induce apoptotic cell death (80). It has been shown to be active against both the extracellular promastigote form and the intracellular amastigote form of *Leishmania* parasites both in vitro and in vivo (88).

Pharmacology. Miltefosine is well absorbed after oral administration and is widely distributed. Minimal pharmacokinetic data are available for humans, but in rats the drug is rapidly taken up and accumulates in the kidney, liver, lung, spleen, and adrenal glands (63). On oral administration of 30 mg of miltefosine per kg of body weight twice per day, concentrations of 155 to 189 nmol/g of tissue are achieved (42). Miltefosine has a long half-life, about 8 days, and is slowly metabolized by phospholipase.

Spectrum of activity. Miltefosine has activity against *Leishmania* spp. and *Trypanosoma cruzi* both in vitro and in vivo, but clinical studies to date have been limited to leishmaniasis. In vitro activity of miltefosine against *Trypanosoma brucei, Entamoeba histolytica,* and *Acanthamoeba* spp. has also been demonstrated (19).

The majority of studies using miltefosine have examined its role against visceral leishmaniasis in India. In clinical trials, miltefosine has been found to have a cure rate of 94 to 97% at 6 months in both adults and children (6, 97). It is generally given at 2.5 mg/kg per day for 4 weeks (6, 28, 42, 96, 97). Recent studies have also examined its efficacy in New World cutaneous leishmaniasis and have found 82 to 94% efficacy against *Leishmania (Viannia) panamensis*, 60% cure for *Leishmania mexicana*, and 33% cure for *L. v. braziliensis* (91, 92).

Adverse effects. In various clinical trials, toxic effects associated with miltefosine have usually been found to be tolerable and reversible, although the therapeutic window appears to be narrow (80). Mild to moderate gastrointestinal side effects, including nausea, vomiting, and diarrhea, occur in up to 60% of patients. Dose-related motion sickness is also reported in up to 40% of patients (92). A mild increase in aspartate aminotransferase and creatinine and/or blood urea nitrogen levels has been reported, with reversible hepatotoxicity and renal damage in a few cases (6, 63).

Miltefosine is abortifacient and teratogenic in animals and should not be used in pregnancy.

Pentamidine

Pentamidine is an aromatic diamidine compound that is used as an antiprotozoal agent in the treatment of leishmaniasis and African trypanosomiasis. Its use has been approved by the FDA (59b).

Mechanism of action. Pentamidine is chemically related to guanidine. Its mechanism of action has not been clearly defined and may not be uniform against different organisms. It is possible that it inhibits dihydrofolate reductase and interferes with aerobic glycolysis in protozoa. It may also interfere with amino acid transport, precipitate nucleotides and nucleotide-containing coenzymes, and inhibit DNA, RNA, and protein synthesis (86).

Pharmacology. Pentamidine is currently available as an isothionate salt. It is poorly absorbed from the gastrointestinal tract. When used for protozoal infections, it must therefore be administered intramuscularly or intravenously, but it can be given via inhalation for prevention of *Pneumocystis carinii* pneumonia. Following intravenous administration of a 4-mg/kg dose, concentrations of 0.3 to 1.4 μg/ml in plasma have been reported (105). The highest concentrations of the drug are found in the kidney, liver, and spleen, and pentamidine penetrates poorly into the central nervous system. It has a short serum half-life of 6.5 to 9 h because it is rapidly and extensively taken up by tissues. Its extensive tissue binding results in prolonged excretion over a period of 6 to 8 weeks, and it is eliminated via the kidneys. Pentamidine should be used cautiously in the presence of renal or hepatic failure, but no dosage adjustment is recommended.

Spectrum of activity. The antiparasitic indications for pentamidine include leishmaniasis and African trypanosomiasis, but its main use is as an antifungal agent for treatment and prophylaxis of *Pneumocystis carinii* pneumonia.

Despite its leishmanicidal activity, pentamidine's toxicity means it is not first-line therapy for leishmaniasis, and it is used predominantly in individuals intolerant of antimonial compounds or in those whose disease is refractory to other treatment. *Leishmania donovani* infections in particular are increasingly resistant to antimonials, and pentamidine can be used as an alternative agent.

Pentamidine is also active against African trypanosomes, but its utility is restricted to trypanosomiasis without central nervous system involvement. Because central nervous system involvement occurs early with *Trypanosoma brucei rhodesiense*, it is used more frequently for *T. b. gambiense* infections.

Adverse effects. Over half of parenteral pentamidine recipients experience some adverse effect from therapy. Administration is associated with a variety of reactions that appear to be unrelated to drug concentrations in plasma (86). Immediate reactions include nausea, anorexia, dizziness, pruritis, and hypotension. Pentamidine can also produce local effects including pain and necrosis at the site of injection. In addition, pentamidine administration is associated with hematologic effects, particularly leukopenia (up to 10% of recipients) and thrombocytopenia (approximately 5%), as well as with electrolyte abnormalities including hyperkalemia, hypomagnesemia, and hypocalcemia. Other severe adverse effects include ventricular arrhythmias, pancreatitis, hypo- or hyperglycemia, hepatotoxicity, and acute renal failure. Pentamidine has also been associated with Stevens-Johnson syndrome. Finally, occasional seizures and hallucinations have been reported.

There are minimal available safety data regarding the use of pentamidine in pregnancy or lactation, and its use is therefore not recommended unless the benefits outweigh potential risks.

Suramin

Suramin is a polysulfonated naphthylamine derivative of urea. Its main indication is in the treatment of African trypanosomiasis. It is not licensed for use in the United States but is available from the CDC via a compassionate-use drug protocol (59b).

Mechanism of action. The mechanism of action of suramin is not fully understood but is thought to be via inhibition of enzymes associated with DNA metabolism and protein synthesis of the trypanosomal parasites (78).

Pharmacology. Suramin is dispensed as a sodium salt and is soluble in water. It is not absorbed when given orally and is usually administered as a 10% solution by slow intravenous infusion. Following intravenous injection, it is rapidly distributed and >99% becomes bound to plasma proteins. It does not cross the blood-brain barrier. Its half-life is 41 to 78 days. It is excreted in the urine. It should not be used in the presence of renal failure or significant hepatic dysfunction.

Spectrum of activity. Suramin is an effective drug for early hemolymphatic stages of *T. b. gambiense* infections. It also has some effect in early *T. b. rhodesiense*, provided that there is no central nervous system involvement (24).

Suramin was used to treat onchocerciasis prior to the development of ivermectin. An advantage of suramin is that it has macrofilaricidal activity, damaging the intestinal epithelium of adult *O. volvulus* worms and resulting in their death (99). However, it is associated with frequent toxic effects at required doses, so it has therefore been replaced by ivermectin. Suramin also has activity against adult *W. bancrofti* worms but is not recommended for this indication (32).

Adverse effects. Potential side effects include an immediate reaction with nausea, vomiting, shock, and loss of consciousness following suramin injection. Other adverse effects include renal impairment, exfoliative dermatitis, and neurological toxicity. It has also been associated with pancytopenia.

Suramin has been reported to be teratogenic in rodents (104). No case of infant malformation in humans has been described, but it is not recommended for use during pregnancy except in circumstances where there is no suitable alternative.

Melarsoprol

Melarsoprol is a trivalent arsenical compound. Its main use is in the treatment of African trypanosomiasis. It is not licensed for use in the United States but is available from the CDC via a compassionate-use drug protocol (59b).

Mechanism of action. Melarsoprol appears to act by binding to essential thiol groups of trypanosomes, and it has a particularly high affinity for the active site for pyruvate kinase. This results in interference with energy generation within parasites, thereby preventing trophozoite multiplication.

Pharmacology. Melarsoprol is absorbed if given orally but is generally administered by the intravenous route. It is prepared as a solution in propylene glycol and is given by slow intravenous infusion. It is estimated that <1% penetrates the central nervous system, and concentrations in CSF are up to

50-fold lower than concentrations in serum (12, 34). However, it is so efficacious as a trypanocidal agent that it can be used for late stages of trypanosomal disease. Melarsoprol has a half-life of approximately 35 h. Its metabolism has not been well studied, but it is excreted predominantly in the urine.

Spectrum of activity. Melarsoprol is active in the treatment of all stages of African trypanosomiasis due to *T. b. gambiense* and *T. b. rhodesiense*. Because of its toxicity, it is generally reserved for use in late stages of disease involving the central nervous system.

Adverse effects. Malarsoprol is commonly associated with significant toxicity. It causes vomiting, abdominal pain, hepatotoxicity, peripheral neuropathy, cardiac arrhythmias, and albuminuria. Administration can also lead to a reactive encephalopathy in up to 10% of patients, and this is associated with significant mortality. Hypersensitivity reactions are also relatively frequent. The injection is very irritating, and extravasation during intravenous administration should be avoided. There are minimal data available regarding potential teratogenic effects.

Eflornithine

Eflornithine is also known as DFMO. Its main indication is for treatment of African trypanosomiasis. Its manufacture was ceased temporarily; although it has been resumed, drug availability is still limited, and it is not available in the United States for systemic use.

Mechanism of action. Eflornithine selectively and irreversibly inhibits ornithine decarboxylase, an enzyme required for the formation of polyamines needed for cellular proliferation and differentiation in parasites. It therefore leads to inhibition of parasite growth (47).

Pharmacology. Eflornithine can be administered by mouth and has >50% oral bioavailability, but significant diarrhea frequently results so it is usually given intravenously. No protein binding of the drug occurs following intravenous administration. It crosses the blood-brain barrier and produces CSF-to-blood ratios between 0.13 and 0.5 (40, 61). The half-life is 3 to 3.5 h, and approximately 80% of the dose is excreted unchanged by the kidneys. Dose reduction is required in patients with significant renal impairment.

Spectrum of activity. Eflornithine is used for treatment of *T. b. gambiense* infection when there is central nervous system involvement. It is ineffective as monotherapy against *T. b. rhodesiense* (18). It also displays some activity against other parasites, including *Plasmodium* species, *Cryptosporidium parvum,* and *Trichomonas vaginalis*, but it is not used for these indications because of toxicity.

Adverse effects. Side effects of eflornithine occur in up to 40% of patients. Common adverse reactions include vomiting, abdominal pain, dizziness, arthralgias, hearing loss, and rash. Bone marrow toxicity resulting in thrombocytopenia and leukopenia has also been described.

Eflornithine has been shown to arrest embryonic development in animals (31). There are no good studies of its safety in pregnancy or lactation, so it should be used only when the potential maternal benefits outweigh the possible risks to the fetus.

Agents for American Trypanosomiasis: Nifurtimox and Benznidazole

The two agents used for treatment of American trypanosomiasis are nifurtimox and benznidazole. Nifurtimox is a nitrofuran, and benznidazole is a 2-nitroimidazole derivative. Neither is approved for use by the FDA, but nifurtimox is available from the CDC for compassionate use (59b).

Mechanism of action. Benznidazole has an inhibitory effect on protein and RNA synthesis in *Trypanosoma cruzi* cells (79). It is thought to cause increased phagocytosis, cytokine release, and production of reactive mitogen intermediates that result in destruction of intracellular parasites (66).

The mechanism of action of nifurtimox seems to be related to its metabolism to chemically reactive radicals that cause production of toxic reduced products of oxygen, such as superoxide, hydrogen peroxide, and hydroxyl radicals (23, 34). These compounds accumulate within trypanosomes, leading to toxic effects including membrane damage and enzyme inactivation. It may also cause direct inhibition of protein synthesis via damage to parasite DNA (38).

Pharmacology. Benznidazole is available for oral administration and has a bioavailability of over 90%. It is approximately 40% protein bound. It has a half-life of approximately 12 h and has good tissue penetration (109).

Nifurtimox is administered orally but has poor oral bioavailability. It is metabolized in the liver and has a half-life of approximately 3 h. Dose reduction is advised for patients with significant hepatic or renal impairment, but no specific guidelines exist.

Spectrum of activity. Both benznidazole and nifurtimox are used for the treatment of acute *Trypanosoma cruzi* infection (Chagas' disease). Neither agent has demonstrated efficacy in late stages of disease. Nifurtimox also has been shown to have some activity in leishmaniasis and African trypanosomiasis, but it is not generally used for these indications.

Adverse effects. Side effects are common with benznidazole and are seen in up to 40% of treated individuals. Common adverse effects include vomiting, abdominal pain, peripheral neuropathy, rash, and pruritis. Bone marrow suppression and neuropsychiatric reactions have also been reported. Nifurtimox has significant side effects that preclude the completion of therapy in many patients. Adverse effects include anorexia, nausea, rash, headache, sleep disturbance, peripheral neuropathy, and myalgias. Less frequent but more severe toxicity includes psychosis and convulsions.

Benznidazole crosses the placenta, but there are minimal data regarding teratogenic effects for either agent in animals or humans (21).

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Mechanisms of Resistance to Antiparasitic Agents

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Parasitic diseases rank among the most prevalent and severe diseases worldwide, and yet their control relies heavily on a single tool: the drugs used for chemotherapy or prophylaxis. This situation exists because no effective antiparasitic vaccines are available and implementation of other control measures often proves to be difficult in countries where parasitic diseases are endemic. This dependence on drugs is compounded by the relative paucity of the current armamentarium of antiparasitic products, a situation attributable largely to a lack of economic incentives for research and development. Furthermore, those drugs that are available are too often used incorrectly in communities and in control programs, a practice that encourages the selection of drug-resistant parasites.

The complex biologic interactions between parasites and their hosts (and at times vectors) influence significantly the emergence and expression of drug resistance. In many cases the observed resistance is true resistance, attributable to biologic characteristics of the parasites that enable them to survive drug concentrations that are lethal to susceptible members of the species. Mechanisms for such true resistance are varied and include a decrease in parasite drug accumulation or modifications in enzyme structure or metabolic pathways. However, various host factors modulate the clinical and parasitologic responses to drug treatment, and the observed responses do not necessarily reflect true parasite resistance or susceptibility. For example, in populations with high rates of exposure to parasitic infections, the resulting high rates of immunity might suffice to eliminate an infection treated with an ineffective drug. Conversely, treatment with a drug to which the parasite is biologically susceptible will not necessarily result in therapeutic success if the host takes an inadequate dose of the drug, absorbs it poorly, or lacks the immune response that might be needed for a successful antiparasitic synergism with the drug. Such host factors may be especially important in the areas where most parasitic diseases prevail, where high rates of parasite transmission result in high rates of immunity in most of the population, and where, conversely, malnutrition and human immunodeficiency virus (HIV) infection frequently decrease the patient's immune status.

Greater understanding of the epidemiology and mechanisms of drug resistance can provide valuable guidance for a better use of existing compounds and for the development of novel products. A selective review of drug resistance in five diseases will illustrate the existing problems and their potential solutions. A summary of the proposed mechanisms of resistance is provided in Table 1.

MALARIA

Overview

Malaria, the parasitic disease with the most visible public health impact, also presents the greatest problem of drug resistance (133, 136). Furthermore, resistance is found mainly in *Plasmodium falciparum,* the most pathogenic of the four species that cause human malaria, responsible for at least one million deaths annually. The 4-aminoquinoline drug chloroquine, a cornerstone of antimalarial chemotherapy since the 1940s due to its low cost, safety, and rapid action, has seen its usefulness seriously eroded by the widespread occurrence of resistant strains of *P. falciparum*. From the original foci in Southeast Asia and South America, resistance has spread relentlessly and is now found in most areas of the world, including Africa, the continent with the heaviest malaria burden. Chloroquine no longer constitutes an appropriate treatment option in most countries where malaria is endemic. Among alternate drugs for use against chloroquine-resistant *P. falciparum,* synergistic combinations of inhibitors of the parasite folate biosynthetic pathway are relatively inexpensive and safe. An antifolate combination often used until recently consists of sulfadoxine and pyrimethamine (S-P), two compounds which sequentially block the parasite dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR), respectively. However, resistance to S-P has emerged and is found in Asia and South America, often at high prevalence, and in many African countries. *P. falciparum* resistant to both chloroquine and S-P can be treated with several other drugs, which include quinine and mefloquine (two quinoline methanols), the tetracyclines, the artemisinin derivatives (a sesquiterpene lactone class of compounds), and the recently introduced combination atovaquone-proguanil. However, resistance against some of these drugs has now been observed, especially in Southeast Asia and South America. This precarious situation is of great concern and is addressed by the recommended use of combination therapies, often containing artemisinin derivatives, that aim at delaying the emergence of resistance (133). The newer drugs or drug combinations might, however, prove too costly

TABLE 1 Summary of proposed mechanisms of resistance to selected antiparasitic drugs

Disease	Drug(s)	Mechanism(s) of resistance
Malaria	Chloroquine	Decreased accumulation of the drug by the parasite, resulting from multifactorial, multigenic mechanisms
	Pyrimethamine	Alteration in binding affinities between the drug and the parasite dihydrofolate reductase-thymidylate synthase, resulting from mutations on the corresponding gene
	Sulfadoxine	Alteration in binding affinities between the drug and the parasite dihydropteroate synthase, resulting from mutations on the corresponding gene
	Atovaquone	Alteration in binding affinities between the drug and the parasite cytochrome *b*
Trichomoniasis	Metronidazole Tinidazole	Aerobic: decreased oxygen scavenging Anaerobic: reduced concentration of enzyme(s)necessary to activate nitro group
Leishmaniasis	Pentavalent antimonials	Decreased active intracellular drug concentration through increased efflux or decreased conversion to active trivalent form
	Amphotericin B, paromomycin, miltefosine	Widespread clinical resistance not currently documented; resistance has been produced in laboratory strains
African trypanosomiasis	Melarsoprol, pentamidine	Mutation/loss of P2 adenosine transporters that facilitate drug uptake
Schistosomiasis	Oxamniquine	Lack of esterase necessary for drug activation
	Praziquantel	Widespread clinical resistance not currently recognized as important problem for public health; resistance documented in laboratory strains
	Artemether	Effective against immature worms; no resistance as yet documented

for many areas, particularly in Africa, where the malaria problem is the worst but where drug resistance has not yet reached the nearly untenable proportions found in Southeast Asia. This situation underlines the importance of using rationally those drugs that are still effective.

In *P. vivax,* the most prevalent species, chloroquine-resistant strains were first reported in 1989 in Papua New Guinea (109) and are now found in other areas where *P. vivax* is endemic. In addition, *P. vivax* infections have been described with decreased sensitivity to primaquine, an 8-aminoquinoline that prevents relapses in this species by destroying dormant liver-stage parasites (hypnozoites) (115).

Mechanisms of Resistance to Selected Antimalarials

Chloroquine resistance in *P. falciparum* appears to have a multifactorial, multigenic basis (132). Epidemiologically, the long delay between the introduction of chloroquine and the emergence of resistance, despite massive use of the drug, suggests that several genetic changes are necessary before chloroquine resistance becomes manifest. Clinically and parasitologically, the existence of different degrees of in vivo and in vitro resistance also suggests the intervention of multiple factors. Chloroquine-susceptible parasites accumulate the drug in their digestive vacuole, where the drug appears to interfere with the polymerization and detoxification of heme (a by-product of the digestion of host cell hemoglobin by the parasite), resulting in the accumulation of heme-chloroquine complexes that are toxic to the parasites (120). In resistant parasites this accumulation is decreased (51). Furthermore, chloroquine resistance can be reversed in vitro by various compounds, including the calcium channel blocker verapamil (81) and the tricyclic antidepressant desipramine (7), apparently through increased accumulation of the drug in the resistant parasites (7, 69). Several mechanisms have been proposed for these hallmark manifestations of chloroquine resistance, including (i) a decreased uptake of chloroquine by alterations in a chloroquine importer (possibly an Na^+/H^+ exchanger) found in the parasite plasma membrane (39, 110); (ii) an alteration in pH in the parasite digestive vacuole, which decreases the interactions between the drug and its heme target (43); (iii) a reduced accessibility of heme for binding with chloroquine (13); or (iv) an increased efflux of chloroquine from the acid digestive vacuole (69).

Chloroquine resistance is linked most closely to point mutations in the *pfcrt* gene found on the parasite's chromosome 7. This gene encodes pfCRT, a transmembrane transporter protein found on the parasite digestive vacuole, which may be involved in efflux of drug/metabolites or pH regulation (50). Laboratory investigations using genetic crosses and field studies with chloroquine-resistant parasites from Asia, Africa, and South America have identified the key role of a mutation affecting codon 76, most often Lys→Thr (K76T) (12, 36, 132). This central mutation is accompanied by additional polymorphisms on the *pfcrt* gene, particularly in the neighboring residues 72 to 75 and residue 220, in variant patterns that reflect the geographic origin of the parasites (84, 126, 132). These additional mutations possibly compensate for a loss of transporter function, derived from the K76T mutation, or they may play an additional role in resistance. The K76T mutation is now a valuable molecular marker used in epidemiologic surveys of chloroquine resistance (1, 35, 85). Its measured prevalence may offer a useful predictor of the clinical efficacy of chloroquine in a given area, provided that appropriate adjustments are made for host factors, particularly immunity, that may result in parasite clearance in spite of treatment with an ineffective drug (36, 37).

Another gene has also been linked, albeit less consistently, to chloroquine resistance. The *pfmdr1* gene, on chromosome 5,

encodes the P-glycoprotein homolog-1 (PGH-1), a *P. falciparum* homologue of the mammalian multidrug resistance gene which increases outflux of drugs. Current evidence indicates that *pfmdr1* does not play a primary, causal role in chloroquine resistance. Initial studies that associated amplification of *pfmdr1* with chloroquine resistance have not been confirmed. In genetic cross experiments, chloroquine resistance has not cosegregated with *pfmdr1* (131). However, field assessments have found associations, albeit variable, between *pfmdr1* polymorphisms and chloroquine resistance (136), thus suggesting that *pfmdr1* may play an adjunct function in resistance. Indeed, transfection experiments have shown that selected mutations in *pfmdr1* can modulate chloroquine resistance in vitro, in concert with mutations in other genes such as *pfcrt* (108).

The *pfmdr1* gene also appears to be involved in resistance to other antimalarial drugs. Experiments using transfection (108) or genetic crosses (42) and field investigations (99, 136) have found that mutations at specific positions of *pfmdr1* alter responses not only to chloroquine but also to quinine, mefloquine, and the unrelated artemisinin derivatives (often in the reverse direction). Selection of mefloquine-resistant *P. falciparum* through drug pressure in continuous culture have resulted in amplification of the *pfmdr1* gene, concomitantly with an increase in resistance to quinine and a decrease in resistance to choloroquine in the selected parasites (25). Field investigations have linked *pfmdr1* amplification with in vitro resistance to mefloquine and artesunate (104) and with in vitro resistance to mefloquine, quinine, and artesunate and increased sensitivity to chloroquine (99). These associations are not absolute, indicating that transporter molecules other than PfCRT and PGH-1 may be involved in resistance to these antimalarial drugs (86).

Pyrimethamine-resistant *P. falciparum* appeared quite rapidly in several locations after the introduction of the drug in the early 1950s (94), suggesting that in contrast to chloroquine, a single gene is involved in pyrimethamine resistance. Differently from bacteria and human tumor cells, gene amplification is not involved in *P. falciparum* resistance to DHFR inhibitors (26). Resistance to DHFR inhibitors has indeed been linked to point mutations in the parasite dihydrofolate reductase-thymidylate synthase gene on chromosome 4 of *P. falciparum* in studies of culture-adapted *P. falciparum* (26, 52, 95) and of field-collected parasites (31, 136) and in transfection experiments (137). These mutations result in alterations of the enzyme binding affinities with the drugs and occur in a sequential pattern, the primary event being an S108N mutation, with subsequent mutations (particularly N51I and C59R) allowing increasing levels of resistance in vitro while maintaining the functionality of the enzyme (101). In vitro resistance to cycloguanil, the active metabolite of proguanil (another DHFR inhibitor), is also linked to various *dhfr* mutations. However, these mutations differ from those associated with resistance to pyrimethamine (52, 95). In similar studies with sulfadoxine using culture-adapted *P. falciparum* and transfection experiments, resistance to sulfonamides/sulfone has been traced to a set of sequential mutations in the gene for dihydropteroate synthase (which also encodes dihydrohydroxymethylpterin pyrophosphokinase) on chromosome 8 (121, 129). The likely initial event consists of an A437G mutation, with subsequent additional mutations conferring increasing degrees of resistance (101). Resistance to the S-P combination appears to require a minimum of three mutations on the *dhfr* gene, accompanied by two additional mutations on the *dhps* gene ("quintuple mutant"), with the clinical outcome being subject to additional host factors such as folate levels, immunity, and absorption and drug metabolism (29, 44, 70, 101, 103). The I164L mutation in *dhfr,* associated to high levels of S-P resistance as well as to decreased sensitivity to chlorproguanil-dapsone, a new antifolate combination similar to S-P, has not been yet reported from Africa (92). Surveys of mutations in the *dhfr* and *dhps* genes in *P. falciparum* now offer a useful tool for monitoring of resistance to S-P and other antifolates and are potentially valuable for assessing the risk of emergence of resistance by detection of prerequisite mutations as they appear in local parasite populations (6, 53, 102, 136).

The recently introduced drug combination Malarone consists of a synergistic association of proguanil (a DHFR inhibitor) and atovaquone (a mitochondrial inhibitor of *P. falciparum*). While resistance develops readily to either compound used alone, their combination aims to overcome this problem. Investigations using in vivo and in vitro resistant *P. falciparum* have linked resistance to avovaquone to mutations in the parasite cytochrome *b* gene, that may decrease the binding affinity of cytochrome *b* for atovaquone (67). Further investigations of atovaquone-proguanil treatment failures in cases of *P. falciparum* infections have associated these failures with mutations at codon 268 of the cytochrome *b* gene (71).

While drug resistance in *P. vivax* has not reached the same magnitude as in *P. falciparum,* investigations on mechanisms of resistance in this species currently examine the potential role of *P. vivax* homologues of *pfcrt* (90), *mdr1* (14), *dhfr* (54), and *dhps* (68).

TRICHOMONIASIS

Infection with *Trichomonas vaginalis* is one of the most common causes of human vaginitis. In addition, recent data have linked *T. vaginalis* infections with preterm delivery, low birth weight, greater susceptibility to infection with HIV, and an increased risk of cervical neoplasia (24, 73, 127). As a result, expedient treatment of this infection has become an important public health measure.

T. vaginalis is a facultative anaerobe, and *T. vaginalis* infections are most commonly treated with 5-nitroimidazoles. Metronidazole, and more recently tinidazole, are the only drugs licensed for treatment of trichomoniasis in the United States. Tinidazole functions in a manner analogous to that of metronidazole and is more active at equimolar concentrations (28). However, strains clinically resistant to metronidazole often have cross-resistance to tinidazole.

Most studies on the mechanism of action of 5-nitroimidazoles and resistance to them have been performed with metronidazole. The drug enters parasites in an inactive form by passive diffusion. Once inside, the low intracellular redox potential generated by trichomonad energy production results in reduction of the nitro group, forming a nitro radical that damages the parasite (61). *T. vaginalis* resistance to metronidazole occurs in two forms, designated according to the oxygen environment under which they can be observed. Anaerobic resistance is the more extreme form and has been associated with reduced activity of ferredoxin, pyruvate:ferredoxin oxidoreductase (PFOR), malic enzyme, and hydrogenase, enzymes in the metabolic pathway responsible for activation of metronidazole to its toxic form (15, 41, 72, 74, 107). Transfection of a drug-resistant trichomonad with ferredoxin increased its susceptibility to metronidazole (75). In contrast, targeted disruption of the ferredoxin gene in *T. vaginalis,* which also resulted in a 95% decrease in PFOR

activity, failed to confer resistance (76). Together, these studies suggest that ferredoxin and PFOR are capable of, but not necessary for, 5-nitroimidazole activation.

Anaerobic resistance is usually seen only in laboratory-derived strains, although one clinical isolate with anaerobic resistance has been described (128). Most isolates from patients who cannot be cured with metronidazole display resistance that is detectable only under aerobic conditions. While the mechanism for aerobic resistance is less well understood, the enzymes responsible for oxygen scavenging in these parasites appear to have lower substrate affinities (139). This results in more free oxygen in the cytosol that accepts electrons from, or in lieu of, metronidazole and decreases the intracellular concentration of active drug (72, 106). Isolates with laboratory-induced aerobic resistance and some clinical isolates also have decreased mRNA levels and lowered protein expression of enzymes involved in trichomonad metabolism (105, 107). However, assessment of ferredoxin, PFOR, malic enzyme, and hydrogenase in 28 clinical isolates revealed no correlations between mRNA levels of these enzymes and resistance to metronidazole under either aerobic or anaerobic conditions (83).

The molecular epidemiology of *T. vaginalis* suggests that clinically resistant isolates are genetically related and stable over time (57, 116, 125). However, despite increasing recognition of metronidazole treatment failures (96, 117), no epidemics of resistant trichomoniasis have occurred. To explain this observation, it has been hypothesized that resistant isolates are less transmittable between sexual partners (122). Support for this hypothesis comes from findings that clinical isolates with resistance to metronidazole have decreased aerotolerance (106).

Treatment of patients who have metronidazole-resistant trichomoniasis often results in an immediate resolution of symptoms and a negative wet mount. However, within 3 to 4 weeks, in the absence of further exposure, symptoms may recur as the number of organisms rises. Thus, it is important to monitor the efficacy of treatment for up to a month and to encourage patients to avoid unprotected intercourse during this time. When metronidazole resistance is encountered, patients are often treated with increased doses for a longer time (80). However, many patients cannot tolerate high doses of metronidazole and such practices may only exacerbate the development of drug resistance. Clearly, alternatives to metronidazole for treatment of *T. vaginalis* are needed. Furazolidone is active in vitro against isolates of *T. vaginalis* that are resistant to metronidazole and tinidazole (88), but it is poorly absorbed from the digestive tract and would require topical application, thus somewhat limiting its efficacy. Nitazoxanide and disulfiram kill metronidazole-resistant trichomonads in vitro but have not been evaluated in treatment trials (2, 10, 19). Alternative topical therapies that may have some benefit for patients with metronidazole-resistant *T. vaginalis* infections are paromomycin sulfate and povidone-iodine (91, 130, 135, 140). Non-5-nitroimidazole treatments are also important for persons who demonstrate hypersensitivity to metronidazole and tinidazole.

LEISHMANIASIS

Leishmaniasis is transmitted to humans by phlebotomine sandfly vectors and manifests itself in a variety of syndromes. Depending in part on the infecting *Leishmania* species, the pathology ranges from a cutaneous lesion that may be self-limiting to the more severe mucosal or visceral forms. The identification of leishmaniasis as an important opportunistic infection in patients with AIDS has presented new challenges for treatment of this disease, with treatment failures occurring in up to 50% of HIV type 1-positive individuals (3). Use of antileishmanial drugs is limited by their high cost, the difficulty of their administration (injection for several weeks), and their associated toxicity. These factors are even more consequential in the developing countries where leishmaniasis is endemic and can lead to premature self-termination of therapy, which in turn may promote increased levels of resistance (48, 55). While true drug resistance has been described for isolates of some *Leishmania* spp., other species or isolates may just differ in their intrinsic sensitivity to certain compounds. True resistance is more likely in anthroponotic forms of leishmaniaisis, such as *Leishmania donovani* and *Leishmania tropica* (27).

Leishmania infections caused by all species and of all clinical forms are most commonly treated with the pentavalent antimonial compounds sodium stibogluconate and meglumine antimoniate (5, 87, 93). Consequently, they are also the only antileishmanial compounds to which widespread clinical drug resistance has been described. In some regions of India, treatment failure is as high as 65%. Development of resistance has been attributed both to the substandard quality of drug produced by some manufacturers and to inappropriate administration of treatment by some caregivers (87, 119). Evidence for true drug resistance in this setting comes from observations that isolates from clinically resistant patients require higher in vitro concentrations of drug to kill the parasites than do isolates from patients who responded to treatment (119). Furthermore, these differences persist when the putatively resistant isolates are maintained in vitro in the absence of drug pressure, suggesting that a stable genetic change confers resistance (40). The mode of action for pentavalent antimonials, as well as the development of drug resistance, is still poorly defined. In vitro studies have suggested that the pentavalent antimonials are reduced to an active trivalent form that disrupts the parasite's metabolism (8, 93). One hypothesized resistance mechanism is a loss in the parasite's reductase activity, resulting in lower concentrations of effective drug (93, 113). Resistance has also been associated with increased production of trypanothione or glutathione that bind with the trivalent antimonials and extrusion of the resulting thiol-drug conjugates by ATP-binding cassette (ABC) transporters (65, 93). Increased expression of the enzymes involved in thiol synthesis or of the ABC transporter promotes resistance to pentavalent antimonials; inhibition of these pathways in resistant strains increases susceptibility to drug (16, 45, 56, 77, 93). Because of the high level of pentavalent antimonial resistance, this treatment is no longer considered useful in India and other drugs are used for primary treatment of visceral leishmaniasis (5, 87, 119). One of these other drugs, amphotericin B, interacts with parasite-specific 24-alkyl sterols and induces pore formation in the parasite plasma membranes (20). The use of amphotericin B has been limited in the past due to its high cost and toxicity; however, new lipid-associated formulations of amphotericin B have greatly reduced toxicities and retain good efficacy even when administered in lower dosages (5, 87). Like the pentavalent antimonials, optimal activity of amphotericin B may require competent host immune responses (33). Lipid-associated formulations of amphotericin B are phagocytized by host monocytes and accumulate in the phagocytic lysosomes where *Leishmania* amastigotes reside (138). Although the ABC transporter associated with resistance to pentavalent antimonials shares the characteristics of membrane transporters that confer multidrug resistance, in vitro studies demonstrate that

antimonial-resistant parasite strains do not display cross-resistance to amphotericin B (16, 34, 40). Similarly, laboratory or field isolates with resistance to pentivalent antimonials are killed effectively by paromomycin and miltefosine, two other drugs in development for treating leishmaniasis (5, 16, 27, 40). Miltefosine is particularly promising, as it can be taken orally and has fewer side effects than most of the parenteral treatments for leishmaniasis. As a result, although the drug is more expensive than other therapies, it can be administered on an outpatient basis and the overall cost for treatment is lower than less costly drugs that require hospitalization (87). It has also shown efficacy for a severely immunocompromised leishmaniasis patient coinfected with HIV-1 (111). Enthusiasm is tempered, however, both because it is teratogenic, thereby limiting its unregulated use in women of childbearing age, and because it has been observed that leishmanial resistance to miltefosine develops easily in vitro (112, 119).

AFRICAN TRYPANOSOMIASIS

Trypanosoma brucei rhodesiense and *Trypanosoma brucei gambiense* are the etiologic agents of sleeping sickness. They are endemic in east and west central Africa, respectively, and are transmitted by tsetse fly bites. Because these parasites possess antigenic switching mechanisms, host immune responses are ineffective and the prospects for the development of vaccines against these organisms are meager. As a result, drug treatment is the only medical intervention available to combat sleeping sickness for the foreseeable future. Recent political unrest with subsequent loss of an effective public health infrastructure in parts of Africa has caused a resurgence of this disease, a situation that has been compounded by increased resistance to the limited armamentarium of efficacious drugs.

Pentamidine and suramin are useful for treatment only of early stages of infection because they are highly ionic and do not cross the blood-brain barrier. Late-stage, central nervous system disease is treated with melarsoprol B. Eflornithine is effective against late-stage *T. b. gambiense* infections that are resistant to melarsoprol, but *T. b. rhodesiense* is not susceptible to this drug (4). Data are somewhat limited but suggest that patients coinfected with HIV are more likely to relapse following treatment with melarsoprol or eflornithine than individuals with trypanosome infection alone (58).

The efficacies of pentamidine and melarsoprol are dependent on their selective accumulation inside the parasite rather than in the host's cells (11). These two drugs share an amidinium-like moiety with amino purines that is recognized and actively taken up by nucleoside transporters in the trypanosome membrane, resulting in a concentration of these compounds within the parasite. Thus, although pentamidine and melarsoprol have different modes of action, resistance to these drugs seems to occur through a similar mechanism due to shared structural motifs (46, 89). Resistance to both drugs has been associated with the loss of the adenosine transporter P2 and accumulation of less drug (8, 11, 18, 89). Other transporters permit parasite uptake of purines and confer survival in the absence of this transporter (17). The P2 transporter has been cloned and expressed in yeast, where it enabled uptake of adenosine and was inhibited by melarsoprol but not pentamidine (82). Sequencing of the P2 transporter from drug-resistant parasites revealed six amino acid changes, at least one of which was shared with a field isolate from a patient with a melarsoprol-resistant infection (82). Two additional transporters have now been shown to contribute to pentamidine uptake, explaining at least in part the high rate of clinical resistance to melarsoprol compared to that of pentamidine (11, 30, 38, 62). Mechanisms of resistance to suramin or eflornithine are unknown but are thought to similarly reflect decreased intraparasite levels of drug accumulation. However, it is likely that uptake of suramin uses other transporters or is based on an alternative mechanism, as trypanosomes that have undergone genetic manipulation of their transporters that renders them 10-fold more resistant to pentamidine are still susceptible to suramin (65).

SCHISTOSOMIASIS

Drug resistance in human helminths is rare, a fact attributed to their long reproduction cycles and to their lack of multiplication inside the human host (the exception being *Strongyloides stercoralis*). Thus, when considering treatment failures in schistosomiasis, it is important to distinguish characteristics leading to reduced drug efficacy from those of true drug resistance. For example, persons with very high levels of infection are less likely to be cured with single-dose therapy than individuals with lower worm burdens (63, 100, 123). This is in part related to the fact that the standard drugs used to treat schistosomiasis, oxamniquine and praziquantel, are effective only against the adult stage of the parasite. Immature worms that may be present at the time of drug treatment, especially in areas of high transmission, are not susceptible to the initial treatment and subsequently develop into patent infections that give the impression of drug resistance. As a result, a minimum of two treatments spaced 4 to 6 weeks apart should be attempted when drug resistance is suspected (100, 123).

Nevertheless, *Schistosoma mansoni* infections that have true oxamniquine resistance have been reported in Brazil and Kenya (23, 32) and praziquantel-resistant infections have been described in Egypt and Senegal (60, 118). While alternative explanations to drug resistance have been offered for the praziquantel treatment failure, eggs obtained from the feces of individuals who could not be successfully treated have been used to establish infections in mice for confirmation of the drug resistance phenotype (47, 59, 60, 79). Nevertheless, despite continued treatment pressure, widespread clinical resistance has not been observed since these foci were described (9).

Oxamniquine is activated by a schistosome esterase, binds the parasite's DNA, and irreversibly inhibits RNA and DNA synthesis (21). This enzymatic activity is not present in *Schistosoma haematobium* (a species not susceptible to oxamniquine) or oxamniquine-resistant strains of *S. mansoni* (21, 98). Classical genetic studies on oxamniquine-resistant strains demonstrate that this resistance is controlled by a single autosomal recessive gene that also confers resistance to hycanthone, an older antischistosome drug that is no longer used clinically (22, 97). Production of oxamniquine has now ceased, and it is no longer available commercially (49).

Resistance to praziquantel is manifested by a reduced effect of the drugs on the tegument of adult schistosomes (59, 78, 134). In drug-susceptible parasites, this damage makes the worms susceptible to attack and killing by the host's immune response. Interestingly, although immature worms are not susceptible to praziquantel treatment, eggs, miracidia, and cercariae from praziquantel-susceptible strains of *S. mansoni* show decreased hatching, drastic morphologic changes, and increased tail shedding, respectively, when exposed to drug in vitro than do these life cycle stages from praziquantel-resistant strains (79). The unique beta subunit of the schistosome calcium ion channel has been proposed as a molecular

target for praziquantel (66). However, no differences in this gene's sequence or expression were observed among a limited number of praziquantel-resistant and -susceptible parasite strains (124). While mathematical models predict that widespread resistance to praziquantel is not imminent, they do anticipate that it will emerge in 10 to 20 years (64). This finding reiterates the need for ongoing drug discovery and testing even for parasites less prone to the development of drug resistance.

One new compound being investigated for the treatment of schistosomiasis is artemether, a derivative of the antimalarial drug, artemesinin. Unlike oxamniquine and praziquantel, artemether is most effective against juvenile worms (114). Thus, it could be used as a prophylactic against patent schistosomiasis for persons with a known exposure or in combination with praziquantel to kill all worms in the human host. Caution about its use, however, has been raised as there is concern that treatment of schistosomiasis with this drug will hasten the occurrence of malarial resistance to it (49).

FUTURE PERSPECTIVES

Several factors contribute to the emergence of drug-resistant parasites. Those parasite species with short life cycles and high multiplication rates that occur in areas of intense transmission are most likely to develop resistant subpopulations. The selection of such populations is encouraged when the parasites are repeatedly exposed to suboptimal drug concentrations. This pattern can result from the use of drugs with long half-lives, or more typically from the frequent, often unjustified use of inadequate doses of drugs, a common occurrence in countries where parasite infections are endemic. Public health interventions to correct these factors have not always been successful and would benefit from a better understanding of the drug resistance mechanisms used by parasites. These mechanisms are very diverse and have been difficult to study, but recent technological advances now provide long-awaited tools that will facilitate the task. The genome sequences for *Plasmodium falciparum, Trichomonas vaginalis, Leishmania major,* and *Trypanosoma brucei* have recently been completed, and sequencing of the *Schistosoma mansoni* genome is ongoing. When these data are used, for example, in combination with microarray technology, where the DNA of drug-resistant parasite strains is compared to that of drug-susceptible strains, identification of the genes that confer resistance should proceed even more rapidly than in the last few years. In addition, genomic data may also be useful in the design of new chemotherapeutic agents as they help researchers identify metabolic processes of parasites that are sufficiently different from those of their human hosts to allow specific attack.

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Susceptibility Test Methods: Parasites

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OVERVIEW

Accurate methods for ascertaining the responses of parasites to antiparasitic drugs can prove useful at several levels. They can assist in the clinical management of individual patients, they can yield epidemiologic information that may guide drug use policies and public health interventions, and they offer crucial research tools for the development of new and better drugs. Drug susceptibility tests fall into four broad categories: in vivo tests, in vitro tests, tests with experimental animals, and molecular tests.

In vivo tests with patients directly assess the clinical efficacies of existing compounds. These tests are performed in actual epidemiologic investigations, and their modest technical requirements make them suitable for use under field conditions in developing countries. The interpretation of in vivo tests is limited by potential interferences by factors related to the host (e.g., immunity or variations in drug intake or metabolism) or to the environment (e.g., reinfections). However, such tests have proven instrumental in guiding drug use policies, particularly for malaria.

In vitro tests circumvent these interferences by isolating the parasites from their hosts and investigating them in culture under controlled laboratory conditions, with opportunities for repeated assessments against multiple compounds (including experimental compounds). In vitro tests, however, are technically more demanding and therefore less amenable to performance under field conditions. They are most informative if they are used to investigate parasites that multiply rapidly in culture, a select group consisting mostly of protozoa. They are of limited use for assessing inactive drug precursors that must be activated by the host or drugs whose antiparasitic activity necessitates the synergistic effect of the host's immune defenses.

Tests with experimental animal models permit investigations of parasites that cannot be grown in culture or of drugs not yet approved for use in humans.

Molecular tests detect genetic variations that are potentially linked with resistance. Such tests offer unique advantages. PCRs can be performed with minute amounts of nonviable parasite genetic material. They can be run in batches, allowing large-scale epidemiologic studies. Molecular analysis at the single-organism level circumvents potential ambiguities associated with the heterologous parasite populations occasionally encountered in in vivo or in vitro tests and allows the dissection of such populations. Because of their short duration (hours), molecular diagnostic procedures can potentially be used to guide patient management. The other categories of tests usually require more time (days to weeks) and yield results that are used mainly for epidemiologic surveillance and experimental chemotherapy studies. Drawbacks of molecular tests reside in their technical requirements and in the need to be certain that the genes evaluated correlate with functional resistance. However, thanks to the development of more practical protocols and more robust automated equipment, as well as a better understanding of the genes that confer resistance, molecular techniques are used in an increasing number of laboratories, including field facilities.

These different categories of tests provide complementary information. At one end of the spectrum, molecular tests analyze parasites at their most basic biologic level, without any outside interference. At the other end, in vivo tests with patients reflect complex interactions between host and parasite, yet are most relevant for clinicians and public health practitioners. While a good correlation between results of various test methods is desirable, some degree of discrepancy should be expected to result from factors linked to the host or the culture conditions. Indeed, a judicious analysis of such discrepancies might provide valuable insights into the mechanisms of drug action and resistance.

These points are illustrated in the following discussions of five parasitic diseases, selected for their particular chemotherapeutic challenges. A summary is provided in Table 1.

MALARIA

Most drug resistance tests in malaria concern *Plasmodium falciparum,* which is, among the four species that infect humans, the most virulent and the most prone to the development of resistance.

Initial observations of drug-resistant malaria occur most often in a clinical context, and their confirmation is frequently sought by in vivo tests. These aim at documenting the parasitologic and clinical response of a malaria infection in a patient treated with a standard dose of the test drug and monitored under controlled conditions. Initially standardized by the World Health Organization (WHO) for the response of *P. falciparum* to chloroquine, in vivo tests have been variously modified for simpler performance

TABLE 1 Selected antiparasitic agents and susceptibility testing methods[a]

Disease and drugs	Testing methods	Remarks
Malaria Chloroquine, sulfadoxine-pyrimethamine, quinine, mefloquine, artemisinin, atovaquone-proguanil, tetracycline, primaquine, others	In vivo tests in patients with *P. falciparum* or *P. vivax* malaria Culture of erythrocytic stages of *P. falciparum;* criteria for assessment: • Microscopic examination: maturation from rings to schizonts; parasite multiplication • Metabolic activity: incorporation of [^{3}H] hypoxanthine; production of pLDH, HRP2 • DNA quantitation PCR-based genetic analysis (mostly, *P. falciparum*) of mutations/amplification in genes putatively involved in resistance to chloroquine *(pfcrt, pfmdr1),* antifolates (*dhfr, dhps* genes), atovaquone (cytochrome B gene), and others	Drug resistance is a major problem, especially in *P. falciparum;* it also occurs in *P. vivax.* Tests are used for epidemiologic assessment as well as for laboratory investigations. Short-term culture tests are also described for erythrocytic stages of *P. vivax.* On an experimental basis, in vitro tests for drug effect on liver stages and sexual stages (gametocytes)
Trichomoniasis Metronidazole, tinidazole	Culture under aerobic and anaerobic conditions; criterion for assessment: parasite mobility	Resistance to metronidazole is relative; testing is performed over a wide range of concentrations.
Leishmaniasis Sodium stibogluconate, meglumine antimoniate, pentamidine, amphotericin B, paromomycin, miltefosine	Culture of promastigotes; criteria for assessment: • Microscopic examination and parasite count • Metabolic activity: incorporation of [^{3}H] thymidine; hydrolysis of *p*-nitrophenyl phosphate; conversion of MTT or Alamar Blue Culture of amastigotes (intracellular or axenic); criteria for assessment: • Microscopic examination and counting of stained intracellular parasites • Luciferase activity of transfected parasites • Flow cytometry using GFP-transfected parasites or intracellular staining with anti-*Leishmania* monoclonal antibody • Flow cytometry based on alterations in membrane potentials (amphotericin B)	Problems with most drugs: high cost, difficulty in administration, and toxicity High level of failure of pentavalent antimonials in some areas Coinfection with human immunodeficiency virus type 1 can compromise therapy results. Tests with intracellular or axenic amastigotes show better correlation with clinical drug efficacy.
African trypanosomiasis Pentamidine, suramin, melarsoprol, eflornithine	Culture of trypomastigotes; criteria for assessment: • Microscopic examination and parasite count • Metabolic activity: hydrolysis of *p*-nitrophenyl phosphate, reduction of Alamar Blue	In vitro tests used mainly for laboratory investigations Ability to detect mutations in transporter responsible for drug uptake may soon allow tests applicable in the field.
Schistosomiasis Oxamniquine, praziquantel	Damage to adult worms from experimental infections with suspected resistant strains (praziquantel) Egg hatching efficiency, miracidial morphology, and cercarial tail shedding (praziquantel)	In vivo animal tests needed for confirmation due to dependence of drug effect on host immune response

[a] See text for details.

and assessments of other drugs and are used in epidemiologic investigations or for monitoring drug resistance (23, 61).

In vitro drug studies of the activities of drugs against the malaria parasite have focused on the asexual erythrocytic stages of *P. falciparum,* for which long-term cultivation techniques are available (53). The microtechnique originally described by Rieckmann et al. (41) offers the simplest test format and has been standardized and adapted to field work by WHO for use with various antimalarial drugs (60). By this technique, a 100-μl fingerprick capillary blood sample is collected from an infected individual and diluted 10-fold in culture medium (RPMI 1640 supplemented with 25 mM HEPES buffer). This blood-medium mixture containing synchronous *P. falciparum* ring stage parasites (the only asexual stage found in patient peripheral blood) is cultured in the wells of a 96-well microtiter plate. The wells are predosed with various concentrations of test drug (including a non-drug control), and the plates are incubated in a candle jar at

37°C. After 24 to 30 h of incubation, thick smears of the wells' contents are made and the smears are stained with Giemsa stain; the maturation of parasites to multinucleated schizonts in drug-containing wells is compared with the maturation of parasites in the control well, and the inhibition of parasite maturation by various concentrations of the antimalarial compound is assessed. In the case of chloroquine, the presence of schizonts in the wells containing 8 pmol of chloroquine indicates in vitro resistance. The results can be alternately expressed as a dose-response curve, and results from several isolates can be pooled to reflect the overall status of malaria parasites in an area.

Beyond this simplest format, other in vitro tests that are not as widely available as the microtechnique but offer valuable advantages have been developed. These more versatile tests are applicable both to field-collected parasites and to the parasites growing asynchronously in long-term laboratory cultures and can also be used for screening potential new antimalarials and for investigations on drug modes of action. Parasites are cultured in suspensions containing fewer cells for a longer period, often 48 to 72 h, allowing the drug to exert its inhibitory effect on all phases of the parasite's full cycle of asexual multiplication. Parasite growth and inhibition can be assessed using different methods. Parasite counts by microscopic examination are simple to perform but cumbersome (33). Measurement of uptake of [^{3}H]hypoxanthine (10) offers a semiautomated, quantitative, high-output approach but necessitates the use of radioactive material and specialized equipment. Measurement of *Plasmodium*-specific products such as histidine-rich protein 2 (HRP2) (34) or parasite lactate dehydrogenase (pLDH) (31) by enzyme-linked immunosorbent assay circumvents the use of radioisotopes but still requires a spectrophotometer. Quantification of parasite DNA by real-time PCR is an additional approach (9).

The successful completion and interpretation of all malaria in vitro tests depend on several factors. Such factors include a recent intake of antimalarial drugs by the patient, which may decrease the test success rates; high parasite inoculums, which can lead to an overestimation of resistance to some drugs (14); and folate and *para*-aminobenzoic acid in the culture medium, which will antagonize the in vitro effect of antifolate drugs (57).

While the great majority of tests assess asexual stages of *P. falciparum*, in vitro drug studies on other species and stages are also possible, albeit substantially more cumbersome. The microtechnique has been adapted for testing of erythrocytic stages of *P. vivax* (52), and progress in continuous culture techniques (17) might allow further improvements. In vitro cultures of the sexual blood stages (gametocytes) and the liver (exoerythrocytic) stages of *P. falciparum* can also be used to assess the antimalarial activity of various compounds on these stages (7, 49).

Potential genetic markers for drug resistance that now offer promising tools for assessing drug resistance have been identified (59). Chloroquine resistance has been linked to point mutations in the *pfcrt* gene of *P. falciparum*, which encodes PfCRT, a transmembrane transporter found on the parasite digestive vacuole, with a Lys→Thr mutation on codon 76 (K76T) playing a central role (13). *P. falciparum* and *P. vivax* resistance to antifolates, such as sulfadoxine-pyrimethamine, has been associated with mutations in the genes encoding parasite dihydrofolate reductase *(dhfr)* and dihydropteroate synthetase *(dhps)* (20, 39). Point mutations and gene amplification of the *pfmdr1* gene encoding Pgh1, the P glycoprotein homologue 1, have been linked to varying extents with *P. falciparum* responses to chloroquine and other drugs such as quinine, mefloquine, and artemisinin derivatives (38). Resistance to atovaquone (a component of the recently introduced drug combination atovaquone-proguanil) has been linked to point mutations in the *P. falciparum* cytochrome B gene (24). Such genetic polymorphisms can be identified through standard techniques, which include mutation-specific nested PCR (13) or nested PCR followed by sequencing (2), restriction fragment length polymorphism analysis (51), or detection with molecular beacons (15). High-throughput methods using real-time PCR (56) or sequence-specific oligonucleotide probes (1) permit the rapid analysis of large numbers of samples and are thus particularly suitable for molecular surveillance. Using these techniques, a large number of field investigations have been conducted to assess linkage between parasite genotypes and resistance; to measure the prevalence of putative molecular markers for resistance; and to predict the risk of future emergence of resistance, based on the number and nature of polymorphisms observed in existing parasite populations.

The judicious use of in vivo, in vitro, and molecular tests can yield valuable information to address drug resistance in malaria (35).

TRICHOMONIASIS

While failures of treatment for *Trichomonas vaginalis* infections have often been regarded as being due to poor patient compliance or rapid reinfection, there is an increasing recognition that true clinical resistance exists and may be increasing (36, 48), thus necessitating accurate drug susceptibility testing of patient isolates. In vitro testing for resistance to 5-nitroimidazoles is indicated following failure of two standard treatments to cure the patient's infection. Metronidazole and tinidazole susceptibility testing for *T. vaginalis* is a simple assay of parasite motility in the presence of drug (29). It is performed under both aerobic and anaerobic conditions to determine which type of resistance is present. Also, differences in in vitro drug sensitivity between clinically resistant strains are more pronounced when the assay is performed under aerobic conditions. Axenic trichomonads are cultured in Diamond's trypticase-yeast-maltose media with serial dilutions (400 to 0.2 μg/ml) of metronidazole or tinidazole dissolved in dimethyl sulfoxide (DMSO) and the appropriate parallel concentrations of DMSO in U-bottom microtiter plates. Plates are incubated at 37°C for 48 h and are then examined microscopically with an inverted phase-contrast microscope. The lowest concentration of drug in which no motile organisms are observed is defined as the minimum lethal concentration (MLC). Aerobic MLCs greater than 100 μg of metronidazole per ml and anaerobic MLCs greater than 3.1 μg of metronidazole per ml have been associated with clinical resistance (32). This assay has been adapted to determine 50% inhibitory concentrations and screen novel compounds for antitrichomonad activity by measuring incorporation of [^{3}H]thymidine (8) or acid phosphatase activity (27). These modifications have not yet been adapted to identify clinically resistant isolates, but theoretically this could be accomplished rather easily.

One difficulty of the in vitro culture method for assessing resistance of *T. vaginalis* isolates is the need to derive axenic cultures from clinical specimens. As individuals with trichomoniasis are often infected with other sexually transmitted disease organisms that grow in Diamond's media, this may require an extended time. Recent studies on the molecular epidemiology of *T. vaginalis* suggest that genetic markers of

metronidazole resistance exist (19, 47, 55). While other studies fail to corroborate this finding (50), the prospect of a molecular marker for resistant *T. vaginalis* is exciting, as it may make possible a PCR-based assay for resistance. This would obviate the need for establishing axenic cultures and may allow direct testing of specimens from patients suspected of harboring a resistant isolate.

LEISHMANIASIS

Treatment failure in leishmaniasis may result from either true drug resistance or patient immunodeficiencies that preclude effective chemotherapeutic action. Differentiation of these two possibilities is important for both individual patient care and general public health. Traditional testing for drug resistance in *Leishmania* is performed by culturing the promastigote form of the parasite with drug in standard cell culture media (Schneider's, RPMI, or M199) supplemented with bovine sera for 42 to 72 h at 26 to 37°C and assessing viability by direct counting (4), [^{3}H]thymidine incorporation (18), enzymatic hydrolysis of *p*-nitrophenyl phosphate (3), conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (43), or reduction of Alamar Blue (30). [^{3}H]thymidine incorporation is perhaps the most sensitive method, but it requires access to radioactive materials and a scintillation counter. Hydrolysis of *p*-nitrophenyl phosphate and conversion of MTT are colorimetric assays, but *p*-nitrophenyl phosphate hydrolysis has high backgrounds caused by medium components and MTT may interact with certain drugs (e.g., meglumine antimoniate). An advantage of Alamar Blue is that it is not necessary to further manipulate the parasites in order to read the assay. Thus, drug activities can be monitored at different time points after initiation of cultures.

Recently, amastigotes have been adapted to in vitro drug testing in both axenic and intracellular assays. Intracellular assays bear the closest resemblance to the in vivo situation, and their results correlate better with the in vivo response to treatment than the results of assays that use promastigotes do (26). Previously, intracellular assays were very labor-intensive, as each condition necessitated preparation of slides, staining of macrophages, and manual counting of infected cells. Stable transformation of isolates with firefly luciferase has facilitated this process and provides an added advantage in that efficacy of drugs can be compared for promastigotes, axenic amastigotes, and intracellular amastigotes (44). Cells are simply lysed, the substrate luciferin is added, and activity is measured on a luminometer.

Assays that use a flow cytometer to evaluate drug efficacy have also been developed. In one of these assays, laboratory or field isolates are transfected with the gene encoding green fluorescent protein (GFP) (45). Promastigotes or amastigotes are then incubated with drug and enumerated on a flow cytometer. In this assay, there is no need for addition of substrates or staining with antibodies because GFP is intrinsically fluorescent.

If transfection with exogenous genes is undesirable, mammalian cells that have been infected with parasites and treated with drug can be fixed and permeabilized. Intracellular amastigotes are then detected with anti-*Leishmania* lipophosphoglycan monoclonal antibody followed by a fluorescence labeled anti-mouse immunoglobulin antibody (12). This assay can be used for assessing drug sensitivity of a variety of *Leishmania* species, and it is more adaptable to use with individual patient isolates. A third flow cytometer-based test that works by measuring parasite membrane potentials has been developed (11). However, this test is useful only for evaluating resistance to amphotericin B. A drawback of tests that employ luminescent or flow cytometric detection is that they require specialized equipment.

Recently, clinical isolates of *Leishmania donovani* with resistance to a pentavalent antimonial (sodium antimony gluconate) were identified by PCR amplification of a novel parasite sequence (46). This assay is of interest, as no manipulation beyond growing parasites is needed. However, it is likely that its applicability is species and drug restricted.

AFRICAN TRYPANOSOMIASIS

As in leishmania drug sensitivity testing, trypanosomes can be cultured in vitro with drug and monitored for viability by enzymatic hydrolysis of *p*-nitrophenyl phosphate (3) or by direct counting (22). Parasites are cultured at 37°C for 24 h in 4 to 5% CO_2 in phenol red-free Iscove's medium containing hypoxanthine, thymidine, glutamine, L-cysteine, pyruvate, β-mercaptoethanol, and heat-inactivated bovine or horse sera. An in vitro lysis assay has been developed for testing sensitivity of *Trypanosoma brucei* subspecies to melarsoprol (62). It requires the ability to culture trypanosomes and a thermostatically controlled recording spectrophotometer. As susceptible organisms die, their absorbance at 500 nm is reduced over the course of 30 min. If control drug-susceptible and drug-resistant strains are available to use as controls, this technique could perhaps be adapted to field use because differences in intensity at this wavelength can be distinguished by the unaided eye. Visible color change can also be observed when trypanosomes are incubated with Alamar Blue (40). However, in contrast to assays for *Leishmania* drug sensitivity, the dye is added after parasites are incubated with drugs for 66 to 72 h, depending on the parasite subspecies.

With the identification of the purine transporter responsible for melarsoprol uptake and the identification of the genetic mutations that confer resistance, it may soon be possible to utilize molecular tools to identify trypanosome isolates that are resistant to melarsoprol. One of the genetic mutations leading to an amino acid change in the transporter abrogates a restriction endonuclease site, while a different mutation creates another one (28). Thus, PCR amplification of the appropriate gene followed by enzymatic digestion and gel electrophoresis results in different DNA banding patterns that could be used to distinguish melarsoprol-susceptible and -resistant isolates. While more clinical drug-resistant isolates must be evaluated to confirm the utility of this test, a similar banding pattern between melarsoprol-resistant laboratory-derived and field isolates (28) suggests that this tool could be useful for both management of individual patients' infections and tracking the spread of resistance in a population (5).

SCHISTOSOMIASIS

Investigation of drug resistance mechanisms of schistosomes is more difficult and time-consuming than those associated with protozoan infections. This is because the parasites are extremely difficult to grow in vitro and the efficacies of the two drugs most commonly used for the treatment of schistosomiasis, praziquantel and oxamniquine, are dependent upon components of the immune system (16, 42). Thus, tests for drug sensitivity generally require investigators to obtain eggs from an unsuccessfully treated mammalian host, infect the snail intermediate host, obtain cercariae from the infected snail, and infect and treat experimental animals.

A protocol to determine 50% effective dose (ED_{50}) values for mice has been developed and demonstrates good reproducibility among different laboratories (6). ED_{50}s can also be estimated in an in vitro assay by assessing contraction of worms following perfusion and incubation with praziquantel (37). Recently, praziquantel-resistant worms obtained from infected mice were shown to suffer less tegumental damage when exposed to praziquantel in vitro than did adult worms from isolates susceptible to praziquantel (21, 58). Although immature worms are relatively invulnerable to praziquantel, differences in egg hatching efficiency, miracidial morphology, and cercarial tail shedding can be observed between praziquantel-resistant and praziquantel-susceptible strains in the presence of drug (25). In theory, because these assays are simple and do not require sophisticated equipment, they should be readily adaptable to field use to assess parasite resistance in infected humans (utilizing eggs or miracidia hatched from eggs obtained from fecal material) or snails. Random amplified polymorphic DNA PCR analysis of praziquantel-susceptible and -resistant *Schistosoma mansoni* strains yields different banding patterns following electrophoresis of PCR products (54). These results suggest that specific primers that will be useful for rapid differentiation of drug-susceptible and -resistant isolates could be designed.

FUTURE PERSPECTIVES

Most susceptibility tests for antiparasitic drugs are not routinely available in clinical diagnostic laboratories, because these procedures are not in frequent demand and present special technical requirements. Such tests are performed mainly in reference or research laboratories or during epidemiologic investigations in areas of endemicity. Thanks to recent advances in laboratory technology and genetic analysis of parasites, available tests are increasingly sophisticated and informative. The development of tests that are robust and simple to use will facilitate field studies that aim at optimizing the deployment of currently available drugs. These field tests usefully complement the more sophisticated procedures used in research laboratories whose main orientation is toward the development of novel antiparasitic compounds.

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